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Multitargeted drug development: Discovery and profiling of dihydroxy substituted 1-aza-9-oxafluorenes as lead compounds targeting Alzheimer disease relevant kinases

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ABSTRACT

Alzheimer disease (AD) turned out to be a multifactorial process leading to neuronal decay. So far merely single target structures which attribute to the AD progression have been considered to develop specific drugs. However, such drug developments have been disappointing in clinical stages. Multitargeting of more than one target structure determines recent studies of developing novel lead compounds. Protein kinases have been identified to contribute to the neuronal decay with CDK1, GSK-3β and CDK5/p25 being involved in a pathological tau protein hyperphosphorylation. We discovered novel lead structures of the dihydroxy-1-aza-9-oxafluorene type with nanomolar activities against CDK1, GSK-3β and CDK5/p25. Structure–activity relationships (SAR) of the protein kinase inhibition are discussed within our first compound series. One nanomolar active compound profiled as selective protein kinase inhibitor. Bioanalysis of a harmless cellular toxicity and of the inhibition of tau protein phosphorylation qualifies the compound for further studies.

Alzheimer disease (AD) is the presently most widespread dementia disease.[1] The possibilities to lower the observed neuronal decay are limited to some acetylcholine esterase (ACE) inhibitors and the *N*-methyl D-aspartat (NMDA) receptor antagonist memantine. The therapeutical benefit of the ACE inhibitors is poor and the NMDA receptor antagonist memantine is supposed to cause neurotoxic effects.[2,3] AD histopathology is characterized by the formation of protein deposites in brain, namely the extracellular, insoluble amyloide plaques (APs) and the intracellular neurofibrillary tangles (NFTs) which consist of hyperphosphorylated tau protein.[4–6] The insoluble APs are discussed to be nontoxic. However, recent studies suggest an influence of the soluble oligomers of the APs precursor the amyloide-β protein on the NFTs formation. The hyperphosphorylation of the tau protein is known to be triggered by the glycogen synthase kinase (GSK) 3-β which is found being overexpressed in neuronal cells with NFTs.[7] Another kinase contributes to the tau protein hyperphosphorylation, namley the cycline dependent kinase (CDK) 5 which is physiologically controlled by the protein p35.[8] High concentrations of p25 which is a truncated subunit of p35 were found in the neuronal cells of AD patients.[9] P25 constitutively activates CDK5 and thus p25 is made responsible for the tau protein hyperphosphorylation mediated by CDK5.[9] The AD progression of the neuronal decay is known to be a multifactorial process.[10,11] Past efforts to develop specifically acting drugs were disappointing in clinical studies because the patient benefit in the improvement of cognition or memory was poor.[11–13] Such drugs have been ACE inhibitors or secretase inhibitors which prevent the formation of the amyloide-β proteins by the inhibition of their cleavage from the amyloide precursor protein (APP).

A novel strategy in the development of perspective AD therapeutics concentrates on the development of drugs which target more than one AD relevant structure which contribute to the neuronal decay.[14,15] So recent studies tried to optimize a combined effect of the multitargeting drug Memoquin **1** as ACE inhibitor with amyloide-β aggregation inhibiting properties.[16]

Inhibitors of GSK-3β have been reported but they all suffered from a limited protein kinase selectivity. Only one inhibitor from the thiadiazolidinone type **2** with micromolar activities reached clinical trials, but nothing has been reported so far about its selectivity (Fig. 1).[17]

Flavoperidol **3** as a nonselective CDK inhibitor with micromolar activities has been investigated in clinical trials but toxic problems occurred due to its nonselectivity of protein kinase inhibition.[18]

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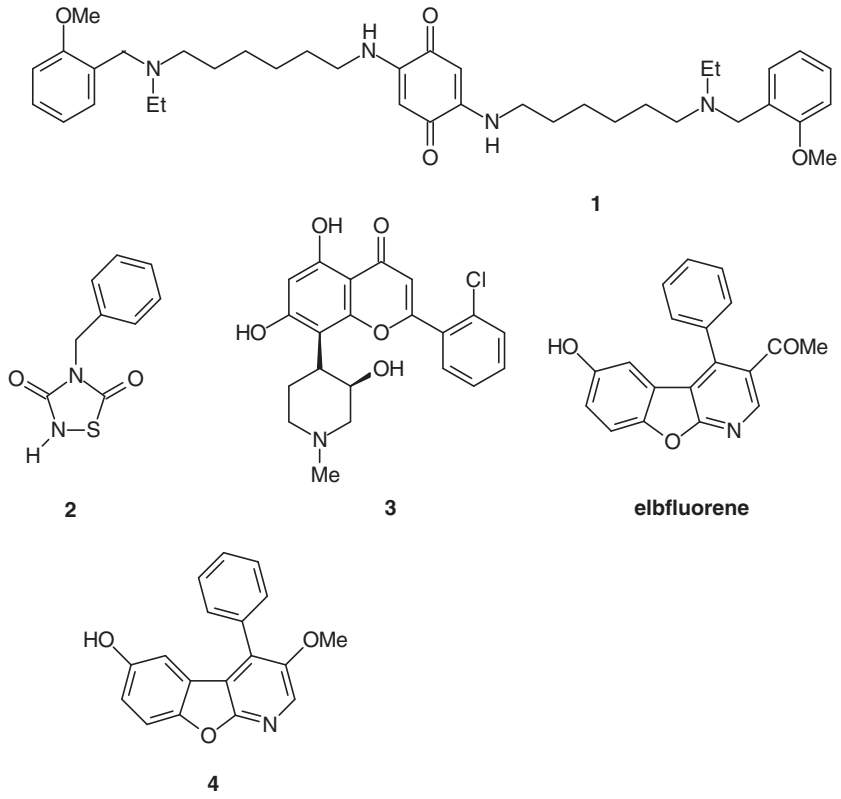


Figure 1. Structures of Memoquin **1** and of protein kinase inhibitors in AD relevant clinical trials, namely thiadiazolidinone **2** and flavoperidol **3**, including elbfluorene and our 1-aza-9-oxafluorene starting structure **4**.

Recent studies proved a certain influence of CDK1 on the decay of neurons in AD brains. CDK1 is found being overexpressed in such vulnerable neurons which reenter the cell cycle and die.[1,19,20]

We tried to develop a multifactorially acting protein kinase inhibitor addressing the AD target structures CDK1, GSK-3β and CDK5/p25 which contribute to the tau protein hyperphosphorylation and to the neuronal death by the later formed NFTs.

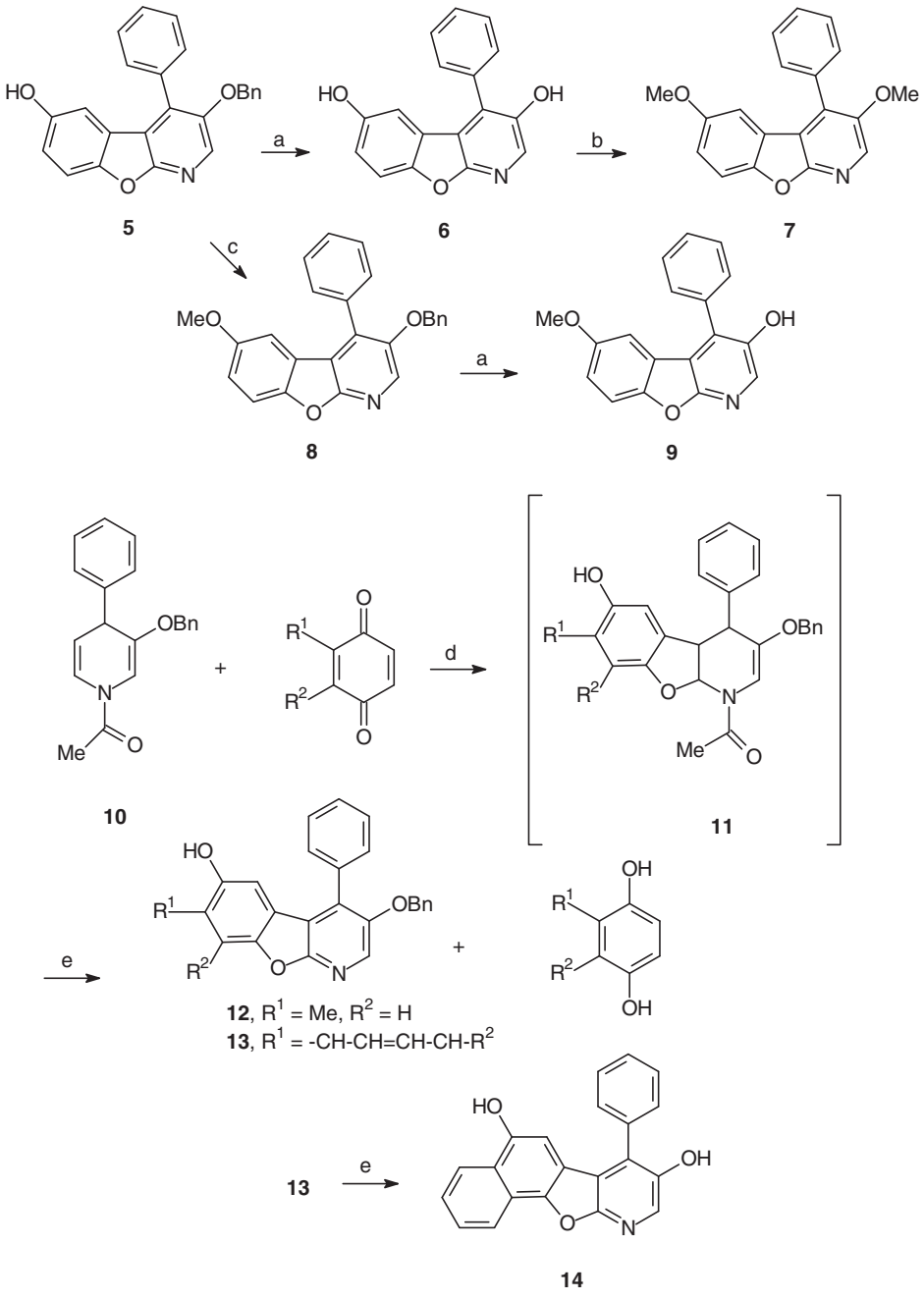
Early 1-aza-9-oxafluorenes with a 3-carbonyl function like elbfluorene showed some CDK1 inhibiting properties.[21]

We started our search from the 3-methoxy-4-phenyl-1-aza-9-oxafluorene **4** and consequently varied the substitution patterns in both the 3- and the 6-position of the molecular scaffold with a first benzo-annelated compound included. The synthesis of all derivatives is shown in Scheme 1.

The varying 1-aza-9-oxafluorenes **5**–**14** have been individually prepared. The 3,6-dihydroxy derivative **6** was given after treatment of the 3-benzyloxy precursor **5** with hydrogen at a pressure of 2 bar using palladium on charcoal (10%).[22] The 3,6-dimethoxy substituted derivative **7** was yielded by a methylation reaction of compound **6** in a small volume of dried THF at room temperature by the use of two equivalents of methyl iodide.[24] Similar reaction conditions were used to yield the 6-methoxy substituted derivative **8** from the 3-benzyloxy derivative **5**. The used methyl iodide excess was only the half of the previous reaction to yield **7**. Finally, the 3-hydroxy-6-methoxy compound **9** was given by the reaction of the 3-benzyloxy derivative **8** with hydrogen and palladium on charcoal similar to the formation of **6**.

The methyl-substituted and the benzo-annelated 1-aza-9-oxafluorenes **12** and **13** were prepared by the reaction of *N*-acetyl-3-benzyloxy-1,4-dihydropyridine **10** with methylbenzoquinone or naphthoquinone in dried dioxane under perchloric acid catalysis (6%). The non-isolated primary formed tetrahydro derivative **11** was oxidized by the addition of portions of the corresponding quinone until no more of this intermediate was detectable by tlc.[25] The debenzylation of **13** followed the procedure of the formation of compound **6**.[26]

Micromolar activities in the inhibition of CDK5/p25 were reported for our starting compound **4**.[27] We actually determined the affinity data of **4** towards CDK1, CDK5/p25 und GSK-3β and the two related serine/threonine kinases CDK2/E and CDK4/D which both are also discussed to contribute to the tau protein hyperphosphorylation.[1,19,20] The K_i values determined in our present assay system are shown in Table 1. We found micromolar affinity data towards CDK1/B and GSK-3β. However, the compound was completely inactive as CDK5/p25 inhibitor. The determined affinities towards CDK2/E and CDK4/D were poor. While the 3-methoxy function in **4** may serve as hydrogen bond acceptor function, the contribution of the 6-hydroxy function to the protein kinase affinity was not clear. Therefore we methylated this functional group. A hydroxyl function may basically serve as hydrogen bond donator or as hydrogen bond acceptor function. Compound **7** as twice-methoxylated derivative showed a complete loss of affinity to all the considered protein kinases. We concluded that the 6-hydroxy function may play a central role in the binding to the protein backbone of the respective protein kinases. A replacement of the 3-methoxy function in derivative **7** with a hydroxy function did not restore the protein kinase affinity as shown with derivative **9** which was also inactive as protein kinase inhibitor. So a methylation of the 6-hydroxy function turned out as unfavourable to increase the protein kinase affinities of our compounds. A replacement of the 3-methoxy group of derivative **4** with a 3-benzyloxy function resulted in similar affinities of the resulting compound **5** towards CDK1/B and GSK-β. Obviously, the more bulky 3-benzyloxy substituent is well tolerated if compared to the 3-methoxy substituent because of some slight affinity improvements especially towards CDK2/E with a micromolar affinity. The 6-meth-



Scheme 1. Reagents and conditions for the preparation of compounds: (a) H_2, Pd/C, MeOH, 2 bar, rt; (b) 2 equiv MeI, THF, 0 °C-rt; (c) 1 equiv MeI, THF, 0 °C-rt; (d) perchloric acid (6%), dioxane, rt; (e) methylbenzoquinone or naphthoquinone, perchloric acid (6%), dioxane, rt; (f) H_2, Pd/C, THF/MeOH, 2 bar, rt.

oxy derivative **8** showed a significant improvement of the GSK-3β affinity to nanomolar ranges. A loss of CDK1/B affinity was accompanied by a CDK5/p25 activity. While a 6-methoxy function in the compounds with a smaller either methoxy or hydroxyl function in the 3-position was unfavourable with respect to the protein kinase affinity, a 6-methoxy function in derivative **8** with the bulky 3-benzyloxy substitution turned out as favourable. So a different binding mode towards the protein kinases especially to GSK-3β was suggested because of the main increase in affinity to nanomolar ranges.

The 6-hydroxy function was favourable for a protein kinase affinity of our derivative with a 3-methoxy function, so that we replaced the 3-methoxy function in derivative **4** with an additional hydroxyl group. Compound **6** turned out to be a good multikinase inhibitor with nanomolar affinities towards CDK1/B and GSK-3β and submicromolar affinities towards CDK5/p25 and CDK2/E. The increased affinities towards CDK5/p25 meant an improvement in activity by a factor of 200 if compared to the first CDK5/p25 inhibitor **8** of our series. Thus we received the first multitargeting candidate for a further evaluation as will be demonstrated below.

We then investigated the influence of an additional 1-aza-9-oxafluorene skeleton substitution by introducing a methyl group into the 7-position. Compound **12** showed similar affinities towards CDK1/B and GSK-3β than the non-methylated derivative **5**. However, some CDK5/p25 affinities were measured. We increased the skeleton substitution by the annelation of a phenyl ring as a more space demanding substituent in derivative **13**. The additional benzo-substitution led to main increases in the CDK1/B affinities

Table 1
Serine/threonine kinase inhibition profiles of target compounds **4**–**14**

	K_i values[a,b] [μM]				
	CDK1/B	CDK2/E	CDK4/D	CDK5/p25	GSK-3β
4	5.3	24.0	93.6	217	14.8
5	2.3	6.4	36.6	n.a. [c]	5.8
6	0.01	0.60	18.5	0.11	0.02
7	n.a.[c]	147	n.a. [c]	n.a. [c]	n.a. [c]
8	n.a. [c]	n.a. [c]	n.a. [c]	26.1	0.02
9	n.a. [c]	n.a. [c]	144	n.a. [c]	n.a. [c]
12	1.3	n.d. [d]	n.d. [d]	25.3	5.8
13	0.09	n.d. [d]	n.d. [d]	2.10	1.60
14	0.70	n.d. [d]	n.d. [d]	0.07	0.01

[a] K_i values have been calculated from determined IC_{50} values of kinase inhibition following described protocols.[31,32]
[b] Standard errors of the K_i values are typically below 20%. In many cases standard errors below 10% are found.
[c] n.a., not active (K_i >1000).
[d] n.d., not determined.

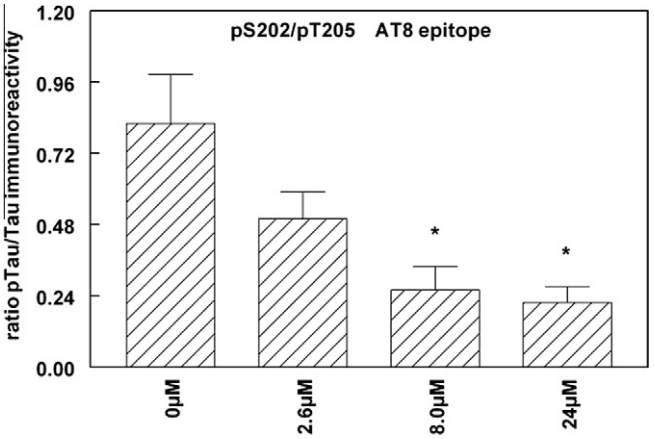


Figure 3. Decrease of phosphorylation of tau amino acids serine 202 and threonine 205 in transfected N2A neuroblastoma cells with increased concentrations of compound **6**.

and micromolar CDK5/p25 affinities were reached if compared to the non-annelated derivative **5** which was completely inactive as CDK5/p25 inhibitor. Moreover, the GSK-3β affinities improved.

The debenzylation of derivative **13** finally led the the 3- and 6-dihydroxy substituted derivative **14** with nanomolar affinities towards CDK5/p25 and GSK-3β and yet submicromolar affinities towards CDK1/B. The additional benzo-annelation in compound **14** proved to be slightly less favourable with respect to the CDK1 affinities, but increased the CDK5/p25 and the GSK-3β affinities if compared to the non-annelated derivative **6**.

So both 3,6-dihydroxy substituted 1-aza-9-oxafluorenes **6** and **14** are excellent multikinase inhibitors of AD relevant kinases with their nanomolar affinities.

Compound **6** was our best CDK1/B and CDK2/E inhibitor. Beside the benefit of affecting also these kinases in a multitargeted AD therapy we investigated possible cell toxic effects of this selected compound which may be caused by a possible cell cycle influencing.

Cell toxic effects have been determined in various assay systems using concentrations up to 90 μM to exclude any possible toxic problems. We investigated the effects in neuronal-like N2A neuroblastoma cells. The mitochondrial function was checked in the MTT assay by determination of the formed formazan crystals by mitochondrial reduction via dehydrogenases.[28] The formed formazan amounts would mainly decrease in the case of a cellular toxicity. We observed no decrease in the formazan formation up to concentrations of 90 μM if compared to the compound-free cells as shown in Figure 2.

The membrane integrity was additionally examined in the lactate dehydrogenase assay (LDH) by determination of the extracellular activity of this enzyme.[28] Also in this assay system concentrations up to 90 μM showed no cell-toxic effects if compared to the compound-free cells.

We then investigated our nontoxic compound **6** to inhibit protein kinases from various protein kinase families of the human kinome. We determined K_i values to PKC isoforms of the PKA family (PKC-α, -γ, -ε and -iota), to kinases of the receptor tyrosine kinase family (EGFR, VEGFR2, ERBB2 and TIE2), to the related CDK6/D1 and to kinases of the Casein kinase (Ck) family (WEE1 and Ck1-α1). Derivate **6** was completely inactive as inhibitor of all these kinase with K_i values >1000 μM. The K_i value towards CDK6/D1 was little below 1000 μM with a K_i value of 773 μM and the K_i value towards EGFR was about 296 μM which meant less than a residual activity.

A final bioanalysis was carried out with compound **6** as potential AD relevant kinases multitargeting inhibitor in N2A neuroblastoma cells to prove the ability of a cellular influence on the tau protein phosphorylation. The neuroblastoma cells have been stably transfected with a 3-repeat tau protein construct.[29] The inhibition of relevant amino acid phosphorylations of tau by the inhibitor has been determined with respective antibodies of the representing

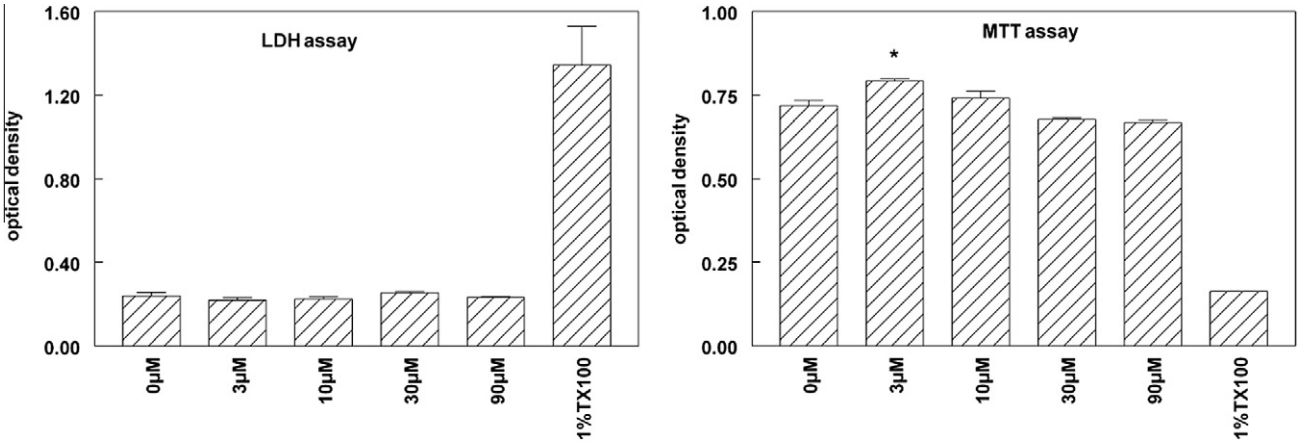


Figure 2. Cellular toxicity of compound **6** in the lactate dehydrogenase (LDH) assay measured as increased LDH activity displayed left and in the MTT assay measured as reduced formazan formation displayed right.

phosphorylated amino acids serine 202 and threonine 205 as shown in Figure 3.

Main reductions of the cellular tau protein amino acid phosphorylation of both serine 202 and threonine 205 were observed at the lowest concentrations. Thus a first proof-of-concept could be given for effective cellular activities of reducing tau protein phosphorylation by the AD relevant protein kinases CDK5/p25 und GSK-3β.

Finally, we succeeded in a lead optimization with the discovery of nanomolar active CDK1, CDK5/p25 and GSK-3β inhibitors of the 3,6-dihydroxy-1-aza-9-oxafluorene type **6** and **14**. We demonstrated nontoxic properties of the selective derivative **6**.

Thus, first multikinase inhibitors for a perspective AD multitargeted therapy were found and will be investigated in further preclinically directed studies. Moreover, our selective inhibitors will help to understand the effect of tau phosphorylation on AD progression.

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22. *Representative procedure for the debenzylation of compounds **5** and **8**:* 4-phenylbenzo[4,5]furo[2,3-*b*]pyridine-3,6-diol (**6**). Compound **5** (0.025 g, 0.068 mmol)[23] was dissolved in dried methanol (20 mL). Palladium on charcoal (0.020 g, 10%) was added and the suspension was shaken under hydrogen atmosphere at a pressure of 2 bar for 3 h. After filtration and evaporation to dryness the residue was purified by column chromatography over silica gel using an eluent mixture of cyclohexane and ethyl acetate (60/40). The collected fractions were unified, the eluent was evaporated and **5** crystallized from diethyl ether overnight in a refrigerator. Yield 0.018 g (93%); mp 214–217 °C; IR (KBr) ν = 3349, 2965, 1466, 1377, 1277, 1250, 1183 cm^{-1}; ^1H NMR (DMSO-d_6) δ = 9.72 (br s, 1H), 9.29 (br s, 1H), 8.07 (s, 1H), 7.61–7.51 (m, 5H), 7.46 (d, J = 8.9 Hz, 1H), 6.89 (dd, J = 8.9, 2.6 Hz, 1H), 6.54 (d, J = 2.6 Hz, 1H); m/z (ESI) 278 ($M+H^+$). Elemental Anal. Calcd. (%) for $C_{17}H_{11}NO_3$: C, 73.64; H, 4.00; N 5.05. Found: C, 73.38; H, 3.85; N 4.77.
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24. *Representing procedure for the methylation of compounds **5** and **6**:* 3,6-dimethoxy-4-phenylbenzo[4,5]furo[2,3-*b*]pyridine (**7**). Compound **5** (0.010 g, 0.036 mmol) was dissolved in dried THF (1 mL) under argon atmosphere. The solution was cooled down to 0 °C in an ice bath. Then a 14-molar excess of sodium hydride (0.012 g, 0.5 mmol) in paraffin oil (60%) was added. After stirring for 15 min the suspension was warmed-up to rt and stirring continued. Then a 6-molar excess of methyl iodide (0.015 g, 0.11 mmol) with reference to both hydroxy functions was added. After stirring for 9 h at rt the reaction mixture was poured into ice water (2 mL) and extracted with chloroform (10/10/5/5 mL) for four times. After drying over sodium sulfate the solvent was removed in vacuum and the remaining oil was purified by column chromatography over silica gel using an eluent mixture of cyclohexane and ethyl acetate (60/40). The collected fractions were evaporated to dryness and the residual oil was dissolved in ethyl ether from which **7** cyrstallized. Yield 0.006 g (54%); mp 143–145 °C; IR ν = 2927, 2836, 1587, 1477, 1435, 1272, 1189, 1089, 1033 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 8.17 (s, 1H), 7.59–7.51 (m, 5H), 7.47 (d, J = 9.0 Hz, 1H), 7.02 (dd, J = 9.0, 2.7 Hz, 1H), 6.68 (d, J = 2.7 Hz, 1H), 3.88 (s, 3H), 3,63 (s, 3H); m/z (ESI) 306 ($M+H^+$). Elemental Anal. Calcd. (%) for $C_{19}H_{15}NO_3$: C, 74.74; H, 4.95; N 4.59. Found: C, 74.56; H, 4.68; N 4.22.
25. Procedure for the formation of the 3-benzyloxy-4-phenylnaphtho[1′,2′:4,5]furo[2,3-*b*]pyridine-6-ol (**13**). 0.85 g of the *N*-acetyl 1,4-dihydropyridine **10** (2.8 mmol)[23] and 0.53 g of the naphthoquinone (3.4 mmol) were dissolved in a minimum volume of dried dioxane. Then a mixture of ten parts of dried dioxane and one part of perchloric acid (70%) were added reaching a final solution reaction volume of 75 mL. After 24 h of stirring at rt the added naphthoquinone partly disappeared according to tlc analysis of the reaction mixture and additional 0.17 g of naphthoquinone (1.1 mmol) were added. The addition of portions of the naphthoquinone continued until no more tlc-detectable tetrahydro derivative **11** was present in the reaction mixture. Then ice water was added (15 mL) and the pH of the solution was adjusted to pH = 9 using a sodium hydroxide solution (1 M). Then the reaction mixture was extracted four times with each 50 mL of chloroform. The organic layers were unified and dried over sodium sulfate. After filtration and evaporation the remaining oil was purified by column chromatography over silica gel using an eluent mixture of cyclohexane / ethyl acetate (80/20). The collected compound-containing fractions were evaporated yielding compound **13** as a brownish powder. Yield 0.18 g (15%); mp 219–222 °C; IR (KBr) ν = 3170, 3056, 3028, 2982, 2917, 1596, 1583, 1374, 1260, 1228 cm^{-1}; ^1H NMR (DMSO-d_6) δ = 10.06 (s, 1H), 8.36 (s, 1H), 8.30 (d, J = 8.3 Hz, 1H), 8.21 (d, J = 8.3 Hz, 1H), 7.71 (t, J = 8.3 Hz, 2H), 7.63–7.59 (m, 5H), 7.32–7.28 (m, 5H), 6.63 (s, 1H), 5.20 (s, 2H); m/z (ESI) 418 [$M+H^+$]. Elemental Anal. Calcd. (%) for $C_{28}H_{19}NO_3$: C, 80.49; H, 4.59; N 3.36. Found: C, 80.13; H, 4.25; N 2.99.
26. Spectroscopical data of 4-phenylnaphtho[1′,2′:4,5]furo[2,3-*b*]pyridine-3,6-diol (**14**): mp 258–260 °C; IR (KBr) ν = 3337, 2977, 1640, 1599, 1582, 1444, 1369, 1256, 1212, 1172, 1070, 1037, 1028 cm^{-1}; ^1H NMR (acetone-d_6) δ = 9.04 (s, 1H), 8.49 (s, 1H), 8.36 (d, J = 8.3 Hz, 1H), 8.33 (d, J = 8.3 Hz, 1H), 8.16 (s, 1H), 7.74–7.70 (m, 1H), 7.67–7.57 (m, 6H), 6.71 (s, 1H); m/z (ESI) 328 [M+H]. Elemental Anal. Calcd. (%) for $C_{21}H_{13}NO_3$: C, 77.05.49; H, 4.00; N 4.28. Found: C, 76.85; H, 4.09; N 4.22.
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28. LDH-assay was performed according to the manufactureŕs recommendations (Roche Diagnostics) in triplicate. 50 μL Medium was complemented with 50 μL reagent mixture and absorbance values were measured at 492 nm using a HT3 microtiter plate reader after 20 min. MTT was added in triplicate to cells to a final concentration of 1 mg/mL and cells were incubated for 2 h. After incubation cell culture medium with MTT was removed, 200 μL DMSO and 25 μL Soerenseńs glycine buffer (0.1 M Glycine, pH 10.5, 0.1 M NaCl) per well were added and absorbance values were measured at 550 nm with the microtiter plate reader. Absorbance values of N2A cells cultures treated with compound **6** were compared to cells treated with 1% Triton X-100 (100% cell death) and untreated cells.
29. Tau phosphorylation was quantified using N2A mouse neuroblastoma cells stably transfected with an 3-repeat tau expression construct. For the incubation of N2A cells with the protein kinase inhibitor cells were maintained in a 96-well plate in DMEM/HAḾs F-12 (pH 7.4) supplemented with 5% (v/v) fetal bovine serum, 2 mM L-Glutamine, 50 μg/mL gentamicin in a 5% CO_2 environment at 37 °C for 24 h. Afterwards cells were maintained for 16 h in 150 μL of the described media with the protein kinase inhibitor at different concentrations diluted from a stock of 25 mM in DMSO. The final DMSO concentration in medium did not exceed 0.4% and was also included in the control treatment group. Extracted proteins were separated by 8% SDS–PAGE and then transferred onto PVDF membrane overnight using a tank blot system. Immunodetection of phosphorylated tau protein was performed as described.[30]
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