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Review

Biochemical and pharmacological characterization of 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline: a biologically relevant neurotoxin?

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Abstract

Acute and long-term effects of exposure to reactive compounds as the result of environmental pollution, workplace conditions or dietary intake are suspected to be involved in the etiology of a variety of disorders, including neurodegenerative disorders such as Parkinson's disease. The recognition in 1970s that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxic by-product of illicit meperidine synthesis, elicits parkinsonian symptoms in primates, including man, prompted the search for naturally occurring analogs which might be involved in human disease. It has been suggested that one candidate, 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo), a potent dopaminergic neurotoxin, might be formed endogenously in humans following the administration of the hypnotic chloral hydrate or after the exposure to the industrial solvent trichloroethylene. Such spontaneous formation has, indeed, been recently reported. The biochemical and pharmacological characteristics of TaClo and related compounds are thus reviewed here, and their potential significance for human neurodegenerative disease discussed.

Keywords: Neurotoxin; (TaClo) 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline; Carboline; Parkinson's disease; (MPTP) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

1. Introduction

The impact of environmental toxins on human health is regarded by both the scientific community and the general public with increasing concern, and the acute and long-term effects of exposure to reactive compounds as the result of environmental pollution, workplace conditions or dietary intake are suspected to be involved in the etiology of a variety of disorders. In particular, it has been suggested that such toxins might be involved in Parkinson's disease, the etiology of which, despite decades of intensive research, remains obscure. This suspicion was strengthened by the recognition at the end of 1970s that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxic by-product of illicit meperidine synthesis, elicits parkinsonian symptoms in primates, including man (Davis et al., 1979; Burns et al., 1983). This finding prompted the search for environmentally relevant structural analogs of MPTP, potential candidates including 4-phenylpyridine and the herbicide paraquat, naturally occurring alkaloids such as 1,2,3,4-tetrahydroisoquinolines and the β-carbolines, as well as various heavy metals (reviewed: Gerlach and Riederer, 1996; Gerlach et al., 1998a; Sian et al., 1999). The results of this search, however, have thus far been inconclusive. Most studies to date have focused upon the acute to medium term direct effects of candidate toxins. The generally late age of onset and the progressive nature of Parkinson's disease, and the failure to identify a specific "risk environment" associated with the disorder, suggest, however, that a more complex mode of toxicity should be considered. For example, a moderately toxic or even innocuous substance might be "activated" by normal metabolic processes in the exposed person, setting in train a cascade of metabolic and biochemical events, the consequences of which are only fully realized years after the primary exposure to the toxin.

It has been recognized for some time that the Pictet–Spengler condensation of nucleophilic 2-arylethylamines with reactive (electrophilic) aldehydes or α-ketoacids to

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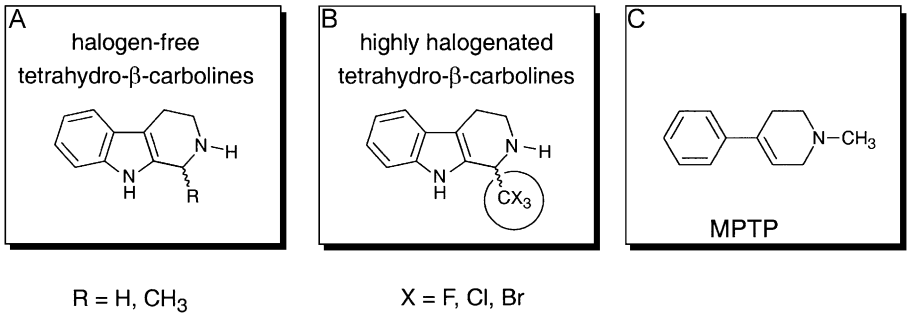


Fig. 1. Structures of the halogen-free tetrahydro-β-carbolines (A), of the highly halogenated tetrahydro-β-carbolines (B) and (for comparative purposes) of the neurotoxin MPTP (C).

give *tetrahydro-β-carbolines* (=tetrahydronorharmanes) can occur spontaneously in man and other mammals in the presence of elevated levels of reactive carbonyls or nitrogen-containing nucleophiles (Bringmann et al., 1986). These ''endogenous alkaloids'' have attracted interest because of their possible influence on the central nervous function; a rise in tetrahydro-β-carboline levels following alcohol ingestion, presumably via condensation of tryptamine with acetaldehyde (Brossi, 1993), was described by Rommelspacher et al. (1980), and tryptoline and eleagnine have been extensively investigated with respect to their involvement in alcohol and opioid intoxication and addiction (Davis and Walsh, 1970; Bringmann, 1979; Melchior and Collins, 1982; Rommelspacher et al., 1984; Myers, 1989). This interest was further stimulated by the recognition of the structural similarity of the tetrahydro-β-carboline molecule framework with that of MPTP. Although no tetrahydro-β-carboline has thus far demonstrated a neurotoxic capacity equal to that of MPTP, members of this group exhibit a number of relevant biochemical properties, including inhibition of monoamine oxidase activity and interference with the neurotransmitter storage, release and uptake (Airaksinen and Kari, 1981a,b). It is of interest that certain tetrahydro-β-carbolines may be neuroprotective under conditions of oxidative stress (Stolc, 1999).

The current report concerns the pharmacology and biochemistry of 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo; Fig. 1), the prototype of a novel class of neurotoxins, the *highly halogenated tetrahydro-β-carbolines*. Members of this class may be biologically significant for two reasons: not only are they potent neurotoxins, they can also arise spontaneously in the living organism under certain circumstances.

2. In vivo formation of TaClo and the neurotoxicity of trichloroethylene

One of our laboratories reported in 1990 that TaClo was readily formed under quasi-physiological conditions (buffered water, pH 7.4, 37°C) from the biogenic amine tryptamine (Ta) and trichloroacetaldehyde (=chloral, Clo; Bringmann and Hille, 1990). The significance of this finding derived from the fact that chloral hydrate is employed as a hypnotic in all age groups, administered at doses of up to 2 g (Rall, 1990; see also Sourkes, 1992). Chloral might also be formed in vivo following exposure to the commonly used industrial solvent trichloroethylene, either in the workplace (for example, in the cleaning and printing industries) or as a consequence of solvent abuse (''sniffing''; Fig. 2). It has also been found to be widely distributed in drinking and other water from various sources in the United States (White et al., 1997), but not in Germany (Spott et al., 1999). Brugnone et al. (1994) found trichloroethylene at relatively high concentrations in the urine and blood of about three-quarters of a sample of the general Italian population. While urine levels were similar for city and country residents, blood level were significantly higher in urban than in rural dwellers; blood levels in workers potentially exposed to industrial solvents were significantly higher than those of either of these groups. Kostrzewski et al. (1993) commented that blood levels of trichloroethylene were a better estimate of exposure than urine concentrations of its metabolites. Pleil et al. (1998) have recently reported that although 78% of trichloroethylene inhaled during acute exposure is eliminated by routes other than exhalation, blood levels could be predicted with high accuracy on the basis of breath elimination curves.

Toxicity associated with trichloroethylene has long been a matter of concern (reviews: Feldman, 1979; Bruckner et al., 1989; Burg and Gist, 1999). The carcinogenicity of trichloroethylene in humans is an unresolved issue (Kaneko et al., 1997) and will not be further discussed in the current paper. Occasional reports of fatalities attributed to acute trichloroenthylene toxicity have been reported (De Baere et al., 1997), but most attention has focused on the effects of chronic exposure. Animal studies have indicated that trichloroethylene produces a loss of myelin in the brainstem and in the temporal and occipital cortices, and damages hippocampal oligodendrocytes (Isaacson et al., 1990); several studies suggest that trichloroethylene modifies membrane lipid metabolism (see references in White et al., 1997). Its employment as an anesthetic during the 1940s was associated with severe and largely irreversible damage

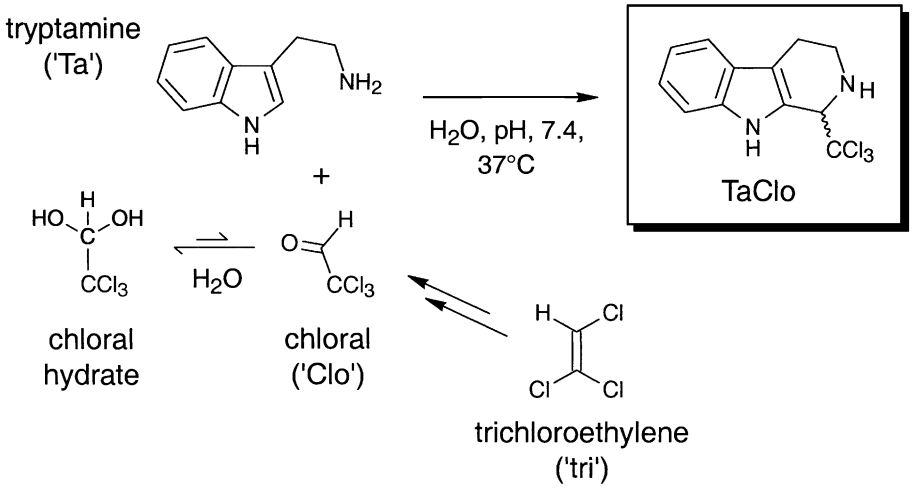


Fig. 2. Condensation of tryptamine with chloral to form TaClo.

to the trigeminal and other cranial nerves (McAuley, 1943; Grasso, 1988). The most common symptoms associated with chronic exposure to trichloroethylene (at concentrations of greater than 40 ppm for periods of 2–15 years) are headache, fatigue and dizziness. Neurological changes have also been identified in a number of studies; McCarthy and Jones (1983) published a detailed report concerning the frequency and consequences of industrial accidents involving trichloroethylene and other solvents, and identified a very high presentation of neurological symptoms in such cases. Workers exposed to a variety of industrial solvents, amongst them trichloroethylene, are reported to exhibit significantly reduced performance in tests of attentiveness, verbal ability, memory psychomotor reaction, and visual–spatial and motor skills, symptoms consistent with toxic encephalopathy and, possibly, early dementia (see references in Grasso, 1988; Simonsen et al., 1994). It is also significant that White et al. (1997) were able to identify a range of neurological and behavioural deficits in three populations chronically exposed to low levels of trichloroethylene in their drinking water; the effects were most marked in those subjects who had been exposed to the toxin during their childhood. Nevertheless, there is no conclusive evidence for measurable neurological deficits in humans chronically exposed to trichloroethylene concentrations of less than 40 ppm.

It has long been recognized that concomitant administration of trichloroethylene and alcohol appears to potentiate the physiological effects of both substances (Lilis et al., 1969; Sellers et al., 1972). Müller et al. (1975) presented evidence which suggested that ethanol inhibits the catabolism of trichloroethylene, significantly elevating its circulating levels. More importantly, the lipophilic nature of trichloroethylene would lead to substantially higher concentrations in the lipid components of the brain. On the other hand, the use of inhalant stupefacients, including trichloroethylene, by teenage "sniffers" in Poland was associated with exacerbation of the psychopathological aspects of alcohol abuse, including cognitive disturbances, aggression, anxiety and self-mutilation; it is, however, conceivable that these effects are attributable to the enhancement of the effect of the trichloroethylene component of the abuse cocktail (Tomczak et al., 1989).

The hypothesis that in vivo formation of TaClo from endogenous tryptamine could be a consequence of elevated blood chloral levels following the exposure to trichloroethylene or chloral hydrate prompted the investigation during the past 10 years of its biochemical and pharmacological properties. The significance of in vivo formation of TaClo or analogous compounds for the toxic effects of trichloroethylene and related chemicals is presently unknown. Kostrzewski et al. (1993) estimated a half-life for trichloroethylene in venous blood of 21.7 h, allowing ample time for in vivo condensation reactions. The large and highly reactive trichloromethylene group, a characteristic shared with the insecticide and well-characterized neurotoxin *p*,*p*′ -dichloro-diphenyl-trichloroethane (DDT), would be expected to render the molecule more lipophilic than non-halogenated tetrahydro-β-carbolines, and hence to enhance its membrane permeability, as well as its inducing free radical synthesis. This was predicted to have the following consequences (Bringmann et al., 1995a):

- enhanced capacity for crossing the blood–brain barrier;
- elevated accumulation in fatty tissue and long-term retention by the organism;
- increased potential for the induction of free radical-mediated damage to cell membranes.

Polychlorinated cyclic compounds are also characteristically resistant to metabolic degradation (Deichmann, 1981), further enhancing their toxic potential.

The structural similarities of TaClo, MPTP and 1-methyl-4-phenylpyridinium (MPP^+) suggested that TaClo might possess neurotoxic properties significant for the

etiology of Parkinson's disease. Reports of parkinsonian symptoms associated with longer term exposure to trichloroethylene remain anecdotal; for example, Guehl et al. (1999) reported the case of a 47-year-old woman who developed parkinsonian symptoms (which responded favourably to L-dihydroxyphenylalanine = L-DOPA) following 7 years of exposure to trichloroethylene in the workplace. This observation prompted them to treat male OF1 mice (8 weeks old) with trichloroethylene (400 mg kg^{-1}, 5 days a week for 4 weeks); a 50% reduction in tyrosine hydroxylase-positive neurons was measured in the substantia nigra pars compacta (Guehl et al., 1999). No other brain regions were examined, so that no comment on the specificity of this effect is possible. Nevertheless, the available evidence suggests the need for a closer examination of the association between trichloroethylene exposure and later presentation of parkinsonian symptoms.

3. Laboratory synthesis and assessment

Bringmann et al. (1992, 1995b) have described methods for the laboratory synthesis of TaClo in detail. The initial preparative technique employed, whereby tryptamine was refluxed with chloral in toluene, was complicated by the necessity for subsequent purification of the target compound by column chromatography. It is more efficient to conduct the reaction of the two substrates in formic acid as solvent, yielding the product *N*-formyl-TaClo without the need for a further purification step. This compound is easily recrystallized from methanol, can be stored without decomposition, and is readily deformylated to TaClo by treatment with methanolic hydrochloric acid (Bringmann et al., 1995b).

In order to assess levels of TaClo in biological matrices, a sensitive and specific gas chromatographic assay was also developed; the technique involves the conversion of TaClo to its volatile trifluoroacetyl derivative and subsequent assessment by gas chromatography in electron capture detection mode (Bringmann et al., 1995b).

4. Metabolism and distribution of exogenous TaClo in the rat

Following intravenous administration of TaClo (8.5 mg kg^{-1}) to a female Wistar rat, a significant portion (35% of the administered dose) was recovered in the bile. Treatment of biliary fractions with the enzymes arylsulphatase and β-glucuronidase increased measured levels by 150%, indicative of a high degree of phase II conjugation (Bringmann et al., 1996b). Following administration of a high dose of TaClo (40 mg kg^{-1} i.p. or p.o.) to female rats, only small amounts were recovered from urine (mainly unconjugated), feces (conjugated and unconjugated) and blood (where it was localized to the erythrocyte fraction, not the plasma), indicative of resorption and rapid metabolism of TaClo by the rat (Bringmann et al., 1996b; Table 1). Given the high levels measured in bile, the low fecal concentrations suggest resorption of TaClo or its phase II metabolites by the small intestine, perhaps because of its lipophilic nature. On the other hand, tissue concentrations (muscle, adipose tissue, lung, heart, kidney, liver) of TaClo following the high dose administration were quite low (less than 50 ng g^{-1} wet tissue); the hepatic concentration was less than 1 μg g^{-1} wet tissue (Bringmann et al., 1996b). These results are consistent with more recent investigations examining the distribution of [^{14}C]TaClo in the rat (0.6 μCi kg^{-1} = 2 mg kg^{-1} i.p.; Fig. 3). The heterocycle was rapidly distributed throughout the organism; radioactivity was slowly excreted within 48 h by renal (~ 35%) and intestinal (~ 65%) elimination. Within the first 6 h, the highest radioactivity was found in the kidneys, liver and small intestine, while only low values were detected in the brain, spleen, heart and

Table 1
Metabolic fate of TaClo administered peripherally to female Wistar rats (based on Bringmann et al., 1996b)

	Intraperitoneal	Per os
Dose	40 mg kg^{-1}	41 mg kg^{-1}
Urine		
Peak concentration	2.0 μg ml^{-1} (0–12 h)	1.1 μg ml^{-1} (24–48 h)
% dose excreted 0–48 h	0.2%	0.1%
Effect of treatment with AS/β-Glu	no effect	no effect
Feces		
Amount of TaClo excreted 0–48 h	42 μg	90 μg
% dose excreted 0–48 h	0.5%	1.0%
Blood		
Concentration of TaClo (2 h after administration)	6 μg ml^{-1}	4 μg ml^{-1}
Bile		
Peak concentration	0.7 mg ml^{-1} (0–2 h)	not done
% dose excreted 0–48 h	35%	
Effect of treatment with AS/β-Glu	Increased by a factor of 2.5	

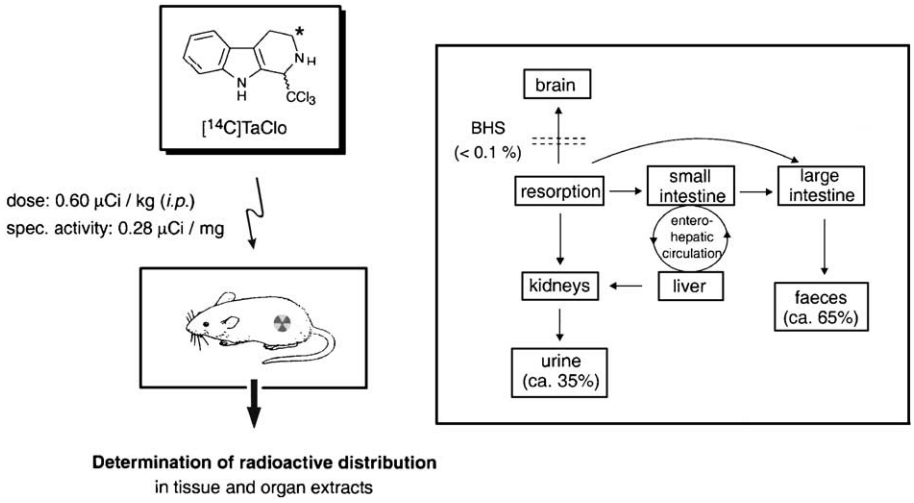


Fig. 3. Distribution of peripherally administered [^{14}C]TaClo in the rat with respect to time (0.60 μCi kg^{-1} = 2 mg kg^{-1}).

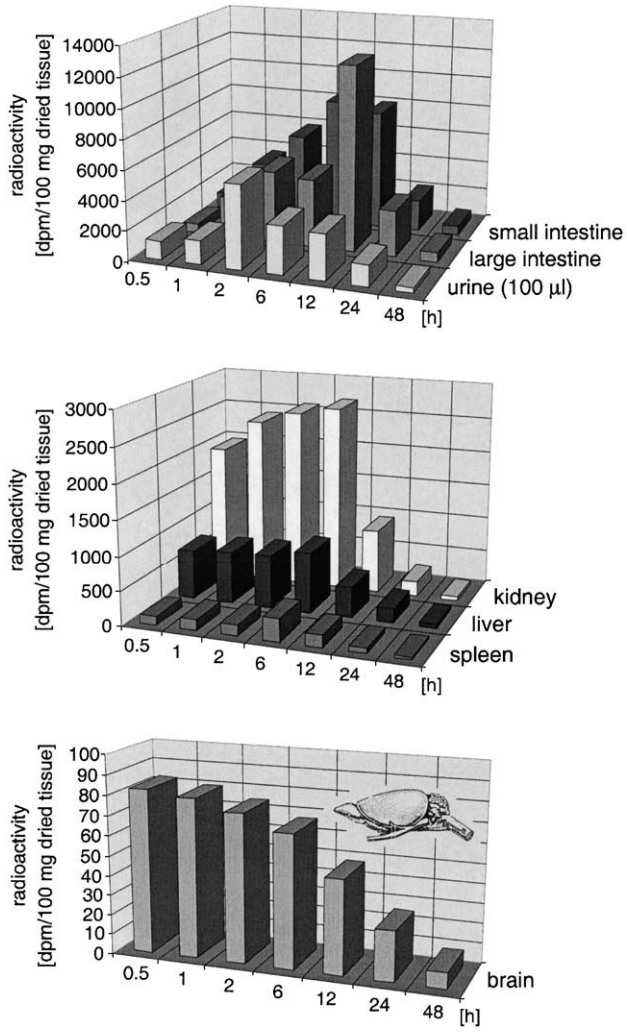


Fig. 4. Distribution of peripherally administered [^{14}C]TaClo in rat brain with respect to time (0.60 μCi kg^{-1} = 2 mg kg^{-1}).

muscle. Fatty tissues adjacent to the kidneys stored radioactivity for 24–48 h (Fig. 3). There is evidence for significant hepatic first-pass metabolism of TaClo, which may explain the low activity in systemic blood and brain. Fractionation of blood samples revealed the low activity in the erythrocytes and only traces in the plasma. It must, however, be borne in mind that these latter investigations do not distinguish between the relative contributions of TaClo and its metabolites to the measured radioactivity.

Of particular interest was the finding that TaClo is able to cross the blood–brain barrier. Rats received 4 mg kg^{-1} TaClo i.p. per day for 6 days; the measured tissue concentration 24 h after the last injection was fairly uniform in all brain regions examined (60–90 ng g^{-1} wet tissue), with the exception of the cerebellum (~ 30 ng g^{-1} wet tissue; Bringmann et al., 1996b). More recent investigations have indicated that after a single i.p. injection of [^{14}C]TaClo (2 mg kg^{-1}), although less than 1% of the applied activity penetrated the blood–brain barrier, the level in the brain tissue declined very slowly between 0.5 and 48 h, so that the possibility of accumulation of endogenously formed TaClo, particularly in the brain, following long-term treatment with chloral hydrate must be taken into account (Fig. 4). These results, however, are to be viewed with caution; doses of 0.2–0.4 mg kg^{-1} TaClo administered daily for 7 weeks have been found to be sufficient to elicit behavioural changes in rats, as discussed below; the effects of the larger doses administered in these distribution experiments may thus differ from those to be expected from occupational or incidental exposure to TaClo or its precursors.

5. De novo synthesis of TaClo in rats and in humans

In vivo synthesis of TaClo in rats following the chronic administration (20 days) of its precursors (chloral hydrate: 10 mg kg^{-1} i.p.; tryptamine HCl: 5 mg kg^{-1} i.p., both five

times per week) has also been demonstrated (Bringmann et al., 1995a, 1996a). As is the case following direct administration of TaClo, only low levels were measured in body fluids (such as whole blood) and tissues (for example, brain), perhaps reflecting its rapid metabolism in the rat.

Endogenous TaClo levels have also been assessed in five parkinsonian patients (age: 65–82 years) who had received chloral hydrate orally over a number of days (250–500 mg day^{-1}, 3–12 days; Bringmann et al., 1999). Gas chromatography with electron capture detection analysis detected the presence of TaClo in low concentrations in the blood of these patients, particularly in the clot fraction, with the intensity of the TaClo signal proportional to the amount of chloral hydrate received; quantities of up to 200 ng per 10-ml sample were measured (range: 2.5–104.9 ng g^{-1}; Bringmann et al., 1999). Leuschner et al. (1998), on the other hand, did not detect TaClo in the plasma of young, healthy volunteers who had received a single 500-mg dose of chloral hydrate. The findings of Bringmann et al. (1999), however, suggested that multiple administration of the hypnotic may be required before levels of TaClo are produced which would be measurable with the relatively insensitive method employed by Leuschner et al. (high performance liquid chromatography with ultraviolet detection); the detection limits of their method was about 5 ng ml^{-1}, whereas the TaClo concentration measured by the Bringmann group after three days of treatment (250 mg chloral hydrate/day) was less than 1 ng ml^{-1}.

6. Structural analogs of TaClo

Bringmann et al. (1995b) described a number of TaClo derivatives which could be formed in vivo, including the presumptive metabolites *N*-methyl-TaClo, tetradehydro-TaClo (each of which resembles MPP^+ more closely than TaClo itself) and 1-CCl_2-tetrahydro-β-carboline (Fig. 5) As the *N*-methylation of tetrahydro-β-carbolines, in particular, has been described in the human brain (Matsubara et al., 1993), it is significant that the *N*-methyl derivative of TaClo is more potent than the parent compound in many of the investigations described in this paper; that *N*-methylation of TaClo might be important for its neurotoxicity would also be consistent with the MPTP-like effects of the other *N*-methyl-tetrahydro-β-carbolines in mice and monkeys (for example, see Neafsey et al., 1989). Analogous heterocycles might also be formed from the condensation of chloral with other precursors, such as with 5-hydroxytryptamine to form 6-OH-TaClo, and with tryptophan to form 1-trichlomethyl-tetrahydro-β-carboline carboxylic acid (3-COOH-TaClo; Fig. 5), the formation of which under quasi-physiological conditions has been reported (Bringmann et al., 1991).

7. Effects of TaClo on mitochondrial function

The inhibition by MPP^+ of complex I of the mitochondrial respiratory chain is believed to underlie the neurotoxic action of MPTP on the dopaminergic neurons (Gerlach et al., 1991; Gerlach and Riederer, 1996). It was thus of great interest that Janetzky et al. (1995, 1999) reported that TaClo specifically inhibits electron transfer from complex I (NADH ubiquinone 1-dehydrogenase) to ubiquinone in both rat brain homogenate and in rat liver submitochondrial particle preparations, and that its potency to do so is an order of magnitude greater than that of MPP^+ (Table 2). Further, the TaClo concentration required to completely inhibit complex I activity could be reduced by 50% in the submitochondrial particle preparations by extending the incubation time from 5 to 30 min. By washing the submitochondrial particle preparation after 5 min of exposure to TaClo, 90% of complex I activity could be recovered, indicating that the association of the toxin and enzyme is reversible. Further, the comparison of its effects with those of non-halogenated analogs indicated that its trichloromethyl group was essential for mitochondrial action of TaClo. That the toxin gains access to the mitochondrion via a passive process is suggested by the finding that oxygen

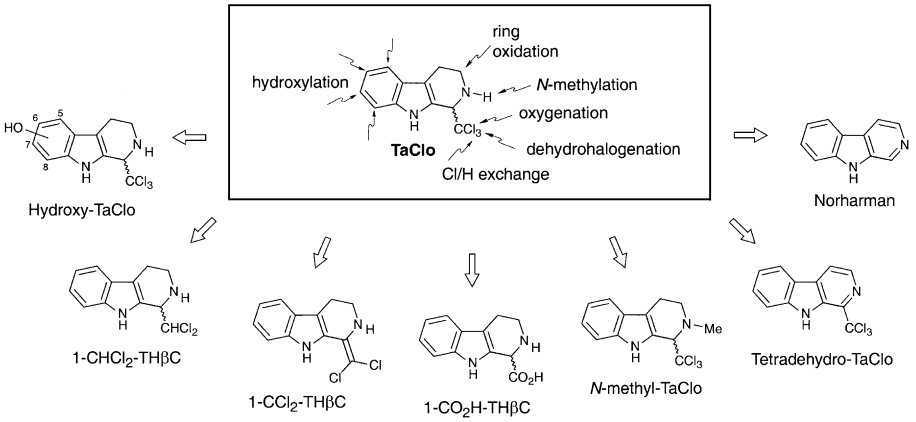
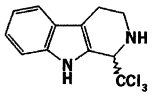
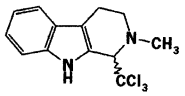
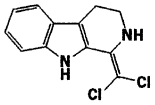
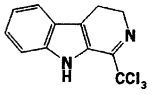
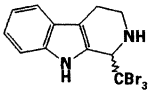
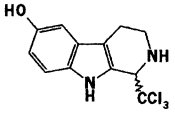
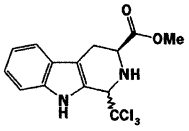
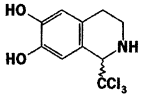
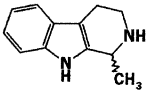
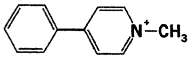


Fig. 5. Potential metabolic derivatives of TaClo. TDH = tetradehydro-TaClo; THBC = tetrahydro-β-carboline.

Table 2
Inhibition of mitochondrial respiratory chain complex I and complex II by TaClo and other halogenated tetrahydro-β-carbolines, by the halogenated isoquinoline 1-trichloromethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (DaClo), by a representative non-halogenated tetrahydro-β-carboline (eleagnine) and by the neurotoxin MPP^+

		Inhibition of mitochondrial complex I		Inhibition of mitochondrial complex II/III
		IC_{50}	IC_{100}	
TaClo		250	700	30% at 500μM
***N*-methyl-TaClo**		150	250	IC_{100}=300
Dichloromethylene-THBC		50	250	IC_{100}=300
TDH-TaClo		200	500	N.D.
TaBro		200	650	N.D.
6-OH-TaClo		300	650	N.D.
3-COOMe-TaClo		200	500	N.D.
DaClo		25% at 1mM		No inhibition <1mM
Eleagnine		N.D.	1200	No inhibition <1mM
MPP^+		7500	3500	No inhibition <1mM

All concentrations are in micromolar (μM). Adapted from Janetzky et al. (1999).
N.D. = not done.

consumption was completely inhibited by the same concentration of TaClo (700 μM) in both the submitochondrial particle preparations and intact mitochondria. In contrast, 7.5 mM MPP^+ effects only 60% of the inhibition of complex I in submitochondrial particles, whereas in intact mitochondria, 50–100 μM MPP^+ suffices for complete inhibition, consistent with the active accumulation of MPP^+ by the mitochondrion (Ramsay et al., 1986; Janetzky et al., 1995).

Several points should be noted in the interpretation of these results. First, the passive entry of TaClo into mitochondria as described above implies that any neurotoxic effects of TaClo are unlikely to be specific for dopaminergic or any other specific cell type. This is of particular interest in regard to Parkinson's disease, where deficits in several neuronal systems apart from the dopaminergic nigrostriatal pathway have been described (Jellinger, 1991; Braak et al., 1995). Second, the reversible association of toxin and mitochondrial protein indicated by these experiments suggests that long-term exposure to the toxin and the maintenance of a minimal local concentration may be required in order for toxic effects to be manifested. This renders the previously discussed issue of accumulation of TaClo in the central nervous system critical; it also implies that a mechanism-based distinction between acute and chronic effects of toxin can be drawn.

Interestingly, Janetzky et al. (1999) reported that *N*-methyl-TaClo was an even more potent inhibitor of complex I than TaClo itself, and also partly inhibited complex II

(succinate dehydrogenase; Table 2). This enhancement of activity by *N*-methylation is consistent with the greater complex I inhibitory capacity of *N*-methyl-tetrahydro-β-carboline and *N*-methyl-tetrahydroisoquinoline with respect to their parent molecules. This fact has been attributed to the enhancement of the lipophilic nature of the molecule by the methyl group (Albores et al., 1990; Sayre et al., 1991; Suzuki et al., 1992). Further, Gerlach et al. (1991) reported that the *N*-methyl group was essential to the neurotoxicity of MPTP.

8. Effects of TaClo on dopaminergic systems: cell culture studies

Rausch et al. (1995) observed significant changes in primary cell cultures of C57/B16 mouse mesencephalon cells (containing dopaminergic neurons) following exposure to TaClo for 24 h. At a TaClo concentration of 100 μM, numbers of both the tyrosine hydroxylase-immunoreactive cells and of astrocytes were reduced by 50%, dopamine uptake was reduced by 43% and dopamine content was reduced by 66%. Morphological changes observed following TaClo treatment included swollen dendrites and cell bodies and the loss of axons and dendrites. In a further study, Janetzky et al. (1999) reported that TaClo, *N*-methyl-TaClo and 1-dichloromethyl-tetrahydro-β-carboline effectively reduced dopamine uptake and the number and size of tyrosine hydroxylase-positive cells in C57/BL6 primary cell cultures (24-h exposure; Fig. 6). These results were consistent with their observations of the effects of halogenated tetrahydro-β-carbolines on mitochondrial respiration, as discussed above. Whereas TaClo-class molecules were more effective inhibitors of mitochondrial respiration than MPTP, their effects on cell culture parameters were not as great; 1 μM MPP^+, but 100 μM *N*-methyl-TaClo, reduced cell size in these cultures by 60%, while cell number was reduced to 30% of control levels by 10 μM MPP^+, but only to 60% by 10 μM *N*-methyl-TaClo (Koutsilieri et al., 1993; Janetzky et al., 1999).

Although it thus appears that TaClo is cytotoxic when directly applied to primary cultures of dopaminergic cells, the significance of this finding for the actions of TaClo in the whole organism awaits clarification; the response of fetal cell cultures to external agents cannot necessarily be extrapolated to the post partum organism, whether juvenile or adult.

Further, the relationship between the cytotoxic effects of TaClo-class compounds and MPP^+ and their respective effects on mitochondrial respiration requires further exploration, as there appears to be no direct correlation between the two effects (Janetzky et al., 1999). The most likely explanation for this phenomenon is the greater affinity of MPP^+ for the dopamine transporter compared to that of β-carbolines (MPTP, ~ 0.4 μM: McNaught et al., 1996; 3,4-β-carbolines, 12–24 μM: Drucker et al., 1990). McNaught

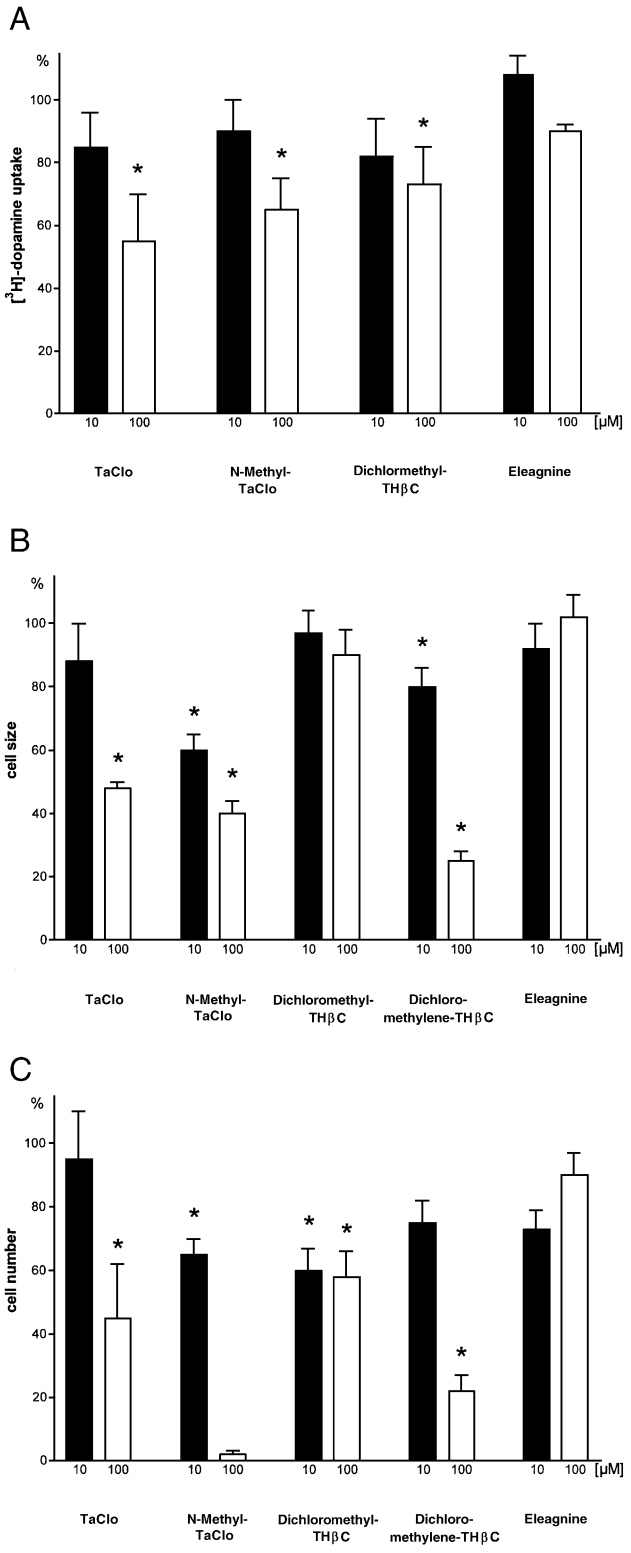


Fig. 6. Effect of various β-carbolines on (A) [^{3}H]dopamine uptake and on (B) size and (C) number of tyrosine hydroxylase-positive cells in C57/BL6 mouse cell cultures. Data are presented as mean value ± S.E.M.; for (A), 100% = 0.8 ± 0.3 pmol [^{3}H]dopamine/15 min/well, for (B) 1500–2000 μm^2, for (C) 4–8 cells per view field (= 200–300 cells/well). $N = 4$ in each case; * $P < 0.05$ by χ^2 test after the Kruskal–Wallis *H*-test. Complete data are presented in Janetzky et al. (1999).

et al. (1996) reported, for instance, that the cytotoxicity of isoquinoline derivatives was correlated with their ability to inhibit [^{3}H]dopamine uptake into PC12 cells. The effects of TaClo and *N*-methyl-TaClo on [^{3}H]dopamine uptake into mouse C57/BL6 cells, on the other hand, were similar, whereas *N*-methyl-TaClo was more potent with respect to both cytotoxicity and inhibition of the complex I activity (Table 2, Fig. 6; Janetzky et al., 1999). Passive uptake into the neuron and mitochondria may thus be the rate-limiting process for the exertion of the neurotoxic effects by TaClo and its derivatives. The lipophilic trichloromethyl group of TaClo would be expected to promote its nonspecific uptake through cellular and mitochondrial membranes; *N*-2-methylation (*N*-methyl-TaClo) or a double bond at C-1 (dichloromethylene-tetrahydro-β-carboline) would further enhance the lipophilic nature of the molecule, consistent with these molecules inhibiting mitochondrial respiration to a greater degree than TaClo itself. Further investigations of the effects of TaClo and its derivatives on mitochondrial respiration in intact cells are required in order to clarify the role of such inhibition in its neurotoxic effects.

9. Effects of TaClo on serotonergic systems

Bringmann et al. (2000a,b) found that TaClo entered JAR cells (a human choriocarcinoma cell line carrying the 5-HT (5-hydroxytryptamine) transporter protein) by a non-saturable, passive process, presumably by virtue of its lipophilic nature. TaClo inhibited [^{3}H]5-HT uptake into these cells (IC_{50} = 59 μM), as did the 5-HT-derived analog 6-OH-TaClo (more potently: IC_{50} = 26 μM; Fig. 7). Reduced transmitter uptake is characteristically observed following treatment with MPP^+ (Koutsilieri et al., 1993), trypan blue staining confirming that both agents were cytotoxic for JAR cells (EC_{50}: 100–500 μM). In control experiments, neither agent influenced the viability of dopamine transporter-bearing IMR-32 cells, nor did the dopamine-based analog DaClo significantly affect the viability of the JAR cells (Bringmann et al., 2000a,b). These results suggest that TaClo does not directly affect 5-HT uptake and that the observed reduction is secondary to reduced cellular viability following the exposure to TaClo or 6-OH-TaClo.

Further, Gerlach et al. (1998b) found that, in pentobarbital-anesthetized rats, acute systemic administration of TaClo (in NaCl; 0.4 mg kg^{-1} i.p.) elicited an immediate, statistically significant increase in extracellular striatal 5-HT concentration (+330%) as assessed by in vivo dialysis, but only a small rise in that of dopamine (+30%). This was followed by a progressive increase in extracellular levels of 2,3-dihydroxybenzoic acid (2,3-DHBA; +190%), the hydroxyl radical adduct of sodium salicylate, reflecting increased free radical formation (Obata and Chiueh, 1992). 3,4-Dihydroxyphenylacetic acid (DOPAC), glutamate and GABA levels were significantly elevated 2–3 h after administration of TaClo (+40–60%). These results are consistent with increased free radical generation secondary to increased release of 5-HT (and, to a lesser extent, of dopamine) induced by acute exposure to a low TaClo dose. The increased amino acid levels were hypothesized to reflect uptake inhibition by these radicals (cf. Berman and Hastings, 1997).

10. Effects of TaClo on dopaminergic systems: intranigral administration of TaClo and its analogs

Bringmann et al. (1996a) reported that stereotactic administration of TaClo into the rat substantia nigra pars compacta (10 μg in 2 μl 0.1 M HCl) resulted in significant declines of both neuronal density (15.9 ± 2.3%) and number (13.4 ± 0.4%) in this region as assessed by Nissl staining. Using in vivo pulse voltammetry in the rat, Grote et al. (1995) found that intranigral injection of TaClo (10 μg in 2 μl saline) effected a 55% reduction of the DOPAC signal in the ipsilateral striatum a week following injection, and a 74% reduction 3 weeks posttreatment (Fig. 8). It should be noted that, contrary to the published report, TaClo was not prepared in saline in these investigations, but in alcohol (personal communication from the authors). A more recent study by this group has detected a progressive decline in the DOPAC levels over a period of 6 weeks following a single

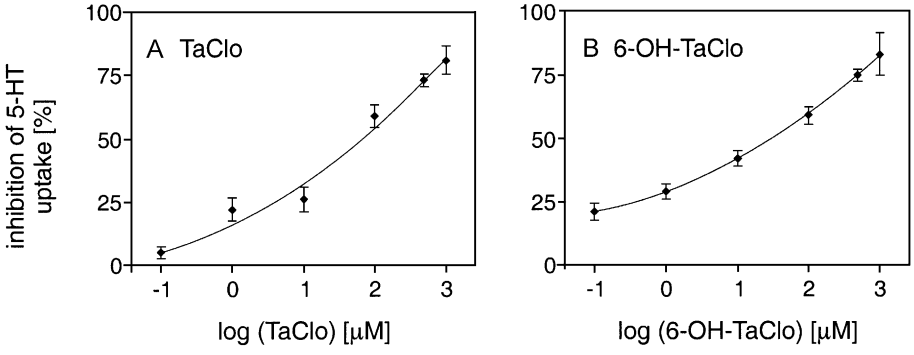


Fig. 7. Inhibition of 5-HT uptake into JAR cells by TaClo and 6-OH-TaClo. Data are presented as mean ± S.E.M. of triplicate determinations.

TaClo dose (Fig. 9). The putative TaClo metabolite *N*-methyl-TaClo was even more effective, producing reductions of 90% and 93% at 1 and 3 weeks respectively (Fig. 8); this response is comparable with that elicited by MPTP (Wesemann et al., 1993). The effects of both TaClo and *N*-methyl-TaClo could be reversed by systemic L-DOPA (100 mg kg^{-1} i.p.). The same group has compared the effects of a number of other tetrahydro-β-carbolines on striatal DOPAC levels; the synthetic halogenated tetrahydro-β-carboline TaBro (Bringmann et al., 2000a,b) elicited reductions of 83% and 77% at 1 and 3 weeks posttreatment, but the effect was only partly reversible in the first week by L-DOPA treatment (Fig. 10). These results suggest that striatal dopamine turnover is progressively reduced following the nigral exposure to TaClo, consistent with TaClo inducing a slowly progressive neurodegenerative process in the nigrostriatal dopaminergic projection. It should, however, be noted that in all cases, the investigated animals were anesthetized with chloral hydrate; this is of some concern, given the possibility that this drug may itself be converted to TaClo in the rat, as discussed above.

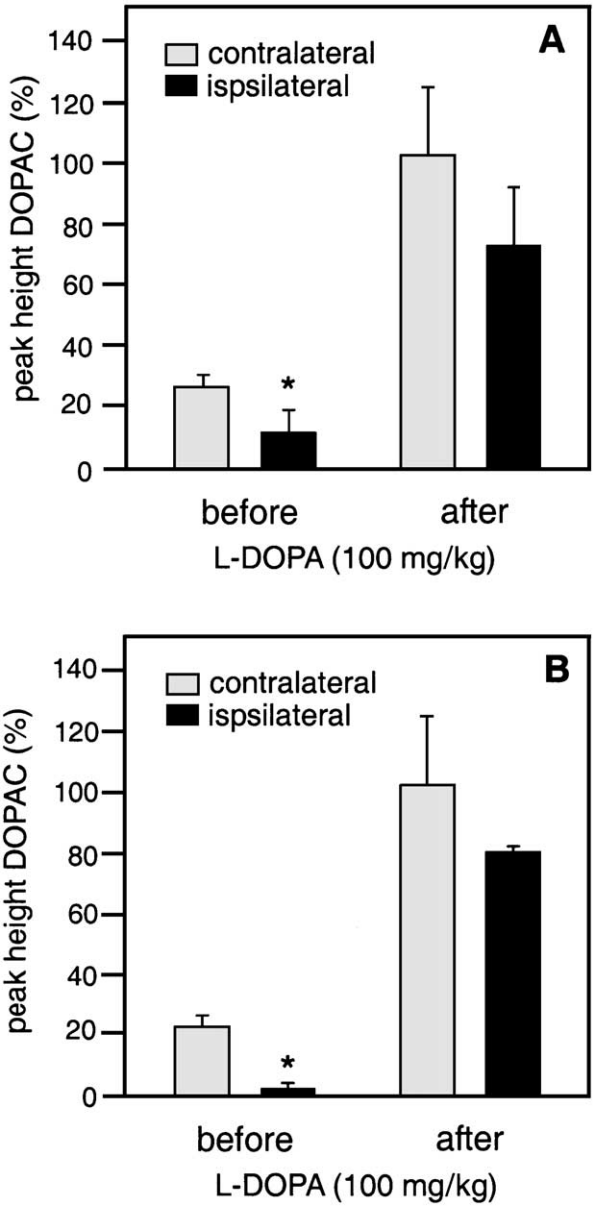


Fig. 8. Effect of (A) 10 μg TaClo or (B) 10 μg *N*-methyl-TaClo applied directly to the substantia nigra pars compacta 1 or 3 weeks prior to the assessment of the extraneuronal DOPAC in rat striatum by in vivo pulse voltammetry. L-DOPA was applied intraperitoneally at a dose of 100 mg kg^{-1}. Data are presented as mean signal (intact side after L-DOPA = 100) ± S.E.M.; $n = 5$ per group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with intact side.

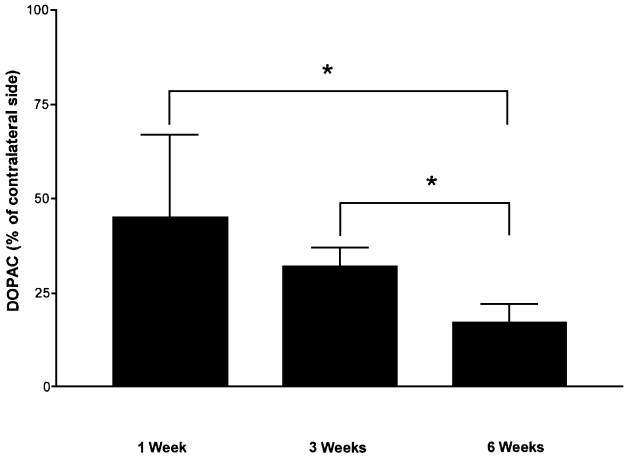


Fig. 9. Progressive neurodegeneration of the rat nigrostriatal pathway following unilateral injection of 10 μg TaClo into the substantia nigra pars compacta. Data are presented as mean signal (intact side after L-DOPA = 100) ± S.E.M.; $n = 5$ per group; $^{*}P < 0.05$ compared with intact side.

11. Effects of TaClo on the whole organism: behavioural studies

As already discussed, interest in TaClo was stimulated by its structural similarity to the specific dopaminergic neurotoxin MPTP and the observation that its large halogenated group could facilitate its toxic effects. Sontag's group identified as early as 1991 that chronic administration of TaClo to rats (0.4 mg kg^{-1} i.p., 4 weeks) induced a behavioural supersensitivity to the dopaminergic agonist apomorphine (0.4 mg kg^{-1} s.c.) comparable with that effected by lesioning of the nigrostriatal tract with the neurotoxin 6-hydroxydopamine (Bringmann et al., 1992).

This observation led to the subchronic treatment of female Wistar rats (3–4 months old) with TaClo (0.2 mg kg^{-1} in 0.9% NaCl i.p., daily, 7 weeks; Sontag et al., 1995). At various time-points after the completion of TaClo treatment, spontaneous motor activity (during the 60-min habituation phase) and the locomotor response to apomorphine (0.4 mg kg^{-1} s.c.) were assessed in a computerized open field apparatus. Four to nine days after termination of

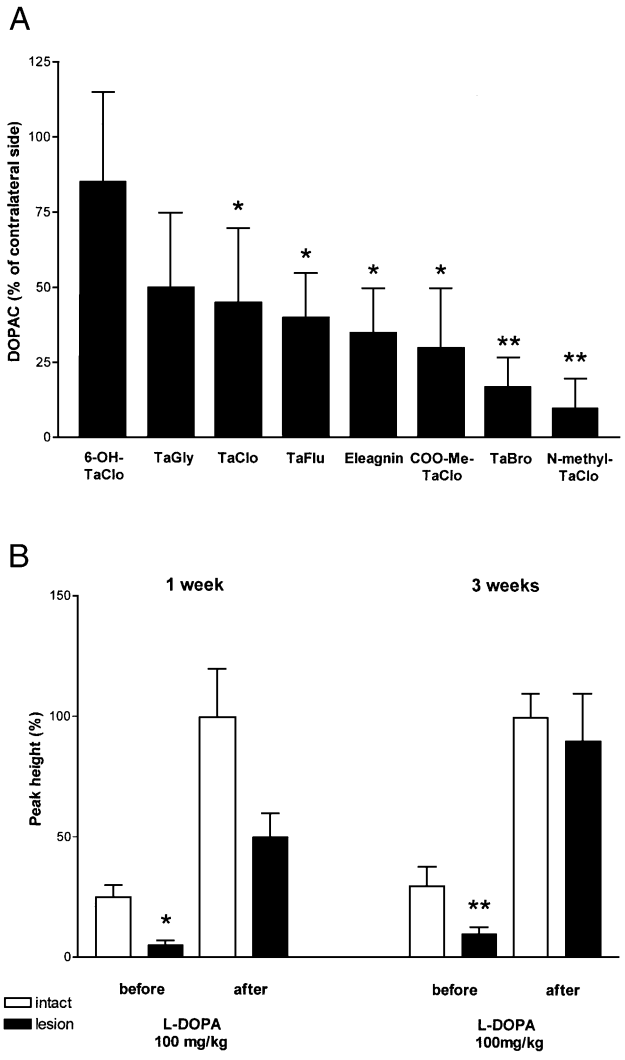


Fig. 10. (A) Effect of the unilateral intranigral injection of a number of TaClo analogs (each 10 μg) on striatal dopamine metabolism. Voltammetric assessment of striatal DOPAC was performed 1 week following the application of agent. (B) Effect of 10 μg *N*-methyl-TaBro applied directly to the substantia nigra pars compacta 1 or 3 weeks prior to the assessment of the extraneuronal DOPAC in the rat striatum by in vivo pulse voltammetry. L-DOPA was applied intraperitoneally at a dose of 100 mg kg^{-1}. Data are presented as mean signal (intact side after L-DOPA = 100) ± S.E.M.; $n = 5$ per group; * $P < 0.05$, ** $P < 0.01$ compared with intact side.

TaClo treatment, spontaneous locomotor activity was increased in the TaClo-treated animals compared with the NaCl-treated rats as indicated by the distance travelled ($P < 0.001$). This increase in activity was possibly indicative of increased dopaminergic activity (cf. O'Neill and Fillenz, 1985). This is also seen with MPTP: one of the acute effects of toxin is the dopamine release (Obata and Chiueh, 1992). At 12 weeks and 9 months, this difference in spontaneous activity was no longer evident (Sontag et al., 1995; Heim and Sontag, 1997a; Fig. 11).

There were no major differences between TaClo-treated and control animals 4–9 days posttreatment with respect to locomotor activity following apomorphine challenge; that is, the differences between the increase in activity elicited by apomorphine was smaller in the TaClo- than in the vehicle-treated animals (Fig. 11; Sontag et al., 1995). As the spontaneous or baseline behaviour of the TaClo-treated animals was higher than that of control animals, this could be interpreted either as a reduced motor response to apomorphine in these animals or as indicative of maximal dopaminergic activity having already been induced by the TaClo treatment. Nine months after treatment with TaClo, however, spontaneous locomotor activity was equal in the two groups, whereas the distance travelled following apomorphine challenge was significantly greater in the TaClo-treated group ($P < 0.01$; Heim and Sontag, 1997a; Fig. 11). While this supersensitivity of the TaClo-treated animals to apomorphine was even more marked 14 months after the end of the treatment with TaClo, basal locomotor activity was reduced in comparison with that at 9 months (Heim and Sontag, 1997b).

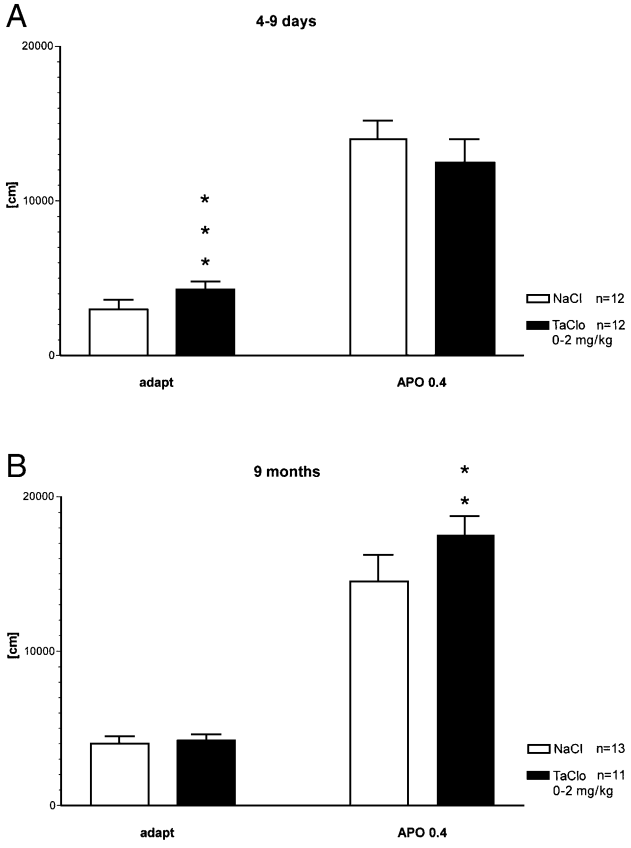


Fig. 11. Spontaneous (adapt) and apomorphine-induced (0.4 mg kg^{-1} s.c.; APO 0.4) locomotor activity in TaClo-treated (0.2 mg kg^{-1} in saline, i.p., daily, 7 weeks) and control (saline-injected) female Wistar rats: (A) 4–9 days and (B) 9 months after termination of TaClo treatment. Results are expressed as mean total distance travelled (60 min) ± S.E.M. Group differences: *** $P < 0.001$ (TaClo vs. control group), ** $P < 0.02$ (TaClo vs. control group) and $P < 005$ (apomorphine-induced increase at 9 months vs. 4–9 days); ANOVA employed for statistical analysis.

In a further experimental series, male rats were treated with a higher dose of TaClo (0.4 mg kg^{-1} i.p., daily, 7 weeks; Heim and Sontag, 1997b). In both control and TaClo-treated animals, spontaneous locomotor activity (as indicated by distance covered) declined between days 4–9 and 14 months after the end of the TaClo or vehicle treatment. Activity of the TaClo-treated animals was less than that of the control group; the significance of the difference, however, declined with age (days 4–9: $P<0.0001$; 9 months: $P<0.05$) and disappeared completely by 14 months post-treatment. The distance covered by TaClo-treated animals following apomorphine challenge was also less than that of the control animals. This parameter began to decline 9 months after the end of the TaClo treatment, whereas a reduced response to apomorphine in control animals was first observed at 14 months. As the response in TaClo-treated animals was similar at 9 and 14 months, the statistical significance of the difference between the TaClo-treated and control animals was also reduced (9 months: $P<0.0001$; 14 months: $P<0.01$). Similar differences were also observed with respect to stereotypic behaviour (sniffing, grooming, scratching, turning and shaking).

Heim and Sontag (1997b) also examined spatial learning abilities in TaClo-treated rats (0.4 mg kg^{-1}, daily, 7 weeks). Ten weeks after completion of TaClo treatment, TaClo-treated rats performed significantly worse than the controls in the Morris water maze test, whereby animals are required to locate a platform hidden in a circular pool. This combination of cognitive and motor deficits resembles the syndrome observed in humans following acute or chronic exposure to trichloroethylene (Bowler et al., 1991; Lindström et al., 1982; Feldman et al., 1985; White et al., 1997).

In summary, subchronic treatment (7 weeks) of rats with TaClo (0.2 or 0.4 mg kg^{-1} i.p.) on a daily basis resulted in statistically significant behavioural changes over a period of 16 months following TaClo treatment. It is difficult, however, to explain the different responses to the two doses employed in the two experimental series (Sontag et al., 1995; Fig. 11). Further work will be required to determine whether this lack of a dose–response effect and the markedly different response patterns must be attributed to the gender of the animals investigated or to other factors. Species- and gender-related differences in the metabolism of trichloroethylene by liver microsomes have, for instance, been reported (Elfarra et al., 1998). The observed effects in both groups, however, suggest either a defect in dopaminergic system activity or an altered balance between various neurotransmitter systems involved in the control of motor activity. It is highly important to note, however, that these changes developed *after* the end of exposure to TaClo. It was concluded that subchronic treatment with TaClo might initiate a series of events which result in a slowly progressive functional lesion of the nigrostriatal dopaminergic neurons. This would lead to disturbance in the balance between the dopaminergic systems involved in apomorphine-induced behaviours, as revealed by elevated behavioural sensitivity to systemically applied apomorphine (cf. Amalric and Koob, 1993). This contrasts with the behavioural changes elicited by MPTP, which are already detectable shortly after the exposure to the drug.

12. Biological significance of TaClo and its analogs as endogenously produced neurotoxins

The investigation of TaClo was stimulated by its structural similarity to the dopaminergic neurotoxin MPTP and by the discovery that it could be formed under physiological conditions from a biological substrate and a not uncommon environmental agent. It was thus conceivable that TaClo might be an endogenously produced neurotoxin, and that it might be of significance in the etiology of neurodegenerative diseases, such as Parkinson's disease. Evidence has been presented here that TaClo and/or its derivative *N*-methyl-TaClo:

1. is produced endogenously in rats and humans following exposure to chloral hydrate (Bringmann et al., 1995a, 1999);
2. has the ability to cross biological membranes, including the blood–brain barrier (Bringmann et al., 1996b, this paper);
3. is a potent inhibitor of mitochondrial complex I in vitro and (possibly) in vivo (Janetzky et al., 1995, 1999);
4. when acutely and systematically administered to rats, elicits the release of striatal 5-HT, with a consequent local rise in oxidative radical production Gerlach et al. (1998b);
5. has neurotoxic effects on the dopaminergic and serotonergic neurons and on astrocytes in cell culture (Rausch et al., 1995; Bringmann et al., 2000a,b);
6. when injected into the SN, reduces dopamine turnover in the striatum (Grote et al., 1995);
7. when subchronically administered to rats, influences motor responses to dopaminergic agents (Sontag et al., 1995; Heim and Sontag, 1997a,b).

Significant is the fact that the functional lesions described under points (6) and (7) are progressive in nature; that is, the magnitude of the lesion elicited by acute or subchronic exposure to TaClo increases with time, even when direct exposure to the toxin has been terminated, so that full realization of its toxic effects is manifested only in the older animal. The TaClo model of neurodegeneration is thereby one of the few toxin-based animal models of parkinsonism which exhibits this feature (cf. iron-induced nigral degeneration: Sengstock et al., 1994; Wesemann et al., 1994); MPTP intoxication, for example, produces a parkinsonian syndrome in primates, but this syndrome is presented shortly after exposure to the toxin and exhibits only a limited progredience (Burns et al., 1983). While

MPTP intoxication reliably reproduces the characteristic parkinsonian symptomatology, the validity of this model with respect to the etiology of the idiopathic disorder has been questioned (Jenner et al., 1986; Mohanakumar et al., 1994). TaClo, on the other hand, maybe not only a neurotoxin which generates a model of nigral degeneration which more closely reproduces the clinical course of neurodegeneration in Parkinson's disease, but also one which may actually play a role in the human disease.

Nevertheless, there have as yet been no published reports concerning the central histological changes induced by TaClo treatment in the whole animal, whether peripherally or centrally administered; it thus remains to be established how closely this toxin models the disease process observed in Parkinson's disease. Further studies are required to determine whether progression of the functional lesion produced by TaClo in animals ultimately results in the dysfunctions and neuropathology characteristic of Parkinson's disease. Irreversible neurotoxic effects — that is, the induction of a consistent pattern of neural dysfunction in a defined neural system — is generally associated with structural changes leading to degeneration of the affected nerve cell. Instances of long-term functional changes without significant morphological abnormalities have, however, also been reported (for example, Spencer and Schaumburg, 1984; Simonsen et al., 1994). This possibility must be entertained when examining the histopathological consequences of trichloroethylene or TaClo exposure, particularly at low concentrations; it is conceivable that functional impairment, particularly compromised cellular energy economy, precedes the distinct histological changes in affected brain region.

Further investigations are required to fully characterize the neurotoxic effects of TaClo on particular neurotransmitter systems in the central nervous system; to date, only particular aspects of the effects of TaClo on neural function have been systematically investigated. Because of the MPTP analogy, investigations of TaClo have thus far been focused upon dopaminergic parameters, but the potential for a range of endogenously produced neuroactive substances which are active at a number of neurochemical loci should be considered. It is possible, for instance, that condensation of endogenous nucleophilic amino acids (DOPA, cysteine, histidine) with chloral also yields novel compounds with psychotropic or neurotoxic properties (Bringmann et al., 1992). The structural similarity of 5-HT and 6-OH-TaClo confers the further investigation of this derivative as a potentially specific serotonergic neurotoxin a special importance.

Despite decades of research, it has not been possible to define a causative agent in the etiology of Parkinson's disease, although tantalizing hints regarding the involvement of environmental toxins have been reported. Epidemiological studies have detected, for example, an increased risk for Parkinson's disease associated with the use of pesticides, herbicides and heavy metals in rural areas of industrialized societies (Barbeau et al., 1985; Spencer and Butterfield, 1995). As Parkinson's disease, however, occurs throughout the world and exhibits a fairly uniform incidence, it is highly unlikely that exposure to a single environmental toxin explains all cases. This difficulty would be partly resolved where an indirect toxic mechanism is involved, such as that proposed here for chloral/trichloroethylene. TaClo possesses a number of qualities which might be expected to promote such a role; its lipophilic nature allows it to access cells and would promote its accumulation by fatty tissue, while the apparent lack of specific uptake would slow initiation of its action while not restricting its effects to a particular neuronal type. This fact is underscored by recent findings of Gerlach et al. (1998b) and Bringmann et al. (2000a,b) regarding the effects of TaClo and 6-OH-TaClo on serotonergic systems.

The potency exhibited by TaClo and certain derivatives with respect to inhibition of mitochondrial complex I activity underlines the central role which the impairment of cellular energy metabolism plays in the action of a range of neurotoxins. Compromised complex I activity has also been implicated in the cellular pathology of Parkinson's disease (Mizuno et al., 1989; Schapira et al., 1990); that this impairment could be effected by an endogenously synthesized agent may be a vital clue in the elucidation of processes underlying not only neurodegeneration but also 'normal ageing'. It is not, of course, suggested that all cases of Parkinson's disease are attributable to TaClo toxicity or, indeed, to toxins of any particular type. The TaClo model, however, represents an additional mechanism by which an environmental toxin might induce neurodegeneration, particularly in individuals with a genetically determined metabolic vulnerability.

The role of TaClo and similar compounds in the neurotoxicity of trichloroethylene requires further investigation; there is thus far no published evidence which demonstrates that trichloroethylene exposure elicits the same biochemical and behavioural changes which have been described in this paper. It is also important that most investigations of the toxicity of trichloroethylene and other industrial chemicals have been based upon acute studies in animals, the principle aim of which was to establish safe standards of exposure in the workplace. It is increasingly clear, however, that long-term exposure to industrial agents or combinations of agents can elicit syndromes distinct from their individual, acute effects (Allen, 1979). Therein lies the special significance of the investigation of TaClo as a potential biological toxin; should the investigations reported here be an indicative of the potential consequences of chronic or even subchronic exposure to trichloroethylene, chloral hydrate or other organic compounds, neurotoxicity attributable to these agents might not become evident until years after primary exposure. Further, the exposure levels which can be regarded as being 'safe' may need to be reassessed in the light of potential long-term and indirect neurotoxic effects of such compounds (cf. White et al., 1997). The investigation of this phenomenon is thus of major significance not

only for workplace safety but also with respect to the general environmental hygiene. The findings summarized in this paper raised the possibility that TaClo and its derivatives might have simultaneous effects at several neurochemical loci; this would render the determination of their individual effects more complicated, but would also be consistent with the fact that neurodegenerative disorders, including Parkinson's disease, are not restricted to single pathways or transmitter systems, but instead involve multiple problems at a number of neural sites.

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