Myricetin Derivatives Ameliorate Deficits in 6-OHDA Animal Model of Parkinson´s Disease

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1 BACKGROUND

Flavonoids are natural polyphenols widely distributed in plants and herbs. Flavonoids, including myricetin possess anti-oxidative, anti-inflammatory, and neuroprotective properties and may therefore reduce the risk of neurodegenerative diseases. Data suggest a protective mechanism of myricetin in animal models of Parkinson Disease (PD) (Ara et al., 2017, Dhanraj et all., 2018, Magalingam at al., 2015).

An Integrated Drug Discovery program at Charles River Discovery Service was designed to synthesize, characterize and examine the efficacy of two novel myricetin derivatives, TA1 and TA2, in a range of therapeutic indications. Here, we present data on their maximum tolerated dose (MTD), pharmacokinetic (PK) properties, and efficacy in a rat model of Parkinson's disease (PD).

2 METHODS

Animals. In total of 78 CD rat (Charles River) and 40 CD1 mice (Charles River) have been used in the studies.

Maximum Toxicity Dose (MTD) assessment of TA1 and TA2 in mice. The MTD was assessed in CD1 mice over 25 days. Each compound was tested at three doses (150, 300, 600 mg/kg) QD or BID (n=5/group). Animals were weighed and observed for any adverse treatment effects and clinical signs.

Pharmacokinetic (PK) analysis of myricetin, and its derivates (TA1 and TA2) in rats. Plasma concentrations of TA1, TA2 and unmodified myricetin given p.o. at 100 mg/kg (1 ml/kg) were assessed at 1h, 3h, 10h and 24h post dose (n=3/group/timepoint). Brain concentration were measured at 6h and 24h post-dosing. Tissues underwent enzymatic hydrolysis to convert the conjugated metabolites into the free forms as described by Dang et. all., 2014. Briefly, plasma samples stabilized with ascorbic acid and methanol were treated with β-glucuronidase and sulfatase to release free myricetin, or myricetin derivatives by means of hydrolysis of their glucuronidated and sulfated conjugates. After hydrolysis, equal amounts of acetonitrile and ethyl acetate were added to remove proteins and extract analytes. Samples were centrifuged (13,000 rpm for 10 min). The supernatant was transferred and evaporated to dryness at 35°C under a gentle stream of nitrogen. The residue were reconstituted with mobile phase. After centrifugation supernatant was injected into liquid chromatography/mass spectrometry (LC-MS/MS) API-4000 system. Brain tissue samples were homogenized by sonication in ascorbic acid containing solution, treated with the enzymes, as above, and centrifuged. The resulting supernatant was used as brain extract sample and diluted 10× prior to injection onto the LC-MS/MS API-4000 system.

TA1 and TA2 efficacy in Parkinson Disease rat model:
The study was designed as prophylactic intervention where myricetin derivatives were administered for 3 weeks, starting at 1 wk before and for 2 wks after model induction with 6-OHDA infusion.

Myricetin derivatives treatment. TA1, TA2 or vehicle (n=15/group) has been dosed starting at 7 days before 6-OHDA infusion and continue for 2 wks after 6-OHDA (QD, 100 mg/kg, p.o.).

Unilateral Intrastriatal Administration of mesencephalic astrocyte derived neurotrophic factor (MANF). Rats were anesthetized with 5% isoflurane (in 70% N_2O and 30% O_2; flow 300 mL/min) and placed in a stereotactic frame. During the surgery, the concentration of the anesthetic was reduced to 1–1.5%. The rectal temperature was maintained at 37.0 ± 1.0 °C with a homeothermic blanket system. The right brain hemisphere was exposed through a small craniectomy to the skull. The dura mater was carefully removed with fine forceps and a stereotaxic injection of 12 µg MANF (Icosagen) in 2 µL PBS or vehicle alone was infused at a speed of 0.5 µL/min and was equally distributed between two sites (1.0 µL/site) in the right striatum using the following coordinates relative to the bregma and dura: AP +1.0, ML +2.8, DV -6.0, and -5.0 mm. The cannula was left in place for another 5 min before being withdrawn.

Unilateral 6-hydroxydopamine (6-OHDA) Lesioning of Medial Forebrain Bundle (MFB). To generate partial retrograde degeneration of dopaminergic neurons in the Substantia nigra (SNc) in all experimental groups, 6-OHDA lesioning was carried out according to Sauer and Oertel with modifications (Sauer and Oertel, Neurosci. 1994) 24 h after MANF/vehicle infusion. Thirty minutes before 6-OHDA injection, desipramine (Sigma-Aldrich; 15 mg/kg, i.p.; 1 ml/kg) was administered to prevent the uptake of 6-hydroxydopamine into noradrenergic nerve endings and thus protect these nerve terminals from destruction. Initial steps of surgical procedure were carried out as described above for MANF infusion. A stereotaxic injection of 6-OHDA (2.5 µg/µl, 10 µg/rat) was made into the right MFB. A total of 5 µL of 6-OHDA solution (20 µg in total) was infused at a speed of 0.5 µL/min and was equally distributed between four sites (1.25 µL/site) at the following coordinates: AP +1.0, ML +2.8, DV -6.0, -5.5, -5.0 and -4.4 mm. The needle was left for another 5 min before being withdrawn from each site.

Analgesia. Fifteen minutes before each infusion, rats received buprenorphine 0.03 mg/kg s.c. Additional dose of buprenorphine 0.03 mg/kg was administered s.c. twice a day during the first 24h after MANF/vehicle infusion, and for 48h after 6-OHDA infusion.

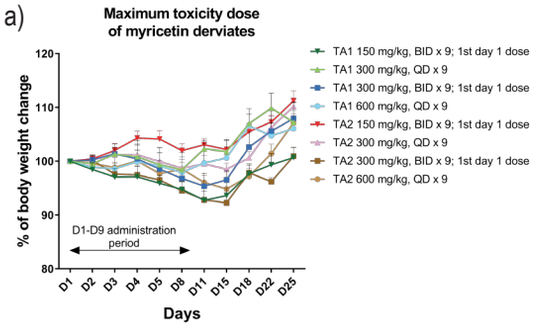
Amphetamine-Induced Rotation. Rats underwent rotational activity measurement on weeks 2, 4 and 6 after 6-OHDA injection. Amphetamine-induced asymmetric rotational activity was monitored in automatic rotameter bowls (TSE Systems, Bad Homburg, Germany). After a habituation for 30 min, a single dose of d-amphetamine (2.5 mg/kg, 10 ml/kg, i.p.) was injected and the number of full (360°) clockwise and counterclockwise turns was recorded for a period of 2 h. Net ipsilateral turns to the lesion were calculated by subtracting the turns to the right from the turns to the left.

Endpoint sampling. At 7 wk after 6-OHDA infiusion rats were terminally anesthetized with pentobarbital (60 mg/kg, Mebunat, Orion Pharma) and transcardially perfused with heparinized (2.5 IU/ml) saline, followed by ice-cold 4% paraformaldehyde in PBS. Brains were isolated and dissected on ice. The posterior midbrain block containing the SNc was dissected and further fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 24 h, cryoprotected in 30% sucrose in 0.1 M PB for 2–3 days, frozen in liquid nitrogen and stored at −80 °C.

TH+ fiber density assessment using unbiased stereology. Midbrain samples were sectioned as 20 µm coronal sections at 200 µm intervals through substantia nigra (SN) and mounted on slides. Sectioning was started at -4.6 mm from bregma and continued to -6.2 mm from bregma in AP axis. The sections were first thawed and air dried. Anti-TH immunohistochemistry was performed with a standard IHC protocol at CRL DRS. The number of TH-positive neurons were determined by counting immunopositive cells through the SN pars compacta (SNpc).

3 RESULTS

a)

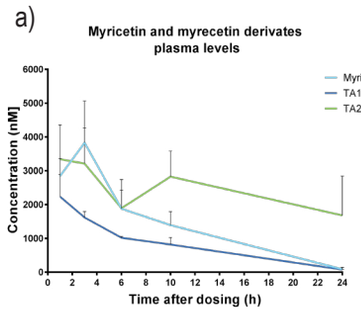


b)

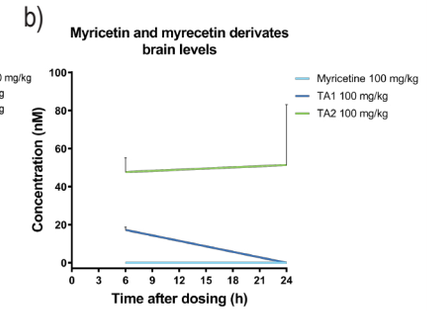
Compound	Dose (mg/kg)	Dosage regimen	Total number of doses	BW NADIR
TA 1	150	bid x 9 first day 1 dose	18	-7.6%
	300	qd x 9	9	-1.5%
	300	bid x 9 first day 1 dose	18	-4.6%
	600	qd x 9	9	-2.4%
TA 2	150	bid x 9 first day 1 dose	18	--
	300	qd x 9	9	-1.6%
	300	bid x 9 first day 1 dose	18	-7.9%
	600	qd x 9	9	-5.3%

Figure 1. A. Changes in body weight over the period of 25 days after 9 days of myricetin derivates administration. Data presented as mean % + SEM of D1. n = 5 /group **B.** Summary of lowest mean body weight for each group (expressed as % of D1) Mean body weight losses for all treatment groups were acceptable and did not exceed 8%. There were no clinical signs related to treatments, no treatment related or non-treatment related deaths.

a)



b)



c)

Compound	Brain:Blood ratio at 6h	Brain:Blood ratio at 24h
Myricetin	N/A	N/A
TA1	0.02	N/A
TA2	0.03	0.03

Figure 2. Pharmacokinetic (PK) analysis of myricetin, and its derivates (TA1 and TA2) in rats. A. Plasma concentration after single 100 mg/kg p.o. dosing of myrecetin and its derivates. The Tmax in the plasma for myricetin was detected after 3 h, for TA1 and TA2 Tmax was at 1 h post-dosing. The PK profile of TA2 suggest lower metabolism ratio as compared to myricetin and TA1. The plasma profile of myricetin is in line with published data by Dang et al., 2014. The peak concentration in the present study (3h after dosing) was 3830 nM (1216 ng/ml), whereas in the published data it was 2611 ng/ml (5,2 h after dosing). **B.** Both TA1 and TA2 were detected in the brain at 6 h after dosing. Interestingly TA2 was still detected in the brain after 24h, whereas the TA1 was not present in the brain at that timepoint. Myricetin was not detected in the brain at neither 6h nor 24h after dosing. **C.** Brain:Blood ratio of myricetin, TA1 and TA2 at 6h and 24h post dosing. Data presented as an mean + SEM, n=3/group/timepoint.

3 RESULTS CONT'D

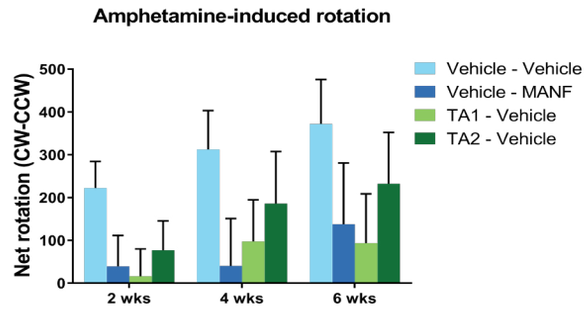


Figure 3. Efficacy on TA1 and TA2 on net rotation following amphetamine administration at 2, 4 and 6 weeks after 6-OHDA infusion. 6-OHDA infusion resulted in rotation asymmetry in all groups and was present at all assessed timepoints. MANF, TA1 and TA2 treatment resulted in tendency towards decreased rotation asymmetry as compared to vehicle treated rats (One-Way ANOVA followed by Dunnet´s post-hock). For TA1 and TA2 treated rats the tendency was particularly present at 2 wks after 6-OHDA that is when TA1 and TA2 were still administered. The tendency decreased at 4 wks and 6 wks after 6-OHDA, when TA1 and TA2 administration was discontinued. Data presented as mean ± SEM, n=15/group.

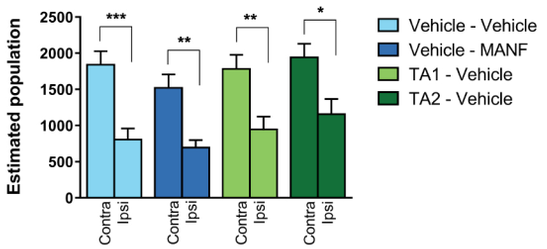


Figure 4. At 7 weeks after unilateral 6-OHDA infusion significant reduction of TH+ cells in SNpc side is present in all groups in the ipsilateral site as compared to contralateral site (* p<0.05, ** p<0.01, *** p<0.001, Paired t-test). TA2 group showed tendency towards increased number of TH+ cells as compared to MANF and TA1 treated group (One-way ANOVA followed by Dunnett´s post-hoc). Data are presented as mean + SEM.

4 CONCLUSIONS

- Novel myricetin derivatives TA1 and TA2 show better PK profile then unmodified myricetin
- Novel myricetin derivatives TA1 and TA2 show lack of pronounced side effects at high doses
- MANF, TA1 and TA2 showed comparable efficacy on ameliorating deficits on amphetamine-induced rotations in 6-OHDA lesioned animals
- TA2 showed tendency for beneficial effect on dopaminergic cell death as evidenced by cell counting of TH positive neurons in SNpc

5 REFERENCES

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3. Dang *et al*., Drug Res (Stuttg). 2014 Oct;64(10):516-22
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