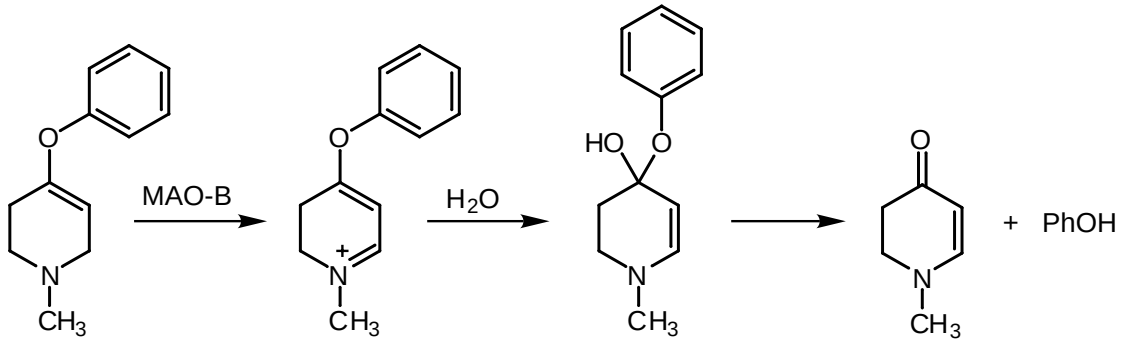
Chapter 4. Evaluation of the neurotoxicity of 1-methyl-4-(4-phenylphenyl)-1,2,3,6-tetrahydropyridine

As previously discussed, MPTP induces an MAO-B mediated selective striatal dopaminergic neurotoxicity in the established C57Bl/6 mouse model (section 1.2). MPTP type neurotoxins must be bioactivated in the brain by MAO-B to form the pyridinium species. Additional requirements for toxicity include the active transport of the neurotoxic pyridinium species first into the striatal nerve terminals via the dopamine reuptake transporter[47] and then into the inner mitochondrial membrane[251] where it inhibits mitochondrial respiration.[252] It must be noted that the active neurotoxin must be transported into the neurons to exert toxicity as MAO-B is located extraneuronally. However MAO-A is located in the mitochondrial membranes present in the nerve terminals. The mechanism of the MAO-B mediated neurotoxicity of MPTP is based in part from the observations that neuroprotection is achieved when MAO-B is inhibited (see section 1.2.3 for details).[47-50] The role of MAO-A in catalyzing MPTP like analogs to neurotoxic species has not been fully characterized due to the lack of a highly selective MAO-A substrate. The MAO-B selective 4-(2-methylphenyl) (**31**, $SC_{B/A}$ = 2.2) and the moderately MAO-A selective 4-(2-ethylphenyl) (**32**, $SC_{A/B}$ = 2.3) MPTP analogs have been used to illustrate the existence of the role of MAO-A in eliciting a neurotoxic effect.[198] The more MAO-A selective 4-phenoxy analogs (Chart 3, Panel B) would be better to evaluate the role of MAO-A in mediating neurotoxicity. The *m*-chlorophenoxy (**102**) and the *m*-phenylphenoxy analogs (**96**) display 6 times and 8.7 times more selectivity for MAO-A than MAO-B, respectively.[95] However, with the phenoxy analogs, the

MAO catalyzed oxidation does not result in a neurotoxic species due to the instability of the dihydropyridinium species (Scheme 14).

Scheme 14. Metabolic Fate of **88** when Incubated with MAO-B.



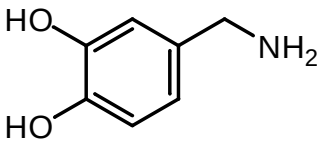
88 **150** **151** **152** **153**

The experiment of interest is to find a selective MAO-A substrate that is neurotoxic. The MAO-A substrate should be oxidized to a neurotoxic species in C57Bl/6 mice and cause the selective loss of nigrostriatal neurons as displayed by dopamine depletion. Subsequently, it should be possible to protect against this neurotoxicity by blocking in vivo the formation of the neurotoxic species using clorgyline, an MAO-A selective inhibitor. (R)-Deprenyl, an MAO-B selective inhibitor, and dopamine reuptake inhibitors should not protect against the MAO-A mediated toxicity. This type of experiment should provide definitive evidence for the role of the dopamine transporter in MAO-B catalyzed neurotoxicity. We have been fortunate to obtain a very MAO-A selective substrate that is catalyzed to a pyridinium species. The MPTP analog 1-methyl-4-(4-phenylphenyl)-1,2,3,6-tetrahydropyridine (**133**) ($SC_{A/B}$ = 74) has one of the highest MAO-A selectivities reported. Compound **133** has been

investigated in vivo in the C57Bl/6 mouse model for the possibility of producing MAO-A mediated neurotoxicity.

4.1. In vivo C57Bl/6 mouse experiments

The toxicity studies were carried out by the systematic i.p. administration of the test compound to 25-30 gram retired male breeder C57Bl/6 mice housed 1 to a cage with free access to food and water. Two weeks after administration of the test compound, the animals were sacrificed by cervical dislocation. The striata were dissected out, weighed, and homogenized with 10 μL/mg wet striata of 5% trichloroacetic acid (TCA) (w:v) containing the internal standard 3,4-dihydroxybenzylamine (DHBA, **154**).



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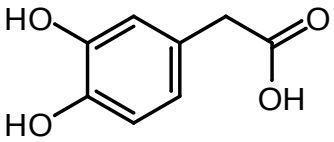

155

The samples were centrifuged for 3 minutes at 14,000 rpm using an Eppendorf microcentrifuge. The resulting supernatant was analyzed for DA and 3,4-dihydroxyphenylacetic acid (DOPAC, **155**) levels by an HPLC (LC-EC) system with a Beckmann pump, a Bas electrochemical detector (for the oxidation of DA and DHBA), a C-18 column, and a Kipp and Zonen strip chart recorder. A typical HPLC tracing is presented in Figure 15. The retention time of DOPAC is 3.6 minutes. The retention times of DHBA and DA are 4.6 and 7.6 minutes, respectively. With each experiment we ran a series of standards containing

known levels of DHBA, DA, and DOPAC, to insure that the instrument was running linearly, to confirm retention times, and to determine the actual levels of the compounds in each sample. An example of the dopamine standard curve is give in Figure 16. All of the data discussed were determined to be statistically significant by the student t-test.

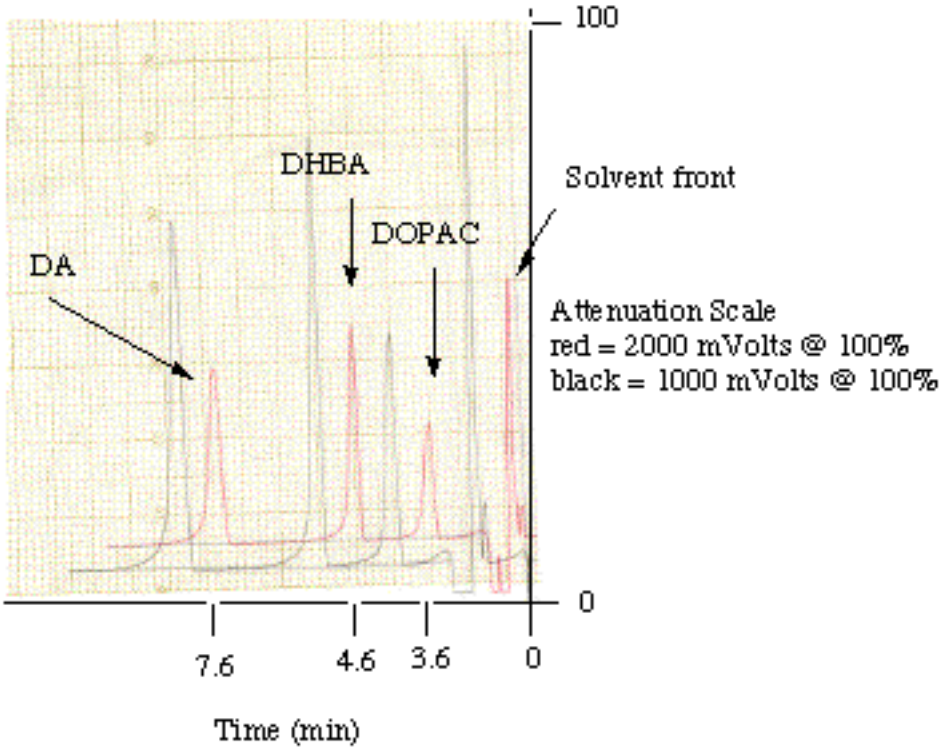

Figure 15. Standard HPLC Tracing for Dopamine Analysis

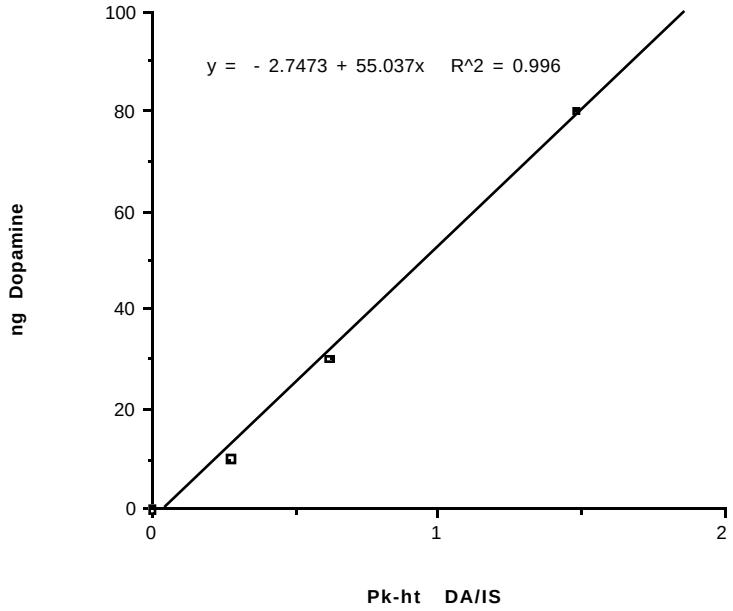

Figure 16. Dopamine Calibration Curve

4.2. Results and discussion

The first set of experiments was designed to establish a protocol for dosing of 1-methyl-4-(4-phenylphenyl)-1,2,3,6-tetrahydropyridine (**133**) in male retired breeder C57Bl/6 mice. As a control experiment, group 2 containing six mice was given a single i.p. dose of 50 mg/Kg (238 μmol/Kg) of MPTP·HCl while group 1, the controls (6 animals), received only saline. As summarized in Table 8b, a single 50 mg/Kg injection of MPTP resulted in a 72% loss in a the striatal DA content (28% remaining DA) as detected by HPLC with electrochemical detection (LC-EC). The animals treated with MPTP displayed no visible signs of sickness as previously discussed in section 2.3.

Table 8b. In Vivo Toxicity of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine at a Dosage of 238 μmol/Kg

Group (n=# of mice)	Pk ht ratio DA/IS (% CV)	ng DA / mg wet tissue	% of control
1 -control (6)	0.22 ± 0.05 (26.9 %)	8.63	100
2 -MPTP treated (6)	0.061 ± 0.87 (23.1 %)	1.40	27.7

MPTP has a V_{max}/K_m of 523 $min^{-1}mM^{-1}$ for MAO-B while the *o*-biphenyl analog has a V_{max}/K_m of 892 $min^{-1}mM^{-1}$ for MAO-A. To establish a dosing protocol for **133** we decided to use first ICR male white mice rather than the model C57Bl/6 mice due to the fact that they are cheaper and less difficult to handle. Based on the relative in vitro activities of MPTP and the *o*-biphenyl analog, we decided on an initial dosage of 40 mg/Kg (117.8 μmol/Kg) for analog **133**. As summarized in Table 9, group 1 received only saline, group 2 received an i.p. injection of **133** at 40 mg/Kg (117.8 μmoles/Kg) for 3 consecutive days, and group 3 received 35 mg/Kg (102 μmoles/Kg) for 3 consecutive days. As with MPTP, there was no visible signs of toxicity in the *o*-biphenyl treated ICR mice. The 40 mg/Kg dosing of the ICR mice gave the best results with a DA depletion of 42%.

Table 9. Preliminary ICR White Mice Experiments with 1-Methyl-4-(4-phenylphenyl)-1,2,3,6-tetrahydropyridine (**133**)

Group (n=# of mice)	Pk ht ratio DA/IS (% CV)	ng DA / mg striata	% of control
1 -control (6)	0.237 ± 0.01 (4.2 %)	9.41	100
2 -**133** treated (3)	0.138 ± 0.04 (28.9 %)	4.93	58
3 -**133** treated (3)	0.214 ± 0.02 (9.4 %)	8.36	91.6

With the established protocol in the ICR mice, we attempted to do the same type of study with the C57Bl/6 mouse model in which we dosed the animals i.p. with **133** at a dosage of 40 mg/Kg. Group 1, the controls (6 mice), was given vehicle (0.9 % saline solution) and group 2, the *o*-biphenyl treated animals (6 mice), was given a 40 mg/Kg dosage of the test compound. Unlike the MPTP treated mice or the ICR mice, the *o*-biphenyl treated animals within 15 minutes after treatment showed signs of sickness including hypothermia (cold to the touch) and impaired movement (stiff tails, jerking motions). After 24 hours, 100% of the treated animals had died (Table 10). The establishment of protocol in ICR mice, was not transferable to C57Bl/6 black mice as with MPTP. Thus, we began to establish a protocol for dosing C57Bl/6 mice with the *o*-biphenyl-tetrahydropyridinyl analog. Based on the findings with a 40 mg/Kg dose of **133**, we decided to do a second experiment using a 20 mg/Kg (58

μmol/Kg) i.p. dosage of the *o*-biphenyl and attempt to give the test compound **133** over a longer period of time. As summarized in Table 11a, group 1 (six controls) received saline and the six treated animals, group 2, received a 20 mg/Kg i. p. dose of the *o*-biphenyl compound given on day 1, day 3, and day 5. The animals were sacrificed by cervical dislocation after 14 days. The brains of the mice were dissected to remove the striata, the samples were processed and analyzed by HPLC for the DA content. There was no statistical difference in DA levels between the controls and the treated mice. In a third experiment we decided to use a slightly higher dose of [25 mg/Kg (73.6 μmol/Kg)] of the test compound for four days consecutively. Using 6 control and 12 treated animals given the *o*-biphenyl compound as described we obtained a 27% loss in the DA content relative to controls (see Table 11b).

Table 10. Dosing Protocols for 1-Methyl-4-(4-phenylphenyl)-1,2,3,6-tetrahydropyridine (**133**) in C57Bl/6 Mice

Dosage μmoles/Kg	Dosing Protocol	general toxicity	% DA Depletion
117.8	one i.p. injection	100 % lethal	NA[a]
58.9	3 alternating days	abnormal activity	0
73.6	4 consecutive days	50 % lethal	27

Each experiment contained 6 control animals and 6-12 treated animals.
[a]Not applicable

Table 11a. Dopamine Depletion Studied Using 1-Methyl-4-(4-phenylphenyl)-1,2,3,6-tetrahydropyridine (**133**) at a Dosage of 58.9 μmoles/Kg

Group (n=# of mice)	Pk ht ratio DA/IS (% CV)	ng DA / mg striata	% of control
1 -control (6)	0.481 ± 0.41 (29.3 %)	10.83	100
2 - **133** treated (6)	0.466 ± 0.10 (19.6 %)	10.41	98

Table 11b. Dopamine Depletion Studied Using 1-Methyl-4-(4-phenylphenyl)-1,2,3,6-tetrahydropyridine (**133**) at a Dosage of 73.6 μmoles/Kg

Group (n=# of mice)	Pk ht ratio DA/IS (% CV)	ng DA / mg striata	% of control
1 -control (6)	0.665 ± 0.08 (11.2 %)	16.1	100
2 -**133** treated (12)	0.485 ± 0.09 (18.6 %)	10.8	73

The general toxicity of analog **133** is a problem in dosing the C57Bl/6 mice so we are limited in the dosing protocol. However, we do observe dopaminergic neurotoxicity (27% dopamine depletion) at the tolerated dose. To determine if this neurotoxicity observed is MAO-A mediated, we performed an experiment in which we used selective MAO-A and B inhibitors. As

summarized in Table 12 the control animals (group A) received saline; group B received i.p. pretreatment with (R)-deprenyl on day one and on day 2-6 and i.p. injection of 25 mg/Kg of **133**; group C received i.p. pretreatment with clorgyline on day one and i.p. injection of 25 mg/Kg of **133** on days 2-6; group D received i.p. pretreatment with (R)-deprenyl and clorgyline on day one and i.p. injection of 25 mg/Kg of **133** on days 2-6; group E received i.p. injection of 25 mg/Kg of **133** only on days 2-6.

Table 12. Neuroprotection Experiment with 1-Methyl-4-(4-phenylphenyl)-1,2,3,6-tetrahydropyridine (**133**)

Test group (n)*	ng DA / mg striata (CV)	% Dopamine remaining
A - Controls (6)	18.9 ± 0.87 (4.6%)	100
B - Pretreated with deprenyl then **133** (4)	14.2 ± 0.48 (3.4%)	75.1
C - Pretreated with clorgyline then **133** (4)	17.5 ± 0.30 (1.7%)	92.6
D - Pretreated with deprenyl and clorgyline the **133** (4)	19.7 ± 0.080 (5.7%)	104
E - Treated with **133** (4)	14.5 ± 0.68 (4.7%)	76.7

*number of animals in each set
Groups B-E were treated with 235 μmoles/kg total of **133**.
Groups B-D were treated with 2.5 mg/Kg of the corresponding inhibitor.

Group E showed a DA depletion of 24%, similar to the neurotoxicity observed in the identical protocol experiment (27%) as reported in Table 10. Group B, pretreated with the MAO-B selective inhibitor (R)-deprenyl also displayed neurotoxicity, like the treated animals, with a DA depletion of 25%. Groups C and D, were protected against the neurotoxicity of **133**, since DA levels were the same as in control animals.

4.3. Conclusions

1-methyl-4-(4-phenylphenyl)-1,2,3,6-tetrahydropyridine in C57Bl/6 mice is neurotoxic, producing a DA depletion of 27%. It has been established from these experiments that the displayed neurotoxicity of **133** is MAO-A mediated and that the selective MAO-A inhibitor clorgyline protects against this neurotoxicity. (R)-Deprenyl alone can not protect against the neurotoxicity of **133**, but clorgyline does. This evidence supports the fact that the observed neurotoxicity is MAO-A mediated and not mediated by MAO-B. This compound however was not as promising as first anticipated due to the fact that there is a problem with general toxicity. There are several biological factors that may contribute to the observed general toxicity. Furthermore, compound **133** may be metabolized by peripheral enzymes. The levels of compound **133** that are reached in the brain are unknown. Because the toxicity is apparent within the first 15-30 minutes after dosing, it would be useful to evaluate the concentration of pyridinium and dihydropyridinium products in the C57Bl/6 mouse brain treated with **133** in order to determine what percentage of the compound injected reaches the brain. What is needed is an analog that is an MAO-A

selective substrate which produces high levels of dopaminergic neurotoxicity but has very little general toxicity. We are currently looking for other MAO-A selective tetrahydropyridines.