appropriate divalent cation could result in "spliced" RNA products being formed (*31*). These and other tests can help to evaluate the validity of the proposed model.

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Inhibition of Dihydropteridine Reductase by Novel 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Analogs

Abstract. *Hydroxylated derivatives of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a nigrostriatal neurotoxin in humans and primates, noncompetitively inhibited dihydropteridine reductase from human liver and rat striatal synaptosomes in vitro at micromolar concentrations. In contrast, MPTP and its chloro- and* nor-*derivatives did not inhibit this enzyme at lower than millimolar concentrations. Dihydropteridine reductase converts dihydrobiopterin to tetrahydrobiopterin, the required cofactor for the hydroxylation of aromatic amino acids during the synthesis of dopamine and serotonin.*

During the summer of 1982, several individuals with a history of drug addiction intravenously injected a "synthetic heroin" that was obtained from an illegal laboratory (*1*). The drug preparation contained 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) (0.3 percent), a meperidine analog that has analgesic properties, and the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (3.2 percent) that was formed presumably as a byproduct during the synthesis of the meperidine analog. Four of these individuals developed persistent parkinsonian symptoms and were studied extensively (*1*).

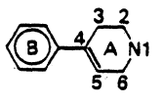
According to another report (*2*), one individual developed parkinsonism after using MPPP (and presumably MPTP) that he had synthesized. This person died of a drug overdose 2 years after the onset of his parkinsonism, and histological examination of his brain revealed marked destruction of the cells in the substantia nigra. In comparison, idiopathic parkinsonism in humans is characterized by a similar degeneration of the nigrostriatal dopaminergic pathway with specific loss of neurons in the substantia nigra accompanied by a marked decrease in the concentration of dopamine and its major metabolite, homovanillic acid, in the caudate nucleus and putamen (*3*). Administration of MPTP to rhesus monkeys has been shown to produce pathological and neurochemical changes that resemble closely idiopathic parkinsonism in humans (*4*), but MPTP does not produce neurotoxicity in the nigrostriatal dopaminergic system in guinea pigs or rats (*5*).

The metabolism and mode of action of MPTP has not been investigated systematically in man or other animals. What is known of the catabolism of xenobiotics by microsomal enzymes (*6*) suggests that MPTP could first be metabolized, either by aromatic hydroxylation or by aromatization of the tetrahydropyridine moiety (*7*) or both, to yield compounds that could react subsequently with one or more components of the nigrostriatal system. Studies in our laboratories have revealed that catechol- or hydroxy-containing aromatic compounds are potent, noncompetitive inhibitors (*8–10*) of DHPR [dihydropteridine oxidoreductase (NADH); E.C. 1.6.99.10]. This enzyme catalyzes the conversion of dihydrobiopterin to tetrahydrobiopterin, the required cofactor for hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine in the rate-limiting step of dopamine synthesis. We tested MPTP and nine of its analogs against DHPR in vitro to determine whether or not they inhibit this enzyme.

The 4-phenyl-1,2,3,6-tetrahydropyridine (PTP) compound and its 4′-chlorophenyl analog were obtained as hydrochloride salts (Aldrich Chemical), and MPTP was obtained as a free base and converted to its hydrochloride salt. The 4′-chlorophenyl derivative of MPTP was prepared via the *N*-methylcarbamate, which was then reduced with lithium aluminum hydride. The various hydroxy-substituted analogs were obtained as described (*11*). All new compounds gave correct combustion analyses, and their structures were confirmed with spectral data.

Enzyme preparations were obtained from human liver purified by ammonium sulfate precipitation and sequential chromatography (on DEAE-Sephacel, Matrex Gel Blue A, and hydroxyapatite) (*8, 12*) and from prepared rat striatal synaptosomes (*13*). Each compound was tested for its ability to inhibit DHPR. After preincubation of each inhibitor with DHPR for 10 minutes at 25°C, the reaction rate of DHPR was determined (*14*)

Table 1. Inhibition of dihydropteridine reductase by 4-phenyl-1,2,3,6-tetrahydropyridines. Human liver enzyme (6 mU or 60 ng of protein) or rat striatal synaptosomes (6.5 mU or 100 μg of P_2 protein) were incubated for 10 minutes with each inhibitor at 25°C. Residual enzyme activity was assayed at 50 μ*M* of each substrate to obtain I_{50} values and at different $qDMPH_2$ concentrations (20 to 50 μ*M*) while the concentration of NADH was kept constant (50 μ*M*) to obtain K_i values.



Ring B	Ring A		I_{50} (molar)	
			Human liver	Rat striatal synaptosomes
———	———	4-Phenyl-1,2,3,6-tetrahydropyridine (PTP)	1.2×10^{-2}	6.6×10^{-3}
———	1-CH_3	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	3.0×10^{-3}	4.6×10^{-3}
4′-Cl	———	4-(4′-Chlorophenyl)-1,2,3,6-tetrahydropyridine	3.4×10^{-3}	4.0×10^{-3}
4′-Cl	1-CH_3	1-Methyl-4-(4′chlorophenyl)-1,2,3,6-tetrahydropyridine	2.7×10^{-3}	6.4×10^{-3}
4′-OH	———	4-(4′-Hydroxyphenyl)-1,2,3,6-tetrahydropyridine	5.9×10^{-6}	3.4×10^{-6}
4′-OH	1-CH_3	1-Methyl-4-(4′-hydroxyphenyl)-1,2,3,6-tetrahydropyridine*	3.0×10^{-6}	2.6×10^{-6}
3′-OCH_3,4′-OH	———	4-(3′-Methoxy-4′-hydroxyphenyl)-1,2,3,6-tetrahydropyridine	7.2×10^{-6}	1.0×10^{-5}
3′-OCH_3,4′-OH	1-CH_3	1-Methyl-4-(3′-methoxy-4′-hydroxyphenyl)-1,2,3,6-tetrahydropyridine	9.3×10^{-6}	5.8×10^{-6}
3′,4′-$(OH)_2$	———	4-(3′,4′-Dihydroxyphenyl)-1,2,3,6-tetrahydropyridine	3.6×10^{-6}	1.9×10^{-6}
3′,4′-$(OH)_2$	1-CH_3	1-Methyl-4-(3′,4′dihydroxyphenyl)-1,2,3,6-tetrahydropyridine†	3.4×10^{-6}	2.5×10^{-6}

*A noncompetitive inhibitor of human liver enzyme ($K_i = 2.8 \times 10^{-6}M$). †A noncompetitive inhibitor of rat striatal synaptosomal enzyme ($K_i = 3.3 \times 10^{-6}M$).

by recording the decrease in NADH absorbance at 340 nm for 2 to 3 minutes on a Gilford 250 spectrophotometer equipped with a 6051 recorder. The inhibitor concentrations that gave 50 percent inhibition (I_{50}) of the enzyme activity were determined, and the dissociation constants (K_i) of the enzyme-inhibitor complexes were calculated from plots of the reciprocal of the apparent maximum velocity against the inhibitor concentration.

The results showed that PTP, MPTP, and their 4′-chloro derivatives do not inhibit DHPR at lower than millimolar concentrations, but all 4′-hydroxy and 3′,4′-dihydroxy derivatives that were tested showed inhibition (I_{50} values) at micromolar concentrations (Table 1). The inhibition produced by the hydroxylated *nor*-compounds was approximately equivalent to or slightly less than that of their corresponding 1-methyl derivatives, but the I_{50} values were of the same order of magnitude. The 1-methyl-4-(4′-hydroxyphenyl)-1,2,3,6-tetrahydropyridine and 1-methyl-4-(3′,4′-dihydroxyphenyl)-1,2,3,6-tetrahydropyridine compounds were the most potent inhibitors among the MPTP compounds. Further analysis of these mono- and dihydroxy MPTP compounds at four concentrations revealed noncompetitive inhibition when quinonoid 2-amino-6,7-dimethyl-4-hydroxydihydropteridine ($qDMPH_2$) was used as a variable substrate. The experimental values of I_{50} and K_i were essentially identical, a result which, for theoretical reasons, would be expected of noncompetitive inhibitors (*15*). All hydroxylated derivatives were approximately equally active against highly purified DHPR from human liver and DHPR in intact striatal synaptosomes from rat brain.

The relevance of these in vitro studies to the observed action of MPTP in vivo must be interpreted with caution. Hydroxylated derivatives of MPTP have not yet been identified in humans or primates after administration of the parent compound. Our studies suggest that hydroxylated derivatives of MPTP, if they occur in vivo, could inhibit DHPR, an effect that would reduce the availability of the biopterin cofactor for tyrosine and tryptophan hydroxylation during biogenic amine synthesis. Although the physiological roles of DHPR and tetrahydrobiopterin have not been elucidated completely, this enzyme is essential for normal brain function. Variant forms of hyperphenylalaninemia that are characterized by a deficiency of dihydropteridine reductase (*16*) or by a defect in tetrahydrobiopterin biosynthesis (*17*) lead to severe and eventually fatal neurological deterioration. Reduced levels of biopterin in cerebrospinal fluid of patients with Parkinson's disease, torsion dystonia, Alzheimer's disease, Steel-Richardson syndrome, and Huntington's chorea have also been reported (*18*).

The molecular species of the derivatives of mono- and dihydroxylated MPTP that are directly responsible for the inhibition of DHPR, and the moieties within the protein to which they bind, are unknown. We have proposed that certain catechols undergo oxidation to quinones that are then attacked by nucleophiles in DHPR molecules (*10*). An oxidation of a phenolic tetrahydroisoquinoline alkaloid to the corresponding quinone methide has been shown and may have biological significance (*19*). Other studies (*20*) suggest that aminochromes, the in vitro auto-oxidation products of catecholamines, are the actual inhibitors of DHPR. However, the enzyme inhibition conditions used in our study differed in that we preincubated inhibitors with DHPR for 10 minutes (*8*) before adding peroxidase-H_2O_2 solutions to measure the residual enzyme activity. Quinone derivatives of dopamine have been proposed to form in vivo in increasing concentrations during the process of aging in mammals (*21*). Dopamine has been found to be cytotoxic in a battery of test systems, including DNA single-strand break analysis and mutagenicity assays; these effects have been attributed to the oxidation of dopamine to reactive semiquinones and quinones (*22*). Further, auto-oxidation products of dopamine have been proposed as potential toxic metabolites in iprindole-treated rats after amphetamine-induced depletion of striatal dopamine (*23*). 6-Hydroxydopamine, which selectively destroys adrenergic nerve terminals and is a potent inhibitor of DHPR (*9*), is known to oxidize to its corresponding *p*-quinone, which is converted into indoline and indole-derivatives (*24*). These compounds can then bind covalently to proteins. On the basis of these discoveries, we propose that mono- and dihydroxy MPTP may undergo oxidation and subsequent binding to DHPR.

Studies in our laboratory have shown that DHPR can be inhibited by a wide variety of compounds containing phenolic or catecholic substitutions, including catecholamines and their metabolites (*9*), tetrahydroisoquinolines (*8*), catechol estrogens (*10*), and aporphines (*25*). Many of these compounds can also inhibit tyrosine hydroxylase (*26*) and, in the case of apomorphine and its analogs, act as dopaminergic agonists (*27*). Moreover, 1-*n*-propyl-3-(3′-hydroxyphenyl)-piperidine, which has some structural similarities to monohydroxy MPTP, is reported to be a potent dopaminergic presynaptic agonist (*28*). These results suggest that hydroxylated derivatives of MPTP may also in-

hibit tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, or bind to dopaminergic receptors.

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Cultured Cells of White Pine Show Genetic Resistance to Axenic Blister Rust Hyphae

Abstract. *Hypersensitive resistance to axenically cultured* Cronartium ribicola *was displayed by subcultured callus of* Pinus lambertiana. *Cellular resistance to a destructive rust disease can now be studied at the macromolecular level through use of cloned cells of both host and pathogen in a system amenable to emerging recombinant DNA technology*

White pine blister rust, caused by *Cronartium ribicola* J. C. Fisch ex Rabenh, is responsible for annual losses of millions of cubic feet of timber from the several five-needle pine species in the United States alone. Selective breeding for genetic resistance is the "most feasible and promising approach" (*1*) to initiate recovery of five-needle pines from this pandemic. A single dominant gene in sugar pine (*Pinus lambertiana* Dougl.) affects the only qualitative and well-defined genetic resistance to this disease (*2*). Macroscopic expression of this resistance mechanism is the appearance of a brown fleck on the needle at the site of rust basidiospore inoculation, indicative of a hypersensitive reaction in the cells beneath.

Traditional field breeding programs for disease resistance, both in forest trees and agronomic crops, are labor-intensive, long-term, and usually dictated by generation times of both host and pathogen. Greenhouse and laboratory whole-plant assays for resistance are supportive but commonly involve natural modes of spore inoculation followed in weeks or months by appraisals of relative resistance in some organ-specific reaction. We now report the development of a rapid in vitro assay for a characteristic rust-resistance reaction displayed at the cellular level; the assay employed monocultures of both host and pathogen to challenge cell cultures (callus) with the vegetative pathogen.

Many blister rust–resistance mechanisms have been described in various host species (*3*) and characterized for breeding programs (*4*). In most cases, modes of inheritance of these resistance mechanisms (typically expressed only after secondary needle development in the 3-year seedling) have remained unresolved. However, the hypersensitive rust-resistance mechanism reported in sugar pine is expressed not only in spore-inoculated cotyledons and primary and secondary needles (*5*) but also in young embryos inoculated with vegetative hyphae of the rust fungus grown in axenic culture (*6*). Characteristic resistance expressed within 2 weeks by these embryos was similar histologically to that seen in the spore-inoculated needle. This suggested the possibility that resistance was being expressed independent of the degree of host tissue organization and thus on the cellular level.

Six callus lines were established from minced germinated embryos of sugar pine seeds (*7*) to test this hypothesis. Three seeds were heterozygous for hypersensitive resistance to blister rust; three were homozygous recessive for resistance and thus susceptible to rust. Callus cultures were maintained on a modified Brown and Lawrence agar-solidified medium (*8*) containing 2.2 μM benzylaminopurine (BAP) and 2.7 μM naphthalene acetic acid in petri plates at 20° ± 1°C under continuous cool white fluorescent light (2000 lux). Cultures were subcultured to fresh medium biweekly for 10 months before use. Axenic rust cultures were established (*8*) from basidiospores produced on the alternate host *Ribes hudsonianum* var. *petiolare* that had been inoculated with rust aeciospores collected in Idaho. Axenic cultures were maintained by subculture of segments excised from established colonies onto freshly prepared medium of the same composition (*8*).

For the infection assay, callus from each line was arranged in three pads (1 cm in diameter, 1.5 mm thick) on fresh pine callus medium. After 6 days of incubation, a smooth, fresh callus surface had formed. At this time two of the pads were inoculated at three sites each with axenic rust hyphae, and the third pad served as an uninoculated control.