Studies into the mechanism of ifosfamide-induced encephalopathy

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Summary

This thesis presents new mechanism-based studies regarding the dose-dependent central nervous side-effects caused by the cytostatic drug ifosfamide (IF), the so-called IF-induced encephalopathy which is observed in 10 to 30 % of all patients receiving IF.

The toxic IF metabolite chloroacetaldehyde (CAA) is probably the crucial factor for the pathogenesis of the neurotoxicity. We therefore studied the influence of CAA on intermediate metabolism in rats and examined whether the protective effect of methylene blue (MB), the redox dye clinically used to prevent and reverse IF-induced encephalopathy, was due to a decreased formation or an enhanced elimination of this toxic metabolite. Furthermore, the hypothesis was tested that some of the expected metabolic effects of CAA might be corrected by MB. We found that MB did not alter the concentration of CAA in plasma following administration of IF and did not stimulate glucose output and fatty acid oxidation of hepatocytes in vitro and in vivo. Failing to see an effect of MB may be due to the fact that disturbances of intermediate metabolism are not critical in IF-induced encephalopathy but that the formation of compounds with central nervous activity which may be modulated by MB is more important.

Based on the high reactivity of CAA we therefore hypothesized that mono-chlorinated tetrahydro-β-carbolines (THβCs), possibly formed by the reaction with endogenous indoleethylamines, might be involved in IF neurotoxicity due to their pharmacological properties such as inhibition of monoamine oxidases, interactions with GABA-ergic receptors and inhibition of the respiratory chain in mitochondria. Three different THβCs were synthesized and characterized, but consequently not found in the urine of patients receiving IF. This suggests that THβCs if they are formed in pharmacologically active concentrations in the brain, do not readily leave the central nervous system or are further metabolized to unknown compounds.

Since the formation of β-carbolines from metabolism of IF might interfere with the metabolism of serotonin, which plays a critical role in modulating mental functions, serotonin was measured in the brain of mice following administration of IF, combined IF and MB or MB alone and was subsequently found to be markedly increased after administration of MB probably due to inhibited monoamine oxidase activity. This increase might play a role in the reversal of IF-induced encephalopathy.

In addition, the IF metabolite S-carboxymethylcysteine (SCMC) was detected in the brain of mice after administration of IF. As SCMC selectively activates AMPA/Kainate receptors,

possibly due to structural similarities with the excitatory neurotransmitter glutamic acid (Glu), this metabolite might contribute to the pathogenesis of IF-induced encephalopathy.

Zusammenfassung

In der vorliegenden Arbeit werden neue Untersuchungen zur Entstehung der zentral nervösen Nebenwirkung von Ifosfamid (IF), der sogenannten IF-induzierten Enzephalopathie vorgestellt, welche in 10 – 30 % aller Patienten nach Gabe von IF auftritt und deren Ursache bis heute unklar ist.

Der toxische IF Metabolit Chloracetaldehyde (CAA) ist möglicherweise der Hauptverantwortliche für die schwere ZNS Nebenwirkung des alkylierenden Zytostatikums. Aufgrund dieser Tatsache wurde in der vorliegenden Arbeit zunächst geprüft welche Auswirkungen dieser Metabolit auf den Intermediärstoffwechsel hat und ob Methylenblau (MB), welches prophylaktisch und therapeutisch im Fall einer IF-Enzephalopathie verabreicht wird und dessen Mechanismus bis heute ungeklärt ist, diese beeinflussen kann. Die Plasmakonzentration von CAA in Ratten nach Gabe von IF war in Kombination mit MB unverändert, ebenso wie die Glukose Produktion und die Oxidation freier Fettsäuren durch Hepatozyten in vitro und in vivo.

In der Folge wurde die Möglichkeit der Entstehung von zentral aktiven Stoffen untersucht. Es wurde davon ausgegangen, dass chlorierte Tetrahydro-β-carboline (THβCs) aus der Reaktion mit CAA und Indolethylaminen enstehen könnten. THβCs besitzen ausgeprägte pharmakologische Eigenschaften, wie zum Beispiel die Hemmung von Monoaminoxidasen, Interaktion mit GABA-ergen Rezeptoren und Hemmung der Atmungskette in Mitochondrien und werden im Zusammenhang mit neurodegenerativen Erkrankungen diskutiert. Drei verschiedene THβCs wurden im Rahmen dieser Arbeit synthetisiert und charakterisiert. Im Urin von IF Patienten wurden sie nicht gefunden, was den Verdacht erhärtet, dass sie in situ im Hirn entstehen, dort ihre Wirkung entfalten und in Form unbekannter Metabolite im Urin ausgeschieden werden.

Es konnte gezeigt werden, dass die Gabe von MB einen Einfluss auf die Neurotransmitter Homöostase in Ratten hat. MB erhöht den Serotoningehalt im Hirn und hemmt die MAO-abhängige Tyraminoxidation. Darin liegt möglicherweise der nützliche Effekt von MB in Fall einer IF-Enzephalopathie.

Im Weiteren wurde der Metabolit S-carboxymethylcystein (SCMC) nach Gabe von IF im Hirn von Mäusen nachgewiesen. Dieses Resultat ist von besonderer Bedeutung, da gezeigt wurde, dass SCMC ausgeprägte agonistische Wirkung an AMPA/Kainat Rezeptoren hat. Wir nehmen daher an, dass SCMC zur Entstehung der IF-induzierten Enzephalopathie beitragen könnte.

Abbreviations

BZD	benzodiazepine
βCs	β-carbolines
CAA	chloroacetaldehyde
CEA	chloroethylamine
CP	cyclophosphamide
CYP450	cytochrome-P-450
DMT	N,N-dimethyltryptamine
ESI	electrospray ionization
ethyl-βC-3-c.	ethyl-β-carboline-3-carboxylate
GABA	gamma–aminobutyric acid
Glu	L-glutamic acid
Glufos	glufosfamide
GSH	glutathione
HPLC	high performance liquid chromatography
3-OH-βC	3-hydroxymethyl-β-carboline
IF	ifosfamide
LCMS	liquid chromatography mass spectrometry
LOD	limit of detection
MAO	monoamine oxidase
MB	methylene blue
Mesna	sodium 2-mercaptoethanesulphonate
OH-THβC-1-clm.	6-hydroxy-1,2,3,4-tetrahydro-β-carboline-1-chloromethyl
PA	pipecolic acid
PO	pipecolic acid oxidase
RDA	Retro-Diels-Alder
S/N	signal to noise
SCMC	*S*-carboxymethylcysteine
SMPs	submitochondrial particles
SS	synaptosome suspension
TDGA	thiodiglycolic acid
TaClo	1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline
THβC-1-clm.	1,2,3,4-tetrahydro-β-carboline-1-chloromethyl
THβC-1-clm.-3-c.	1,2,3,4-tetrahydro-β-carboline-1-chloromethyl-3-carboxy
THβCs	1,2,3,4-tetrahydro-β-carbolines
Trofos	trofosfamide

1 Introduction

1.1 Oxazaphosphorines

Oxazaphosphorines are nitrogen mustard derivatives. During World War I, chemical warfare with mustard gases (chemical name: bis (2-chloroethyl) sulfide; $C_4H_8Cl_2S$) produced leukopenia, bone marrow aplasia and destruction of lymphatic tissue. Gilman and others investigated the underlying mechanism and shortly after, the first modern clinical testing of chemotherapy led to the discovery of the first cyclic nitrogen mustard analogue cyclophosphamide (CP) [Gilman 1963]. Ifosfamide (IF) was developed in the middle of the 1960s and was introduced as an anticancer drug in the early 1970s [Loehrer 1992, Kamen et al. 1995]. Because phosphoramidase enzymes were thought to be more abundant in tumours compared to normal tissue, oxazaphosphorines were designed to be cleaved by these enzymes in order to provide nitrogen mustard selectively to malignant cells. Today it is evident that oxazaphosphorines act as prodrugs, but the pharmacological route to DNA alkylation does not involve activation by phosphoramidases [Boddy and Yule 2000]. The cytotoxic effect of oxazaphosphorines is believed to be caused by the nitrogen mustard. After intracellular activation, both chloroethyl groups are converted to reactive electrophilic alkyl groups ($R\text{-}CH_2^+$), which in turn react with the nucleophilic moieties of the bases in DNA. Due to their bifunctional character the primary adducts go on to form cross-links through reaction of the second chloroethyl arm of the mustards. The different intramolecular distance between the chloroethyl groups in CP and IF mustards results in a different range of cross-linked DNA [Springer et al. 1998]. A major advantage of IF over its isomer CP is its lower myelotoxicity, allowing the use of higher doses [Brade et al. 1986]. Moreover, IF is successfully used in CP resistant solid tumors and is thus most important for the clinic [Dechant et al. 1991].

1.2 Ifosfamide: metabolism

The oxazaphosphorine IF is among the most widely used cytostatic drugs in the treatment of myeloproliferative and lymphoproliferative disorders as well as for solid malignancies [Sladek 1988]. The pharmacologically inactive prodrug IF is converted into its primary active form 4-hydroxyifosfamide (4-hydroxy-IF) which exists in equilibrium with its tautomeric form, aldoifosfamide (aldo-IF). Aldo-IF is either dehydrogenated to the inactive metabolite carboxyifosfamide, or spontaneously decomposes to form the pharmacologically active alkylating metabolite isophosphoramide mustard and acrolein. Inactive metabolites are produced by oxidation of 4-hydroxy-IF to 4-ketoifosfamide or by sulfur conjugation of the intermediate compound to 4-thioifosfamide. IF is also subject to deactivation pathways that involve the removal of the chloroethyl side-chains (N-dealkylation or N-dechloroethylation) from either the exo- or endocyclic nitrogen atom to form the non-toxic metabolites 2- and 3-

dechloroethylifosfamide (dechloro-IF). An equimolar amount of chloroacetaldehyde (CAA) [Kaijser 1994] is released as represented in figure 1. CAA is most likely the key candidate responsible for the neurotoxicity observed in 10-30 % of all patients receiving IF [Cerny and Küpfer 1992].

IF activation (4-hydroxylation) is catalysed by multiple cytochrome-P-450 (CYP450) enzymes. The involvement of CYP3A4 in the activation pathway was shown in human liver microsomes [Walker et al. 1994]. Roy and co-workers showed an additional participation of CYP2A6, CYP2B6 and CYP2C9 [Roy et al. 1999a]. Regarding the N-dechloroethylation pathway it was repeatedly demonstrated that mainly CYP3A4 but also to some extend CYP2B6 are involved in the degradation reaction [Walker et al. 1994, Granvil et al. 1999, Roy et al. 1999b, Huang et al. 2000].

A detailed study of IF metabolism in human liver microsomes, including the co-determination of keto- and carboxyifosfamide and considering the free and protein-bound acrolein, showed that 92 % of the turnover of IF in vitro was accounted for by 4-hydroxylation (CYP3A4 and CYP2A6 mediated) and 8 % by N-dechloroethylation (CYP3A4 mediated) [Preiss et al. 2002]. In vivo, other investigators found 9.8 % of the total dose of IF administered as dechloroethylated metabolites in the urine of patients receiving intravenous infusions of 2 or 3 g/m^2 IF over 1 or 2 days [Kerbusch et al. 2001]. In addition, it was shown that a time-dependent increase in urinary dechloro-metabolites, which is caused by auto-induction of hepatic oxidative metabolism [Boddy et al. 1995], results in 13.5 % dechloroethylated metabolites following a dose of 1.5 g/m^2 daily over 5 days [Kurowski and Wagner 1997]. Thus, a substantial fraction of IF is metabolized to CAA.

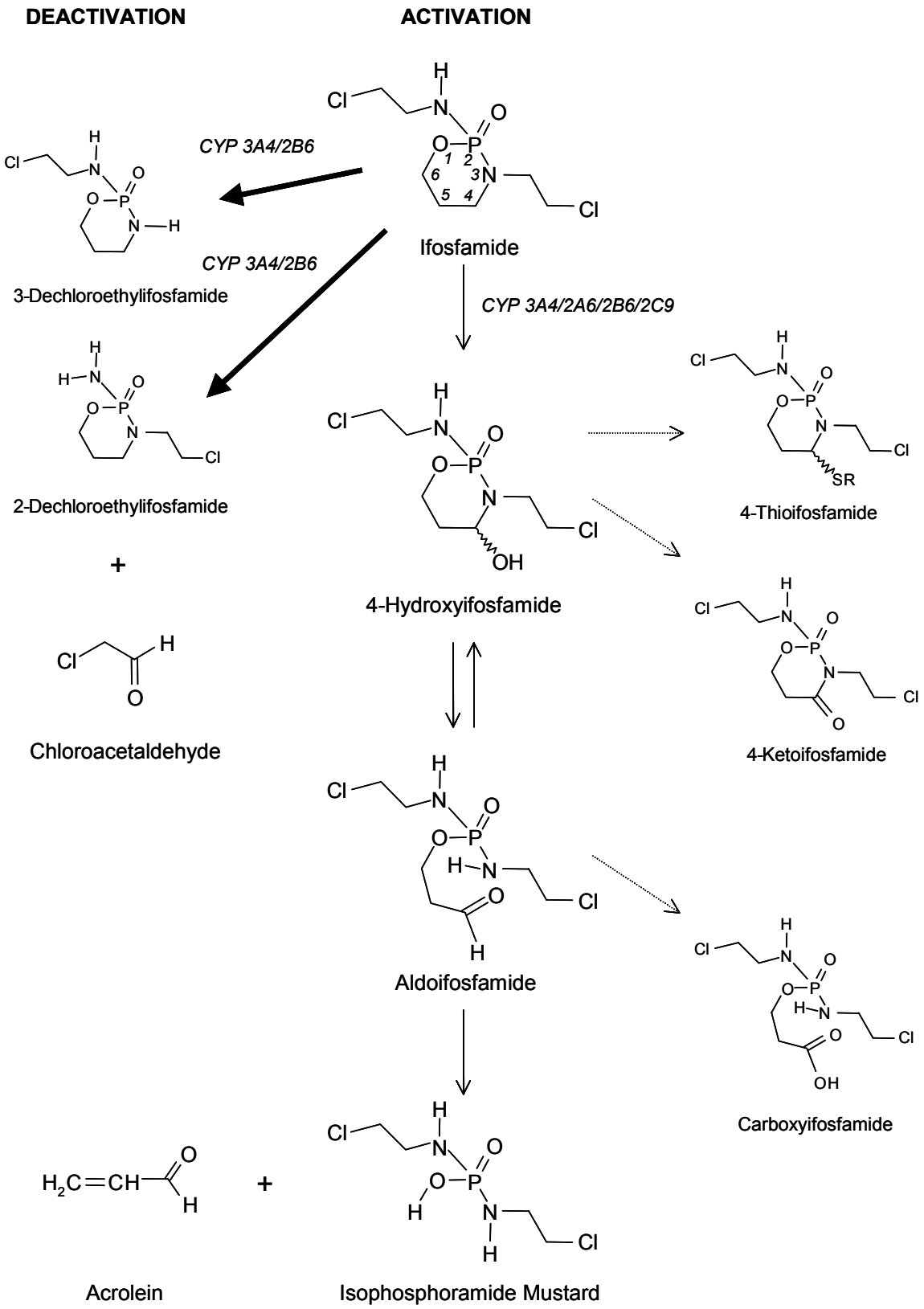


Figure 1: Metabolism of IF: bold arrows indicate degradation pathways (N-dechloroethylation) which result in the formation of the potentially neurotoxic compound chloroacetaldehyde (CAA), plain arrows indicate bioactivation pathways and dashed arrows represent pathways leading to pharmacologically inactive compounds.

Other oxazaphosphorine agents have been developed among them trofosfamide (Trofos) and glufosfamide (Glufos). The latter is an isophosphoramide mustard coupled to glucose and does therefore not require bioactivation. This might be associated with a lower risk of neurotoxicity. Whether CAA is generated from Glufos or not remains to be demonstrated. Further data regarding the metabolism and the pharmacokinetics of Glufos is awaited. Trofos which has 3 chloroethyl groups may be metabolised to a 4-hydroxy metabolite of either CP or IF and is thus a substrate for the same CYP450 enzymes [Hempel et al. 1997, May-Manke et al. 1999].

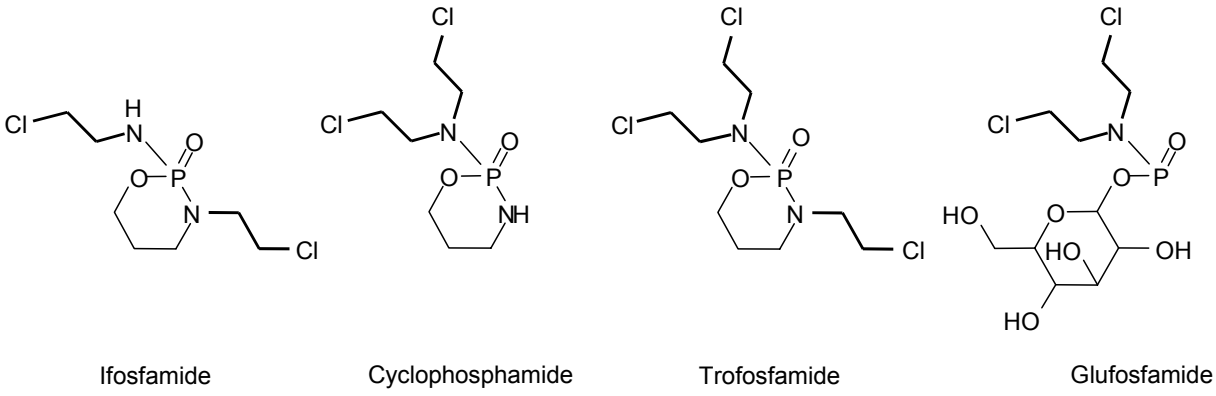


Figure 2: The four oxazaphosphorines ifosfamide (IF), cyclophosphamide (CP), trofosfamide (Trofos) and the aliphatic glufosfamide (Glufos). Chloroethyl side chains are marked as bold lines.

1.3 Ifosfamide neurotoxicity: facts

Central nervous system toxicity occurs in a dose-dependent manner in 10 to 30 % of all patients receiving single-agent treatment with IF and limits the dose that can be administered. The IF-induced encephalopathy is characterized by somnolence, hallucinations, confusion, anxiety, seizures and in some cases by coma. Most encephalopathies are reported to be reversible but cases of a fatal outcome are described, mainly in children [Pratt et al. 1986].

Predisposing factors for IF-associated encephalopathy are oral administration, decreased renal and hepatic function, previous chemotherapy with cisplatin, low serum albumin and brain metastases [Meanwell et al. 1986]. With intravenous use a fast rate appears to be a risk factor [Cerny et al. 1990]. A higher incidence was also observed in women and elderly patients [Alonso et al. 1996]. DiMaggio presented a series of 6 patients with neurotoxic symptoms after IF. Five of these six were women [DiMaggio et al. 1994]. In another study, acute encephalopathy following treatment with IF and Mesna (sodium 2-mercaptoethanesulphonate) was observed in four women and one man in a group of totally 28 patients [Merimsky et al. 1992]. Watkin and co-workers also describe a higher incidence

of IF-neurotoxicity in women [Watkin et al. 1989]. This might be due to gender differences in the IF N-dechloroethylation, as it was shown in a study with human liver microsomes [Schmidt et al. 2001]. Urotoxicity, which often manifested itself as haemorrhagic cystitis, used to be the dose-limiting toxicity for IF. Today urotoxicity is prevented by the co-administration of Mesna, which binds to the highly reactive 4-hydroxy metabolites of oxazaphosphorines (including acrolein) to produce inert thioesters [Bryant et al. 1980]. The uroprotective Mesna does not cause CNS toxicity when administered as a single agent, since IF encephalopathy was reported even before Mesna became available [Cantwell and Harris 1985]. 4-hydroxy metabolites are believed to be responsible for the urotoxicity, although CAA has also been implicated [Brock et al. 1979, Shaw et al. 1983]. CAA has also been proposed to be responsible for the nephrotoxicity observed with IF [Springate 1996]. Predictive factors for a nephrotoxicity are mainly young age, cumulative IF dose and concurrent administration of cisplatin [Loebstein and Koren 1998]. IF may induce renal Fanconi syndrome, which has never been observed following administration of CP. This proposal is consistent with the fact that much more CAA is formed after IF administration than after CP administration.

Among the many metabolites of IF, CAA is the prime candidate responsible for the neurotoxicity. A correlation between CAA plasma levels following IF administration and the incidence of neurotoxic symptoms was shown in children by Goren and co-workers [Goren et al. 1986]. In contrast to IF, no neurotoxic adverse effects are described following CP and Trofos. Differences in the metabolism of various oxazaphosphorines to CAA may account for this observation. The key enzyme responsible for the oxidation of the side-chain of CP was identified as CYP3A4 [Bohnenstengel et al. 1996]. Differences in hepatic metabolism between the two drugs IF and CP are attributed to their structural differences, which involve a shift of one chloroethyl group from the exocyclic nitrogen to the nitrogen of the oxazaphosphorine ring, as can be seen in figure 2. In contrast to CP, which has two chloroethyl side-chains at the exocyclic nitrogen, one of the two chloroethyl groups of IF is placed at the endocyclic nitrogen. The spatial separation of the chloroethyl groups on the IF molecule slows the rate of activation via ring hydroxylation, because of steric inhibition by the chloroethyl moiety on the adjacent endocyclic nitrogen [Dechant et al. 1991]. This is also most likely the cause for larger CAA concentrations following administration of IF than following CP. Encephalopathy has also been observed following the administration of high-dose thiotepa (N,N',N''-triethylenethiophosphoramide) [Wolff et al.1990]. Urinary profiling of a patient receiving thiotepa revealed the existence of the IF metabolites CAA, chloroethylamine (CEA), S-carboxymethylcysteine (SCMC) and thiodiglycolic acid (TDGA) [Höfer 1995]. This might be due to opening of the aziridine rings, which are ethyleneamino groups, and consequent attachment of chlorine to form CEA, CAA and consequently SCMC and TDGA,

similar to what is reported from IF. Küpfer and co-workers showed that IF performs a rapid and reversible intramolecular rearrangement which results in aziridino-IF (2-aziridino-3-(2-chloroethyl)-tetrahydro-2H-1,2,3-oxazaphosphorine-2-oxid), structurally similar to thiotepa [Küpfer et al. 1990].

Brain and co-workers recently proposed modulation of the different liver CYP450 isoenzymes with various CYP450 inducers and inhibitors, shifting IF metabolism from CAA to 4-hydroxy-IF in order to decrease the generation of CAA [Brain et al. 1998]. However, it was shown that CAA itself possesses cytotoxic effects against solid tumour cells [Börner et al. 2000, Brueggemann et al. 2002]. Therefore, inhibition of the metabolic pathway leading to CAA, although possibly beneficial as a mean of reducing neurotoxic response, might be associated with a reduction in antitumor effect.

1.4 Ifosfamide neurotoxicity: hypotheses

Till today the processes leading to IF-induced encephalopathy are unclear. No consistent pattern of clinical or laboratory abnormalities has been found so far. Various hypotheses have been proposed, but no mechanism has been convincingly demonstrated. Nevertheless, the crucial factor is most likely CAA which is a highly reactive and toxic compound. Only a few pharmacokinetic studies have been performed on CAA because of analytical problems due to short half-life of the compound in biological samples ex vivo [Cerny et al. 1990].

CAA causes a rapid depletion of glutathione (GSH) in plasma after administration of IF/Mesna. This could contribute to the development of encephalopathy, particularly since the glutathione regeneration capacity of the liver is much higher than that of the brain. It was shown that plasma cysteine, homocysteine and GSH fell below 20 % of the starting value after 5 days of IF chemotherapy [Lauterburg et al. 1994]. This depletion of endogenous sulfur compounds may be a risk for the development of the IF-associated neurotoxicity.

Besides GSH depletion, inhibition of acyl-CoA dehydrogenase-dependent processes in the mitochondrial respiration chain by CAA and reaction products may result in neurotoxicity. It was shown that elevated levels of the urinary dicarboxylic acid glutaric acid and of sarcosine are found in patients receiving IF [Küpfer et al. 1994]. This urinary pattern is similar to what is seen in glutaric aciduria type II, a multiple acyl-CoA dehydrogenase deficiency. It results from impaired flavin-mediated transfer of electrons between mitochondrial matrix dehydrogenases and the electron transport chain caused by electron transfer flavoprotein (ETF) deficiency. Methylene blue (MB) has been used successfully in patients with glutaric aciduria type II, possibly because it acts as an alternative non-physiological electron acceptor, replacing

inhibited flavoproteins and thus restoring the mitochondrial respiratory chain [Küpfer et al. 1996].

CAA might react with endogenous substrates to form compounds with central nervous activity. One proposal is the formation of tetrahydro-β-carbolines (THβCs) via reaction with indoleethylamines, another suggestion is the formation of tetrahydro-isoquinolines from reactions with phenylethylamines. THβC compounds might undergo further reactions to form β-carbolines (βCs) or methylated THβCs.

CAA and its associated oxidation/reduction products chloroacetic acid and chloroethanol, respectively, undergo conjugation with cysteine to form S-carboxymethylcysteine (SCMC) which is further metabolised to thiodiglycolic acid (TDGA) [Hofmann et al. 1991]. TDGA is excreted in large quantities in patients receiving IF [Visarius et al. 1998]. It was recently shown that SCMC exhibits distinct agonistic effects on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/Kainate receptors, a excitatory ionotropic glutamate receptor [Chatton et al. 2001], but the presence of SCMC in brain after IF therapy has not been demonstrated so far. Whether SCMC forms a cyclic metabolite which inhibits pipecolic acid oxidase (PO) is speculative.

The oxidation of CAA to chloroacetic acid by aldehyde dehydrogenase is accompanied by the simultaneous reduction of NAD^+ to NADH. The formation of other aldehydes, such as acrolein and aldo-ifosfamide [Kaijser 1994], by CYP450 in the liver might further decrease the availability of NAD^+ which is the co-factor for aldehyde dehydrogenases [Forth et al. 1996]. The resulting shift of the NAD:NADH ratio might impair cellular processes and might contribute to the encephalopathy. In addition, in the absence of GSH and cysteine for conjugation, chloroacetic acid might interrupt, like other haloacetic acids, the Krebs cycle at the level of isocitrate formation by the mechanism of so-called lethal synthesis [Peters 1952] and might further impair cellular metabolism.

IF is a chiral drug with an asymmetric phosphorus atom and exists in two enantiomeric forms, (R)-IF and (S)-IF. In clinical practice IF is administered as racemic mixture of the two enantiomers. The 2- and 3- dechloro-metabolites (figure 1) are also chiral and exist in R- and S-enantiomers. In humans and in rats the metabolism of IF is enantioselective where (S)-IF is more extensively cleared by the N-dechloroethylation pathway. This was shown in various studies and is reviewed in the paper of Granvil and co-workers [Granvil et al. 1999]. As both IF-enantiomers undergo N-dechloroethylation it seems unlikely that the administration of pure enantiomer would overcome the problem of IF-associated encephalopathy.

MB is successfully used to prevent and treat IF-encephalopathy. A comprehensive review of all published cases of IF-encephalopathy and its management with MB till 2000 is found in the paper by Pelgrims [Pelgrims et al. 2000]. Although several hypotheses to explain the protective effect of MB have been proposed its mode of action remains to be elucidated. It was shown that MB inhibits monoamine oxidase (MAO) in vitro [Aeschlimann et al. 1996], thereby reducing the formation of CAA from the monoamine CEA that is generated by hydrolysis from IF. It is also known that MB oxidises NADH to NAD^+ and it is thereby able to reverses some of the changes in the intracellular redox state occurring in ethanol fed animals [Madison et al. 1967]. Others have demonstrated that MB inhibits aldehyde dehydrogenase in cytosol and mitochondria from human and rat liver, respectively [Cronholm 1993, Helander et al. 1993]. MB was also able to compensate for the effect of CAA on long-chain fatty acid oxidation, thus possibly preventing encephalopathy [Visarius et al. 1999]. Figure 3 gives an overview over the mentioned hypotheses regarding the IF-induced encephalopathy.

1.5 Aim of the thesis

Although IF has been used successfully for over 30 years in the treatment of patients with various malignant diseases there is still a need for a mechanism-based understanding of its neurotoxicity, as this side-effect of IF is severe and dose-limiting. It is the aim of this thesis to contribute to the understanding of IF-induced encephalopathy.

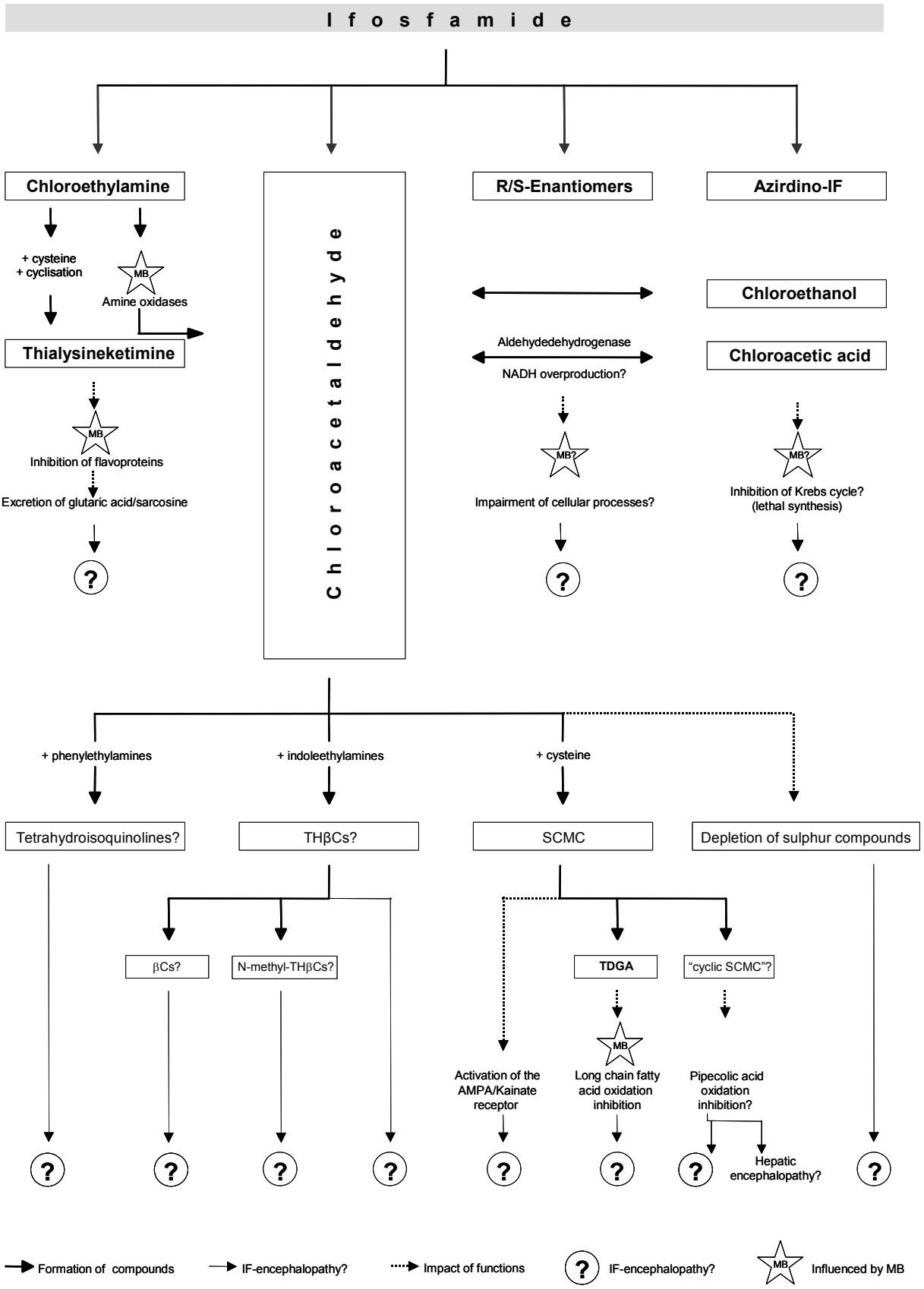


Figure 3: Overview over hypotheses regarding the IF-induced encephalopathy. CAA is most likely the key to resolve the problem of the neurotoxicity. Bold compounds are unambiguously detected as IF-metabolites.

2 Disturbance of intermediate metabolism by ifosfamide and its metabolite chloroacetaldehyde. Effects of methylene blue

2.1 Summary

This part of the thesis was designed to examine whether the protective effect of MB in IF-induced encephalopathy was due to a decreased formation or an enhanced elimination of the toxic metabolite CAA. Furthermore the hypothesis was tested that some of the expected metabolic effects of CAA might be corrected by MB.

It seems that MB does not reverse effects caused by CAA in the intermediate metabolism. Cell viability as well as glucose production and oxidation of free fatty acids was unaffected by the addition of MB. However, the damaging effect of CAA on cell viability and on hepatic GSH concentration was demonstrated. In vivo CAA levels were unaffected following additional MB.

2.2 Introduction

CAA is generated by CYP450 N-dechloroethylation of IF [Goren et al. 1986, Walker et al. 1994, Yu and Waxman 1996, Brain et al. 1998, Granvil et al. 1999, Huang et al. 2000] and by oxidation from CEA [Highley et al. 1995, Aeschlimann et al. 1996]. It accumulates in blood after oral or intravenous administration of IF and is considered to be the metabolite responsible for the IF-associated encephalopathy (see chapter 1). The oxidation of CAA to chloroacetic acid by aldehyde dehydrogenase is accompanied by the simultaneous reduction of NAD^+ to NADH. An increase in the NAD:NADH ratio in the liver during CAA oxidation might result in a decreased hepatic gluconeogenesis. In addition it is known that a high NAD:NADH ratio inhibits the rate of β-oxidation, although this inhibition is only significant at high ratios [Bremer and Wojtczak 1972]. Moreover, CAA might inhibit the Krebs cycle. Chlorocitrate, a metabolite of its oxidation product chloroacetic acid (figure 4) may enter the Krebs cycle where it might result in an inhibition, like fluorocitrate which irreversibly and lethally inhibits the cycle at the level of aconitase [Brand et al. 1973, Hayes et al. 1973, Bosakowski and Levin 1987] which converts citrate to isocitrate (figure 5).

MB, a thiazine dye with oxidation/reduction properties [The Merck Index 1996] might be able to normalise the NAD:NADH ratio, that is disturbed by the accumulation of CAA. Thereby, it might accelerate the metabolism of CAA. In addition, the generation of CAA from CEA might be decreased by the inhibitory effect of MB on amine oxidases (figure 4) [Aeschlimann et al. 1996].

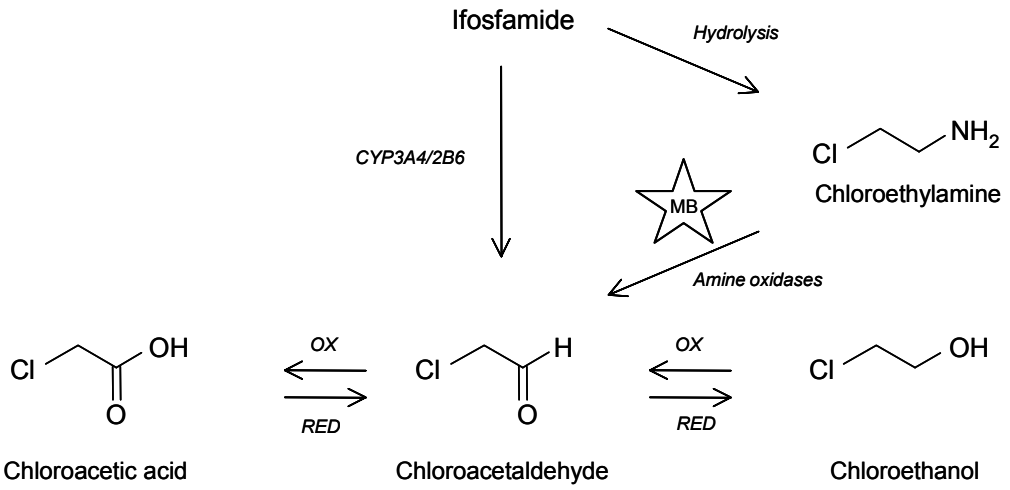


Figure 4: Formation of CAA and the two redox-products chloroethanol and chloroacetic acid. Chlorocitrate, a metabolite of chloroacetic acid might enter the Krebs cycle. Methylene blue was shown to inhibit plasma amine oxidases in vitro and might therefore decrease the formation of CAA from CEA.

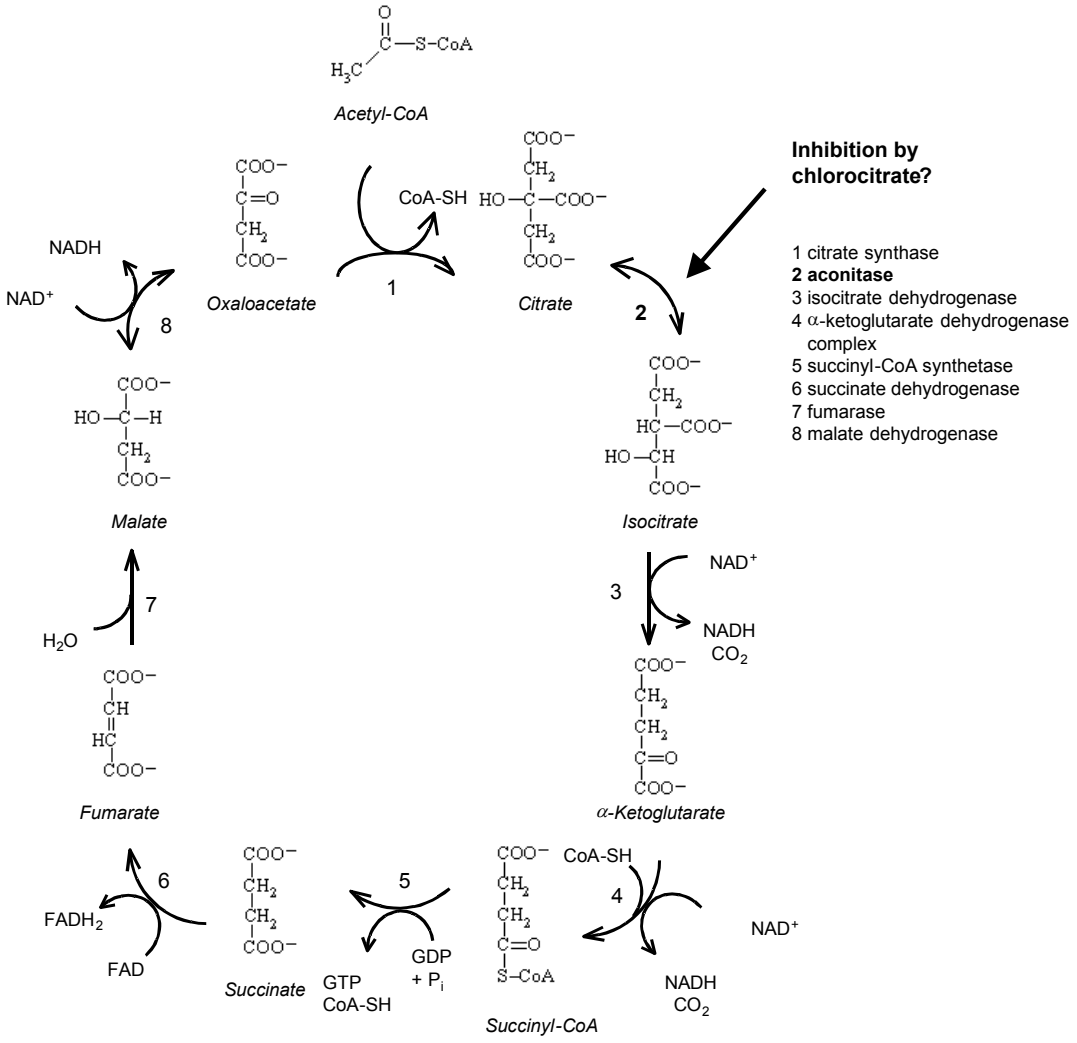


Figure 5: Krebs cycle and possible aconitase inhibition by chlorocitrate.

In the present work the effect of MB on the plasma concentration of CAA following administration of IF and on the metabolic effects of CAA was studied in intact animals and isolated hepatocytes.

2.3 Material and methods

Chemicals.

CAA-solution 45% (wt/vol) in water and CEA hydrochloride were obtained from Fluka (Buchs, Switzerland). Ifosfamide (Holoxan®) was from Asta Medica (Wangen, Switzerland). Methylene blue (methylthionini chloridum Ph.H.VI) was from Hänseler AG (Herisau, Switzerland). Collagenase was purchased from Gibco (Basel, Switzerland). [^{14}C]acetate sodium salt, specific activity 2.11 GBq/mmol (9.25 MBq/1.25 ml), was obtained from Amersham Bioscience (Buckinghamshire, UK). Palmitic acid sodium salt, adenosine and 1,N^6-ethenoadenosine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, USA), glucose monohydrate, alanine and HPLC grade acetonitrile from Merck (Darmstadt, Germany). The water was of Purelab Option E (Labtec, Wohlen, Switzerland) quality (filtered and UV light treated). Control serum N (human) was obtained from F. Hoffmann-La Roche (Basel, Switzerland).

Animals: IF administration.

Two groups of 5 male Wistar rats (180-200 g) were pre-treated with 0.5 g/L phenobarbital (PB) in the drinking water during 4 to 7 days in order to induce the CYP450 dependent dechloroethylation of IF. For the experiment they were anaesthetised with pentobarbital (50 mg/kg i.p.). A carotid artery was cannulated for multiple blood sampling. Thirty minutes before the administration of IF group 1 received 5 mg/kg (15.5 μmol/kg) MB i.p.. Both groups obtained an i.p. injection of 300 mg/kg (1.15 mmol/kg) IF at time point 0'. At the same time point the first group received another 5 mg/kg MB. Blood samples (250 μl) were withdrawn from the catheter at each of the 10 time points (0, 5, 15, 30, 60, 90, 120, 180, 240 and 300 minutes). The volume of blood collected was replaced with an equal volume of 0.9 % saline. Blood samples were mixed with heparin and an aliquot of 150 μl was pipetted to 30 μl of 10 mmol/L formaldehyde in 0.9 % saline. Further preparation was performed as described by Huang and Waxman 1999. CAA plasma levels were determined by HPLC.

Determination of CAA by HPLC.

The assay to determine CAA is based on the formation of the fluorescent adduct 1,N^6-ethenoadenosine upon reaction of CAA with adenosine (figure 6).

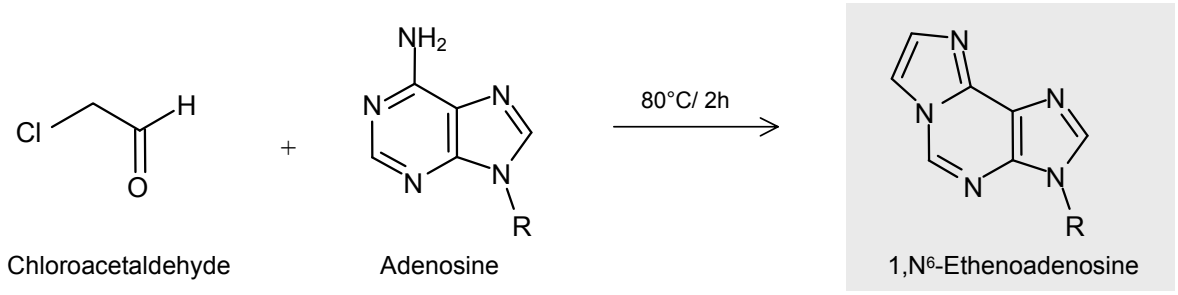


Figure 6: Formation of the highly fluorescent compound 1-N^6-Ethenoadenosine from the reaction between CAA and adenosine.

For the chromatographic analysis a Hewlett Packard series 1100 quaternary pump and fluorescence detector as well as a Agilent 1100 series automatic sample injector were used. The chromatographic system consisted of a Nucleosil 100-5 C_{18} RP column (150*4.6 mm i.d.) (Macherey-Nagel AG, Oensingen, Switzerland). All samples were eluted with 11 % acetonitrile in water in isocratic mode with a flow rate of 0.8 ml/min. The fluorescence detector was set to an emission wavelength of 270 nm and to 411 nm for excitation. For the analysis of the chromatograms a HP ChemStation software was used.

Animals: CAA administration.

Two groups of 3 male Wistar rats (180-200 g) were anaesthetised with pentobarbital (50 mg/kg i.p). A carotid artery was cannulated to allow for multiple blood sampling. The first group received an injection of 5 mg/kg (15.5 μmol/kg) MB i.p. in 0.9 % NaCl at time point -30'. At time point 0' both groups received 90 mg/kg (1.15 mmol/kg) CAA in 0.9 % NaCl. At the same time point the first group received an additional i.p. injection of 5 mg/kg MB in 0.9% NaCl. Blood samples (250 μl) were withdrawn from the catheter at each of the 8 time points (-30', -15', 0, 5, 15, 30, 45 and 60 minutes) and the volume of blood collected was replaced with an equal volume of 0.9 % saline. Plasma was obtained by centrifugation. Plasma levels of glucose and free fatty acids were determined.

Isolation of rat hepatocytes.

Female Wistar rats (ca. 250 g) fed a standard chow diet and water ad libitum, were used in all experiments. Hepatocytes were obtained by collagenase perfusion of the liver. In brief, livers were perfused with 10 mmol/L HEPES/NaOH buffer (pH 7.4, gassed with 100 % O_2) containing 0.2 mmol/L EGTA (ethyleneglycol-bis-(β-aminoethyl ether)-tetra-acetic acid), 143 mmol/L NaCl and 7 mmol/L KCl. After 15 min, the perfusate was switched for approximately another 20 min to 50 mmol/L HEPES/NaOH buffer (pH 7.6, gassed with 100 % O_2) containing 100 mmol/L NaCl, 7 mmol/L KCl, 5 mmol/L $CaCl_2$ and 0.1 % (w/v) collagenase. After adequate digestion was achieved, the liver was removed from the animal, placed in a Petri dish and the capsule was stripped. The resulting crude suspension was incubated for

10 min at 37°C in a 250 ml round-bottom flask in 50 mmol/L HEPES/NaOH (pH 7.4, equilibrated with room air) containing 60 mmol/L NaCl, 40 mmol/L KCl, 1 mmol/L $CaCl_2$, 2 mmol/L $MgSO_4$ and 1 mmol/L Na_2HPO_4. Thereafter, the suspension was filtered through a 100 μm mesh nylon filter. The hepatocytes were further purified by two low g centrifugation washes (2 min, 50 g). Cells were suspended at a density of $5*10^6$ cells/ml in 25 ml Erlenmeyer flasks rotating in a water bath maintained at 37°C in 50 mmol/L HEPES, 60 mmol/L NaCl, 40 mmol/L KCl, 1 mmol/L $CaCl_2$, 2 mmol/L $MgSO_4$, 1 mmol/L Na_2HPO_4.

Incubation of rat hepatocytes.
Isolated hepatocytes from three animals were diluted so that $5*10^6$ cells/ml were obtained. The effect of increasing concentrations of CAA (1.5, 5 and 10 mmol/L) was studied in the presence and absence of MB (30 μmol/L). The total volume of an incubation mixture was 4 ml. All samples were gently shaken at 37°C. MB was added to the cell suspension 15' prior to CAA. Alanine (10 mmol/L final concentration), a precursor for gluconeogenesis, was added 1' prior to CAA.

Determination of glucose.
Glucose was measured with the Unimate 5 GLUC HK Kit from F. Hoffmann-La Roche (Basel, Switzerland), which is an enzymatic UV-test that is based on the glucose dependent enzymatic generation of NADH and a consequent change in absorption at a wavelength of 340 nm [Bergmeyer 1974]. Calibration was performed with glucose and controlled with control serum N (human).

Determination of free fatty acids.
Free fatty acids were measured with a kit (Free fatty acids, Half-micro test) from Roche Diagnostics GmbH (Mannheim, Germany) as a function of β-oxidation. The red colouring was measured at a wavelength of 546 nm. Calibration curves were obtained using palmitic acid.

Viability.
The viability of the isolated cells was determined by measuring the exclusion of trypan blue. It is based on the observation that viable cells do not take up the dye trypan blue, while non-viable and severely damaged cells take up the dye and turn blue [Phillips 1973]. Immediately after isolation, light microscopic examination of the hepatocyte suspension provides information on the quality of the cell preparation. Damaged hepatocytes may show blebbing of the plasma membrane. Bleb formation is a sign of fluid uptake by hypoxic or anoxic cells, which is often followed by cell death. The viability was assessed prior to incubation and was more than 80 % in all cases (4 fold measurement of each cell preparation). In addition the test was performed at the time points 0' and 60' of the incubation.

Determination of glutathione.

The concentration of total glutathione in hepatocytes was measured in deproteinated samples (10 % perchloric acid) according to the method of Tietze [Tietze 1969]. The cell suspension volume taken at each time point was 100 µl.

Metabolism of acetate by isolated hepatocytes (CO_2 trapping).

Hepatocytes were prepared from three female Wistar rats. Hepatocytes ($5*10^6$ cells/ml) were 30 minutes pre-incubated in stoppered flasks at 37°C with and without MB at a final concentration of 100 µmol/L and with CAA or CEA at concentrations of 100 µmol/L and 1 mmol/L. All samples were gently shaken. The reaction was started by adding [^{14}C]acetate (final concentration 8 µmol/L) and stopped after 60°C by adding perchloric acid through the side arm of the flask. The $^{14}CO_2$ generated and liberated by the acid was trapped in a well containing a filter paper soaked in 0.1 N NaOH. All samples were counted for radioactivity (decays per minute) in a Beta scintillation counter (Kontron instruments) with automatic quench correction.

Statistical analyses.

Results are expressed as mean values +/- SD. Statistical significance of the differences between groups was assessed by one a way ANOVA (analysis of variance) and Bonferroni-t-test.

2.4 Results

The first question we tried to answer with the present experiments was whether MB would influence the formation or disposition of CAA generated from IF. Following the administration of IF the concentration of CAA increased over a period of 90 minutes and then gradually decreased (figure 7). Although there was a considerable interindividual variability (table 1) the average concentration of CAA in the group treated with MB was similar to the one without MB, indicating that MB does not inhibit the formation of CAA and does not stimulate its elimination.

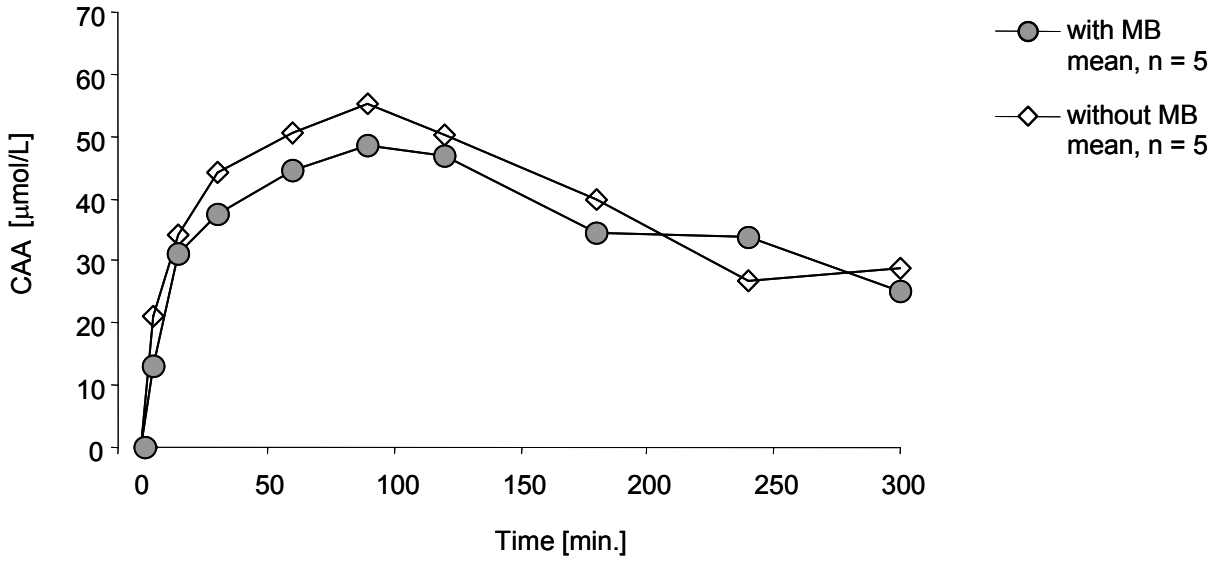


Figure 7: Time course of plasma CAA concentration in 5 rats following administration of IF with and without pre-treatment of MB. Closed circles represent mean values obtained from the rats treated with IF plus MB, while the other curve shows the data from the ones treated with IF only.

Time [min.]	CAA [µmol/L]	CAA [µmol/L]
	without MB	**with MB**
0	0.00	0.00
5	21.03 +/- 16.12	13.08 +/- 11.47
15	34.12 +/- 20.79	31.02 +/- 22.13
30	44.28 +/- 30.46	37.43 +/- 25.43
60	50.73 +/- 25.04	44.63 +/- 31.50
90	55.34 +/- 33.50	48.49 +/- 31.65
120	50.19 +/- 25.09	46.88 +/- 44.12
180	39.97 +/- 16.03	34.35 +/- 21.18
240	26.69 +/- 15.01	33.90 +/- 12.47
300	28.88 +/- 11.54	25.24 +/- 7.72

Table 1: Mean CAA values measured in the plasma of rats after IF or IF/MB over a time of 5 hours. Large interindividual differences resulted in high SD (n = 5). There are no statistical differences between the two groups.

The next question we tried to answer was whether MB would correct some of the CAA-induced changes in intermediate metabolism, in particular the metabolism of glucose, fatty acids and the activity of the Krebs cycle. Figure 8 demonstrates the time course of plasma glucose in rats treated with CAA or CAA and MB, respectively. It can be seen that within one hour the plasma glucose level steadily decreases to less than 50 % from the starting value (time point 0'). There is no significant difference observed between the group of rats with MB and the ones without MB.

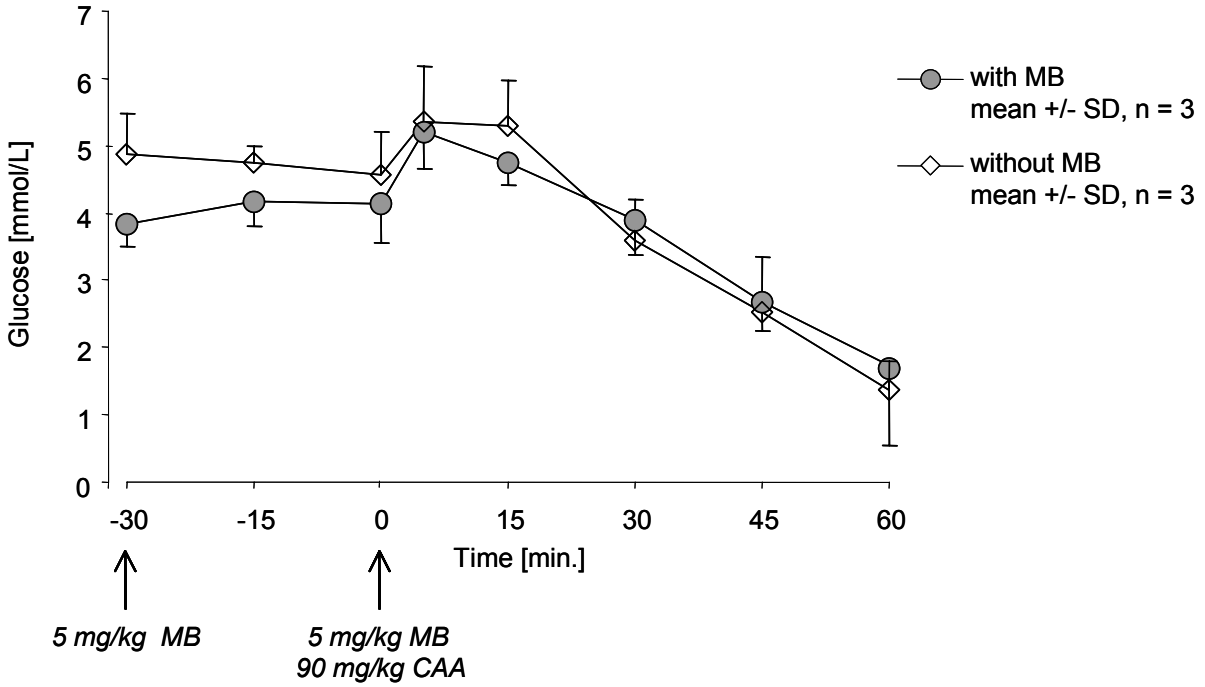


Figure 8: Time course of plasma glucose in rats treated with CAA or CAA and MB, respectively. No significant differences are observed. Values are means +/- SD, n = 3.

Time [min.]			Glucose [mmol/L]			Glucose [mmol/L]
			without MB			**with MB**
-30			4.87 +/- 0.6	*5 mg/kg MB*	→	3.83 +/- 0.3
-15			4.73 +/- 0.3			4.17 +/- 0.4
0	*90 mg/kg CAA*	→	4.57 +/- 0.6	*5 mg/kg MB* *90 mg/kg CAA*	→	4.13 +/- 0.6
5			5.37 +/- 0.8			5.20 +/- 0.5
15			5.30 +/- 0.7			4.73 +/- 0.3
30			3.60 +/- 0.6			3.90 +/- 0.5
45			2.53 +/- 0.8			2.67 +/- 0.4
60			1.37 +/- 0.4			1.73 +/- 1.2

Table 2: Mean values +/- SD for plasma glucose concentration in the two groups (n = 3). There are no statistical differences between the two groups, but both groups are at time point 60' different from the starting value (P = 0.05).

Table 2 lists the absolute values obtained from the described experiments. Administration of MB alone does not seem to influence the glucose level, as can be seen in the group with MB (-30' to 0').

In order to determine whether a decreased gluconeogenesis was responsible for the decreasing plasma concentrations of glucose the release of glucose by isolated hepatocytes was studied next. Up to 60 minutes of incubation the concentration of glucose in the medium increased in a linear fashion and tended to be higher in the presence of CAA (figure 9). The addition of MB had no effect on the glucose concentration.

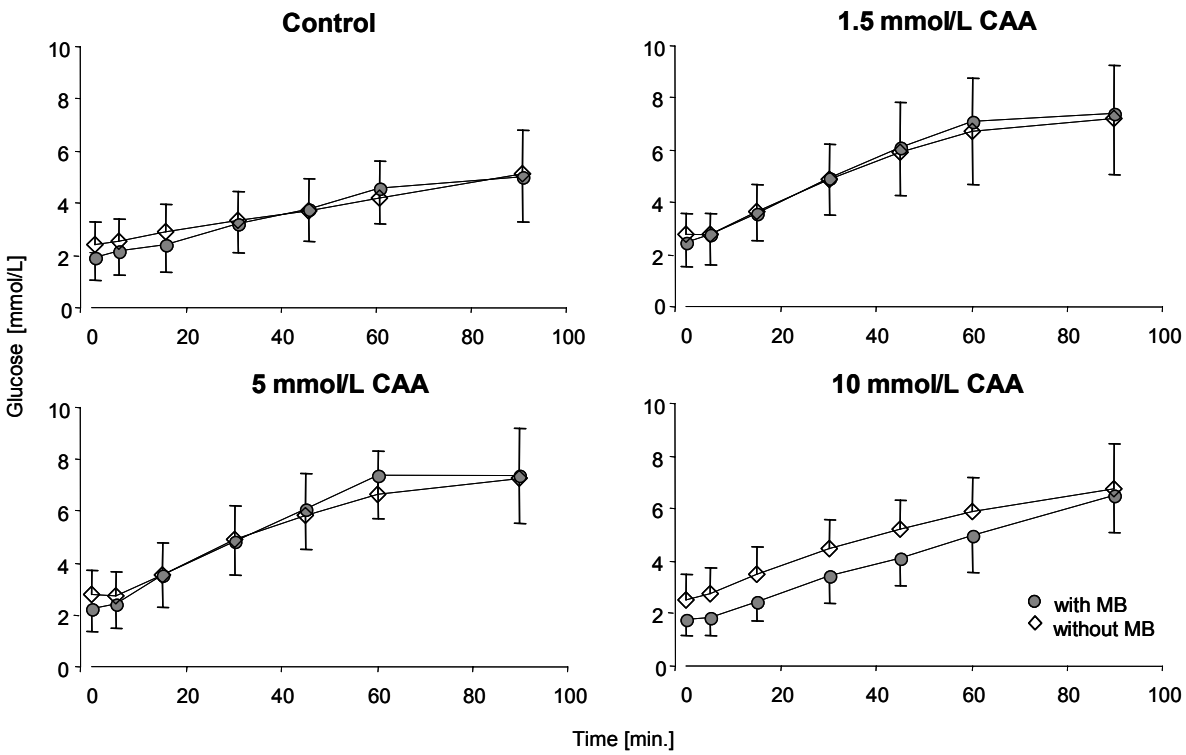


Figure 9: Time course of glucose concentration in the medium of isolated rat hepatocytes from three different experiments with and without addition of MB. There is no difference between incubations with and without MB. However, all endpoint levels are significantly ($P < 0.05$) higher than the corresponding starting value. No difference was found between the control and the CAA experiments. Values are means +/- SD, n = 3.

The maintenance of the glucose production is surprising since the viability of the hepatocytes as assessed by trypan blue exclusion was decreased in the presence of CAA (figure 10) and GSH was rapidly depleted (figure 11).

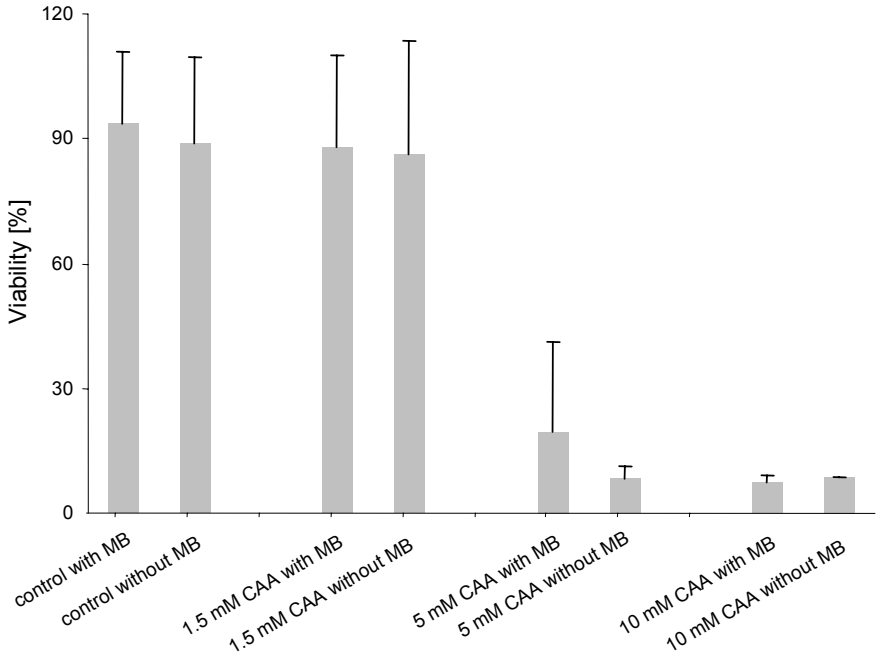


Figure 10: CAA-induced cytotoxicity in isolated rat hepatocytes after an incubation time of 1h. The viability of cells exposed to 5 mmol/L CAA or more is markedly decreased. Moreover, this bar graph shows that there is no difference between the cells with or without MB. Values are means +/- SD, n = 3.

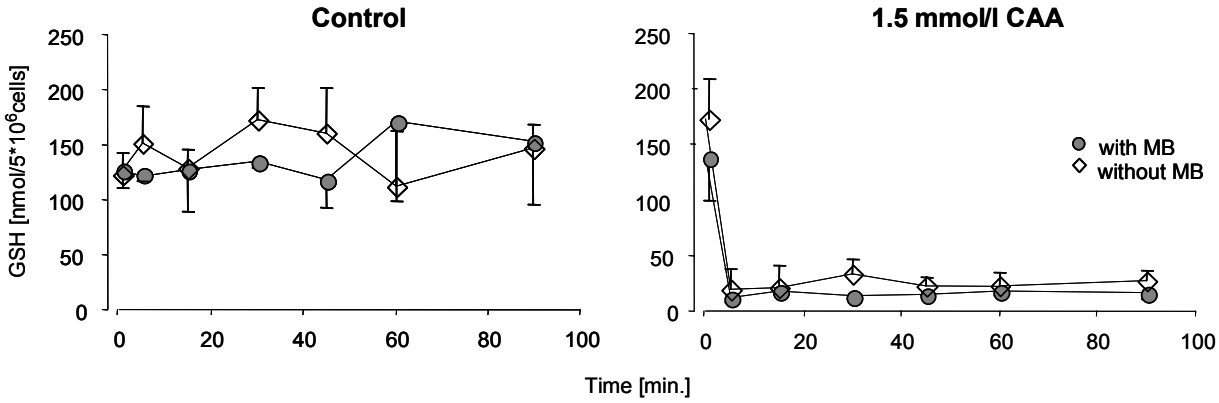


Figure 11: GSH levels were rapidly decreased in the presence of 1.5 mmol/L CAA compared to controls and significantly ($P < 0.05$) different from the starting values. No difference was found with or without MB. Values are means +/- SD, n = 3.

In the intact animals the concentration of free fatty acids in plasma decreased even more precipitously than the concentration of glucose (figure 12). There was no effect of MB on initial time course of the free fatty acids.

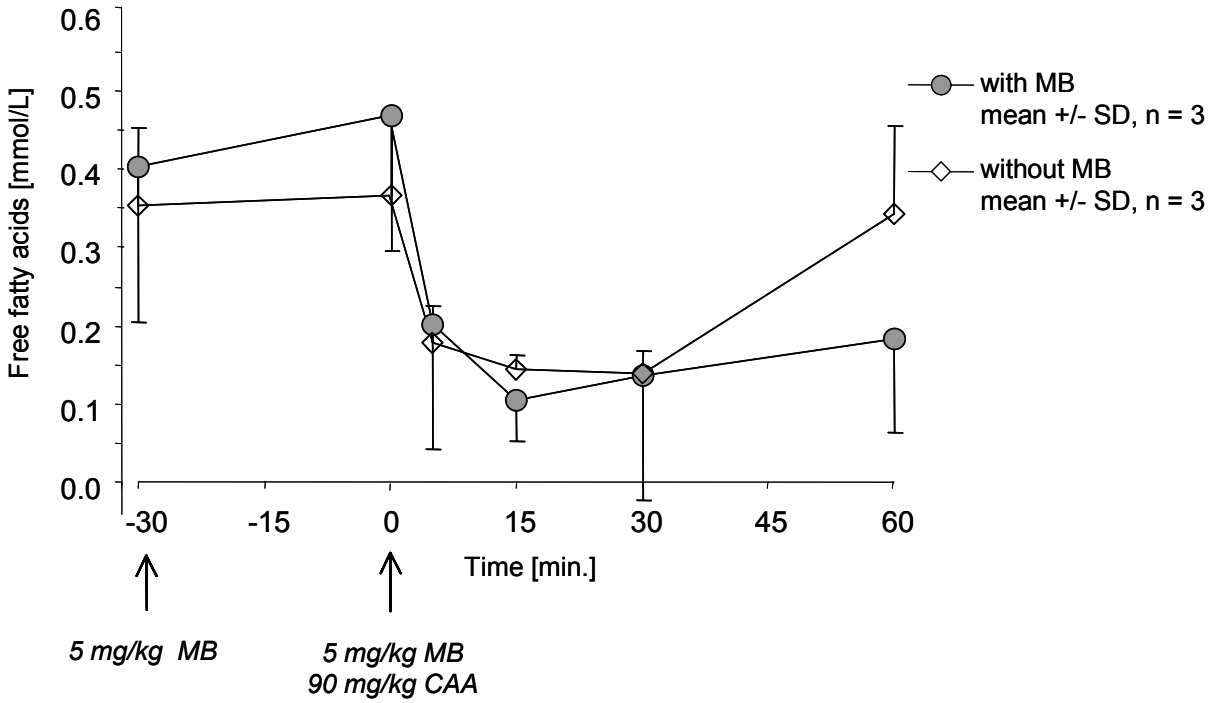


Figure 12: Time course of plasma free fatty acids in rats after administration of CAA or CAA/MB. A rapid decrease is observed within 5 minutes following addition of CAA. There are no statistical differences between the two groups. Values are means +/- SD, n = 3.

Time [min.]			free fatty acid [mmol/L]			free fatty acid [mmol/L]
			without MB			**with MB**
-30			0.35 +/- 0.10	*5 mg/kg MB*	→	0.40 +/- 0.20
0	*90 mg/kg CAA*	→	0.37 +/- 0.10	*5 mg/kg MB* *90 mg/kgCAA*	→	0.47 +/- 0.17
5			0.18 +/- 0.06			0.20 +/- 0.16
15			0.14 +/- 0.02			0.11 +/- 0.05
30			0.14 +/- 0.03			0.13 +/- 0.16
60			0.34 +/- 0.11			0.18 +/- 0.12

Table 3: Values for plasma free fatty acids in the two groups without and with MB. There are no statistical differences between the two groups. Values are means +/- SD, n = 3.

The influence of CAA and CEA with or without MB on oxidation of [^{14}C]acetate was studied in three different hepatocytes preparations. The oxidation was measured by trapping the CO_2 generated in the Krebs cycle. In figure 13 can be seen that there is a significant difference between incubations with or without MB in all groups, except in the 1 mmol/L CAA group. The oxidation of acetate was consistently inhibited by MB, which is contrary to the expectation.

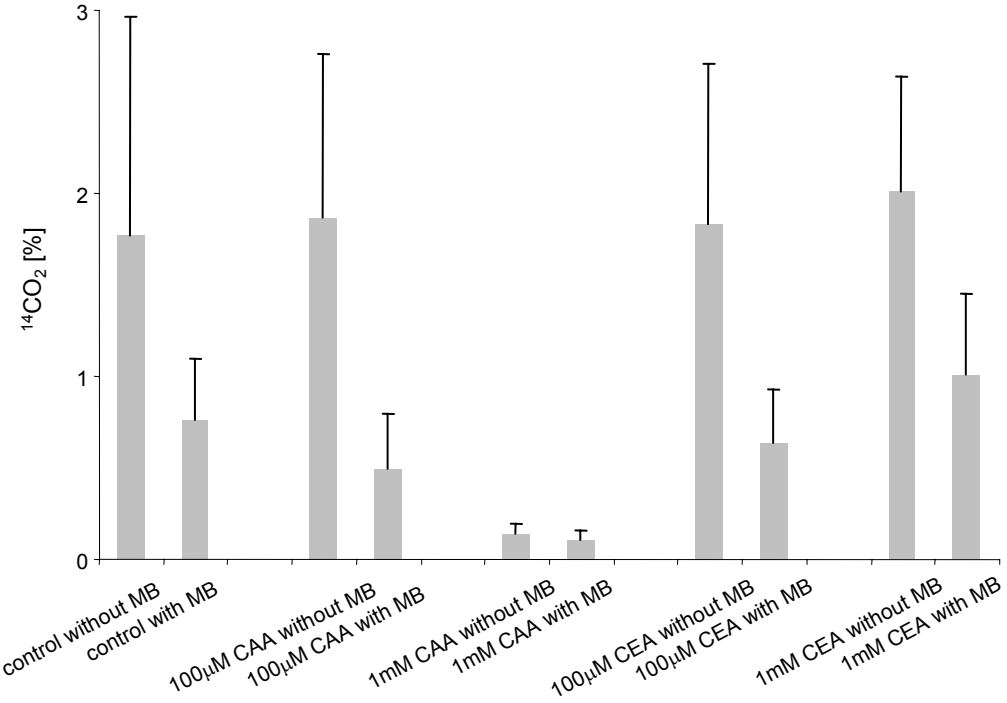


Figure 13: [^{14}C]acetate oxidation in isolated hepatocytes is impaired by MB. There is a statistically significant ($P < 0.05$) difference between incubations with or without MB, except when 1 mmol /L CAA were used. A concentration of 1 mmol/L CAA results in a strong inhibition. Values represent means +/- SD, n = 3.

2.5 Discussion

The present data show that the circulating concentration of CAA reaches substantial levels in rats after administration of IF. There was a large interindividual variability which may in part be due to analytical problems. CAA has a very short half-life ex vivo. Although meticulous attention was paid to rapid stabilization of CAA with formaldehyde and rapid derivatization the instability of CAA may in part account for the variability of the results. MB which could inhibit the formation of CAA from CEA by inhibiting MAO and which could stimulate the elimination of CAA by correcting the NAD:NADH ratio did not have any apparent effect on the plasma concentrations of CAA.

In order to test the hypothesis that MB could correct CAA induced disturbances of intermediate metabolism glucose and fatty acid metabolism was studied next. An increase in the NAD:NADH ratio as it may result from the metabolism of CAA may affect gluconeogenesis and β-oxidation. Oxidation of CAA by decreasing NAD^+ and increasing NADH could depress the oxidation of α-ketoglutarate to succinate, malate to oxaloacetate. Second, the availability of pyruvate for conversion to oxaloacetate and then to phosphoenolpyruvate might also be decreased by the rapid reduction of pyruvate to lactate

in the presence of increased concentrations of NADH. Madison showed that the intravenous administration of NAD^+-dependent precursors of glucose such as α-ketoglutarate and glutamate fail to increase hepatic glucose production when suppressed by ethanol [Madison et al. 1967]. Acetaldehyde inhibits gluconeogenesis in perfused dog liver and MB, due to its redox ability, reverses this perturbation [Madison et al. 1967, Madison 1968].

The observed fall in circulating glucose following administration of CAA is consistent with a decreased gluconeogenesis or an increased shift of glucose into cells. The experiments in isolated hepatocytes did not show a decreased synthesis of glucose from alanine in the presence of CAA, thus arguing against a decreased gluconeogenesis. However, this does not rule out the possibility that in vivo a limited availability of fatty acids may be responsible for the gradual decline in plasma glucose. Indeed, there was a marked decrease in free fatty acids following the administration of CAA. This was somewhat unexpected because an inhibition of β-oxidation and thus an increase in free fatty acids was expected based on previous experiments [Visarius et al. 1999]. The activity of the Krebs cycle as reflected by the in vitro oxidation of acetate was markedly impaired by CAA. Also the intracellular concentration of GSH was rapidly depleted by CAA. This finding is in agreement with other studies in isolated rat hepatocytes [Sood and O'Brien 1994].

MB was not able to correct any of the CAA-induced disturbances of intermediate metabolism, sulfhydrile homeostasis or cell viability. The failure to demonstrate an effect of MB could be due to the chosen experimental conditions that do not closely reflect the situation in patients treated with IF that may be different in regard to the concentrations and the time course of the various compounds of interest. However, failing to see an effect of MB in the presented experiments may also be due to the fact that disturbances of intermediate metabolism are not critical in IF-induced encephalopathy but that the formation of compounds with central nervous activity which may be modulated by MB is more important. To test this hypothesis the following experiments were performed.

3 Ifosfamide and mono-chlorinated 1,2,3,4-tetrahydro-β-carbolines

3.1 Summary

Tetrahydro-β-carbolines (THβCs) possess central nervous activity and are probably formed after administration of IF by reaction with CAA and indoleethylamines. Therefore, mono-chlorinated THβCs were synthesized from the toxic IF metabolite CAA and tryptophan, tryptamine and serotonin, respectively. The compounds were characterized by chromatographic and spectral analysis, ESI-MS and ESI-MSMS and NMR. The presence of THβC in the urine of patients receiving IF could not be demonstrated. Animal experiments revealed that after i.p. administration the unchanged THβC formed from tryptamine is excreted in urine to 38 % whereas after IF administration no THβCs were found with the methods used.

3.2 Introduction

CAA is considered to be responsible for the neurotoxic adverse effects of IF. It is hypothesized that CAA reacts with endogenous substrates to form compounds with central nervous system activity. Other aldehydes, such as acetaldehyde, formaldehyde and chloral hydrate, are known to react with indoleethylamines, such as tryptophan, tryptamine and serotonin to form pharmacologically active THβCs [Brossi et al. 1973, Bringmann et al. 1991, Adachi et al. 1993]. THβCs are naturally occurring tricyclic nitrogen heterocycles formed by the Pictet-Spengler condensation [Hahn and Ludewig 1934, Soerens et al. 1987, Kuehne et al. 1985, Laronze et al. 1991]. The chemical reaction consists of the formation of a Schiff base or imine formation between the aldehyde and the amino acid or biogenic amine followed by spontaneous cyclisation to give the nitrogen containing THβC (figure 14). Structurally the THβCs are alkaloids. Harmine and harmaline are naturally occurring β-carbolines (βCs) widely distributed in plants, originally isolated from *Peganum harmala* and *Banisteriopsis caapi* [Rätsch 1998]. The presence of βC type compounds has also been reported in food [Adachi et al. 1991, Herraiz 1999, Herraiz 2000a, Herraiz 2000b, Diem and Herderich 2001] and in rat brain [Barker et al. 1979]. βCs have been investigated increasingly in humans [Airaksinen and Kari 1981a, Airaksinen and Kari 1981b, Schouten and Bruinvels 1985, Adachi et al. 1991, Rommelspacher et al. 1991, Stohler et al. 1993, Horiuchi et al. 1994, Tsuchiya et al. 1995, Musshoff et al. 1996, Pari et al. 2000], where they were found to be endogenously present in trace amounts. They are therefore considered to represent so-called mammalian alkaloids. βCs exhibit a variety of pharmacological effects that could therefore be involved in the pathogenesis of different diseases. βCs have been studied in relation to alcoholism [Adachi et al. 1993] and their participation in the

pathogenesis of neurological symptoms has been postulated [Janetzky et al. 1999, Bringmann et al. 2000]. βC derivatives have also been implicated in the pathogenesis of Parkinson's disease [Collins and Neafsey 1985, Bringmann et al. 2002]. Some THβCs are described to be inhibitors of complex I of the respiratory chain in mitochondria (see chapter 6). Moreover, they are known to have some potential to inhibit monoamine oxidase (see chapter 4) and to interact with benzodiazepine receptors (see chapter 5).

Since the plasma concentrations of CAA reach values of up to 0.22 mmol/L in patients receiving IF [Cerny and Küpfer 1989] and the indoleethylamines tryptamine, tryptophan and serotonin are widely distributed in humans, the formation of chlorinated THβCs seems probable in these patients. Moreover, there is evidence that tryptophan is a precursor for βC-type compounds [Fekkes et al. 2001]. We hypothesize that CAA like acetaldehyde and formaldehyde reacts with amino acids and biogenic amines to form THβCs in vivo as indicated in figure 14. Following the consumption of ethanol that results in the formation of THβCs the concentration of acetaldehyde is orders of magnitude lower. If CAA is either generated in the brain or able to pass the blood-brain barrier chlorinated THβCs might be formed in the human brain, where they might be responsible for the central nervous adverse effects of IF.

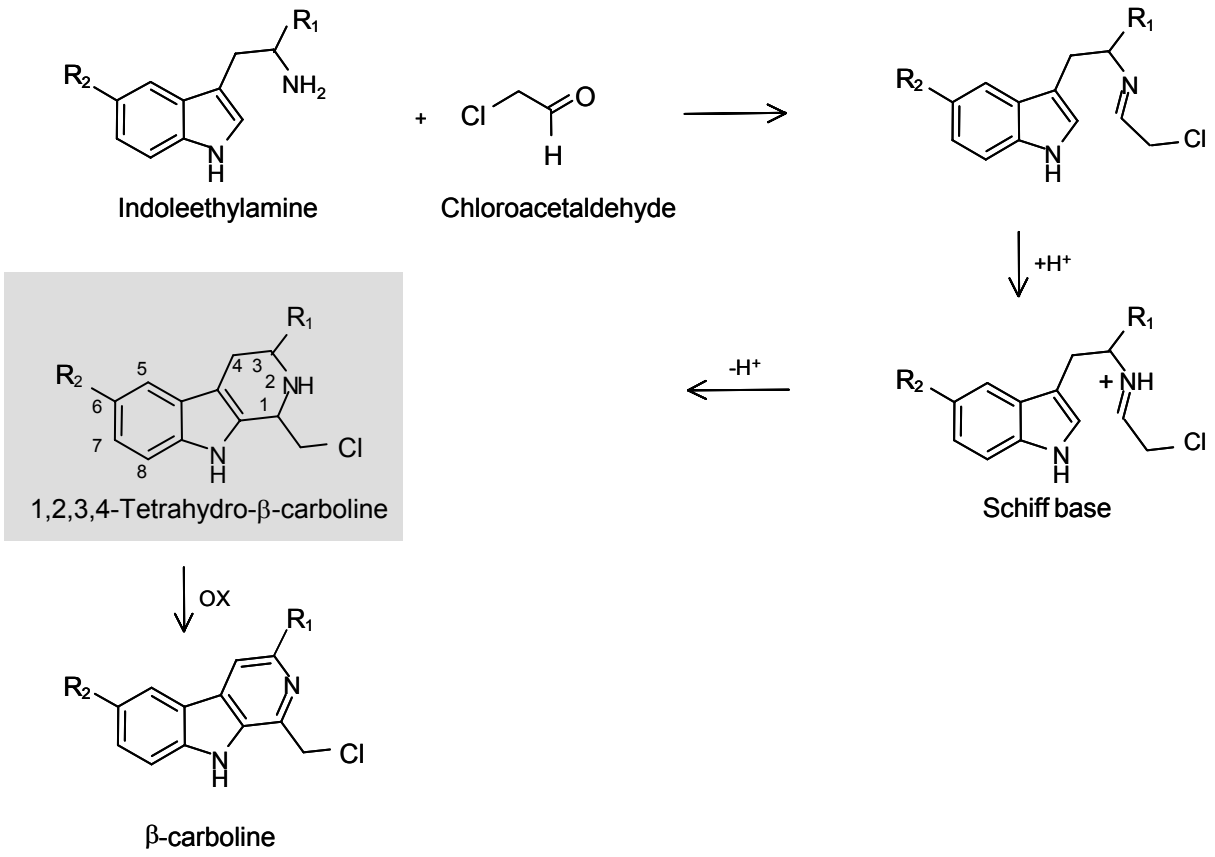


Figure 14: Pictet-Spengler reaction between an indoleethylamine and CAA to give a THβC. Tryptophan R_1 = COOH, R_2 = H; tryptamine R_1 = H, R_2 = H; serotonin R_1 = H, R_2 = OH. Oxidation of THβCs results in βCs. Compounds with aromatic pyrido rings are called βCs and compounds with a reduced pyrido ring THβCs [Herraiz 2000b]. βCs have a nitrogen at position 2 of the ring compared to α- or γ-carbolines which have the nitrogen at position 1 and 3, respectively.

3.3 Material and methods

Chemicals.

L-tryptophan was purchased from Merck (Darmstadt, Germany), serotonin hydrochloride and tryptamine hydrochloride were from Fluka (Buchs, Switzerland) as well as CEA hydrochloride and CAA-solution 45 % (w/v) in water. Harmaline and harmine, commercially available model carbolines, were from Aldrich (Sigma-Aldrich, Buchs, Switzerland). Acetonitrile and methanol (supra-gradient quality) were from Biosolve Ltd. (Valkenswaard, The Netherlands), formic acid 100 % p.a. and acetic acid glacial 100 % p.a. from Merck (Darmstadt, Germany). The water was of Purelab Option E (Labtec, Wohlen, Switzerland) quality (filtered and UV light treated).

Synthesis.

All three THβC from the reaction between CAA and tryptophan, tryptamine and serotonin, respectively, were prepared according to Julia [Julia et al. 1973].

Chromatographic and spectral analysis of the THβCs.

Fluorescence excitation and emission spectra of the three synthesized THβCs as well as of harmine and harmaline were performed on a PerkinElmer luminescence spectrophotometer LS 50 B (PerkinElmer Analytical Instruments, Shelton, USA) . All compounds were dissolved in methanol. From these results the wavelength excitation was set to 270 nm, the emission to 350 nm for the HPLC-fluorescence analysis of the THβCs.

The analysis of the synthesized THβCs by RP HPLC and fluorescence detection was performed using a Nucleosil C_{18} RP 100-5 column (250*4 mm i.d.) (Macherey-Nagel AG, Oensingen, Switzerland), a Waters chromatography pump model M-6000 (Waters, Milford, USA), a variable-wavelength fluorescence detector (Hitachi F-1000, San Jose, USA) and a ChemStation (Hewlett Packard, Palo Alto, USA) software. The mobile phase was acetonitrile-water (20:80) with 0.1 % formic acid and was delivered at a flow rate of 0.8 ml/min.

ESI-MS and LC-ESI-MS.

MS measurements were performed on a Finnigan LCQ ion trap instrument (Finnigan MAT, San Jose, USA) equipped with an electrospray-ionization (ESI) (Finnigan) source that was run in the positive ion mode (4.5 kV). Sheath gas (N_2) flow rate was set at 30 arbitrary units. The temperature of the heated capillary was 200°C. The instrument was computer controlled using the XCalibur 1.0 software Finnigan. Full scan mass spectra were acquired for positive ions in the mass range up to 400. Automatic gain control (AGC) was employed using 3 microscans and a maximum injection time of 200 ms. MSMS was performed with an isolation width of 2 and a relative collision energy of 25 %. For the identification and characterisation of the synthesized mono-chlorinated THβCs stock solutions of 100 μmol/L in methanol-water (50:50) with 0.1 % acetic acid were used.

For the LC-ESI-MS experiments a Hewlett Packard 1100 series quaternary pump and a Agilent 1100 series automatic sample injector were used. The chromatographic system consisted of a Nucleosil C_{18} RP 100-5 column (250*4.6 mm i.d.) (Macherey-Nagel AG, Oensingen, Switzerland). The mobile phase was acetonitrile-water (25:75) with 0.1 % formic acid and was delivered at a flow rate of 0.8 ml/min. The eluate from the HPLC column was directly passed into the ionization region.

NMR analysis.

All experiments were performed in the Department of Organic Chemistry, University of Berne, and carried out by Dr. F. Fischer.

Animals.

Experiment 1: CEA is a precursor of CAA during the activation/detoxification of IF and is probably partly converted into CAA via monoamine oxidase [Aeschlimann et al. 1996]. As CAA is a highly irritant substance its precursor CEA was chosen for this experiment, in particular because the animals were not anaesthetised. 200 mg/kg (1.7 mmol/kg) CEA hydrochloride was given to three rats (in 0.9 NaCl i.p.) as a potential source of CAA and accordingly also of βC type compounds. The amount of CEA corresponds to the amount of CAA expected to be generated following administration of approximately 1 g/kg IF. The rats were placed in a metabolic cage and urine was collected over 24 h and examined for the presence of the THβCs formed from tryptophan, tryptamine or serotonin by HPLC with fluorescent detection (all parameters are described in *Chromatographic and spectral analysis of the THβCs*). Three untreated rats (0.9 % NaCl i.p.) served as control.

Experiment 2: In a second experiment three rats received 337 mg/kg tryptamine hydrochloride (1.7 mmol/kg tryptamine) in addition to CEA. Three untreated rats (0.9 % NaCl) served as control. Analysis were performed as described in experiment 1.

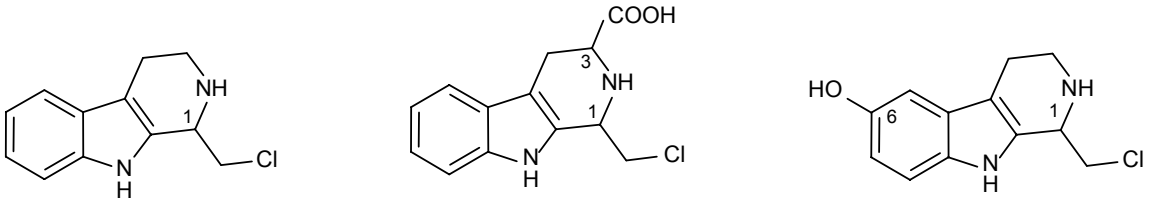
Experiment 3: One rat was i.p. injected with 50 mg/kg (226 μmol/kg) 1,2,3,4-tetrahydro-β-carboline-1-chloromethyl (THβC-1-clm.). Another rat received 800 mg/kg (3.07 mmol/kg) IF p.o.. The amount of THβC corresponds to the amount of CAA expected to be generated from the administrated dose of IF. Both animals were kept in metabolic cages for 6 h. The collected urine was centrifuged and the supernatant was cleaned with a Waters C_{18} SepPak cartridge. Briefly, the cartridge was conditioned with 2 ml methanol and 2 ml water. A measure of 1 ml urine was then loaded onto and slowly drawn through the cartridge. Prior to elution with 2 ml methanol the SepPak cartridge was washed with 2 ml of water. The eluate was dried under a stream of air and re-dissolved in 1 ml of methanol-water (50:50) with 1 % formic acid. The sample was then examined for the presence of THβC-1-clm. with LC-ESI-MS.

Patients.

The urine of three patients receiving IF was examined for the presence of THβCs by HPLC with fluorescence detection as well as with LC-ESI-MS/MS under the conditions described.

3.4 Results

The formation of the mono-chlorinated THβCs was achieved by incubation of tryptophan, tryptamine or serotonin with two equivalents of CAA (figure 15).



1,2,3,4-tetrahydro-β-carboline-1-chloromethyl (THβC-1-clm.)

1,2,3,4-tetrahydro-β-carboline-1-chloromethyl-3-carboxy (THβC-1-clm.-3-c.)

6-hydroxy-1,2,3,4-tetrahydro-β-carboline-1-chloromethyl (OH-THβC-1-clm.)

Figure 15: Chemical structures of the sythesized THβCs.

The newly synthesized THβCs were identified and characterized by spectral and chromatographic analysis (fluorescence, HPLC), ESI-MS and LC-ESI-MS and NMR. The determination of the excitation and emission wavelength maxima served as rough estimation for the subsequent analysis by HPLC. The fluorescence detector was accordingly set to 270 nm for the excitation and 350 nm for emission for the following separation of the new THβCs.

compound	*maximum excitation [nm]*	*maximum emission [nm]*
THβC-1-clm.-3-c.	219	345
THBC-1-clm.	221	349
OH-THβC-1-clm.	230	358
Harmaline	243	418
Harmine	371	478

Table 4: Excitation and emission wavelength for the three synthesized mono-chlorinated THβCs as well as for the two naturally occurring compounds harmaline and harmine. All THβCs exhibited a smaller second excitation peak with the wavelength 270 nm, which was subsequently used for all analyses.

All mono-chlorinated THβCs as well as the starting compounds serotonin, tryptophan and tryptamine were found to be baseline separated with the HPLC method used.

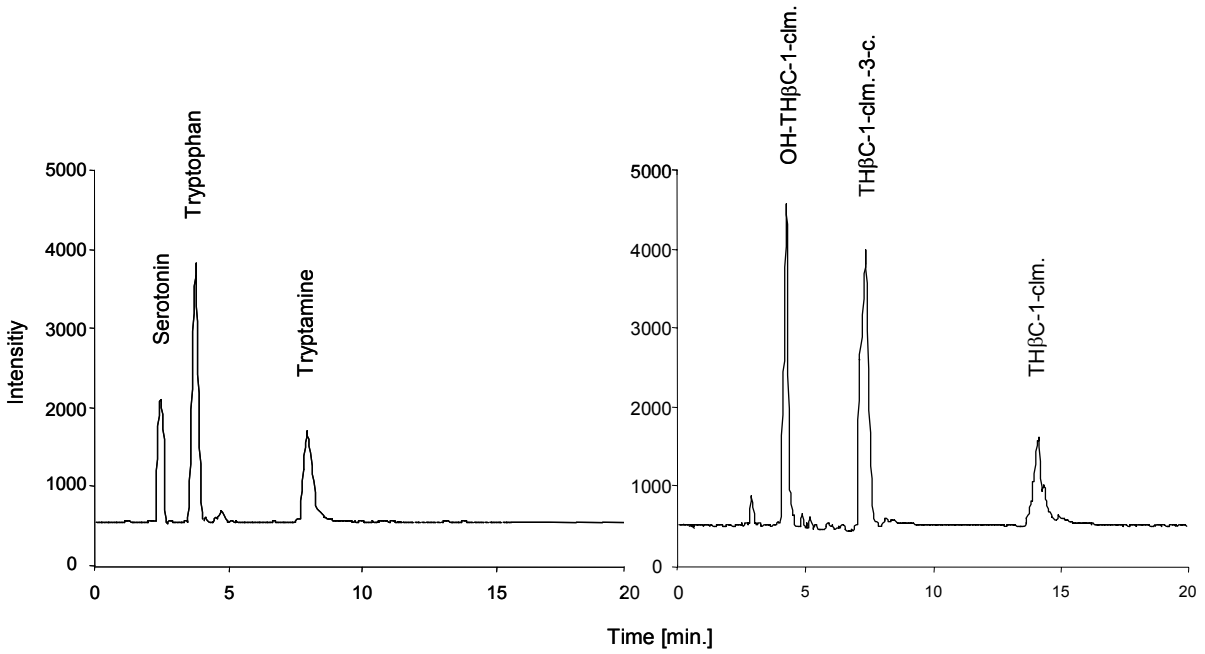


Figure 16: Chromatographic analysis of mono-chlorinated THβCs. In the left fluorescence chromatogram, the three starting substances tryptophan, serotonin and tryptamine are baseline separated, as well as the three mono-chlorinated THβCs in the right chromatogram.

The synthesized THβCs were unambiguously identified and characterized by means of ESI-MS and ESI-MSMS experiments. All solutions were continuously delivered at a flow rate of 10 μl/min by means of a syringe pump system. Mass trace measurements revealed intact protonated parent masses. MSMS experiments of the THβCs resulted in the typical Retro-Diels-Alder (RDA) fragmentation of the tetrahydropyrido moiety with concomitant neutral loss of 73 amu for the tryptophan derived THβC [Gutsche and Herderich 1997, Gutsche et al. 1999]. Accordingly, Pictet-Spengler condensation products from CAA with tryptamine or serotonin exhibited typical product ions by neutral loss of 29 amu, as can be seen in figure 17.

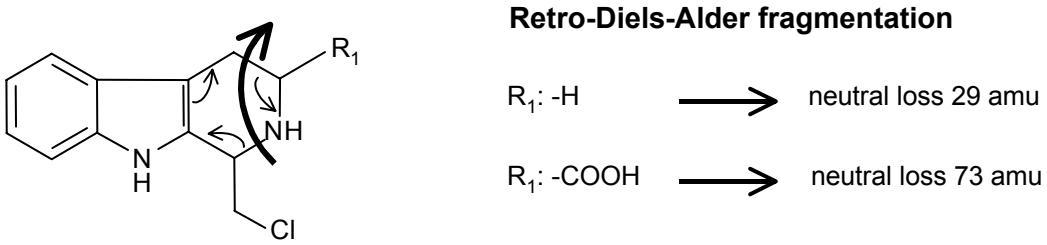


Figure 17: Molecular structure of THβCs and characteristic RDA fragmentation. Tryptamine and serotonin THβCs result in a loss of 29 amu, whereas the tryptophan THβC looses 73 amu.

The following three figures (figures 18-20) represent the ESI-MS and ESI-MSMS spectra for the three synthesized THβCs. Figure 18 provides details for THβC-1-clm.-3-c..

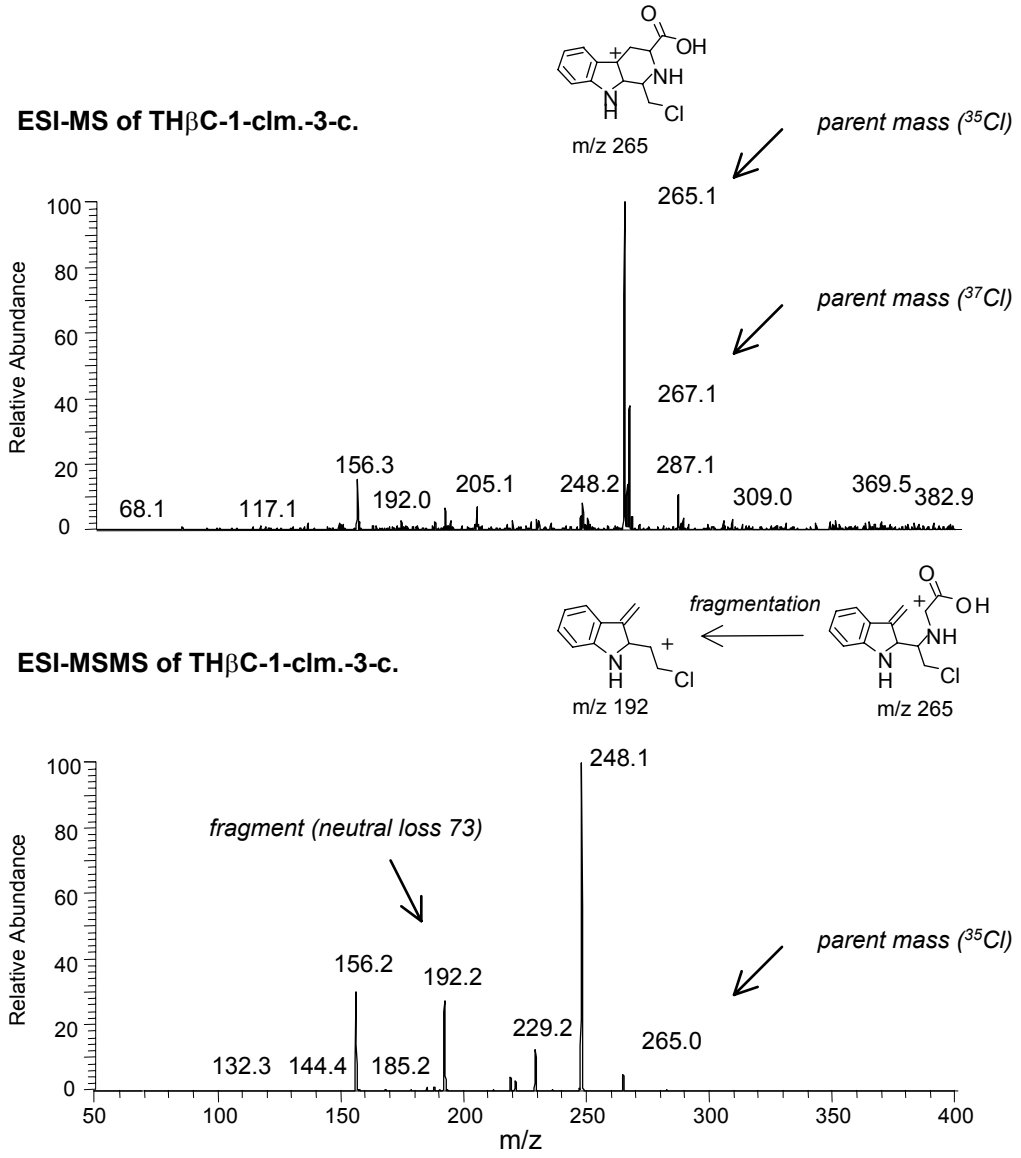


Figure 18: MS spectrum (top graph) and MSMS spectrum (bottom graph) of 1,2,3,4-tetrahydro-β-carboline-1-chloromethyl-3-carboxy. The RDA fragmentation of the protonated parent mass results in the characteristic product ion m/z = 192 (neutral loss of 73).

The most abundant peak in the upper part, the parent mass 265 m/z, represents a THβC-1-clm.-3-c. molecule with a chlorine atom of the mass 35. The natural isotope distribution of chlorine is 75 % for ^{35}Cl and 25 % for ^{37}Cl which can be seen with the masses m/z 265 and 267. The MSMS spectrum shows, among others, the transition m/z 265 → 192 which originates from the protonated molecule (m/z 265, 267 respectively) by the neutral loss of the iminoacetic acid $C_2H_3NO_2$ (-73) via RDA fragmentation.

RDA reaction of the THβC formed from tryptamine and CAA resulted in the loss of the CH_2=NH moiety (-29) as can bee seen in figure 19.

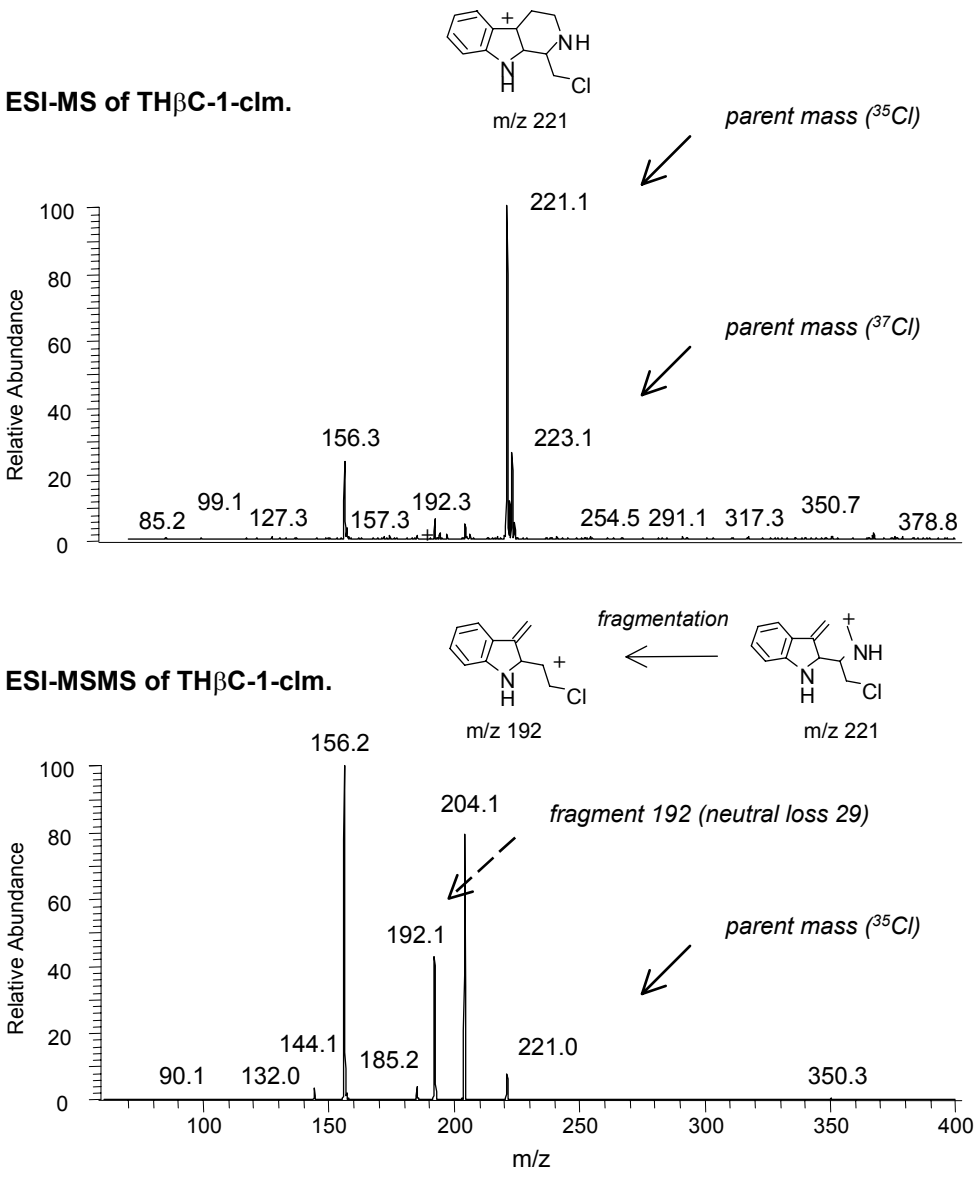


Figure 19: MS spectrum (top graph) and MSMS spectrum (bottom graph) of 1,2,3,4-tetrahydro-β-carboline-1-chloromethyl. A neutral loss of 29 results in the product ion m/z 192.

Figure 20 provides the ESI-MS and ESI-MSMS spectra for the THβC from the reaction of serotonin with CAA.

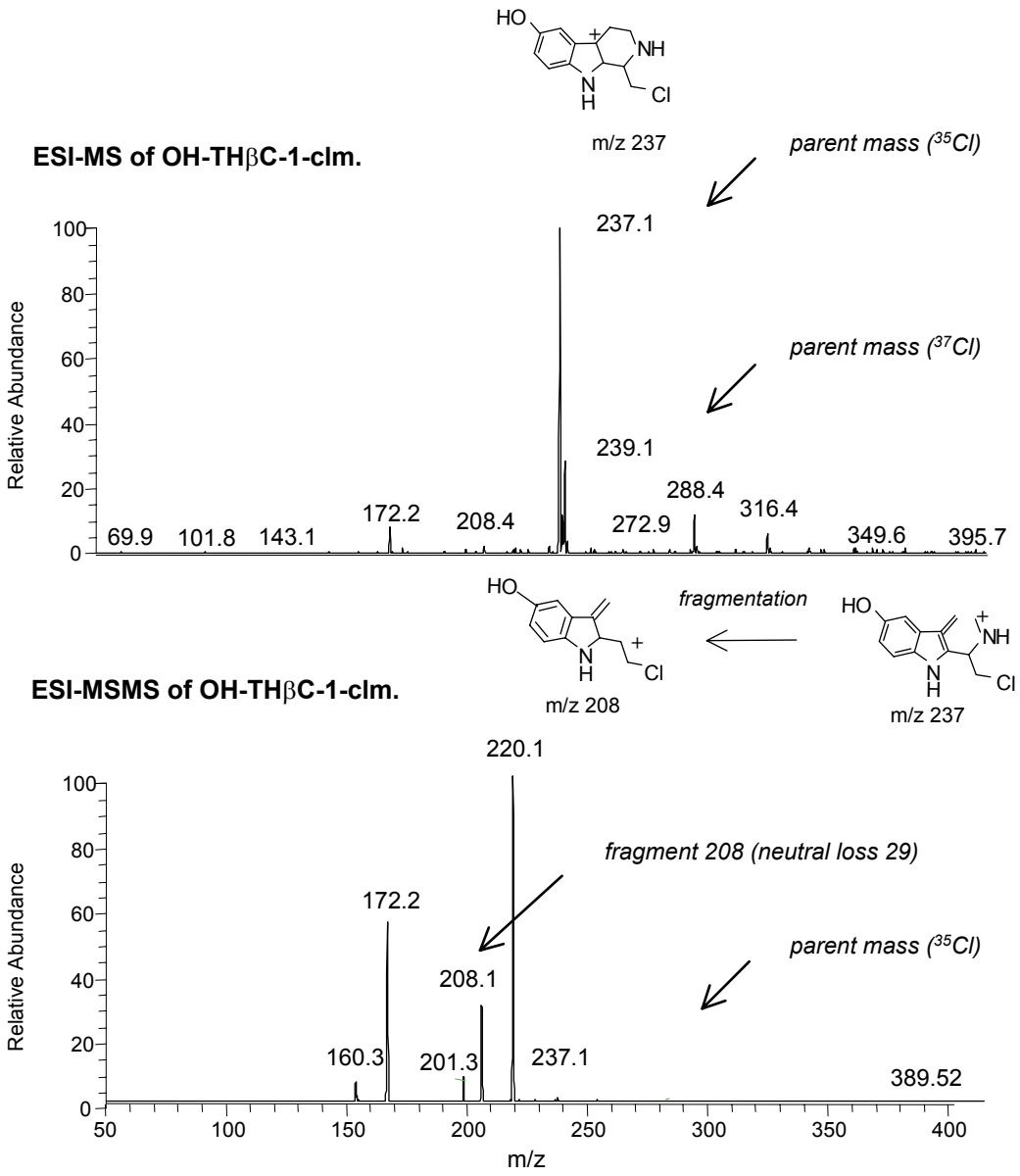


Figure 20: ESI-MS and ESI-MSMS data obtained for the THβC from the reaction with serotonin and CAA. RDA fragmentation results in a loss of 29 amu.

Preparation and data of THβC-1-clm. ($C_{12}H_{13}ClN_2$, M = 220.70):

6.4 g of tryptamine and 400 ml H_2O were mixed. 44 ml 2 N HCl was added and stirred at room temperature till tryptamine was dissolved. After filtration 6.6 ml CAA (45 % in water) were added and the mixture was stirred at 80°C during 1h. The reaction solvent was stored for 48 h and there-after pale brown tin needles were isolated. ^{1}H-NMR (300 MHz, d_6-DMSO): δ = 11.36 (s, 1H), 10.3 (bs, 1H), 9.55 (bs, 1H), 7.49 (d, *J* = 7.7 Hz, 1H); 7.4 (d, *J* = 8.1 Hz, 1H), 7.17 (td, *J* = 8.1/1.1 Hz, 1H), 7.04 (td, *J* = 7.7/0.7 Hz, 1H), 5.1 (bd, *J* = 4.4, 1H), 4.47

(dd, *J* = 12.5/2.9 Hz, 1H), 4.30 (dd, *J* = 12.5/7.0, 1H); 3.61 (dt, *J* = 10.6/4.8 Hz, 1H); 3.35 (m, 1H); 2.9 (m, 2H); ^{13}C-NMR (75 MHz, d_6-DMSO): δ = 137.3, 127.5, 126.6, 123.2, 120.1, 119.2, 112.4, 108.5, 53.7, 43.6, 41.6, 40.8, 18.3; ESI-MS: M+1 221 (100), 223 (37); ESI-MSMS 221 (8), 204 (80), 192 (43), 156 (100), 144 (4).

Preparation and data of THβC-1-clm.-3-c. ($C_{13}H_{13}ClN_2O_2$, M = 264.71):

2.04 g L-tryptophan was mixed with 100 ml H_2O, 11 ml 2 N HCl-solution and 1.65 ml CAA (45 % in water) were added. After stirring for 12 h at room temperature the clear pale yellow product solvent was freeze dried and re-crystallised twice in EtOH at -20°C and white material (38 % yield after one re-crystallisation) was collected. ^{1}H-NMR (300 MHz, d_6-DMSO): δ = 10.94 (s, 1H), 7.43 (d, *J* = 7.7 Hz, 1H), 7.33 (d, *J* = 8.1 Hz, 1H), 7.07 (td, *J* = 7.0/1.1 Hz, 1H), 6.98 (td, 7.7/1.1 Hz, 1H), 4.49 (bd, 1H), 4.25 (dd, *J* = 11.0/2.9 Hz, 1H), 3.97 (dd, *J* = 11.0/6.6 Hz, 1H), 3.69 (dd, *J* = 11.0/4.0 Hz, 1H), 3.0 (ddd, *J* = 15.1/3.9/1.3 Hz, 1H), 2.64 (ddd, *J* = 15.1/11.0/2.6 Hz, 1H); ^{13}C-NMR (75 MHz, d_6-DMSO): δ = 173.7, 136.3, 132.2, 126.6, 121.4, 118.9, 118.0, 111.3, 108.3, 55.7, 53.8, 47.3, 25.1; ESI-MS: M+1 265 (100), 267 (38); ESI-MSMS 265 (8), 248 (100), 192 (28), 156 (30).

Preparation and data of OH-THβC-1-clm. ($C_{12}H_{13}ClN_2O$, M = 236.70):

2.12 g serotonin HCl and 100 ml H_2O were mixed, 10 ml 1 N HCl and 6 ml HCl 2 N were added and stirred at room temperature till serotonin was dissolved. Then 2.88 ml CAA (45 % in water) were added and the mixture stirred at 80°C/1h. The reaction solvent was stored 48 h. No solid reaction products were obtained. Therefore the reaction mixture was freeze dried and characterized.

ESI-MS: M+1 237 (100), 239 (29); ESI-MSMS 237 (1), 220 (100), 208 (27), 172 (69).

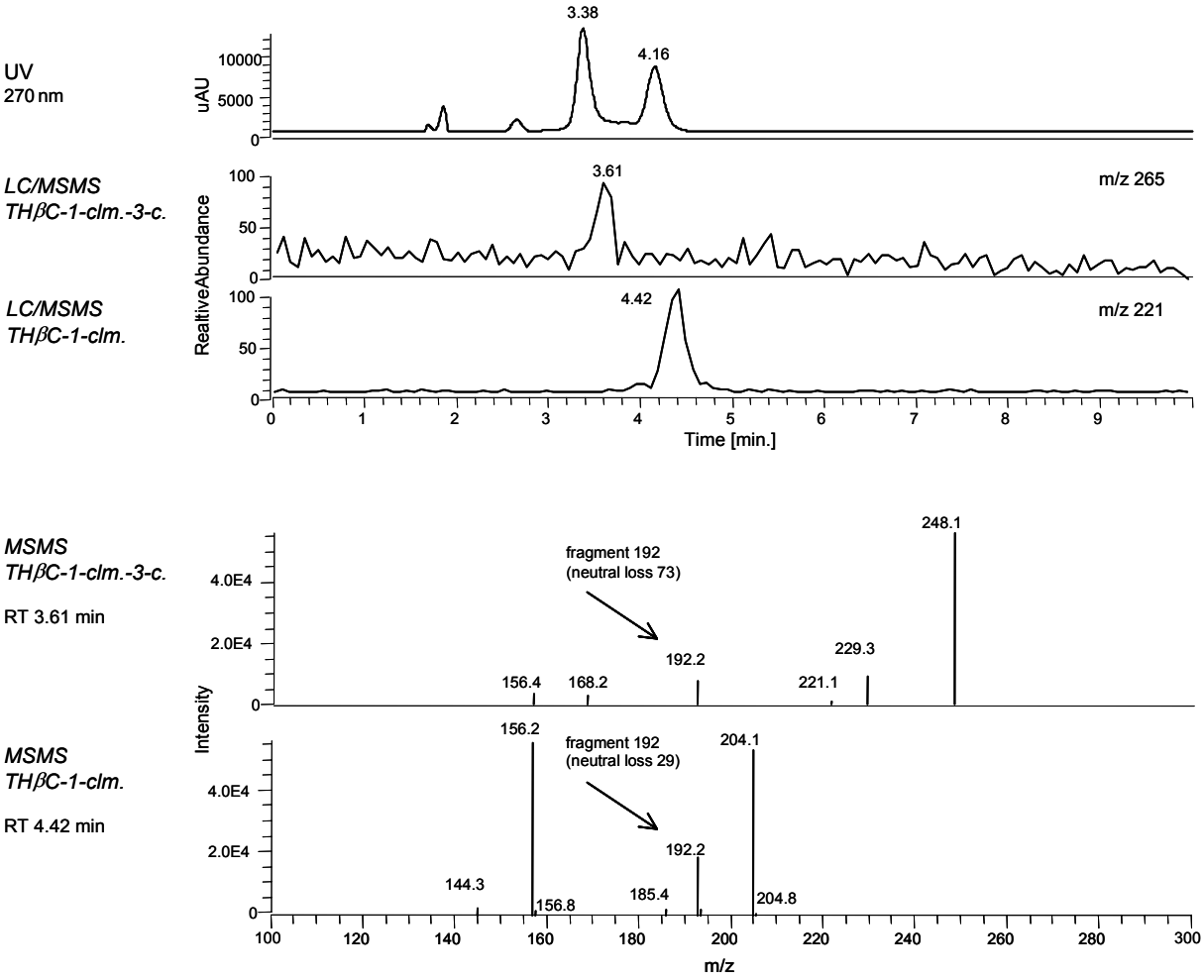


Figure 21: HPLC-UV (wavelength 270 nm) and LC/MSMS chromatograms of the THβCs originating from tryptamine (M+1 = 221) and tryptophan (M+1 = 265) and corresponding MSMS spectra. This experiment was the only one with UV detection, thus related parameters are missing in the material and methods section.

Animals.

Experiments 1 and 2: In the urine of rats receiving the CAA precursor CEA no THβCs were detected and no major difference between the sample and the control group was observed. No THβCs were found in the urine of rats receiving additional tryptamine.

Experiment 3: The recovery from the C_{18} SepPak extraction was more than 90 % (n = 3) for the THβC from tryptamine and CAA. No THβC-1-clm. was found in a 6 h urine from a rat that had received 800 mg/kg IF p.o.. On the other hand a fraction of 37.6 % of the dose of THβC-1-clm. was excreted over 6 h by a rat that had received 50 mg/kg of the synthesized THβC from the reaction of tryptamine with CAA. Although this result is obtained from a single experiment it suggests that a considerable fraction is excreted unchanged whereas it seems

unlikely that THβC-1-clm. is excreted after a high single dose of IF. Following IF administration unchanged IF was found but not quantified (data not shown).

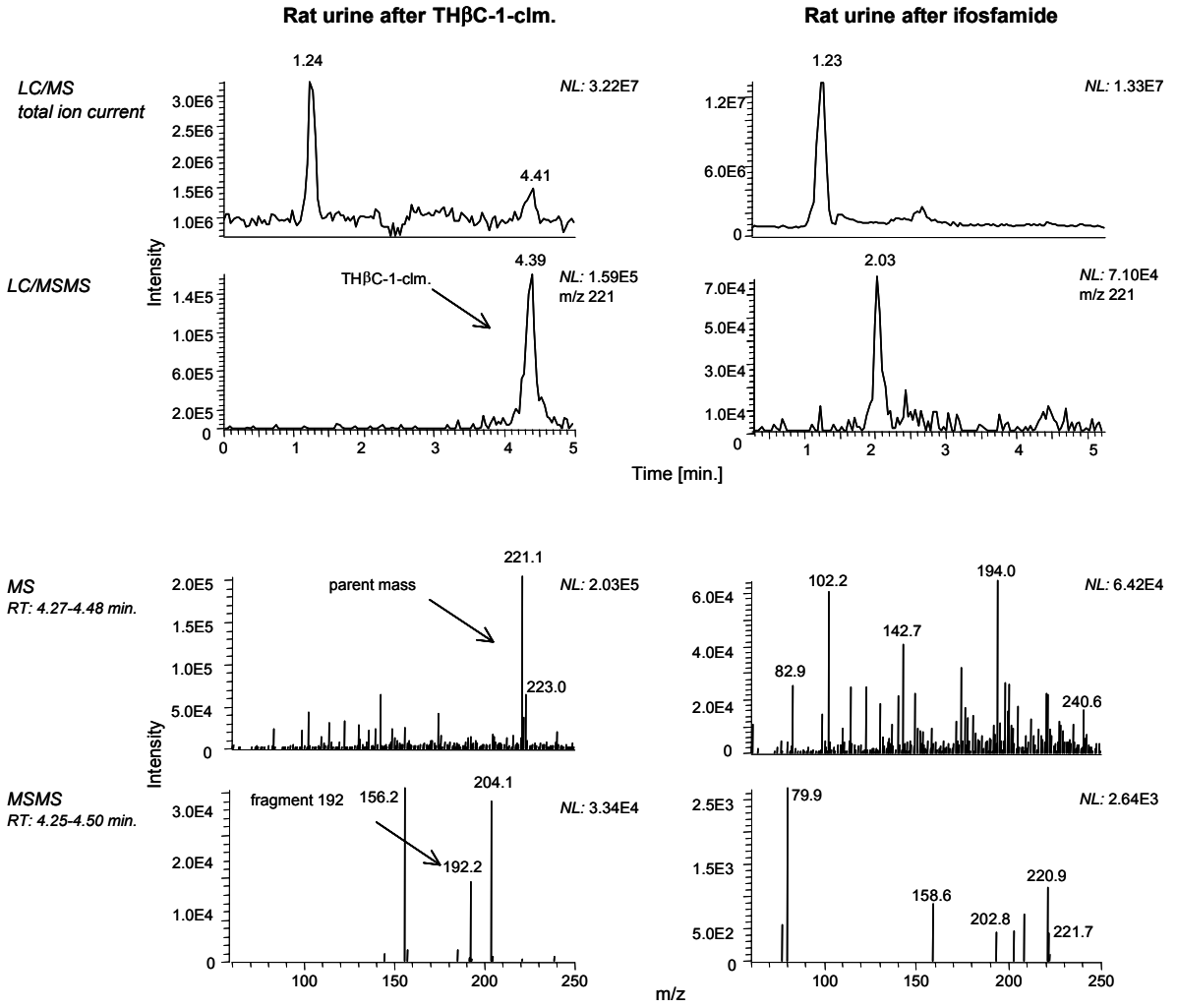


Figure 22: The left part of the graph gives details for the urine from a rat that had received 50 mg/kg THβC-1-clm. i.p., on the right data obtained from the urine of a rat that had received 800 mg/kg IF i.p. The upper part includes the chromatograms from the LC-ESI-MS (total ion current) and the LC-ESI-MSMS experiments. The lower part shows the MS and MSMS spectra. It can be seen that THβC-1-clm. is excreted only after administration of the authentic compound (retention time 4.39), whereas following IF administration no such substance is found. *NL* stands for the peak intensity of the highest peak height in the chromatogram**.**

The presence of mono-chlorinated THβC in the urine of patients receiving IF could not be demonstrated with the analytical conditions used. Urine samples of a male patient receiving a five day course of treatment with 3.3 g/m^2 IF in a 5 % glucose infusion over 24 h with concomitant Mesna with not encephalopathy, urine of a female patient receiving a two day course of treatment with 6.0 g/m^2 and Mesna exhibiting an encephalopathy 24 h after the

second infusion and there-after receiving a single dose of 100 mg methylene blue (MB) i.v. and urine samples of a 12 year old child receiving a five day treatment with 2.1 g/m^2/d as a 2 h infusion and concomitant Mesna were analysed. For details see table 5.

Patient	*IF*	*additional chemotherapy*	*Mesna*	*encephalopathy*	*samples*
female, 58 years (small cell lung cancer)	2 days 6.0 g/m^2/d over 24 h day 4, 100 mg MB		4.0 g/m^2/d	yes	24 h urine, after the last infusion, 24 h urine after MB
male, 49 years (thymom)	5 days 3.3 g/m^2/d over 24 h	Etoposid	3.7 g/m^2/d	no	24 h urine from each day
child female, 12 years (Ewing sarcoma)	5 days 2.1 g/m^2/d over 2 h	Etoposid	0.4 g/m^2/d	no	24 h urine, after the last infusion

Table 5: Characteristics of patients whose urine was examined for the presence of mono-chlorinated THβCs.

3.5 Discussion

Mono-chlorinated THβCs were successfully synthesized and characterized by spectral and chromatographic analysis, NMR and ESI-MSMS experiments. βC-type compounds show very high native fluorescence which facilitates their analysis. Previous studies regarding the analysis of βC type compounds have revealed that such substances are effectively ionized by electrospray processes in the positive mode yielding exclusively protonated molecule ions. Consequently all mono-chlorinated THβCs could be identified unambiguously by means of HPLC-MSMS with the help of their characteristic product ion spectra showing the specific RDA fragmentation of the tetrahydropyrido moiety [Gutsche and Herderich 1997]. For identification purposes an ion trap mass spectrometer represents a useful tool to study mono-chlorinated THβCs due to high selectivity and specificity. With the possibility to perform MS^n-measurements ($n \leq 9$) the identity of substances can be determined unmistakably. The presence of chlorine in the molecule is easily detectable and instantly recognisable due to the presence of the chlorine isotopes ^{35}Cl and ^{37}Cl. Neutral loss experiments might be an elegant and useful tool in the search for other THβCs by MS experiments. But such studies are preferentially performed on a triple-quadrupole MS instead of an ion trap MS, where studies of neutral loss are physically and not only arithmetically feasible.

The fate of the IF-metabolite CEA in vivo is not known, but it is assumed that it is partly converted to CAA which is the key candidate for the IF-encephalopathy. Nevertheless, we failed to demonstrate the in vivo formation of THβCs in rats following administration of the precursor compound CEA alone or after combined administration of CEA and an exogenous

indoleethylamine (tryptamine). Intact THβC-1-clm. was found in the urine of a rat receiving the compound i.p.

The presence of chlorinated βC type compounds in patients receiving IF is yet to be demonstrated, but is predictable based on the existence of substantial concentrations of CAA and indoleethylamines. Whether THβCs undergo further reactions to form fully aromatic βC or methylated THβCs is not yet known, nor if the chloro functionality which is sensitive toward nucleophiles remains or undergoes substitution. Thus, it is possible that once formed, these substances are metabolized to other THβC or βC by many pathways. The observation that only 38 % of the dose of the THβC from tryptamine and CAA was excreted intact in urine argues for further metabolism of the compounds. Moreover, THβC might be trapped in the brain after in situ formation of THβC resulting in pharmacologically active concentrations.

All newly synthesized mono-chlorinated THβCs were considered to be pure enough to perform the subsequent experiments.

4 Inhibition of rat liver monoamine oxidase by mono-chlorinated 1,2,3,4-tetrahydro-β-carbolines

4.1 Summary

In the present work the capacity of six different βC type compounds to inhibit rat liver monoamine oxidase (MAO) was studied. The oxidation of the MAO substrate [^{14}C]tyramine was examined in the presence and in the absence of the potential inhibitors. It was shown that the two compounds tetrahydro-β-carboline-1-chloromethyl-3-carboxy (THβC-1-clm.-3-c.) and ethyl-β-carboline-3-carboxylate (ethyl-βC-3-c.) did not exhibit any inhibition, not even at high concentration. In contrast, tetrahydro-β-carboline-1-chloromethyl (THβC-1-clm.), 3-hydroxymethyl-β-carboline (3-OH-βC), harmine and harmaline inhibited the oxidation of tyramine at a concentration of 200 μmol/L to 38 ± 1.7 % (THβC-1-clm.), 51 ± 1.9 % (3-OH-βC), 56 ± 8.9 % (harmine) and 60 ± 11.9 % (harmaline). Moreover, we confirmed that MB inhibits MAO in vitro.

4.2 Introduction

Reports of hallucinations following the administration of the cytostatic drug IF are frequent, but as adverse effects poorly understood [Cerny and Küpfer 1992, Miller and Eaton 1992, DiMaggio et al. 1994, Pelgrims et al. 2000]. The accumulation of the metabolite CAA during IF treatment [Goren et al. 1986, Cerny and Küpfer 1989, Kurowski and Wagner 1993] and the possible formation of THβCs [Brossi et al. 1973] could provide the biochemical basis for the understanding of the hallucinations as neurotoxic symptoms of the encephalopathy. The βCs harmine, harmaline and the substance N,N,-dimethyltryptamine (DMT) are the characteristic ingredients found in the Amazonian hallucinogenic beverage known as *ayahuasca*, *hoasca*, *caapi*, *daime*, *yagé*, *natem* and so on, depending on geographic area [Rivier and Lindgren 1972]. *Ayahuasca* is a psychoactive plant mixture typically composed of the βC-rich *Banisteriopsis caapi* vine [Brenneisen 1992, Rätsch 1998] and the tryptamine-rich, hallucinogenic plant *Psychotria viridis* [Rätsch 1998, Freedland and Mansbach 1999]. Harmine and harmaline are the main alkaloid components of *Banisteriopsis caapi* and are reported to be most effective inhibitors of purified MAO A [Kim et al. 1997, Iurlo et al. 2001]. This action increases central and peripheral serotonergic activity. Beside the βCs, large amounts of the hallucinogenic DMT from the plant *Psychotria viridis* are an essential component of the psychoactive beverage. The inhibition of MAO by harmine and harmaline facilitates the psychoactivity of DMT by suppressing its deamination. Hypotheses concerning the transmethylation of endogenous indolealkylamines propose, that due to enzymatic disturbances higher amounts of methylated indolealkylamines, such as DMT, could be produced in humans [Pomilio et al. 1999]. Inhibition of MAO alone or combined with

increased levels of methylated indolealkylamines might by reasons for the reported hallucinations in patients receiving IF chemotherapy. In addition some βCs are known to be hallucinogenic themselves [Grella et al. 1998].

MAO A and MAO B are ubiquitous flavin-containing enzymes which catalyse the oxidative deamination of biogenic amines in the central nervous system as well as in peripheral tissue [Sharman 1976]. The enzymes are located on the outer mitochondrial membrane and play an important role in the metabolism of catecholamines, serotonin and tryptophan derivatives [Weyler et al. 1990, Forth et al. 1996]. There has been great interest in MAO since its inhibition shows efficacy in the treatment of depressive disorders. We hypothesize that an inhibition of the MAO system and of tryptamine metabolism by metabolites of IF, similar to what is reported to happen after intake of *ayahuasca*, could explain the episodes of hallucinations and could thus give new insights into the mechanism of IF-induced encephalopathy. We therefore measured the oxidation of [^{14}C]tyramine, a mixed MAO A/MAO B substrate [Tipton et al. 1976], in rat liver homogenate in the presence and absence of βC type compounds. The influence of MB on the MAO activity was studied additionally.

4.3 Material and methods

Chemicals.
THβCs were synthesized and characterized (see chapter 3). 3-OH-βC and ethyl-βC-3-c. were obtained from Biomol Research Labs. Inc. (Plymouth Meeting, USA). Harmine and harmaline were from Aldrich (Sigma-Aldrich, Buchs, Switzerland). [^{14}C]tyramine was purchased from NEN Research Products (Boston, USA), specific activity 1.48-2.22 GBq/mmol (1.85 MBq/0.5 ml). Irga-Safe plus from Packard Instrument B.V. Chemical Operations (Groningen, The Netherlands). Methylene blue (methylthionini chloridum Ph.H.VI) was from Hänseler AG (Herisau, Switzerland).

Rat liver homogenate.
A 10 % homogenate (wt/vol) of rat liver in 0.1 M phosphate buffer, pH 7.4, was prepared in a glass homogenizer with a tissue grind pestle (Kontes Glass company, Vineland, USA) at 4°C. Aliquots of 200 µl were frozen at –70°C prior to use.

Assay.
A stock solution of the [^{14}C]labelled tyramine was prepared in sodium phosphate buffer 0.1 M, pH 7.4, so that 50'000 dpm/20 µl were obtained. For the assay 20 µl of the 10 % homogenate was added to 100 µl of 0.1 M sodium phosphate buffer, pH 7.4, 20 µl [^{14}C]tyramine stock solution and 100 µl of the potential inhibitor (final concentrations: 2, 20 and 200 µmol/L) or 100 µl of buffer (control). The mixture was incubated at 37°C for 30

minutes. The reaction was stopped with 100 μl of 2 M citric acid and the deaminated products were extracted into 3 ml toluene/ethyl acetate (1:1) [Glover et al. 1980]. One ml of the organic phase was added to 10 ml of Irga-Safe plus scintillation solution. All samples were counted for radioactivity in a Beta scintillation counter (Kontron instruments) with automatic quench correction.

Statistics.

The results are presented as mean values +/- SD. Statistical significance of the differences between groups was assessed by one way ANOVA and Bonferroni-t-test.

4.4 Results

Control incubations served as samples with maximal enzymatic activity under the conditions described. Tables 6 to 8 list all βC type compounds studied. From table 6 can be seen that 2 μmol/L of the naturally occurring alkaloids harmine and harmaline decrease the MAO activity to 65 % and 74 % compared to the control. At a concentration of 200 μmol/L the [^{14}C]tyramine oxidation is inhibited by 56 % (harmine) and 60 % (harmaline).

	concentration [μmol/L]	*[^{14}C]tyramine [dpm]*	*MAO activity [%]*
Control		5982 +/- 333	100
Harmine	200	2651 +/- 237	44*
	20	3771 +/- 268	63*
	2	3883 +/- 536	65*
Harmaline	200	2375 +/- 282	40*
	20	3747 +/- 167	63*
	2	4413 +/- 249	74*

Table 6: Harmine and harmaline, naturally occurring βC type compounds, inhibit [^{14}C]tyramine oxidation in a 10 % rat liver homogenate at concentrations of 2, 20 and 200 μmol/L. All values obtained for harmine and harmaline are significantly * ($P < 0.001$) lower compared to the control. Values represent means +/- SD, n = 3.

Table 7 provides details for the mono-chlorinated THβCs. The THβC originating from tryptamine and CAA shows an inhibition of 38 % at a concentration of 200 μmol/L, while the one from the reaction between tryptophan and CAA seems to be an inactive compound.

	concentration [μmol/L]	*[^{14}C]tyramine [dpm]*	*MAO activity [%]*
Control		5982 +/- 333	100
THβC-1-clm.	200	3738 +/- 64	62*
	20	5532 +/- 152	92
	2	6127 +/- 293	102
THβC-1-clm.-3-c.	200	6399 +/- 384	107
	20	6329 +/- 184	106
	2	6325 +/- 82	106

Table 7: The THβC formed from CAA and tryptamine inhibits the MAO by approximately 40 % at a concentration of 200 μmol/L, whereas the one formed from tryptophan exhibits no inhibition at the same concentration. Values represent means +/- SD, n = 3. * = significant difference versus control ($P < 0.001$).

As can be seen from table 8, which lists the results for the fully aromatic βCs, 3-OH-βC is a slightly stronger inhibitor of rat liver MAO than the chlorinated THβC from tryptamine at a concentration of 200 μmol/L. Ethyl-βC-3-c. is an inactive compound.

	concentration [μmol/L]	*[^{14}C]tyramine [dpm]*	*MAO activity [%]*
Control		5982 +/- 333	100
Ethyl-βC-3-c.	200	5970 +/- 1240	100
	20	6022 +/- 81	101
	2	6415 +/- 210	107
3-OH-βC	200	2937 +/- 57	49*
	20	4864 +/- 346	81**
	2	6071 +/- 507	101

Table 8: The commercially available, fully aromatic βC 3-OH-βC shows a 51 % inhibition of the MAO at a concentration of 200 μmol/L. A significant difference between 3-OH-βC and the control was found. Ethyl-βC-3-c. is an inactive compound. Values represent means +/- SD, n = 3. ** (P < 0.050), * (P < 0.001).

Figure 23 summarizes the findings from this work. Harmine and harmaline as well as the fully aromatic 3-OH-βC and the mono-chlorinated THβC formed from tryptamine decrease the oxidation of tyramine.

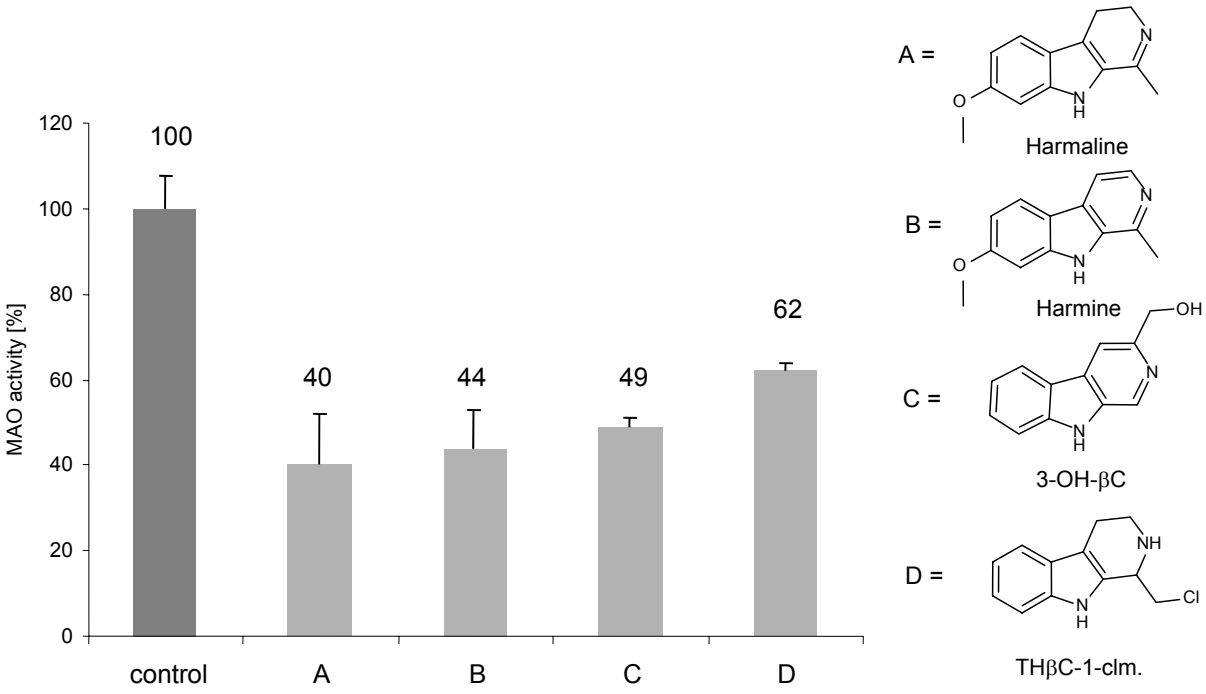


Figure 23: Harmaline, harmine, OH-βC and THβC-1-clm. inhibit rat liver MAO at a concentration of 200 μmol/L by around 40 to 60 %. Values represent means +/- SD, n = 3.

Figure 24 shows the data obtained from the experiments performed with MB. MB seems to be the strongest inhibitor examined in this work. The MAO inhibition caused by MB is in agreement with other studies [Soares-da-Silva and Caramona 1988, Aeschlimann et al. 1996].

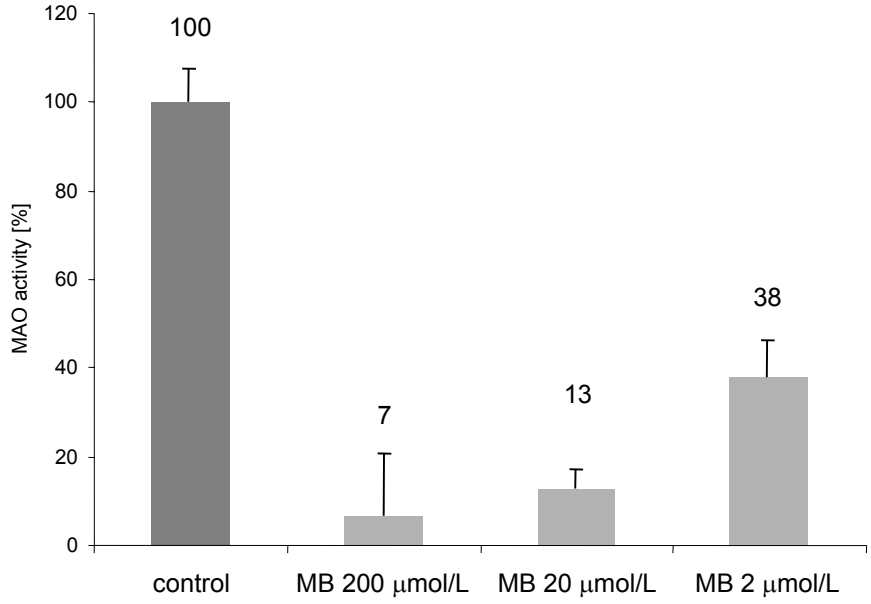


Figure 24: This graph summarizes the results obtained from the experiments with MB. Values represent means +/- SD, n = 3. MB in the range of 2 to 200 μmol/L is significantly ($P < 0.001$) different from the control.

4.5 Discussion

The purpose of this study was to examine the inhibitory effect of βCs on rat liver MAO. From the present results a structure-activity relationship for the inhibition of rat liver MAO caused by βC type compounds can be derived. Figure 25 shows the basic molecular structure of the examined substances. They differ in the extent of oxidation of the third ring as well as in their pattern of substitution at the positions 1, 3 and 7.

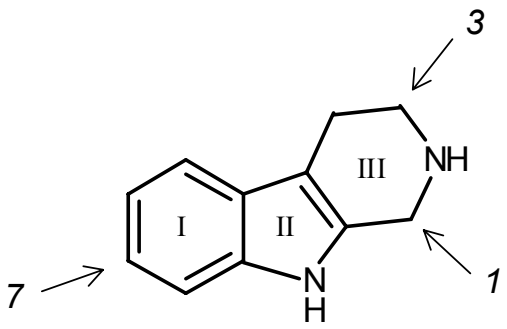


Figure 25: βC type compound with the positions 1, 3 and 7 for substituents described in this work. Numbers I, II, and III stand for the three rings.

The introduction of a large substituent, such as a carboxy- or an ethyl-carboxylate-group at position 3 results in an inactive compound, as can be seen from the data obtained from THβC-1-clm.-3-c. and ethyl-βC-3-c. On the other hand, the introduction of a hydroxy-methyl-group at the same position does not seem to affect the oxidation of tyramine in the same extend. Although 3-OH-βC is not as potent as harmine and harmaline it is nevertheless a definite inhibitor of tyramine oxidation. A larger substituent than a methyl-group at position 1 seems not to be very favourable for the oxidation of tyramine but we still observed a reduced MAO activity with THβC-1-clm. A substituent at position 7, such as in the case of harmine and harmaline, seems not to influence the tyramine oxidation. Planarity of the molecule which is obtained when the third ring is fully hydrogenated, does not seem to be a compelling criteria for an inhibition of the oxidation of tyramine in rat liver. Therefore, mono-chlorinated THβCs undergoing oxidation to aromatic βCs are assumed to cause an inhibition similar to what is observed when the third ring is saturated. Our results are in agreement with other studies and they demonstrate that βC type compounds are an interesting class of MAO inhibitors [Ho et al. 1968]. Whether they play a role as MAO-inhibitors in the development of an IF-encephalopathy is however to be shown.

Some βC, which possess an indolealkylamine element within their three ring structure, are known to be hallucinogenic themselves in humans [Grella et al. 1998]. Classical hallucinogens can be categorized as phenylalkylamine hallucinogens or as indolealkylamine hallucinogens. The phenylalkylamine can be further divided into the phenylethylamines, such as mescaline, and phenylisopropylamines, such as 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane. The indolealkylamines are subdivided into the tryptamines, such as DMT, and the lysergamides, such as lysergic acid diethylamide [Glennon 1996]. Hallucinogenic properties of βCs have also been linked to their activity at serotonin receptors [Nelson et al., 1999 Glennon et al. 2000] and imidazoline receptors [Husbands et al. 2001]. βCs are also known to bind to dopamine receptors [Glennon et al. 2000]. Whether the mono-chlorinated THβCs that are putatively generated from metabolites of IF interact with any serotonin, imidazoline or dopamine receptors is not known.

5 Interactions of mono-chlorinated 1,2,3,4-tetrahydro-β-carbolines with benzodiazepine receptors in rat brain

5.1 Summary

A debilitating encephalopathy occurs frequently during treatment with high doses of IF. Mono-chlorinated THβCs are postulated to be formed in humans from the highly reactive compound CAA, which accumulates during IF therapy, and indoleethylamines, such as tryptophan or tryptamine. βCs type compounds are described to interact with benzodiazepine (BZD) receptors. The report of a successful treatment of IF-induced encephalopathy with diazepam provides the basis for the hypothesis that THβCs might be responsible for the neurotoxicity due to interactions with BDZ receptors.

In the present study we examined βCs regarding their ability to compete with [^{3}H]flunitrazepam for the binding to the BZD receptor. It was shown that only the fully aromatic βCs 3-OH-βC and ethyl-βC-3-c. exhibit a competition whereas the THβCs had no effect on BZD binding. We conclude from these experiments that THβCs are not likely to be responsible for the diazepam-sensitive encephalopathy following IF. The present data, however, suggest that their in vivo oxidation to the fully aromatic βCs might provide an explanation for IF-associated encephalopathy.

5.2 Introduction

Gamma–aminobutyric acid $(GABA)_A$ receptors are pentamers comprising α-, β- and γ-subunit assemblies that mediate some of the effects of BDZs, barbiturates and neuroactive steroids. The binding of a BZD induces conformational changes in the receptor that enhances the affinity for GABA and thus the frequency of GABA-gated chloride channel openings. The opening probability of the receptor is thereby increased and results in a lowered membrane resistance and hyperpolarisation. Consequently, the postsynaptic neuron is inhibited [Forth et al. 1996]. Positive $GABA_A$ modulators, such as the BDZs diazepam and triazolam and the barbiturate pentobarbital, have anxiolytic, muscle relaxant, sedative and anticonvulsant effects. Negative modulators, such as β-carboline-3-carboxylates, have anxiogenic and convulsant effects [McMahon and France 2001].

There are several reports describing the affinity of βCs to BDZ receptors, first published by Braestrup and co-workers in 1980 [Braestrup et al. 1980]. βC derivatives, which are chemically unrelated to the BDZs, interact with the BZD receptors displaying the full spectrum of agonist, inverse agonist and antagonist properties according to their molecular substituents [Polc et al. 1982, Borea et al. 1988, McMahon and France 2001, Sarter et al.

2001]. β-carboline-3-carboxylic acid ethyl ester for example, was shown to bind to BDZ binding sites with high affinity, reducing various effects of BDZs, thus acting as a BZD antagonist [Braestrup et al. 1982, Shannon et al. 1988]. Various structure-activity studies conclude that βCs display high binding affinity for the BZD receptor when the following conditions are fulfilled: a) presence of an esteric, sometimes amidic, function in position 3, capable of accepting hydrogen bonds from a hypothetical donor of the receptor; b) complete aromaticity of the three ring system, thus full planarity; c) no substituents in position 1 [Borea et al. 1988]. It was demonstrated that βCs were generally more potent than THβC analogues, which exist in a twist chair conformation [Cain et al. 1982].

The article published by Simonian and co-workers in 1993, describing a diazepam-sensitive IF-induced encephalopathy caught our attention due to the fact that we assume THβCs to be formed during IF therapy. There are two cases described where the mental status of IF-treated patients changed six hours after the onset of the IF-infusion [Simonian et al. 1993]. A 20-minute electroencephalogram (EEG) showed continuous, generalised irregularities without definite rhythmic or repetitive patterns. These abnormalities markedly improved within 4 minutes of a 5 mg dose of intravenous diazepam. Concurrent with the EEG improvement, the patients' mental status returned to normal. The symptoms of impaired motor function and decreased consciousness that characterize the syndrome of hepatic encephalopathy might be caused by an increased inhibitory neurotransmission mediated by GABA [Basile et al. 1991, Jones 1991, Mullen 1991]. However, the mechanism responsible for the increased GABA-ergic tone in hepatic encephalopathy is uncertain, too.

In the present work we determined the direct interaction between two mono-chlorinated THβCs, formed from tryptophan and tryptamine, respectively, and BZD receptors by measuring the inhibition of binding of a radioactive ligand ([^{3}H]flunitrazepam) by the compound. We hypothesized that the THβCs could compete with the labelled ligand for the same binding site on the receptor in this assay. We therefore prepared synaptosomes from rat brain. Synaptosomes are small spherical particles of sealed nerve terminals in which transmitter processes can be monitored.

5.3 Material and methods

Chemicals and material.

THβCs were synthesized and characterized (see chapter 3). [N-methyl-^{3}H]flunitrazepam, specific activity 2.59-3.33 TBq/mmol (9.25 MBq/250 μl), was purchased from Amersham Bioscience (Buckinghamshire, UK), Protosol was from NEN Research Products (Boston,

USA) and Econofluor-2 from Packard Instrument B.V. Chemical Operations (Groningen, The Netherlands). Circular glass microfibre filters GF/A, 25 mm diameter, were from Whatman International Ltd. (Maidstone, UK). 3-OH-β-C and ethyl-βC-3-c. were obtained from Biomol Research Labs. Inc. (Plymouth Meeting, USA).

Preparation of rat brain synaptosomes.

A 10 % homogenate (wt/vol) of rat brain in 0.1 M sodium phosphate buffer, pH 7.4, was prepared using a Polytron (Kinematica, Luzern, Switzerland). The homogenate was centrifuged at 0°C during 10 minutes at 1'000 g (high speed SS-34 centrifuge, Kontron instruments, Watford, UK). Further centrifugation of the supernatant at 20'000 g during 15 minutes resulted in a pellet which contains synaptosomes. The pellet was re-suspended in 100 ml 0.1 M sodium phosphate buffer, pH 7.4. Aliquots were kept at –20°C until use. After thawing and prior to the work to be performed, the synaptosome suspension (SS) was homogenized again.

Receptor assay.

A stock solution of [^{3}H]labelled flunitrazepam in 0.1 M sodium phosphate buffer, pH 7.4, was prepared so that 22'000 dpm/100 μl were obtained. Determination of linearity was performed with increasing volume of the SS. The assay was there-after performed with 1000 μl of SS. The incubation mixture (final volume 1320 μl) consisted of 1 μmol/L of the THβC solution, 100 μl of a 60 μmol/L cold flunitrazepam solution, 1000 μl of the SS and 200 μl of the [^{3}H]labelled flunitrazepam solution. In the case of total binding 100 μl of buffer was used instead of cold flunitrazepam solution. All mixtures were incubated at 4°C for 15 minutes prior to filtration through GF/A Whatman glasfiber microfilters, which were after that washed with 2200 μl of buffer. The filters were then transferred to a counter tube and 1 ml of Protosol was added to dissolve the filter material during 60 minutes at room temperature. Finally 10 ml of scintillation fluid (Econofluor) was added and the samples were counted for radioactivity in a Beta scintillation counter (Kontron instruments) with automatic quench correction. In all experiments specific binding was defined as the difference between total and unspecific binding.

5.4 Results

This study was carried out to assess the capacity of tetrahydro-, dihydro- and β-carbolines to compete with flunitrazepam for the binding to the BZD receptor in rat brain synaptosomes. In the case of the tetrahydro-compounds the two mono-chlorinated THβCs from the reaction of tryptamine and tryptophan, respectively, with CAA were studied and compared with the naturally occurring alkaloids harmine and harmaline and the commercially available fully aromatic β-carbolines 3-OH-βC and ethyl-βC-3-c. (figure 26).

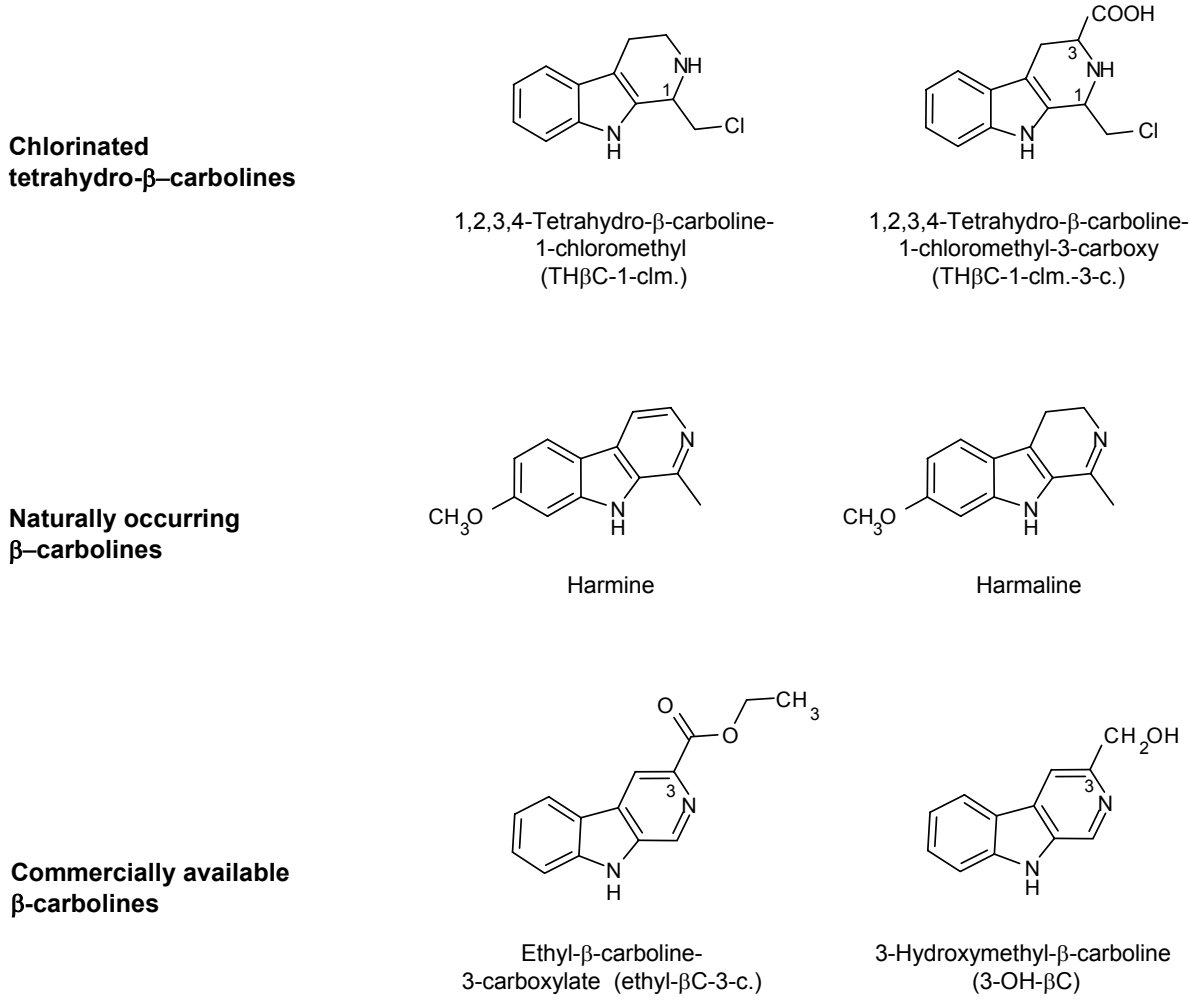


Figure 26: The two mono-chlorinated THβCs formed from CAA by condensation with tryptophan and tryptamine, respectively, were compared with the naturally occurring compounds harmine and harmaline and the commercially available aromatic βCs 3-hydroxymethyl-β-carboline (3-OH-βC) and ethyl-β-carboline-3- carboxylate (ethyl-βC-3-c).

Control incubations served as samples with maximal binding capacity under the conditions described. Figure 27 provides details for the 1-point screening of the substances mentioned at a concentration of 1 μmol/L.

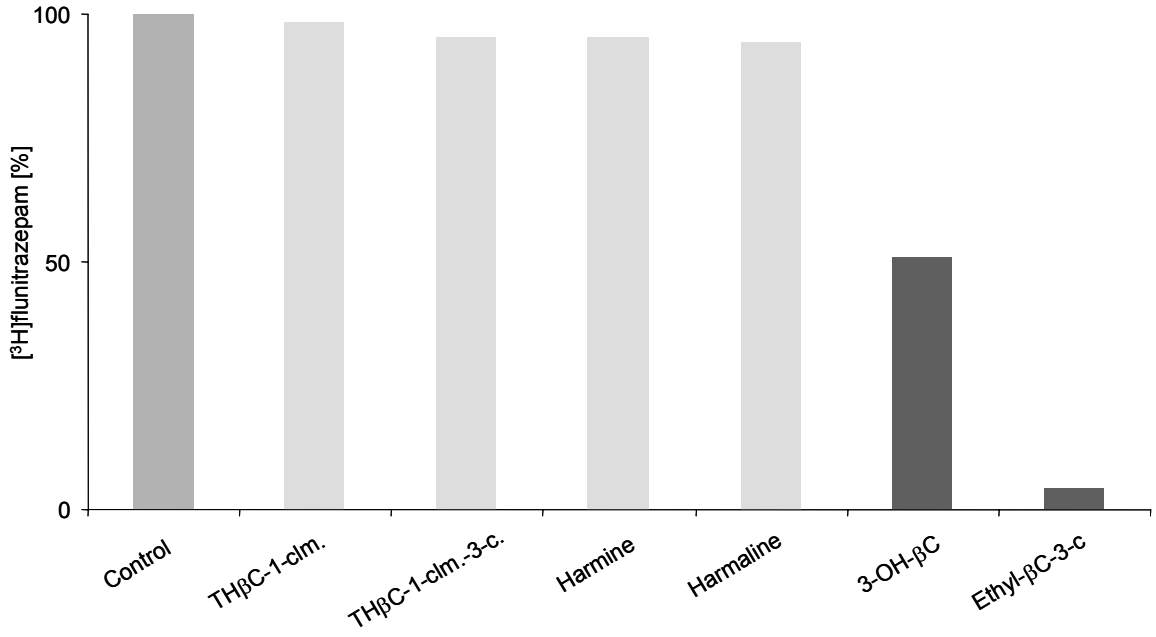


Figure 27: One-point screening: a displacement of [^{3}H]flunitrazepam from the receptor is observed with the fully aromatic β-Cs 3-OH-βC and ethyl-βC-3-c. The tetrahydro- and dihydro-compounds and harmine are inactive compounds. The concentration of the compounds was 1 μmol/L; n = 1.

Consistent with the literature the aromatic βCs were found to displace flunitrazepam from its binding site [Cain et al. 1982]. The other compounds studied seem to be inactive regarding the competition with flunitrazepam.

5.5 Discussion

Our results demonstrate that the mono-chlorinated THβCs examined do not displace flunitrazepam from its receptor in rat brain synaptosomes. In contrast, the βCs examined interact with BZD receptors as expected. It was previously shown that the pattern of substitution of βC type compounds as well as the degree of aromaticity of the three ring system is essential for competition with the BZD flunitrazepam at the receptor. The complete three-ring skeleton is essential for optimal binding to the BDZ receptor, although a two-ring planar structure too seems to have some affinity for the receptor [Cain et al. 1982]. It is reported that compounds possessing a carbonyl at position 3 have higher affinity whereas alcohol substituents result in decreased binding [Borea et al. 1988]. Moreover, modifications at position 1 also result in decreased binding. A small substituent results in small, larger substituents in a more dramatic loss in affinity. Surprisingly, harmine which is a fully aromatic βC does not inhibit the binding of the ligand at all, although the criterion of full planarity of the molecule is fulfilled. It needs to be mentioned that harmine as well as harmaline possess a CH_3O-group attached to the first ring (figure 26) which could result in sterical hindrance for

the binding to the BZD receptor. Our findings concerning the competition with [^{3}H]flunitrazepam are consistent with the literature. Whether βCs and their derivatives act as a) agonists (which are, inter alia, anxiolytic), b) inverse agonists (anxiogenic) or c) antagonists (without any per se biological effect but preventing the interaction of agonists and inverse agonists with the receptor) on the BDZ-receptor requires further studies. Although our results only provide a very rough estimation of the ability of βC type compounds regarding their attitude towards BZD receptors they yield essential basic information.

We conclude from our studies that it seems unlikely that THβCs formed from the toxic IF-metabolite CAA and endogenous indoleethylamines are responsible for IF-associated encephalopathy via the interaction with BZD receptors.

6 Influence of mono-chlorinated 1,2,3,4-tetrahydro-β-carbolines on the respiratory chain in rat liver mitochondria

6.1 Summary

We assume that due to the accumulation of the toxic IF-metabolite CAA the formation of mono-chlorinated THβC in vivo is likely and that this might provide an explanation for IF-induced encephalopathy. In the present study the capacity of three different mono-chlorinated THβCs to inhibit mitochondrial respiration was examined. The THβC formed from serotonin and CAA inhibits complexes I and II, whereas the THβC originating from tryptophan only affected complex I. The THβC formed from CAA and tryptamine has no influence on the respiration in mitochondria or submitochondrial particles (SMPs). It seems that a hydroxy group at position 6 in mono-chlorinated THβCs leads to a pronounced inhibition of complex I and II in the respiratory chain of mitochondria.

6.2 Introduction

Because of the neuropharmacological effects of THβCs these alkaloids have attracted much attention. In particular several xenobiotic βCs (harman, norharman, harmine and their N-methylated derivatives) have been described to exhibit neurotoxic effects on the dopaminergic system [Albores et al. 1990]. Similar to MPP^+ (N-methyl-4-phenylpyridinium), the active metabolite of the parkinsonism-inducing compound MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), these βCs are described to inhibit complex I (NADH:ubiquinone oxidoreductase), some also partially complex II (succinate:ubiquinone oxidoreductase) of the electron transport chain in mitochondria. Such inhibitors act by binding electron carriers in the transport chain. The electron transport chain, located in the inner mitochondrial membrane, transfers electrons from either NADH or $FADH_2$ to molecular oxygen and produces a transmembranous proton gradient used for the generation of ATP and for other reactions. Therefore, an inhibition of this gradient not only leads to a damaged electron transport with concomitant impairment of ATP synthesis, but also results in a reduced uptake of substrates for mitochondrial metabolism. An adequate mitochondrial function is exceedingly essential for the survival of a cell. Janetzky and co-workers showed in 1995 that the highly halogenated THβC 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) is an inhibitor of complex I [Janetzky et al. 1995]. This potent toxin originates in vitro from the biogenic amine tryptamine and the aldehyde chloral hydrate (trichloroacetaldehyde) [Riederer et al. 2002]. It has been postulated that this heterocycle is formed endogenously in humans after administration of the hypnotic drug chloral hydrate or after exposure to the industrial solvent trichloroethylene by spontaneous ring closure reaction. This assumption has been confirmed by analyzing the blood of patients treated orally with chloral hydrate

[Bringmann et al. 1999]. We assume that due to the accumulation and the high reactivity of the IF-metabolite CAA, chlorinated THβCs might be formed in mammalian organism similar to what has been reported following administration of chloral hydrate. Serotonin is an indoleethylamine most widely distributed in mammals and its reaction with CAA would produce OH-THβC-1-clm. (chapter 3, figure 15) [Beck et al. 1982].

This part of the thesis was designed to assess the significance of three mono-chlorinated THβCs on complex I and II of the electron transport chain in rat liver mitochondria. The function of these complexes can be studied in isolated mitochondria by studying oxidative metabolism.

6.3 Material and methods

Chemicals.
Three different mono-chlorinated THβCs were synthesized and characterized (see chapter 3). All other chemicals were purchased from Sigma Chemical Co. (Buchs, Switzerland).

Preparation of intact mitochondria and submitochondrial particles (SMPs).
Mitochondria were isolated from the liver of Wistar rats as described by Hoppel et al. 1979. Rats were killed by decapitation. Livers were quickly removed and placed in ice-cold MSM buffer (220 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L 4-morpholinopropanesulfonic acid [MOPS], pH 7.4). Livers were rinsed, weighed, minced and washed with ice-cold MSM buffer. A 10 % suspension (wt/vol) of minced liver containing 2 mmol/L EDTA (ethylene diamine tetra-acetic acid) was prepared in a Potter-Elvehjem glass homogenizer with a loose fitting pestle. Mitochondria were isolated by means of differential centrifugation. As shown previously, this method yields mitochondria of good quality with only minor contamination by peroxisomes and lysosomes [Krähenbühl et al. 1994]. The resulting pellet was diluted to contain approximately 100 mg protein per ml. Protein concentration was determined by the biuret reaction using bovine serum albumin as standard [Gornall et al. 1949]. SMPs were prepared according to Rafique [Rafique et al. 2001]. Protein content was determined by the method of Lowry reaction using bovine serum albumin as standard [Lowry et al. 1951].

Determination of complex activities.
O_2 uptake in intact mitochondria and SMPs was measured in a thermostatically controlled incubation chamber at 37°C equipped with a Clarke-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, USA), in air-saturated reaction medium at a final volume of 2.5 ml. The incubations contained 1 mg/ml mitochondrial protein or 0.3 mg/ml SMPs protein in 100 mmol/L KCl, 5 mmol/L MOPS, 1 mmol/L EGTA and 5 mmol/L KH_2PO_4. The concentrations of the substrates used in mitochondrial incubations were 4 mmol/L for β-

hydroxy-butyrate, 8 mmol/L for succinate and in the SMPs experiments 0.4 mmol/L for NADH. In the case of mitochondrial experiments measurements were performed in the presence (state 3) or absence (state 4) of 0.2 mmol/L ADP. O_2 consumption was expressed in natoms O_2/min/mg protein. The respiratory control ratio (RCR) was determined as a marker of functional integrity, i.e. coupling of oxidative phosphorylation in mitochondria. The RCR is the ratio of the rate of oxygen consumption during state 3 and state 4. State 4 respiration is characterized by minimal, state 3 respiration by maximal oxygen uptake.

Statistics.

Results are expressed as mean values +/- SD. Differences between control incubations and incubations containing THβCs were analyzed using one way ANOVA and Bonferroni-t-test. Two tailed probabilities of less than 0.05 were considered to be significant.

6.4 Results

The effects of the THβC derivatives on oxidative metabolism in intact mitochondria using β-hydroxy-butyrate is shown in table 9. β-hydroxy-butyrate is metabolised by hydroxy-butyrate dehydrogenase to acetoacetate and NADH which is oxidised by complex I of the electron transport chain. Electrons are then transferred to complex III and IV. An impaired state 3 respiration after addition of β-hydroxy-butyrate could therefore reflect a defect in complex I, III or IV. The concentration of the examined THβCs was 800 μmol/L.

In the presence of β-hydroxy-butyrate the THβC derived from serotonin and CAA revealed a marked inhibition of state 3 oxygen consumption (reduced to 45 %). Moreover, the respiration was reduced to 77 % following incubation with the THβCs originating from tryptophan and CAA. No inhibition of complex I was found with THβC-1-clm. Table 9 also shows that the state 4 respiration is unaffected following incubation with THβCs.

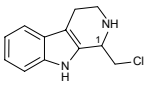
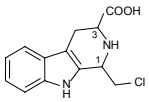
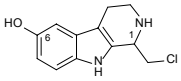
		β-hydroxy-butyrate 4 mmol/L	
		State 3	State 4
		natoms O_2/min/mg protein	
	Control	140 +/- 13	31 +/- 3
	THβC-1-clm.	142 +/- 11	30 +/- 3
	THβC-1-clm.-3-c.	109 +/- 5*	27 +/- 2
	OH-THβC-1-clm.	63 +/- 12*	34 +/- 12

Table 9: Oxidative metabolism of β-hydroxy-butyrate in intact rat liver mitochondria. The concentration of the THβCs was 800 μmol/L. Results are given as mean values +/- SD of two individual mitochondrial preparations run in triplicates. * = significant (P < 0.01) difference versus control.

Due to an impaired state 3 respiration the values for the RCR of OH-THβC-1-clm. as well as of THβC-1-clm.-3-c. are significantly (P < 0.05) different compared to the control as can be seen in figure 28.

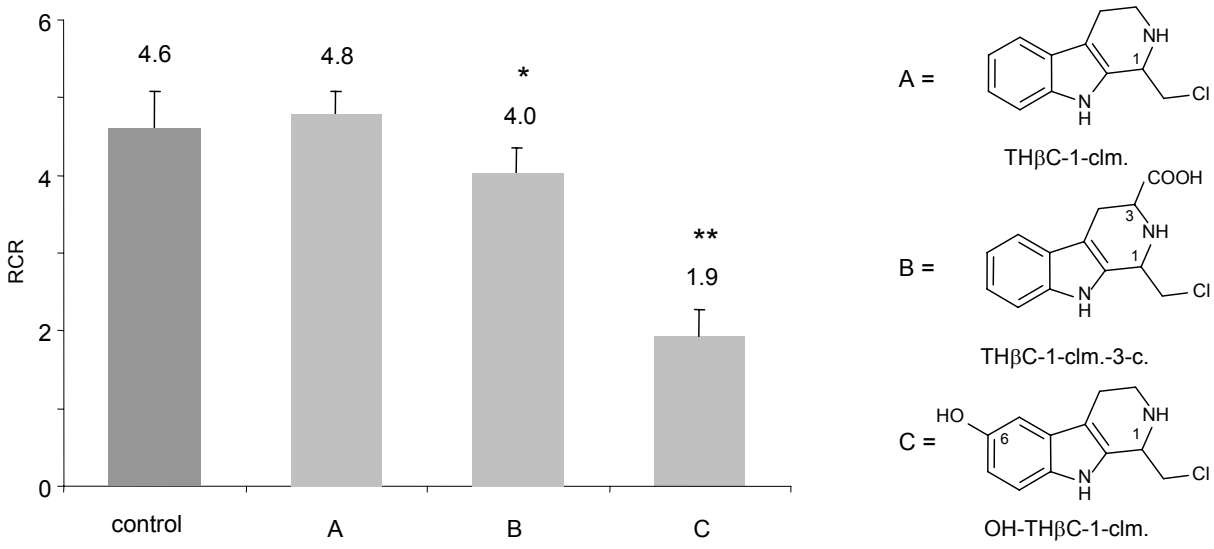


Figure 28: Oxidation of β-hydroxy-butyrate in intact mitochondria. The respiratory control ratio (RCR) is calculated by dividing state 3 by state 4 respiration. Data are given as mean values +/- SD of two individual mitochondrial preparations run in triplicates. B and C are significantly different from the control. * ($P < 0.05$), **($P < 0.001$).

To confirm the results obtained from the mitochondrial preparations the NADH oxidation in SMPs was studied. NADH can not be used with intact mitochondria as this substrate is not transported across the mitochondrial membranes and is active only when the structural integrity of the mitochondria is breached. In addition, the possibility that the observed inhibition is not due to an inhibition of the dehydrogenation of β-hydroxy-butyrate can be excluded with SMPs experiments. Moreover, the inhibitory effects on the electron transport chain in mitochondria can be localised. In broken mitochondria the substrates used can interact directly with the complexes of the electron transport chain. As shown in table 10 the serotonin THβC showed a strong inhibition of the oxidation of NADH.

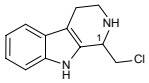
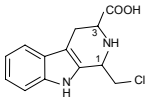
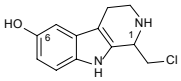
		NADH 4 mmol/L
		natoms O_2/min/mg protein
	Control	35 +/- 2
	THβC-1-clm.	35 +/- 1
	THβC-1-clm.-3-c.	29 +/- 1*
	OH-THβC-1-clm.	6 +/- 3**

Table 10: Oxidative metabolism of NADH in SMPs. OH-THβC-1-clm. inhibits NADH oxidation by 83 % compared to the control. The concentration of the THβCs was 800 μmol/L. Data are given as mean values +/- SD of two individual SMPs preparations run in triplicates. Significant difference versus control: * (P < 0.05), ** (P < 0.001)

Figure 29 illustrates the dose-dependent inhibition of the oxidation of NADH in SMPs caused by OH-THβC-1-clm.

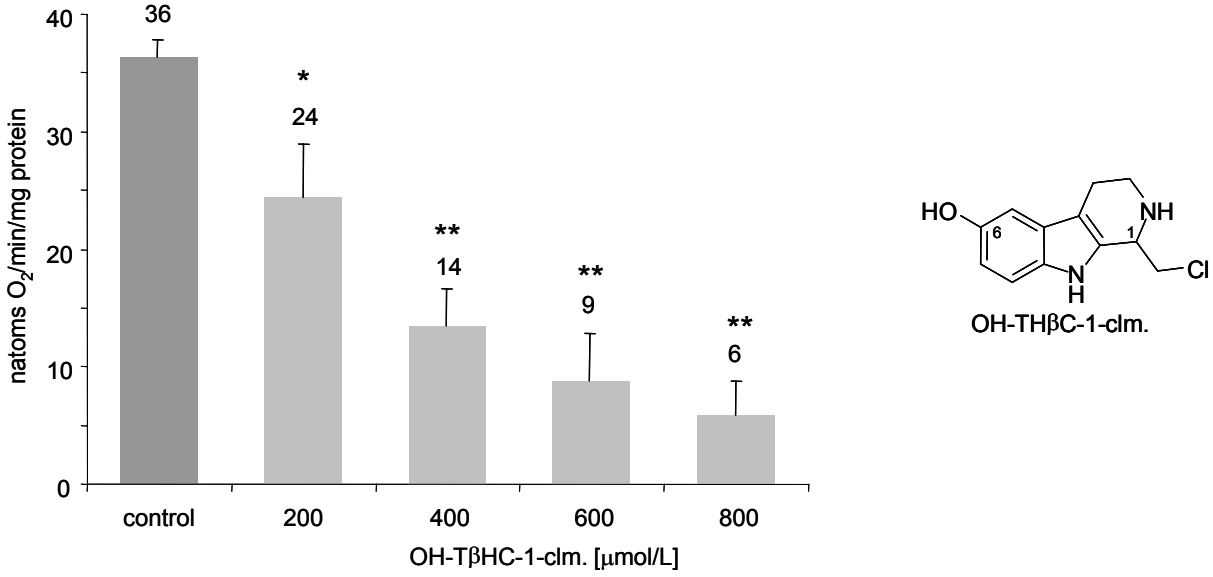


Figure 29: Dose dependent inhibition of NADH linked oxidation in SMPs caused by OH-THβC-1-clm. Results are given as mean values +/- SD of three individual SMPs preparations run in triplicates. Significant difference in comparison to the control: * (P < 0.05), ** (P < 0.001)

These data suggest that the THβC formed from CAA and serotonin induces mitochondrial dysfunction as shown by the impaired state 3 respiration after administration of β-hydroxy-

butyrate as well as by the inhibition of NADH oxidation in SMPs. Moreover, THβC-1-clm.-3-c. seems to be an inhibitor of complex I as demonstrated in mitochondria and SMPs. As qualitatively similar results were obtained in intact mitochondria and SMPs, it is unlikely that the inhibitors tested interfere with the transport of substrate into the mitochondrial matrix.

Table 11 shows the results obtained from the studies performed on complex II respiration with succinate as substrate in SMPs. It can be seen that it is again the serotonin THβC that exhibits a decrease in substrate oxidation. We observed a reduction of 54 % at a concentration of 800 μmol/L.

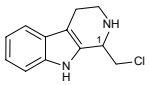
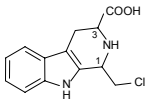
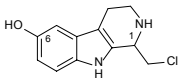
		Succinate 8 mmol/L
		natoms O_2/min/mg protein
	Control	15 +/- 2
	THβC-1-clm.	14 +/- 2
	THβC-1-clm.-3-c.	13 +/- 1
	OH-THβC-1-clm.	8 +/- 0.5*

Table 11: Succinate linked oxidation in SMPs. The concentration of the THβCs was 800 μmol/L. Values are means +/- SD of two individual SMPs preparations run in triplicates.* is significantly (P < 0.001) different from the control.

This qualitative structure-activity relationship study reveals that in the case of the chlorinated THβCs studied, a hydroxy group at position 6 seems to be favourable for an inhibition of complex I and II respiration in rat liver mitochondria and in SMPs.

6.5 Discussion

In this study we found the THβC originating from the condensation of the IF-metabolite CAA with serotonin to be a strong inhibitor of complex I as shown with the impaired state 3 respiration in intact mitochondria as well as with the decreased NADH oxidation in SMPs. Janetzky measured a total inhibition of complex I caused by TaClo at a concentration of 800 μmol/L [Janetzky et al. 1995]. This concentration is 5 to 10 times lower than the one needed with MPP^+. With the measurement of the NADH- and succinate-linked oxidation in

submitochondrial particles we clearly confirmed the results obtained from the mitochondrial experiments. Complex I activity measured via NADH-linked oxidation was strongly inhibited by the serotonin THβC, while the succinate-linked oxidation was less affected. THβC-1-clm.-3-c. was also found to inhibit complex I without influencing complex II. None of the examined THβCs seems to uncouple oxidative phosphorylation since state 4 respiration was not affected in our experiments with intact mitochondria. It seems that a hydroxy group at position 6 in THβCs is favourable for an inhibition of complex I and II.

The observed inhibition by OH-THβC-1-clm. may account for the central nervous side effects following administration of IF. Whether concentrations that might trigger an inhibition are reached in brain following IF remains to be demonstrated. Several complex I inhibitors are under discussion as possible neurotoxins. In the case of Parkinson's disease a close relation between the inhibition of complex I and the pathogenesis of that disorder was predicted [Janetzky et al. 1999]. But not only the understanding of that disease also other neuropathologies such as the IF-induced encephalopathy might be explained and understood with the identification and subsequent characterisation of THβCs. It can not be ruled out at the moment that a presumable formation of mono-chlorinated THβCs in humans receiving IF might possibly be one of probably other causative factors for the induction of IF neurotoxicity.

7 Influence of methylene blue on serotonin levels and on MAO activity in the brain of mice after ifosfamide

7.1 Summary

In the clinic MB is administered in cases of IF-induced encephalopathy and reverses neurotoxic symptoms. The purpose of this study was to measure the serotonin concentration as well as tryptophan and dopamine in the brain of mice treated with IF alone or in combination with MB. These results were compared with controls and mice treated with MB alone. Significant differences were observed between the IF and IF/MB group, as well as between the control and the MB group. In addition, MAO activity was found to be decreased following administration of MB and IF/MB. These findings indicate that MB increases the concentration of serotonin in the brain of mice probably due to inhibition of MAO.

7.2 Introduction

A marked improvement and in some cases a rapid reversal of IF associated central nervous toxicity after the administration of MB has been described as reviewed in the paper of Pelgrims and co-workers [Pelgrims et al. 2000]. Although several hypotheses have been proposed to explain the protective effect of MB its mode of action remains to be elucidated. MB possesses various properties (see chapter 1.4) that could partake in the reversal of IF-encephalopathy. MB could interfere with the metabolism of monoamines. The serotonergic and dopaminergic neurotransmitter systems regulate emotion, mood, reward and cognition. Perturbations in the neurotransmitter systems are thought to contribute to the pathophysiology of several common neuropsychiatric disorders, such as schizophrenia, bipolar disorders, depression and drug addiction.

Since the formation of βCs from metabolism of IF might interfere with the metabolism of serotonin, which plays a critical role in modulating mental functions, serotonin, tryptophan, the precursor amino acid for serotonin, and dopamine were measured in the brain of mice following administration of MB, IF or combined IF/MB and compared with control animals. In addition, the oxidation of tyramine, a substrate for MAO A and B was studied in the presence and absence of IF, MB or IF/MB.

7.3 Material and methods

Chemicals.

Ifosfamide (Holoxan®) was from Asta Medica (Wangen, Switzerland). Methylene blue (methylthionini chloridum Ph.H.VI) was from Hänseler AG (Herisau, Switzerland). Serotonin hydrochloride was from Fluka (Buchs, Switzerland). [^{14}C]tyramine, specific activity 1.48 - 2.22

GBq/mmol (1.85 MBq/0.5 ml), was purchased from NEN Research Products (Boston, USA). Acetonitrile and methanol (both HPLC gradient grade), formic acid 100 % p.a., toluene, ethylacetate and citric acid were from Merck (Darmstadt, Germany). The water was of Purelab Option E (Labtec, Wohlen, Switzerland) quality (filtered and UV light treated). Irga-Safe plus from Packard Instrument B.V. Chemical Operations (Groningen, The Netherlands).

Animals.
Swiss female mice (approximately 25 g) fed a standard chow diet and water ad libitum were used in all experiments. Eight animals were injected with 1 g/kg (3.8 mmol/kg) IF in 0.9 % NaCl i.p.. A group of four mice received 10 mg/kg (31 μmol/kg) MB in 0.9 % NaCl 30 minutes prior to 1 g/kg of IF. Four animals received 10 mg/kg MB, eight served as control (equal volume of 0.9 % NaCl). After 4 hours the mice were killed by decapitation. Brains were quickly removed and frozen in liquid nitrogen. Brains were examined for the presence of serotonin, tryptophan and dopamine by HPLC/fluorescent detection.

Preparation of brain samples.
Brain homogenates (1:3) in water were freshly prepared using a Polytron (Kinematica, Luzern, Switzerland). For the analysis 50 μl of homogenate were mixed with 200 μl of acetonitrile, sonicated and centrifuged. 100 μl of the supernatant were dried under a stream of air and dissolved in 50 μl methanol-water (50:50). 10 μl were injected into the HPLC system.

HPLC equipment, instrumental and analytical conditions.
The analysis of the neurotransmitters by RP HPLC and fluorescence detection was performed using a Nucleosil C_{18} RP 100-5 column (150*4.6mm i.d.) (Macherey-Nagel AG, Oensingen, Switzerland), a Waters chromatography pump model M-6000 (Waters, Milford, USA), a variable-wavelength fluorescence detector (Hitachi F-1000) and a ChemStation (Hewlett Packard) software. The mobile phase was acetonitrile-water (15:85) with 0.1 % formic acid and was delivered at a flow rate of 0.8 ml/min. The fluorescence detector was set to an excitation wavelength of 270 nm and an emission wavelength of 350 nm.

MAO Assay.
A stock solution of [^{14}C]labelled tyramine was prepared in sodium phosphate buffer 0.1 M, pH 7.4, so that 50'000 dpm/20 μl were obtained. For the assay 20 μl of a 1:9 brain homogenate in water was added to 100 μl of 0.1 M sodium phosphate buffer, pH 7.4, and 20 μl of the [^{14}C]tyramine stock solution. The mixture was incubated at 37°C for 30 minutes. The reaction was stopped with 100 μl of 2 M citric acid and the deaminated products were extracted into 3 ml toluene/ethyl acetate (1:1) [Glover et al. 1980]. One ml of the organic phase was added to 10 ml of Irga-Safe plus scintillation solution. All samples were counted for radioactivity in a Beta scintillation counter (Kontron Instruments) with automatic quench correction. Protein

content of the brain homogenates was determined by the method of Lowry [Lowry et al. 1951].

Statistics.
Results are expressed as mean values +/- SD. Statistical significance of the differences between groups was assessed by one way ANOVA and Bonferroni-t-test.

7.4 Results

The HPLC analysis revealed a significant difference in the brain concentration of serotonin between the control animals and the ones that had received MB. Serotonin was not decreased in mice following administration of IF compared to controls. Mice following IF/MB had the highest levels of serotonin compared to the other three groups. Figure 30 shows representative chromatograms obtained for each group.

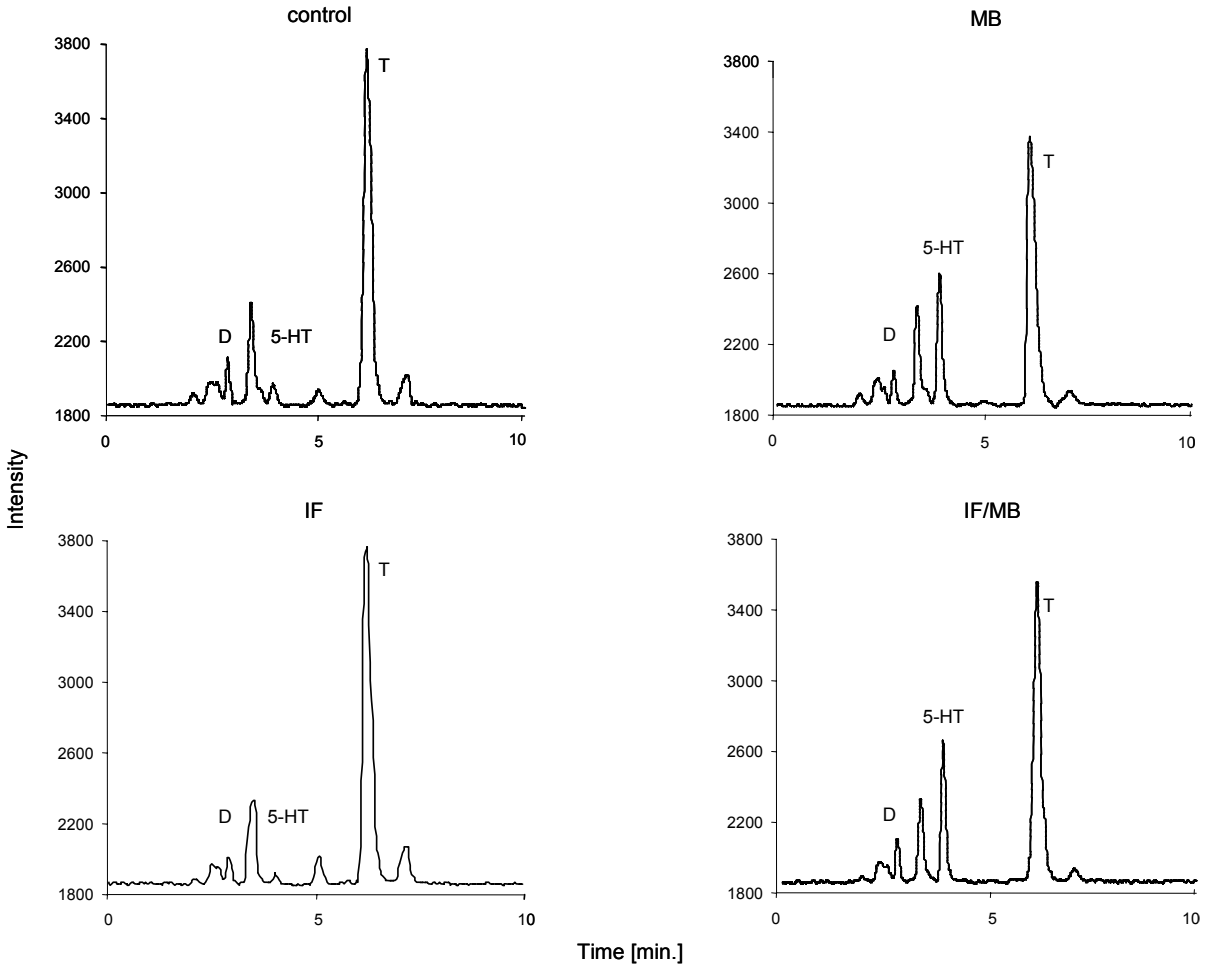


Figure 30: Representative chromatograms of brain homogenates following administration of MB, IF or IF/MB and of control brain homogenates. A marked increase in serotonin was found after MB and after IF/MB. D = dopamine, 5-HT = serotonin, T = tryptophan.

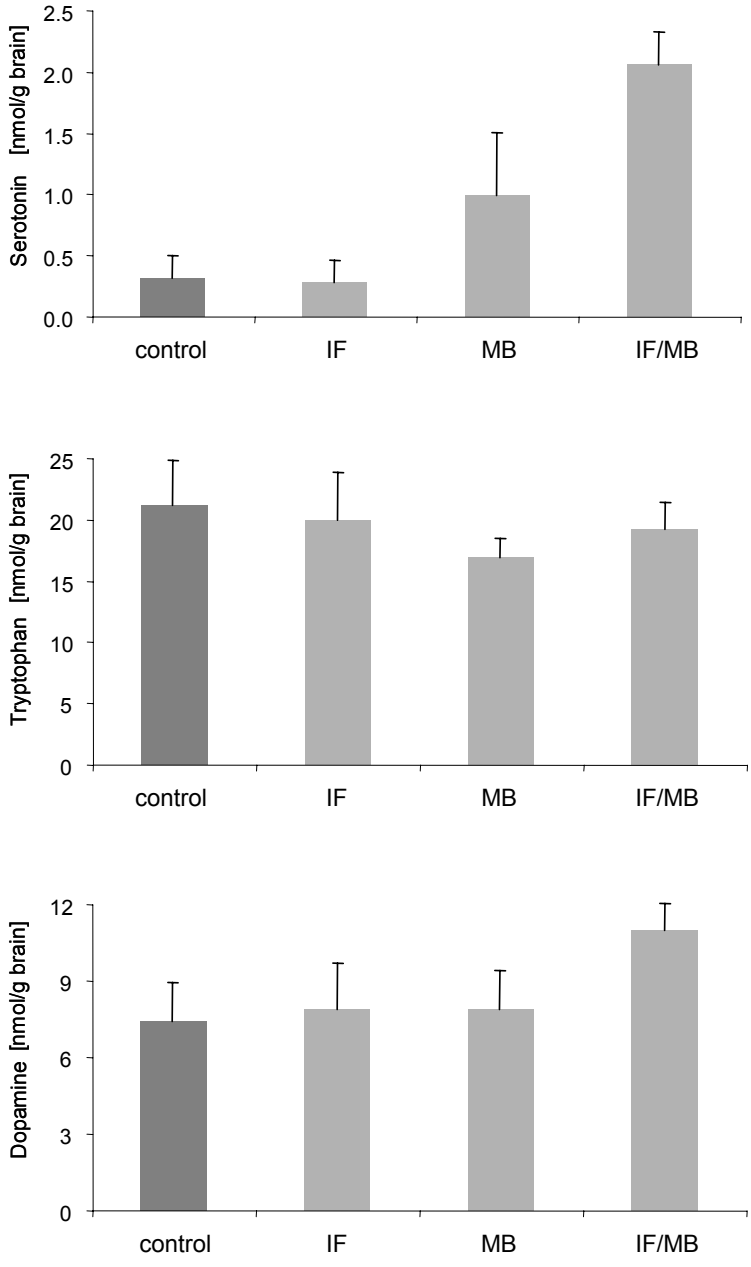


Figure 31: Serotonin, tryptophan and dopamine levels in the brain of mice: All serotonin levels are significantly ($P < 0.01$) different from each other, except controls versus IF mice. Tryptophan levels are not different from each other. The dopamine level in the brain homogenate of animals receiving IF/MB was found to be significantly ($P < 0.05$) higher compared to the control group. Bars represent mean values of four experiments +/- SD.

These findings indicate that MB increases the level of serotonin in the brain of mice compared to controls ($P < 0.01$). A significant difference was found between mice with IF and those with additional MB ($P < 0.01$). No difference regarding the serotonin level was measured between controls and IF-mice. Tryptophan levels were unaffected, dopamine was increased in animals receiving combined IF/MB.

Figure 32 summarizes the activity of MAO in the brain homogenates of the same mice. It can be seen that mice treated with MB exhibit an impaired tyramine oxidation, an effect that is even more pronounced when combined with IF. The administration of IF alone does not seem not to influence MAO activity.

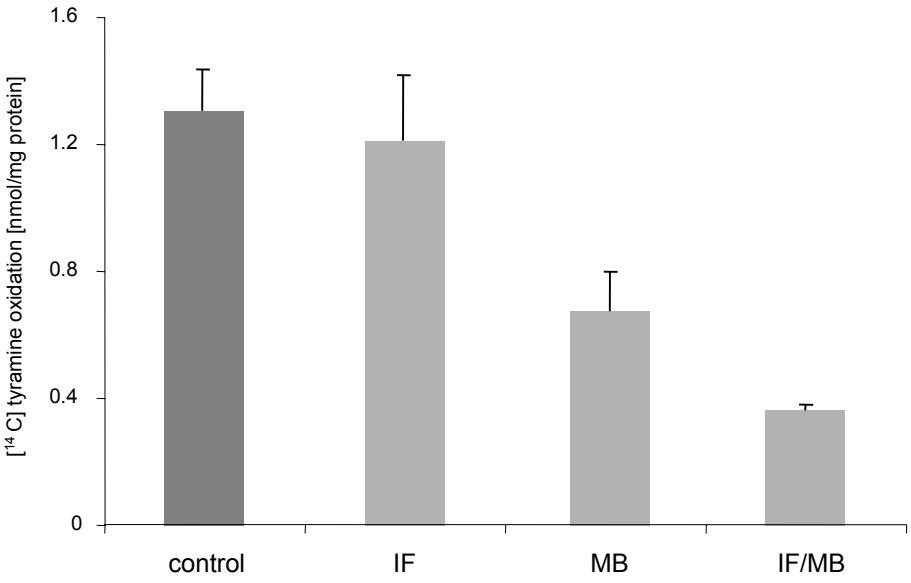


Figure 32: Influence of IF, MB and their combination on the deamination of tyramine, a substrate for both types of MAO. The MB and IF/MB groups are significantly ($P < 0.001$) different compared to the control. The IF and IF/MB groups are significantly ($P < 0.001$) different from each other. As well as the MB group from the IF/MB group. Bars represent mean values +/- SD, n = 4.

7.5 Discussion

The present data indicate that the administration of MB markedly increases the concentration of serotonin in brain. One possible explanation for this finding is, that MB inhibits the disposition of serotonin. As shown by additional experiments MB inhibits MAO which plays a major role in the metabolism of serotonin. That MB which as been shown to inhibit MAO in vitro inhibits the enzyme also in vivo has not been documented previously. The highest concentration of serotonin was indeed found in animal with the lowest activity of MAO. Neither tryptophan levels nor dopamine was found to be changed after MB. Serotonin is preferentially oxidized by MAO A, whereas dopamine is a substrate for both MAO forms. Whether MB selectively influences MAO A oxidation needs to be studied in more detail.

Another explanation for the increased serotonin levels might be a stimulating effect of MB on amino acids decarboxylation in the brain. Acetaldehyde is known to displace pyridoxal 5'-

phosphate (PLP) [Mezey 1985], a cofactor of L-amino acid decarboxylase (AADC), from its binding protein, thereby making it susceptible to hydrolysis. High levels of CAA following administration of IF might also disturb the availability of PLP, resulting in a decreased decarboxylation of amino acids such as tryptophan. Serotonin is synthesized from tryptophan in a two-step enzymatic pathway. First, tryptophan is 5-hydroxylated into 5-OH tryptophan by tryptophan hydroxylase, then decarboxylated to serotonin by AADC. A decreased decarboxylation of 5-OH tryptophan due to PLP deficiency might result in lower levels of serotonin.

In preliminary experiments serotonin levels were found to be decreased after the administration of IF. This finding could not be reproduced in the present set of experiments with a single large dose of IF, suggesting that a deficiency of serotonin is not responsible for the encephalopathy. Administration of IF over a longer period of time could well result in lower serotonin levels, and an increase in brain serotonin following MB might provide an explanation for the beneficial effect of MB observed in patients.

MB is known to possess some additional effects on neurotransmitters. It was shown that it affects iron-containing enzymes [Kelner et al. 1988], such as nitric oxide synthase (NOS) and soluble guanyl cyclase (sGC) and it is suggested that it therefore has an inhibitory effect on the NOS-NO-cGMP pathway. In neurons NO is produced from L-arginine by calmodulin-dependent NO synthase (nNOS) and it fulfils some of the important criteria for a neurotransmitter [Knowles and Moncada 1994]. NO activates sGC via binding to the heme part of the enzyme, causing elevations in cGMP. Considering the important role of this pathway in the brain, studies were performed to support the empirical use of MB in psychiatry. They showed that MB possesses anxiolytic and antidepressant properties in a range of 7.5 - 30 mg/kg in rats [Eroglu and Caglayan 1997].

Our experiments have shown that MB inhibits the oxidation of tyramine and increases the concentration of serotonin in the brain in vivo. Whether these effects are responsible for the reversal of IF-induced encephalopathy by MB remains to be elucidated.

8 Direct determination of S-carboxymethylcysteine in brain of mice after administration of ifosfamide by HPLC/electrospray ionization mass spectrometry

8.1 Summary

S-carboxymethylcysteine (SCMC) is described to have agonistic effects on the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/Kainate receptor and is assumed to be a metabolite of IF. So far, the presence of SCMC following administration of IF has not been shown neither in the brain nor in plasma or urine. We report that SCMC is formed in the brain of mice after a high dose of IF. This observation could provide new insights into the IF-induced encephalopathy.

8.2 Introduction

SCMC is a possible metabolite of the alkylating oxazaphosphorine IF since it is a potential precursor of thiodiglycolic acid (TDGA) that was found in human urine after IF [Visarius 1998]. SCMC selectively activates AMPA/Kainate receptors, possibly due to structural similarities with the excitatory neurotransmitter glutamic acid (Glu) and induces cellular acidification in mouse cortical neurons [Chatton et al. 2001]. This finding raises the question whether SCMC could be responsible for the IF-associated encephalopathy. A number of molecules generated by the reaction of the toxic IF-metabolite CAA with endogenous compounds could be candidates for the neurotoxic adverse effects, among them SCMC. CAA is assumed to penetrate the blood brain barrier [Sood and O'Brien 1996] and the consequent reaction with glutathione or cysteine to form SCMC is thus hypothetically possible. Figure 33 shows the proposed formation of SCMC.

It is unlikely that SCMC crosses the blood-brain barrier, as SCMC is routinely prescribed as an oral mucolytic agent, which has not been described to cause central nervous toxicity. Moreover, SCMC administered to mice does not produce observable CNS effect. This may be due to the impermeability of the blood brain barrier to SCMC whereas in the case of IF SCMC might be formed in situ. High concentrations of CAA up to 0.22 mmol/L [Cerny and Küpfer 1989] might well result in the local formation of SCMC in the brain. We therefore examined brain of mice following administration of IF for the presence of SCMC. Whether MB which is used to prevent and treat IF-induced encephalopathy [Küpfer et al. 1994, Zulian et al. 1994, Küpfer et al. 1996, Pelgrims et al. 2000] influences the proposed formation of SCMC in the brain was studied as well.

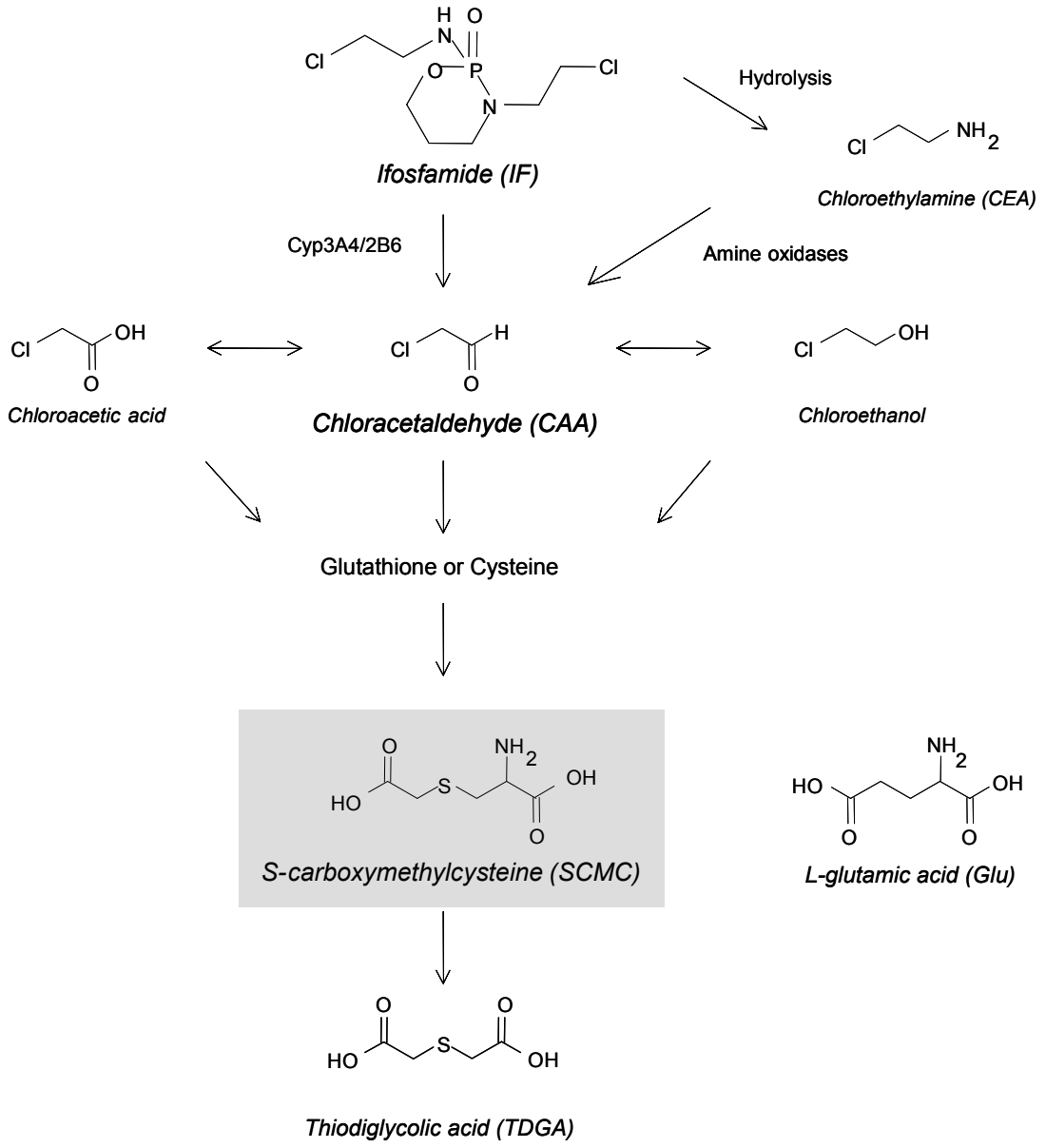


Figure 33: Proposed formation of SCMC from a two-carbon metabolite of IF such as CAA or its associated oxidation/reduction products chloroethanol or chloroacetic acid and glutathione or cysteine. SCMC is a precursor of TDGA that was found in human urine after IF administration. Glu is structurally similar to SCMC.

8.3 Material and methods

Chemicals.

S-carboxymethyl-L-cysteine and L-glutamic acid monosodium salt were provided by Sigma Chemical Co. (Buchs, Switzerland). Ifosfamide (Holoxan®) was from Asta Medica (Wangen, Switzerland). Methanol HPLC of supra-gradient quality was purchased from Biosolve Ltd. (Valkenswaard, The Netherlands), trifluoroacetic acid from Merck (Darmstadt, Germany). The water was of Purelab Option E (Labtec, Wohlen, Switzerland) quality (filtered and UV light treated).

Animals.
Swiss female mice (ca. 25 g) fed a standard chow diet and water ad libitum were used in all experiments. Twelve animals were injected with 1 g/kg (3.8 mmol/kg) IF in 0.9 % NaCl i.p., a group of eight mice received 840 mg/kg (4.7 mmol/kg) SCMC in 0.9 % NaCl i.p., four animals received 10 mg/kg (31 μmol/kg) MB in 0.9 % NaCl, 30 minutes prior to 1 g/kg of IF. The IF and SCMC doses were chosen assuming that one molecule IF forms 1.2 molecules of SCMC. After 4 hours the mice were killed by decapitation. Brain and liver were quickly removed and frozen in liquid nitrogen. Brains were examined for the presence of SCMC and Glu by LC/MS. A separate group of four animals received 840 mg/kg SCMC i.p. which were killed after one hour. Liver and brain were examined for the presence of SCMC.

Brain sample preparation.
Brain homogenates (1:3) in water were freshly prepared using a Polytron (Kinematica, Luzern, Switzerland). For the analysis of SCMC and Glu 50 μl of homogenate were mixed with 200 μl of acetonitrile, sonicated and centrifuged. 100 μl aliquots were dried under a stream of air and dissolved in 50 μl methanol-water (50:50), 10 μl of which were injected into the LC/MS system.

HPLC/MS equipment, instrumental and analytical conditions.
A Hewlett Packard 1100 series quaternary pump and a Agilent 1100 series automatic sample injector were used for all analyses. The chromatographic system consisted of a Nucleosil 100-5 SB column (250*4.6 mm i.d.) (Macherey-Nagel AG, Oensingen, Switzerland). The mobile phase was methanol-water (50:50) with 0.1 % trifluoroacetic acid and was delivered at a flow rate of 0.4 ml/min. The eluate from the HPLC column was directly passed into the ionization region. A similar approach has been proposed for the measurement of SCMC in human plasma [Anacardio et al. 1997].

MS measurements were performed on a Finnigan LCQ ion trap instrument (Finnigan MAT, San Jose, USA) equipped with an ESI (Finnigan) source that was run in the positive ion mode (3.5 kV). Sheath gas (N_2) pressure was set at 60 arbitrary units. The temperature of the heated capillary was 200°C. The instrument was computer controlled using the XCalibur 1.0 software Finnigan. Full scan mass spectra were acquired in the mass range of 50-200. Automatic gain control (AGC) was employed using 3 microscans and a maximum injection time of 200 ms. MSMS was performed with an isolation width of 2 and a relative collision energy of 25 %.

Standard solutions.
Stock solutions of SCMC and Glu of 10 mmol/L were prepared and further diluted to give working standard solutions of 200, 100, 50, 25 and 12.5 μmol/L for SCMC and 10.0, 4.0, 2.0, 1.0, 0.5 and 0.25 mmol/L in the case of Glu 50 μl of each standard solution was mixed with

200 µl of acetonitrile. 100 µl of it were dried under a stream of air and kept at –20°C till use. Before analysis the standards were re-dissolved in 50 µl methanol-water (50:50) and 10 µl were injected.

Validation.
The intermediate precision (between-day or inter-assay precision) was evaluated by measuring SCMC standard solutions of 100 and 25 µmol/L over 9 days. The repeatability (intra-assay precision) of the method was determined by five replicate analyses of worked-up SCMC standard solutions of a concentration of 200 µmol/L. The specificity of the assay was evaluated by analysing brains from untreated animals. The detection limit (LOD) was set to a signal to noise ratio (S/N) of 3. All validation parameters were selected according to the review of Peters and Maurer [2001].

Determination of glutathione.
The content of total glutathione in the brain and liver was measured on deproteinated samples according to the method of Tietze [1969].

Statistics.
The results are expressed as mean values +/- SD. Statistical significance of the differences between groups was assessed by a one way ANOVA and Bonferroni-t-test.

8.4 Results

Following the injection of IF the animals exhibited impaired co-ordination and seizures whereas the behaviour of the mice treated with SCMC was unchanged. Also, the analysis of the brains showed a major difference between the two groups. Mice treated with IF exhibited a large peak corresponding to SCMC in the MSMS chromatogram (figure 34). Figure 34 gives details for Glu, which served as ‘endogenous standard’, as well. The two groups are equal relative to Glu but different in relation to SCMC. The structural confirmation of the SCMC peak was performed by MSMS and comparison of the spectrum of authentic SCMC with the spectrum of the peak of interest in the brain of a mouse treated with IF. The two spectra are congruent, as can be seen from the typical fragmentation pattern, with the main masses 162.9 and 133.9 (figure 35).

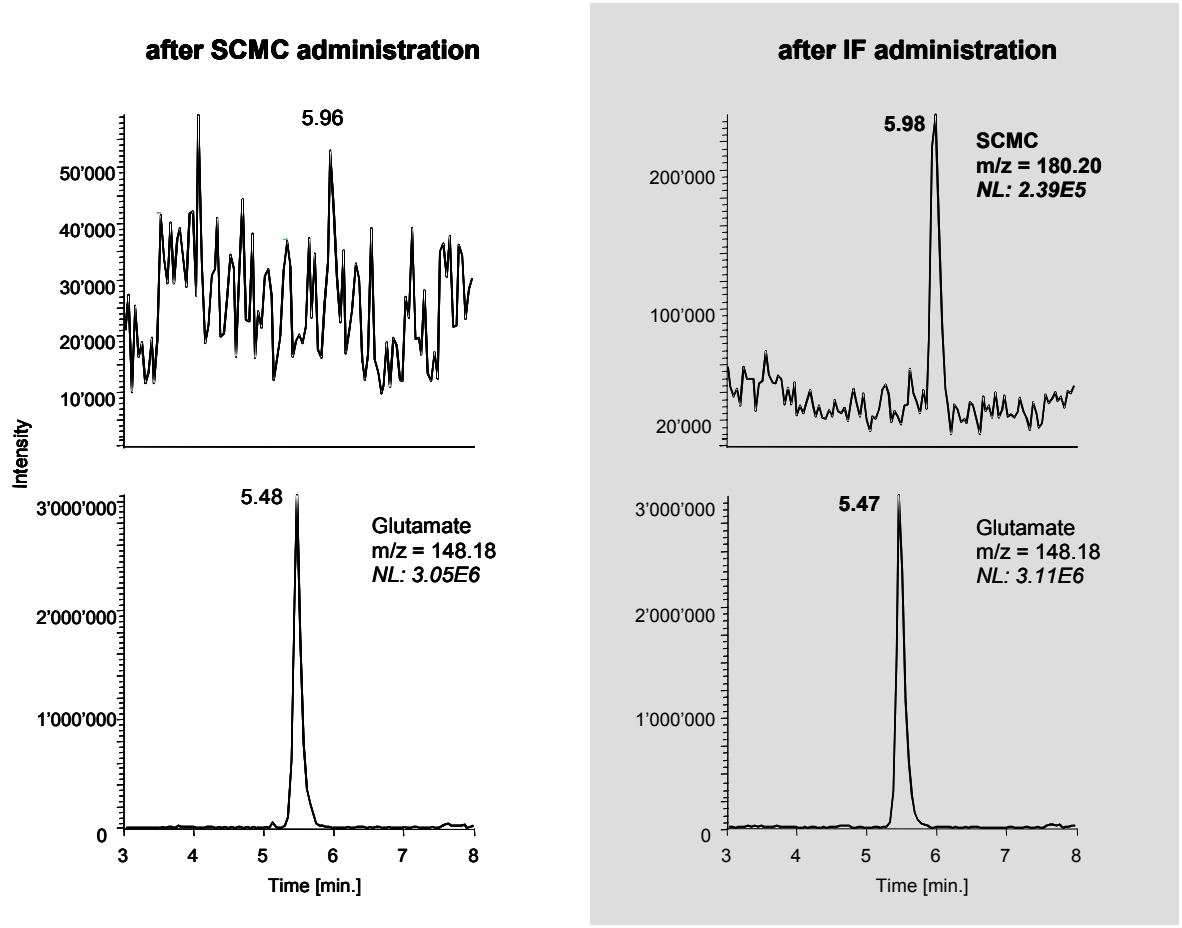


Figure 34: MSMS chromatograms (upper trace SCMC, lower trace Glu) of brain samples following administration of SCMC (left) and IF (right). *NL* stands for the peak intensity of the highest peak height in the chromatogram.

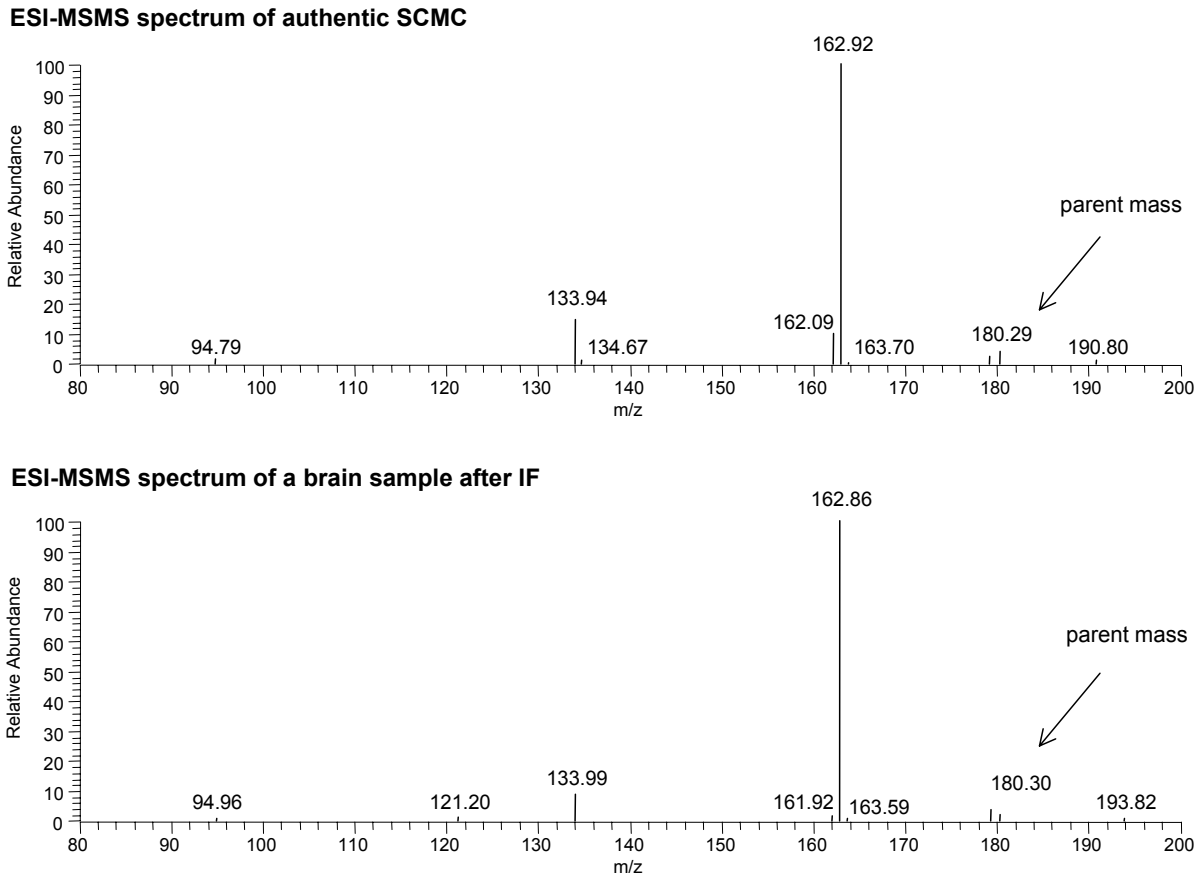


Figure 35: Comparison of MSMS spectra of a standard solution of authentic SCMC and a brain sample from a mouse treated with IF. The fragmentation pattern (180.3 → 162.9 → 133.9) is identical in both spectra.

The LOD was 12.5 μmol/L in standard solutions. The brain concentrations were calculated from the results obtained with 1:3 homogenates in water. In control animals (i.p. injection of 0.9 % NaCl) no SCMC was detected (data not shown). The interday RSD (n = 9) found for peak areas was not more than 16 % in standard solutions. The intraday RSD was determined to be 2.6 % for 200 μmol/L SCMC in a brain homogenate of a native mouse that was spiked with SCMC.

Compound	**Interday RSD (%)**
	n = 9
SCMC [μmol/L]	peak area RSD (%)
100	**16.0**
25	**14.1**

Compound	**Intraday RSD (%)**
SCMC 200 μmol/L	n = 5
peak area	peak area RSD (%)
11'406'217	
11'544'264	
11'598'160	
12'110'157	
11'971'775	**2.6**

Table 12: Interday RSD (%) of standard solutions in water and intraday RSD (%) of SCMC in a brain homogenate spiked with SCMC.

Comparison of calibration curves in water, as used for all measurements, with a calibration curve obtained with a brain homogenate spiked with SCMC showed no major difference. Consequently all quantifications were performed with standard curves in water.

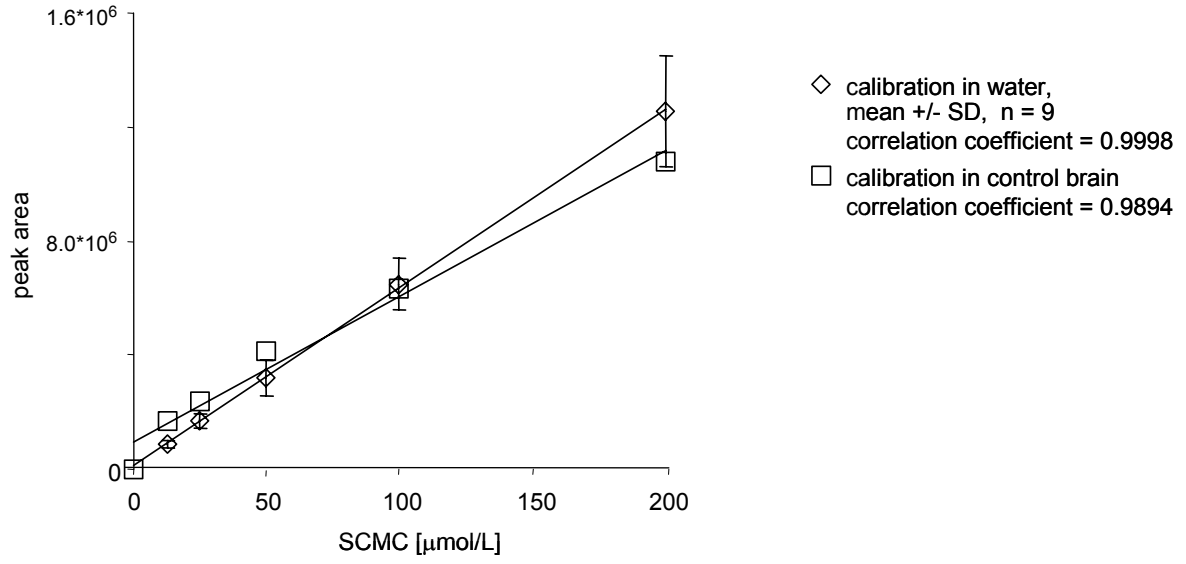


Figure 36: Comparison of calibration curves in water and in control brain fortified with SCMC.

Figure 37 illustrates the results obtained from the brain of mice examined for the presence of SCMC.

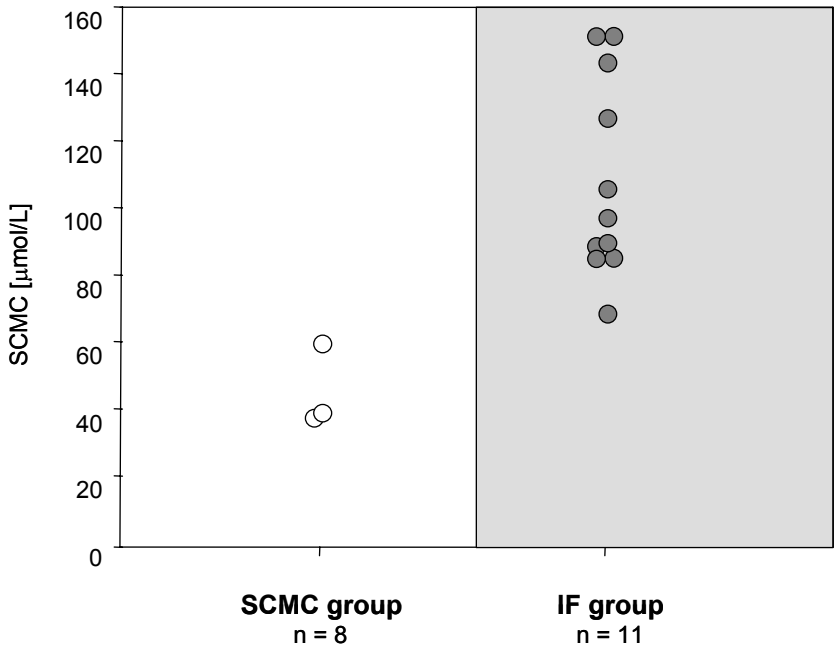


Figure 37: Point plot of all samples. In five of eight mice following SCMC administration no SCMC was detected. One of twelve IF animals shortly died after IF injection.

Table 13 gives details for each animal regarding the concentration of SCMC and Glu in the brain. In five of eight mice following SCMC administration no SCMC was detected. The concentrations measured in three brains following SCMC were all lower than the any concentration of SCMC following IF administration.

mouse	*SCMC group* n = 8 **SCMC nmol/g**	*SCMC group* n = 8 **Glutamate μmol/g**
1	n.d	10.76
2	n.d.	8.74
3	59.36	8.38
4	n.d.	7.36
5	37.27	10.89
6	38.73	11.31
7	n.d.	11.87
8	n.d.	13.89

mouse	*IF group* n = 11 **SCMC nmol/g**	*IF group* n = 11 **Glutamate μmol/g**
1	96.84	10.36
2	105.49	9.27
3	143.19	11.17
4	126.63	12.66
5	151.11	15.47
6	151.04	12.44
7	68.29	10.68
8	84.94	16.60
9	88.41	15.88
10	84.75	17.28
11	89.44	16.95

n.d. = not detected

Table 13: Concentrations of SCMC and Glu in the brain of mice following administration of SCMC or IF.

The administration of MB thirty minutes prior to IF did not influence the SCMC concentration in the brain as can be seen from figure 38.

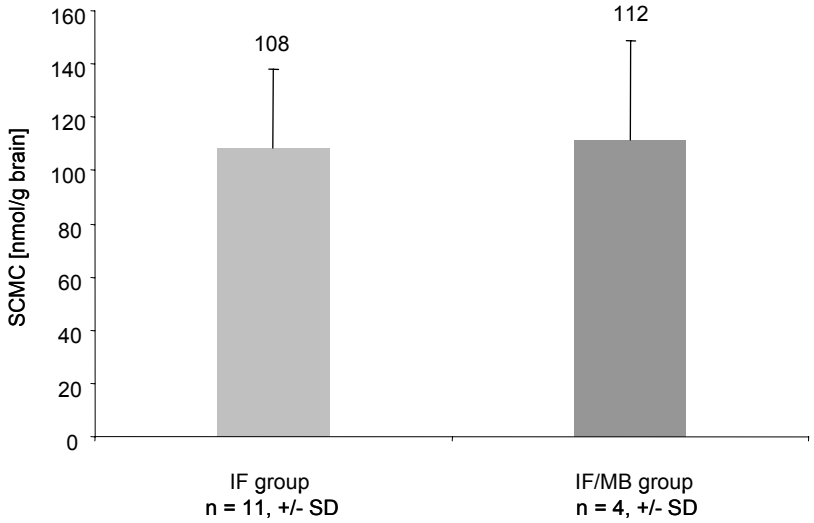


Figure 38: SCMC in the brain of mice following administration of IF or MB followed by IF.

The failure to detect SCMC in the brain four hours after administration of SCMC does not rule out the possibility that SCMC was present at earlier time points but had been eliminated by four hours. Therefore, SCMC was measured in a group of mice one hour after administration of SCMC. Average concentration of SCMC in the liver amounted to 855 μmol/L. Thus, approximately 17.8 % of the total SCMC dose were found in the liver.

mouse	***brain SCMC*** [nmol/g]	***liver SCMC*** [nmol/g]
1	64.69	724.75
2	69.89	590.62
3	65.98	1144.09
4	72.37	960.61
mean +/- SD, n = 4	68.2 +/- 2.7	855.0 +/- 288.7

Table 14: SCMC concentrations in the brain and the liver of four mice one hour after i.p. administration of 4.7 mmol/kg SCMC.

In agreement with the work performed by Sood and O'Brien cerebral GSH was not significantly depleted after administration of IF [Sood and O'Brien 1996]. In contrast, hepatic GSH was markedly decreased after IF compared to the mice that had receiving SCMC.

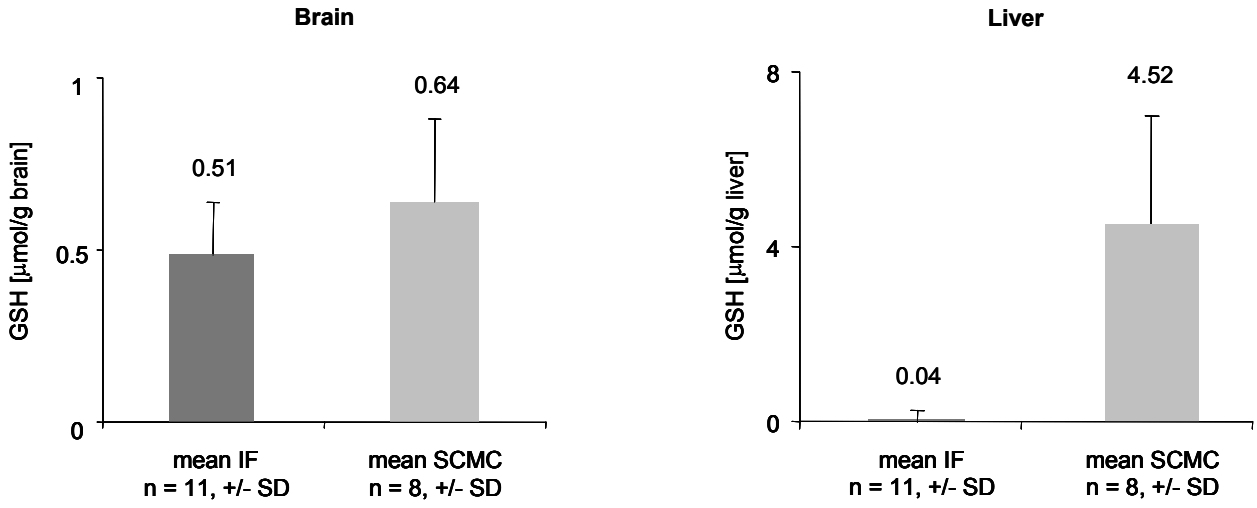


Figure 39: Total GSH was measured according to the method of Tietze [1969]. The bars represent mean values +/- SD. Liver GSH levels are significantly ($P < 0.001$) different from each other whereas there is no statistical difference between the IF and the SCMC group regarding their brain GSH.

8.5 Discussion

The present data demonstrate the presence of SCMC in brain of mice following administration of IF. Small amounts of SCMC seem to be present after administration of SCMC, though they are below a S/N ratio of 10 with the analytical method used. It seems possible that a considerable fraction of the IF dose is converted into SCMC as each molecule IF possesses two chloroethyl side chains which result in CAA by CYP450 dependent dechloroethylation [Dechant et al. 1991, Kaijser et al. 1992, Kurowski and Wagner 1993, Walker et al. 1994, Granvil et al. 1999] or by enzymatic conversion of IF-produced CEA [Aeschlimann et al. 1996]. As suggested by the effects of SCMC on mouse cortical neurons [Chatton et al. 2001] SCMC can interfere with normal central nervous system functions. Whether the concentrations observed in our experiments are sufficient to trigger an activation of the AMPA/Kainate receptors remains to be demonstrated. The concentrations measured in this work are relatively low (68-151 μmol/L) in comparison to EC_{50} for the Ca^{2+} response (1.3 +/- 0.1 mmol/L) in the work of Chatton [Chatton et al. 2001]. Since CAA concentrations of up to 0.22 mmol/L were measured in the plasma of patients receiving IF, it is conceivable that still higher concentrations of SCMC are achieved during a continuous infusion of IF over 5 days. Indeed, the excretion of TDGA, a metabolite of SCMC, increases over 5 days of continuous IF infusion, probably due to auto-induction of CYP450 [Boddy et al. 1995, Visarius et al. 1998]. Considering that up to 8.49 % of the total IF dose were found as TDGA in human urine [Visarius et al. 1998] and that approximately 20 % of SCMC are described to be metabolized to TDGA [Steventon 1999], 42.5 % of a dose of IF might be converted into SCMC.

The activation of the AMPA/Kainate receptor could be of high relevance for the understanding of the encephalopathy associated with IF treatment. The design of the present study does not allow to asses the significance for the clinic but the identification of SCMC in the brain may represent a novel pathway of neurotoxicity for a number of different compounds that are able to generate the same metabolic products in vivo. It has been shown that TDGA, a metabolite of SCMC [Waring and Mitchell 1982, Hofmann et al. 1991, Steventon 1999] is formed after exposure to 1,2-dichloroethane [Payan et al. 1993], 1,2-dibromoethane [Wormhoudt et al. 1998], 2-(chloroethyl)nitrosoureas [Godeneche et al. 1993] and vinyl chloride [Müller et al. 1976, Müller and Norpoth 1977, Draminski and Trojanowska 1981] and SCMC itself is described to be a metabolite of the industrially important compounds acrylonitrile [Fennell et al. 1991, Kedderis et al. 1993] and 1,1-dichloroethylene [Reichert et al. 1979]. The toxicity profile of these substances is characterized by a significant component of neurotoxicity [Liubchenko et al. 1997] and may be due to the in situ generation of SCMC.

Concentrations of up to 1.1 mmol/L SCMC were found in the liver of mice one hour after the administration of 4.7 mmol/kg SCMC i.p. whereas the concentration in brain was at least one order magnitude lower. The observation that in the case of SCMC administration a minor fraction is found in the brain (1.4 % after 1h, less after 4 h) whereas a large amount is found in the liver suggests that the blood-brain barrier is poorly permeable for SCMC. The finding of SCMC in brain following IF but not following SCMC then indicates that SCMC is formed in situ after the administration of IF. The additional administration of MB did not influence the concentration of SCMC in the brain of mice following IF.

In conclusion, the present study has identified and quantified SCMC as a metabolite of the cytostatic drug IF in the brain of mice. This may contribute to the understanding of IF-associated encephalopathy and might reveal new insights into the pathophysiology of other neurotoxicities.

9 Conclusion and outlook

The investigation of the mechanism responsible for a drug-induced encephalopathy is an ambitious and difficult undertaking. In patients exhibiting symptoms of encephalopathy the brain can only been studied from a distance, hoping that the analysis of blood, urine or electrical signals from the brain will provide some clues about the ongoing processes in the central nervous system. In animals, where many variables can be controlled such processes can be studied more closely since the brain can be sampled directly. However, the signs and symptoms of the encephalopathy occurring in the clinical setting are difficult to assess and to document objectively in an animal model. Moreover, the metabolism of the drug under investigation may differ considerably from humans. Thus, clues rather than definite answers regarding the pathogenesis of IF-induced encephalopathy can be expected from this work.

We have shown that considerable concentrations of CAA are reached following administration of IF to rats. Subsequent studies on intermediate metabolism were expected to disclose effects of this toxic metabolite. The expected increase of NADH resulting from the dehydrogenation of CAA was thought to induce changes in cellular metabolism and MB was expected to correct these metabolic disturbances. CAA resulted in hypoglycemia and a decrease in circulating fatty acids and inhibition of the Krebs cycle but MB had no effect on any of these processes and did not decrease the plasma concentration of CAA following IF administration. These findings suggest that systemic metabolic processes, although in part disturbed by the administration of IF, are not likely to be responsible for the development of IF-induced encephalopathy. The subsequent experiments therefore focused on the formation of potentially psychoactive IF metabolites in the brain.

Due to the high reactivity of CAA and the availability of endogenous substrates necessary for the formation of THβCs which exhibit remarkable pharmacological properties and which could be responsible for hallucinations, anxiety, seizures and other manifestations of IF encephalopathy their formation was considered in more detail. Three mono-chlorinated THβCs were synthesized and their effects on mitochondrial function, MAO activity and their interaction with BZD receptors were studied. The examined THβCs inhibited MAO-dependent tyramine oxidation as well as respiration in rat liver mitochondria but could not be identified following administration of IF. Future studies will have to look for unsaturated, methylated and possibly dechlorinated metabolites of the THβCs and tetrahydroisoquinolines, the reaction products of CAA and phenylethylamines. Endogenous βC type compounds and tetrahydroisoquinolines are particularly neurotoxic when activated into N-methyl compounds [Naoi et al. 1989, Matsubara et al. 1992] and have been proposed as possible causative candidates triggering parkinsonism. Nicotinamide N-methyltransferase

(NNMT) seems to be the main enzyme involved in the methylation of azaheterocylic amines and is elevated in patients with Parkinson's disease. This suggests that NNMT might be implicated in the pathogenesis of Parkinson's disease [Matsubara et al. 2002] and might also be a critical factor in other neuropathologies such as IF-associated neurotoxicity.

Chatton and coworkers have suggested that the IF-metabolite SCMC might be involved in the development of IF-neurotoxicity [Chatton et al. 2001]. As a result of the striking findings presented in the above work, namely the activation of AMPA/Kainate receptors in mouse cortical neurons by SCMC, we examined brains of mice for the presence of this critical compound and subsequently showed evidence for its presence following IF administration. Although no firm conclusion can be drawn with respect to the clinical situation from this experiment, it provides an instructive example for the in situ formation of a potentially toxic compound rather than its formation in the liver or some other organ with consequent penetration through the blood-brain barrier into the brain. Based on our studies it is conceivable that SCMC might be one of the triggering factors for the IF-induced encephalopathy acting on neuronal receptors.

Whether SCMC once formed in the brain might trigger further neurotoxic symptoms following metabolic activation will be subject of further experiments. SCMC might react to form a cyclic mono-carboxylate derivative, namely 6-oxo-1,4-thiazane-2-carboxylic acid which is structurally similar to 6-oxo-pipecolic acid, a possible metabolite of pipecolic acid (PA) (figure 40). PA, a six-carbon cyclic imino acid, is formed as an intermediate in the degradation of lysine and possesses distinct GABA-ergic effects. In most mammalian tissues the principal route of lysine degradation proceeds via L-saccharopine. However, the saccharopine pathway is not operative in the central nervous system, where the major pathway for lysine catabolism occurs through PA. Patients with hyperpipecolic acidemia or with the neurodegenerative disorder glutaric aciduria type II, accumulate PA [Dodt et al. 2000]. PA is also elevated in the plasma of patients with chronic liver diseases, especially in cases of hepatic encephalopathy [Fujita et al. 1999, Matsuda et al. 2000]. Because PA and its metabolites possess central nervous activity [Dodt et al. 2000], these compounds might have a role in the pathophysiology of such disorders. In glutaric aciduria type II sarcosine is elevated additionally, because the mitochondrial sarcosine dehydrogenase is blocked. Lately it was shown that pipecolic acid oxidase (PO) is similar to sarcosine oxidase [Dodt et al. 2000]. Sarcosine and glutaric acid levels were also found to be elevated in patients receiving IF [Küpfer et al. 1994]. Zabriskie and Liang found that the compound 1,3-thiazane-4-carboxylic acid (figure 40) causes a time-dependent irreversible inactivation of primate PO [Zabriskie and Liang 1997]. Possible thiazane metabolites of IF might therefore be inhibitors

of brain PO, too, leading to increased levels of pipecolic acid and consequent inhibitory neurotransmission via the GABA-ergic system.

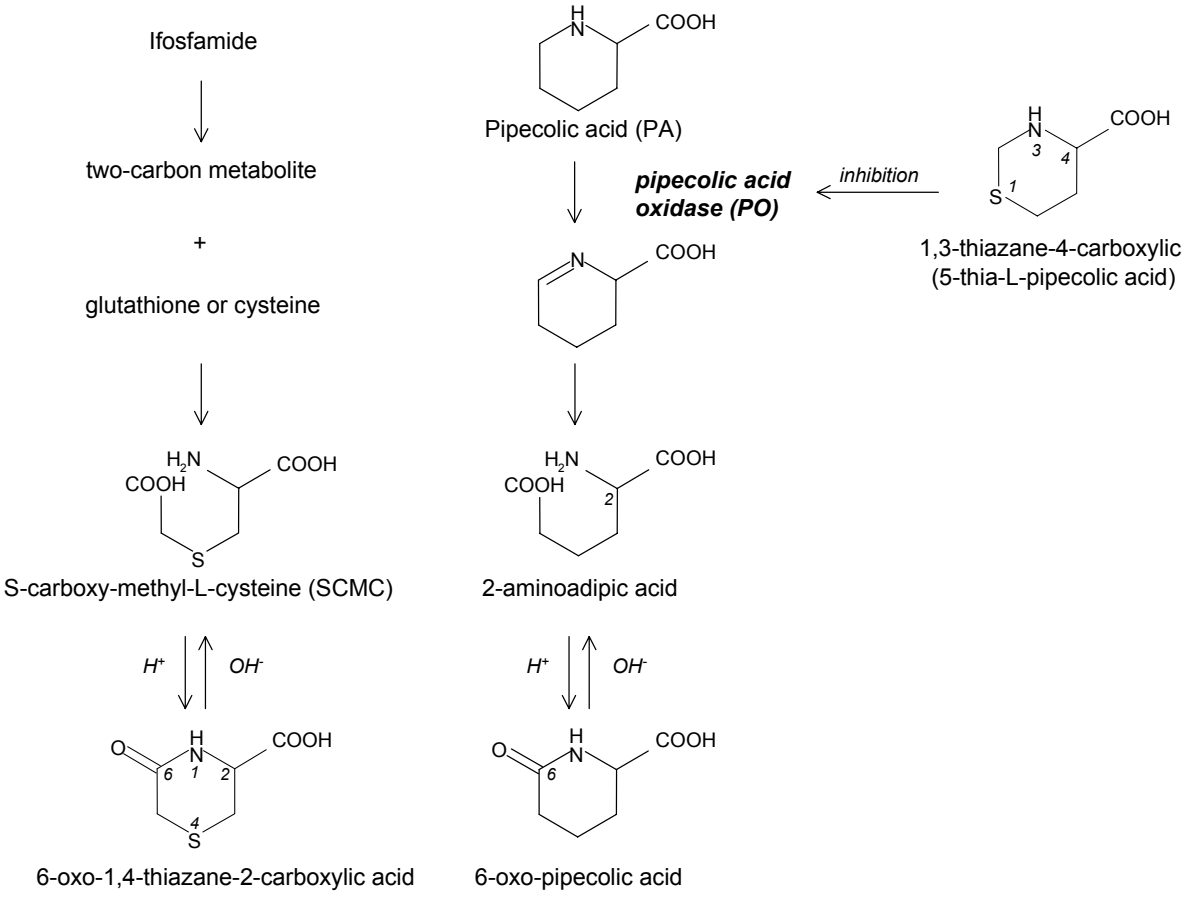


Figure 40: SCMC was shown to be formed in the brain of mice following administration of IF. Its cyclic derivative 6-oxo-1,4-thiazane-2-carboxylic acid might act similarly to 5-thia-L-pipecolic acid, a sulfur containing analogue of PA, that was found to be an excellent substrate for Rhesus monkey liver L-PO and to irreversibly inactivate the enzyme.

The metabolism of the prodrug IF is a prerequisite for its cytotoxic actions. The metabolic products are responsible for both tumour killing as well as for the adverse effects. Paradoxically, the mixed function oxidase CYP3A4 performs both, the desired 4-hydroxylation and the unwanted dechloroethylation resulting in CAA. Since CAA possesses cytotoxic effects of its own modulation of CYP3A4 provides no solution to the prevention of neuropsychiatric side effects. MB is still the only compound with convincingly demonstrated efficacy in the treatment and prevention of IF-induced encephalopathy. While our studies regarding the mechanism of action of MB can not claim to ultimately resolve the questions concerning its mode of action our studies nevertheless provide new information. Our results reveal a significant inhibition of MAO-dependent tyramine metabolism in rats receiving MB in vivo. Concomitant with the reduced activity of MAO in rat brain following MB administration

we found markedly elevated serotonin concentrations compared with controls. An increase in brain serotonin following MB might provide an explanation for the beneficial effect of MB observed in patients with IF-induced encephalopathy. Questions regarding serotonin homeostasis in patients with IF-encephalopathy will have to be pursued in future studies.

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