Toxicity and Biotransformation of 1,1,1,3,3-Pentafluoropropane, 3,3,3-Trifluoropropionic acid and 1,1,1,3-Tetrachloropropane

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“ You never conquer the mountain,

You only conquer yourself ”

Jim Whittacker (born 1929)

first American to climb Mt. Everest;

May 1, 1963

Meinen Eltern
Anita und Josef

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List of Abbreviations

AA	amino acid
ALP	alkaline aminotransferase
ATP	adenosintriphosphate
BAL	dimercaprol
BFCs	bromofluorocarbons
b.p.	boiling point
b.w.	body weight
BSA	bovine serum albumin
$CDCl_3$	deuteriated chloroform
CFCs	chlorofluorocarbons
CFC-11	trichlorofluoromethane
CFC-12	dichlorodifluoromethane
CFC-13	chlorotrifluoromethane
CFC-113	1,1,2-trichloro-1,2,2-trifluoroethane
CFC-114	1,2-dichloro-1,1,2,2-tetrafluoroethane
CFC-115	1-chloro-1,1,2,2,2-pentafluoroethane
CFC-116	hexachloroethane
CK	creatine kinase
CKMB	creatine kinase isoenzyme
CoA	coenzyme A
COSY	correlation spectroscopy
CPT	carnitine palmitoyltransferase
d	duplet
DCTH	5,6-dichloro-4-thia-5-hexenoic acid
DFA	difluoroacetic acid
DFAc	2,2-difluoroacrylic acid
DMA	dimethylamine
DMEM	Dubelcco’s modified Eagles Medium
D_2O	deuterium oxide
EGTA	ethylene glycol-bis-(2-aminoethylether)-N,N,N’,N’-tetraacetic acid)
ELISA	enzyme linked immunosorbent assay

$FADH_2$	flavine adenine dinucleotide
FBS	fetal bovine serum
fig	figure
g	gravities
GC/MS	gas chromatography coupled with mass spectrometry
GGT	gamma-glutamyl transferase
GOT	glutamate oxalacetate transaminase, aspartate amino transferase (AST)
GPT	glutamate pyruvate transaminase, alanine amino transferase (ALT)
GWP	global warming potential
HCFCs	hydrochlorofluorocarbons
HCFC-22	chlorodifluoromethane
HCFC-123	1,1-dichloro-2,2,2-trifluoroethane
HCFC-141b	1,1-dichloro-1-fluoroethane
HFCs	hydrofluorocarbons
HFC-23	trifluoromethane
HFC-125	pentafluoroethane
HFC-134a	1,1,1,2-tetrafluoroethane
HFC-227ea	1,1,1,2,3,3,3-heptafluoropropane
HFC-245fa	1,1,1,3,3-pentafluoropropane
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
Hz	hertz
i.d.	inner diameter
kg	kilogram
α-KG	α-ketoglutarate
kPa	kilopascals
LCA	leucocyte common antigen
LD_{50}	lethal dose, which shows 50% lethality in a defined group
LDH	lactate dehydrogenase
m	multiplet
mAU	million absorption units
min	minute
mio	million

m.p.	melting point
ms	milli seconds
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
m/z	mass to charge relation
NADH	ß-nicotinamide adenine dinucleotide 2´-phosphate
NADPH	ß-nicotinamide adenine dinucleotide 2´-phosphate, reduced tetrasodium salt
NOESY	nuclear overhauser effect spectroscopy
NMR	nuclear magnetic resonance spectroscopy
o.d.	outer diameter
OD	optical density
ODP	ozone depletion potential
PAFT	Programme for Alternative Fluorocarbon Toxicity Testing
PBS	phosphate buffered solution
PCA	principal component analysis
ppm	parts per million
PMS	phenazine methosulfate
PTP	permeability transition pore
rpm	revolutions per minute
rt	retention time
s	singulet
SIM	single ion monitoring
t	triplet
TAMO	trimethylamine-N-oxide
TCA	tricarboxylic acid
TPP	thiamine pyrophosphate
TSP	sodium 3-trimethylsilyl-(2,2,3,3,-$^{2}H_4$)-1-proprionate
TTFA	thenoyltrifluoroacetone
UBUN	blood urea nitrogen

1 INTRODUCTION

The assessment and effects of chemical substances on humans plays a major role in the field of toxicology. The mode of action as the initiator of the toxicological response is probably the most important question to answer. In general, chemical substances can be grouped together with respect to their molecular structure, their mode of action or the resulting biological response. Investigations on 1,1,1,3,3-pentafluoropropane (HFC-245fa) were initiated by the industrial sector in order to take advantage of its characteristics as a potential halocarbon replacement. Such non-chlorine containing compounds have been developed, with the advantage that they do not deplete the ozone layer. Most of these chemicals are hydrofluorocarbons. One subject of this thesis -1,1,1,3,3-pentafluoropropane- is volatile with a boiling point of 15°C and shows the characteristics of a reduced global-warming potential and also of a low potential for ozone depletion.

The ozone layer is the protective shield in the stratosphere and separates the latter from the troposphere. It plays an important role in reducing the amount of ultraviolet B radiation reaching the surface of the earth. Chlorofluorocarbons interfere with the ozone layer as soon as they reach the stratosphere. In the stratosphere, the solar radiation with its short wavelengths and higher energy is sufficient to break down the carbon-chlorine bond. As a consequence, the formed halogen radicals rapidly enter a catalytic ozone destruction cycle in which chlorine atoms cycle to chlorine monoxide and molecular oxygen; arising chlorine monoxide interferes with the catalytic formation of ozone by reacting with an oxygen atom and results in a reduction of stratospheric ozone concentrations. The steady state concentration of ozone is also disturbed by a variety of other factors, for instance the existence of chemically active species such as nitrogen (II) oxide, carbon monoxide, carbon dioxide, methane or reactive oxygen species (Reichl, 2002).

As seen with several other hydrofluorocarbons, 1,1,1,3,3-pentafluoropropane has a low potential for toxicity and is neither mutagenic nor teratogenic (Kawano et al., 1995; Collins et al., 1995). In a 90-day rat study all organs were considered normal; the only histopathological lesion observed in rats exposed to 1,1,1,3,3-pentafluoropropane by inhalation was an increased incidence of diffuse myocarditis with areas of

mononuclear inflammatory cell infiltration and degenerated myocardial fibers, which was seen in all animals exposed to 50 000 ppm and in the majority of animals exposed to 10 000 ppm 1,1,1,3,3-pentafluoropropane (all for 6 hours/day and 5 days/week) (Rusch et al., 1999).

Endpoint myocarditis is, however, considered to be rare for hydrofluorocarbons following repeated administration and has only been observed for 1,1,1,3-tetrachloropropane (Kolesar et al., 1995). Myocarditis is known to occur with drugs like doxorubicin, sulfonamides and furthermore with chemicals like carbon monoxide, lead or phosphorus (Marquardt and Schaefer, 2004). Hydrochlorocarbons however, such as 1,2-dichloropropane, are renowned for their influence on the physiology of the heart which is described as myocardial sensitization. This effect occurs after inhalation to very high concentrations and is due to the unmetabolized parent compound. Moreover, the cardiac sensitization has not been associated with histopathological lesions in the heart. The main target organs for most chemicals from this group are the liver and kidney, e.g. after exposure to trichloroethene, 1,1,2,2-tetrachloroethene and halothane. The toxicity of these compounds is dependent on the biotransformation to reactive metabolites, or on the formation of stable, but toxic products such as halogenated acids.

To elucidate a possible mechanism of 1,1,1,3,3-pentafluoropropane in inducing mild myocarditis, its biotransformation was investigated *in vivo* and *in vitro*. In the first part of the study, experiments addressed the mechanism of formation of the major metabolite of 1,1,1,3,3-pentafluoropropane using rat liver microsomes as *in vitro* system. Due to the unexpected potent toxicity of the stable metabolite 3,3,3-trifluoro-propionic acid following a single oral administration, a second series of experiments was performed in rats. In this context, 3,3,3-trifluoropropionic acid has been considered to be involved in cardiotoxicity.

In order to investigate the basic mechanisms and relevant intermediates which trigger the cardiotoxicity, experiments were designed with 1,1,1,3-tetrachloropropane for comparison. Kolesar *et al* observed in *in vivo* experiments effects on the heart and liver after treatment with 25 ppm of 1,1,1,3-tetrachloropropane and a dose related increase in the incidence of multifocal/focal myocyte degeneration, necrosis with adjacent to areas of chronic myocarditis and a single-cell hepatocyte necrosis with mononuclear cell infiltration. The mechanism of action remained unclear (Kolesar et al., 1995).

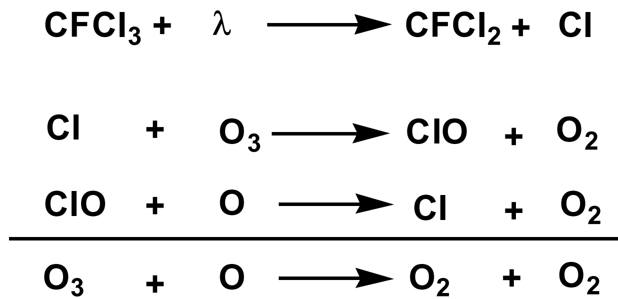
2 LITERATURE REVIEW

2.1 TOXIC EFFECTS OF HALOGENATED ALKANES

Halon gases, chlorofluorocarbons (CFCs) and bromofluorocarbons (BFCs) are widely used in industry and are a part of everyday life. They are used in refrigeration, the cleaning of metal and electronics, as blowing agents, as propellants and as a major component of fire extinguishants. These applications are based on the beneficial characteristics of CFCs as being stable, non-reactive, non-flammable, inexpensive to produce and having a convenient boiling point and a low critical pressure for liquefaction. The large number of these compounds in use implies a low toxicity to human health during production, use and disposal. The impact on the environment however, of this class of chemicals, is of concern because of their ozone depletion potential (ODP) and global warming potential (GWP). Since the ozone layer is the most important protective shield for ultraviolet radiation reaching the surface of the earth, the “Montreal Protocol of 1987 on Substances that deplete the ozone layer” called for the phasing out of both chlorofluorocarbons and bromofluorocarbons.

The ozone depletion potential is a rating system to compare compounds by their effect on the ozone layer and is based on the effect of dichlorodifluoromethane (CFC-12) on the ozone layer as one of the earliest CFCs being used.

CFCs are volatile and, because of their non-reactivity, they can reach the stratosphere in regions close to the equator. As they enter the stratosphere the chlorofluorocarbons are broken down by the intense solar radiation in a photolysis reaction. Chlorine is formed and interferes by the ozone-oxygen cycle. The cycle describes a steady state of ozone formation and degradation with the reaction of atomic oxygen with oxygen molecules to form ozone. The ozone molecule is also unstable and split by ultraviolet light to a molecule of oxygen and atomic oxygen. This depletion of ozone is triggered by the chlorine radicals (c.f. Scheme 1) (Molina, 1974).



Scheme 1: Reaction of chlorofluorocarbons, e.g. trichlorofluoromethane in the stratosphere (for $\lambda < 250$ nm) and interference of resulting chlorine with the ozone-oxygen cycle.

In addition, CFCs support the anthropogenic greenhouse effect causing the tendency of global warming. They contribute to the increase of greenhouse gases in the troposphere because they accumulate and cannot be broken down, although they are stable and inactive in the troposphere.

Table 1: Production quantity and environmental characteristics of major greenhouse gases.

	Industrial/human output (emissions 1995) [Mio tons]	Predicted contribution to manmade global warming	Atmospheric life/years	ODP	GWP at 100 years (CO_2)
Carbon dioxide	26,030	71%	500	0	1
Methane	375	21%	14.5	0	21
Nitrous oxide	9	7.5%	120	0	310
CFC-12	0.189*	<0.5%	102	1	8500
HCFC-22	0.224	<<0.5%	13.3	0.055	1700
HFC-134a	0.020	<< 0.5%	14	0	1300

ODP: ozone depletion potential, GWP: global warming potential; CFC-12: dichloro-difluoromethane, HCFC-22: chlorodifluoromethane, HFC-134a: 1,1,1,2-tetrafluoroethane (Houghton et al., 1996; AFEAS, 1995).

The main alternatives to CFCs are hydrochlorofluorocarbons (HCFCs). As they contain hydrogen, HCFCs break down more easily in the troposphere before reaching the ozone layer and show a shorter environmental half-life than CFCs.

Therefore, HCFCs have a less ODP (reduced by app. 90%), in addition to a lower GWP. Nevertheless, HCFCs will be subject to a phase-out by 2015 under European regulation. Considered as further replacements are hydrofluorocarbons (HFCs), which are molecules with fluorine as a substitute only. They retain the beneficial characteristics of CFCs, are highly energy efficient and of low toxicity (Houghton et al., 1996). A major advantage is that the stratospheric ozone depletion is not catalyzed by fluorine. However, HFCs still have a GWP which depends on the number of fluorine substitutions. Besides the naturally occurring greenhouse gases methane, nitrogen oxide and carbon dioxide, CFCs contributed to approximately 20% of the man-made greenhouse effect (Boutonnet, 1999). The future contribution of HFCs is calculated to be minimal, 1 to 2% (c.f. Table 1). Table 2 shows a selection of common CFCs and alternatives used or used to be used worldwide.

Table 2: Chlorofluorocarbons and hydrochlorofluorocarbons

Code*	**Name**
CFC-11	trichlorofluoromethane
CFC-12	dichlorodifluoromethane
CFC-13	chlorotrifluoromethane
CFC-113	1,1,2-trichloro-1,2,2-trifluoroethane
CFC-114	1,2-dichloro-1,1,2,2-tetrafluoroethane
CFC-115	1-chloro-1,1,2,2,2-pentafluoroethane
CFC-116	hexachloroethane
HCFC-22	chlorodifluoromethane
HCFC-123	1,1-dichloro-2,2,2-trifluoroethane
HCFC-141b	1,1-dichloro-1-fluoroethane
HFC-23	trifluoromethane
HFC-125	pentafluoroethane
HFC-134a	1,1,1,2-tetrafluoroethane
HFC-227ea	1,1,1,2,3,3,3-heptafluoropropane
HFC-245fa	1,1,1,3,3-pentafluoropropane

* Numerical code for chlorofluorocarbons was established by the American Society of Refrigeration Engineers (American Society of Refrigerating Engineers (1957).

Examples of well characterized and established replacements of CFCs are 1,1-dichloro-2,2,2-trifluoroethane (HCFC-123), used as an alternative to Halon 1211, a fire extinguishant, 1,1,1,2-tetrafluoroethane (HFC-134a) used instead of CFC-12 and HFC-245fa instead of trichlorofluoromethane (CFC-11) and 1,1-dichloro-1-fluoro-ethane (HCFC-141b).

In order to use the replacements, the toxicity of CFC replacements was reviewed and extensive investigations have been performed. The “Programme for Alternative Fluorocarbon Toxicity Testing” (PAFT), evaluated, for example, the toxicological effects of HFC-134a as one of the first alternatives to replace Freon 12 (CFC-12) (Collins et al., 1995).

As representatives of chlorofluorocarbons, CFC-12 and its replacement HFC-134a, have been characterized and compared in their toxicity. Due to the lipophilic character of CFC molecules, they distribute quickly into lipid membranes and effects are mainly seen in the central nervous system, resulting in a narcotic effect, which is reversible after the end of exposure. The chronic toxicity is dependent on the formation of reactive metabolites, which varies between the CFCs. In general, the extent of biotransformation is very low and no bioaccumulation has been observed. Narcotic effects have been observed after inhalation of more than 200 000 ppm CFC-12 and cardiac sensitization has only been observed after exposure to high doses (Dekant, 1996b). Solvent-induced cardiac arrhythmia can be induced experimentally by most halocarbon solvents (e.g., carbon tetrachloride and chloroform) and hydrocarbon solvents (e.g., cyclopropane, propane, iso-octane, and n-hexane) or propellants (e.g. CFC-11, 1,2-dichloro-1,1,2,2-tetrafluoroethane (CFC-114), trichloroethane), but not by unsaturated halocarbons.

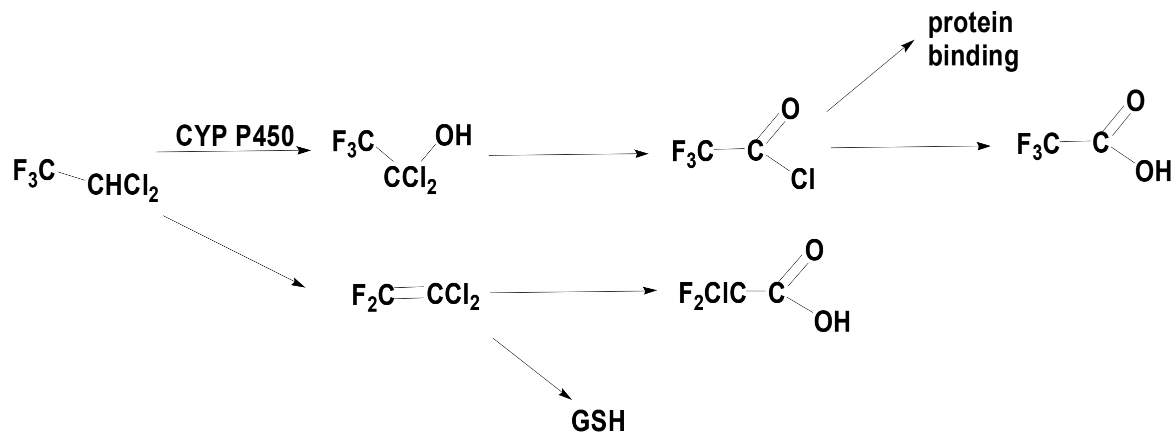
2.2 TOXIC EFFECTS OF CHLOROFLUOROCARBON REPLACEMENTS

Compared with other chemicals, CFC replacements have been extensively tested. Acute, subchronic, chronic and environmental toxicity as well as carcinogenic effects have been studied in animals for human risk assessment. The toxicity of CFC replacements is based mainly on their biotransformation and formation of breakdown products as reactive intermediates.

The acute, subchronic, chronic, developmental and genetic toxicity of HFC-134a has been studied after oral and inhalation exposure. The absorption in the lungs and gastrointestinal tract is rapid and maximal blood concentrations were observed after five minutes. Salmon et al., 1980 showed rapid excretion, mostly as the unchanged parent compound HFC-134a and only 0.34-0.40% had been metabolized (Ellis et al., 1993). Studies with rat-liver microsomes confirmed that HFC-134a is oxidized by the cytochrome P-450 2E1 system to trifluoroacetic acid (Olson et al., 1991). HFC-134a has a very low acute toxicity and a lethal concentration range from 567 000 ppm to 750 000 ppm in rats. Clinical signs of toxicity included lethargy, labored and rapid respiration, foaming at the nose, tearing, salivation, and weight loss (Silber et al., 1979; Rissolo et al., 1967). The potential for skin and eye irritation was observed as low. In chronic toxicity studies with HFC-134a, an increase in organ weight of the testes and an increased incidence of Leydig cell hyperplasia and benign Leydig cell tumors was observed (Dekant, 1996b). Animal studies indicate that HFC-134a has a low level of systemic toxicity with a serum half-life of 4 to 11 minutes, implying a low human toxicity risk and therefore its use as a propellant in metered-dose inhalers has been accepted. Developmental toxicity was found to be low and cardiac sensitization is ranked as acceptable at a threshold of 75 000 ppm. A deficit in neurobehavior was observed at a range of 40 000 to 470 000 ppm after acute exposures to HFC-134a and CFC-12, but more rapidly and at lower concentrations of HFC-134a (Ritchie et al., 2001).

In contrast, based on their extensive metabolism, a different spectrum of toxicity was observed, for example for HCFC-123 and for halothane (2-bromo-2-chloro-1,1,1-trifluoroethane). Both compounds were metabolized to mainly trifluoroacetic acid via the reactive metabolite trifluoroacetyl chloride, which also binds to proteins. The metabolite chlorodifluoroacetic acid was formed only after HCFC-123 exposure, most likely via 1,1-dichloro-2,2-difluoroethene that also formed S-conjugates with glutathione and finally mercapturic acids (c.f. Scheme 2).

Following a chronic exposure (24 month) of HCFC-123 an increased liver weight, minimal histopathological changes such as multifocal random degeneration and necrosis, induction of peroxisomal ß-oxidation and a decrease in serum cholesterol and the triglyceride level were observed (Rusch et al., 1994; Malley et al., 1991; Hoet et al., 1997; Hoet et al., 2001). Liver and kidney have been proven as being target organs (Rusch, 1997).



Scheme 2: Biotransformation of HCFC-123 via cytochrome P-450 to trifluoroacetic acid or alternatively via 1,1-dichloro-2,2-difluoroethene to chlorodifluoroacetic acid (Dekant, 1996b).

The liver in general, is an important target organ of the toxicity of xenobiotics because of its unique and diverse metabolism (Jaeschke et al., 2002; Forkert, 2001). In the following, an overview of relevant reactions of biotransformation of foreign compounds is given (Raucy et al., 1993; Anders, 1991; Lieber, 1997).

A model substrate of the major biotransformation pathway by cytochrome P-450, but also a unique substrate, has been halothane, which can undergo oxidative and reductive biotransformation, both catalyzed by cytochrome P-450. For both metabolic pathways, the liver is the target organ. The oxidative pathway, catalyzed by the cytochrome P-450 iso-enzymes 2E1 and 2A6, results in trifluoroacetylation of proteins and immune-based fulminant hepatic necrosis. Whereby at the end of the predominant reduction pathway, by the cytochrome P-450 isoforms 3A4 and 2A6, 2-chloro-1,1,1-trifluoroethane and 2-chloro-1,1-trifluoroethene were formed. The latter initiates microsomal lipid peroxidation by covalently binding to phospholipids (Spracklin et al., 1997; Manno et al., 1992; Kharasch et al., 2000). Additionally hydrogencarbons are metabolized, initiated by glutathione S-conjugation formation through the cysteine conjugate ß-lyase pathway. HCFCs cannot be metabolized as substrates for glucuronyl- or sulfonyltransferases, but possibly after passing the phase I pathway.

2.3 BIOTRANSFORMATION AND MODE OF ACTION OF 1,1,1,3,3-PENTAFLUOROPROPANE *IN VIVO*

Previous studies on the evaluation of toxicity and ecotoxicity of 1,1,1,3,3-pentafluoropropane as the replacement for HCFC-141b and CFC-11 have been performed by various groups, e.g. Rusch et al., 1999 and reported by the “National Industrial Chemical Notification and Assessment Scheme” (NICNAS, 2001). 1,1,1,3,3-Pentafluoropropane is a colorless liquid or gas, a non-flammable agent and is used as a foam blowing agent, as propellant for refrigeration, as solvent for precision cleaning, for personal care and medical products, e.g. the generation of pharmaceutical aerosols. In the atmosphere, 1,1,1,3,3-pentafluoro-propane degrades over 7.2 years resulting in carbon dioxide and hydrogen fluoride. 1,1,1,3,3-pentafluoropropane has no ODP but its GWP is 790 compared to carbon dioxide for an integration time horizon of 100 years (NICNAS, 2001). This is discussed in contrast to a GWP e.g. for CFC-11 of 4 000 or for HCFC-141b of 600. In experimental animals, 1,1,1,3,3-pentafluoropropane has a low order of acute inhalation toxicity. In a 28-day inhalation toxicity study in rats a NOAEL was established at 50 000 ppm and a NOEL at 500 ppm. In a 90-day inhalation toxicity study in rats (6 hours/day and 5 days/week), the NOAEL was observed at 2 000 ppm but an increase in myocarditis was observed at 10 000 ppm or 50 000 ppm (Rusch et al., 1999). The areas of mononuclear inflammatory cell infiltration and degenerated myocardial fibers were the only histopathological lesions observed. At these concentrations, various hematology parameters were increased, i.e. the packed cell volume, hemoglobin, red blood cell count, neutrophils, monocytes and large unstained cells. The blood chemistry parameters creatine phosphokinase, aspartate amino transferase (AST), alanine amino transferase (ALT), potassium and inorganic phosphorous were increased. Urinalysis showed an increased urine volume in all females and males dosed with 10 000 and 50 000 ppm. Correspondingly, a decrease in specific gravity and protein levels were observed (NICNAS, 2001). An increased incidence of diffuse myocarditis in all animals exposed to 50 000 ppm and in the majority of animals exposed to 10 000 ppm of 1,1,1,3,3-pentafluoropropane was observed. As expected for this class of chemicals, biodegradation and bioaccumulation of 1,1,1,3,3-pentafluoro-

propane has been minimal (Rusch et al., 1999). The toxic effect of 1,1,1,3,3-pentafluoropropane however is rather unusual. To elucidate the possible role of biotransformation in 1,1,1,3,3-pentafluoropropane toxicity, the metabolism of 1,1,1,3,3-pentafluoropropane was studied in rats after inhalation. Metabolites in urine were identified using ^{19}F-NMR and quantified using GC/MS after exposure levels of 2 000, 10 000 or 50 000 ppm. Trifluoroacetic acid and inorganic fluoride were identified as major urinary metabolites of 1,1,1,3,3-pentafluoropropane; 3,3,3-trifluoropropanoic acid and 1,1,3,3-pentafluoropropane-2-ol were identified as minor metabolites (c.f. Table 3). Trifluoroacetic acid is a metabolic breakdown product of halothane and various HFCs, e.g. HFC-134a and 1,1,1,2,3,3,3-heptafluoropropane (HFC-227ea), and can be responsible for toxicological effects. Trifluoroacetic acid is resistant to biodegradation and because of the low lipophilic properties, it is rapidly excreted in humans. The half-life in humans was determined as 16 hours, and no metabolism has been observed (Solomon et al., 2003; Frank et al., 1996; Zehavi et al., 1996; Berends et al., 1999; Boutonnet, 1999). Based on this background, trifluoroacetic acid is not considered to contribute to toxic effects, seen after inhalation of 1,1,1,3,3-pentafluoropropane.

Table 3: Recovery of metabolites of 1,1,1,3,3-pentafluoropropane in rats, exposed by inhalation for 6 hours. Sum of metabolites excreted within 72 hours after exposure in nmol.

Exposure concentration	**Trifluoroacetic acid (nmol)**		**3,3,3-Trifluoropropanoic acid (nmol)**	
	Males	**Females**	**Males**	**Females**
50 000 ppm	4 820 ± 441*	3 945 ± 415	79.3 ± 14.3	9.1 ± 3.3
10 000 ppm	310 ± 41	296 ± 34	27.4 ± 3.5	7.0 ± 1.9
2 000 ppm	96.6 ± 14.1	86.6 ± 11.8	13.3 ± 4.6	2.3 ± 0.4

* Extrapolated, based on half-life of urinary excretion of 30.5 hours.

The mechanism of observed selective toxicity of 1,1,1,3,3-pentafluoropropane on the heart is not known. In the literature, chemically induced myocarditis is an uncommon finding in experimental animals, and persistent cardiac lesions have not been reported with other chlorofluorocarbon substitutes. In general, however, selective organ damage, seen after long-term administration of chemicals, has been often

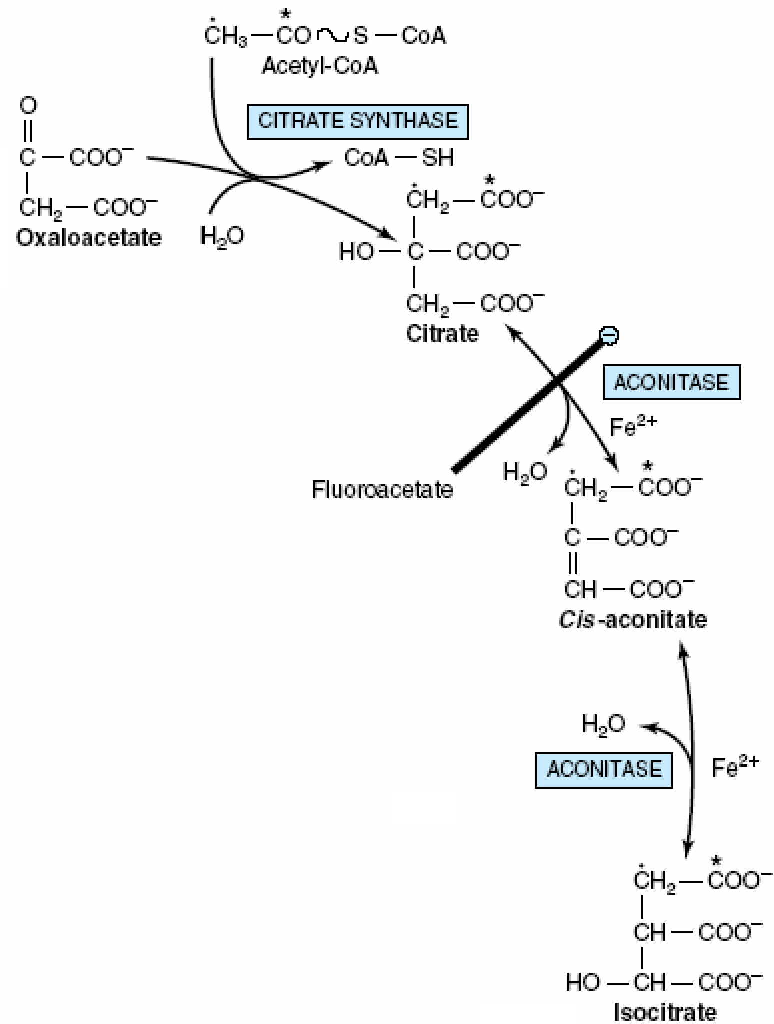
associated with biotransformation to reactive electrophiles or to stable, but toxic, metabolites.

2.4 BIOTRANSFORMATION AND MODE OF ACTION OF 1,1,1,3-TETRACHLOROPROPANE *IN VIVO*

1,1,1,3-Tetrachloropropane is a feedstock used in the production of chlorinated silicone fluids, of fluorosiloxane rubbers and oils, characterized by high thermal stability and is used in the aircraft industry. It is also used in preparing the mainly industrial used chemical trichloropropene. Repeated inhalative exposure of rats to 225 ppm of 1,1,1,3-tetrachloropropane resulted in toxicity to the heart, a target organ not yet observed with other chlorinated hydrocarbons but well known for monochloroacetic acid and monofluoroacetic acid. 1,1,1,3-Tetrachloropropane is known to induce focal myofiber degeneration and necrosis resulting in myocarditis (Kolesar et al., 1995). Likely, the organ-specific toxicity of 1,1,1,3-tetrachloropropane to the heart is based on the biotransformation to stable, but toxic metabolites and the fate comparably to 1,1,1,3,3-pentafluoropropane.

2.5 BIOTRANSFORMATION OF HALOGENATED SHORT CHAIN CARBOXYLATES

The toxicological profile of 3,3,3-trifluoropropionic acid is unknown. However, some halogenated short chain carboxylates are highly toxic. Examples are sodium monofluoroacetate, methyl fluoroacetate and fluoroethanol (Gribble, 1973). Monofluoroacetate is toxic to the organism because of its ability to inhibit the tricarboxylic acid (TCA) cycle (c.f. Scheme 3). Monofluoroacetate is converted to fluorocitrate, which interferes with the TCA cycle by inhibiting aconitase, which catalyzes the conversion of citric acid to isocitric acid. Consequently, citric acid accumulates in the tissues and therefore ATP formation in the TCA cycle is inhibited. Symptoms include convulsion and death due to cardiac failure (James et al., 2002; Naito, 2002).



Scheme 3: A section of the tricarboxylic acid cycle: aconitase as a target of mono-fluoroacetate (Murray et al., 2003).

The accumulation of citric acid results also in inhibition of the enzyme phosphofructokinase, which is responsible for the transformation of fructose-6-phosphate to fructose-1,6-phosphate and plays a major role in the regulation of glycolysis (Ballard et al., 1967; Engel et al., 1957; Reichelt, 1979; Feldwick et al., 1994). Peters *et al* has shown *in vitro* that fluorocitrate, but not monofluoroacetic acid inhibits aconitase (Peters et al., 1969; Peters, 1952). Compounds, which can be metabolized to fluorocitrate *in vivo* are therefore also considered as being toxic. The toxicity of monofluoroacetate and fluorobutyrate has been described as fluoroacetate diabetes because of hyperglycemia as the observed symptom. However, mechanistic studies have shown that there is no damage to pancreatic ß-cells.

The basic mechanism of toxicity of dichloroacetate is similar to that of fluoroacetate. This compound enters the TCA cycle and is converted to glyoxylate by glutathione-

S-transferase and inhibits maleyl-acetoacetate-isomerase, an enzyme of tyrosine metabolism. It has been speculated that dichloroacetate induces the inhibition of tyrosine catabolism, which may account for its toxicity. Long-term administration of dichloroacetate to animals causes hepatotoxicity and neoplasia. However, in humans, dichloroacetate is used clinically to treat lactic acidosis and is known to enhance cellular energy metabolism (Stacpoole, 1998a; Stacpoole 1998b). Trichloroacetate has been characterized as a peroxisome proliferator in rodents and causes liver tumors by this pathway. However, besides liver tumors, no other toxic effects of this compound have been described, except caustic effects when in contact with tissues in high concentrations.
As a further example of fluorinated compounds, toxicity of 1,3-difluoro-2-propanol has been observed by Feldwick et al., 1998. It is based on its biotransformation to 1,3-difluoroacetone, (-) erythrofluoro-citrate and free fluoride. The accumulation of formed citrate in the kidney was considered to be responsible for the toxic effects.

2.6 MODE OF ACTION OF ß-OXIDATION INHIBITORS AND THEIR TOXICITY

Gluconeogenesis is also dependent upon fatty acid oxidation and therefore any impairment in the fatty acid oxidation pathway may lead to hypoglycemia (Hue et al., 1986). The ß-oxidation of fatty acid takes place in the mitochondria. Long-chain fatty acids pass the mitochondrial membrane via carnitine-shuttle transporters, whereas short- and medium chain fatty acids reach the mitochondrial matrix by diffusion.

Figure 1 shows an overview of the metabolism of fatty acids (Rinaldo, 2001). The metabolism is characterized by the oxidation of fatty acids at the ß-carbon position of the fatty acyl coenzyme A (CoA) molecule through the sequential removal of two carbon units by oxidation. Each round of ß-oxidation produces NADH, $FADH_2$ and acetyl-CoA. Acetyl-CoA represents the end product of ß-oxidation and enters the TCA cycle, where it is further oxidized to carbon dioxide with the concomitant generation of NADH, $FADH_2$ and ATP. NADH and $FADH_2$ are able to enter the respiratory pathway for the purpose of ATP production. The oxidation of fatty acids yields significantly more energy per carbon atom than does the oxidation of

carbohydrates and is therefore very important for the energy supply of the organism. During high rates of fatty acid oxidation, which occurs primarily in the liver, large amounts of acetyl-CoA are generated. If the capacity for consumption of acetyl-CoA in the TCA cycle is exceeded, an alternative metabolic pathway takes place, known as ketogenesis. This results in the synthesis of the ketone bodies acetoacetate, ß-hydroxybutyrate and acetone.

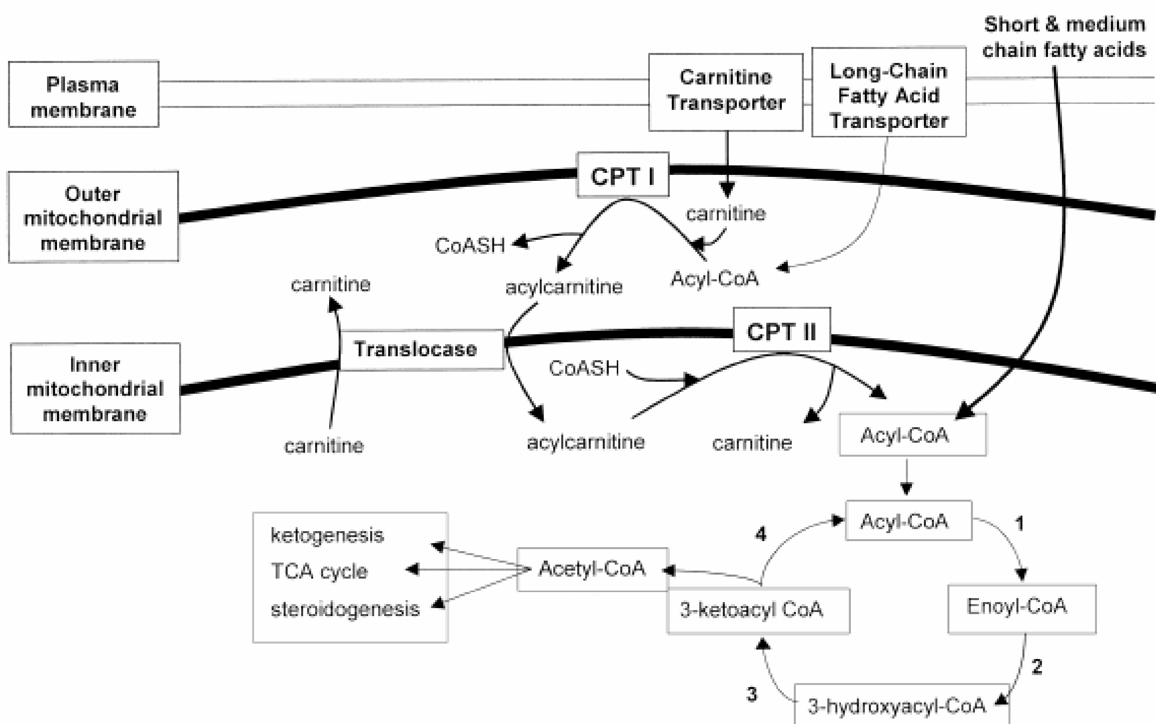


Figure 1: Pathway of fatty acid metabolism from the transport of long-chain fatty acids via the carnitine-shuttle across the plasma membrane leading to the production of the final product acetyl-CoA. Medium- and short-chain fatty acids do not require an active transport mechanism to reach the mitochondrial matrix. Enzymes of the carnitine cycle shuttle of long-chain fatty acids across the mitochondrial membranes are carnitine palmitoyltransferase CPT I and CPT II. The fatty acid-oxidation spiral includes an FAD-dependent acyl-CoA dehydrogenase step (1), followed by a 2,3-enoyl-CoA hydratase reaction (2), the NAD-dependent 3-hydroxyacyl-CoA dehydrogenase step (3), and the thiolase cleavage reaction (4) (Rinaldo, 2001).

Ketone bodies are built in the liver and are used as fuel by other tissues. As for example in early stages of starvation, heart and skeletal muscle consume primarily

ketone bodies to preserve glucose for use by the brain. After long-term starvation or diabetes mellitus, an increased ketone body production in the liver (ketosis) causes ketoacidosis.

The majority of clinical problems that are related to fatty acid metabolism are associated with the process of oxidation. Disorders are categorized first as deficiencies of carnitine that lead to an inability to transport fatty acids into the mitochondrial matrix for their oxidation. Secondly deficiencies of carnitine palmitoyltransferase I (CPT I) which affect primarily the liver, leading to reduced fatty acid oxidation and ketogenesis. Sulfonylurea drugs such as tolbutamide and glyburide for example, inhibit this enzyme. Finally, deficiencies in various acyl-CoA dehydrogenases which result in the impairment of ß-oxidation. Consequently, fatty acids are excreted in excess in the urine, which leads to symptoms such as vomiting, lethargy and frequently coma. In patients with the rare inherited disorder Refsum's Disease, there is a lack of the mitochondrial α-oxidizing enzyme. Consequently, the patients accumulate large quantities of phytanic acid in their tissues and serum. This leads to severe symptoms, including cerebellar ataxia, retinitis pigmentosa, nerve deafness and peripheral neuropathy. The inhibition of the mitochondrial β-oxidation of fatty acids has been described in the literature for the following compounds: 4-pentenoic acid, hypoglycin, pirprofen, valproic acid and tianeptine (Geneve et al., 1987; Fromenty et al., 1989; Freneaux et al., 1988; Sherratt, 1986). In addition, 4-bromocrotonic acid (Schulz, 1987) and 3-mercaptopropionic acid (Sabbagh et al., 1985) are potent inhibitors of the ß-oxidation pathway.

The inhibition of ß-oxidation has been shown in *in vitro* experiments of mitochondrial incubations with radio labeled palmitic acid as substrate of mitochondrial ß-oxidation. For example, incubations of 2 mM pirprofen with [^{14}C] palmitic acid, ATP, carnitine and CoA, result in a decreased formation of [^{14}C] acid soluble β-oxidation products and a decreased formation of [^{14}C] carbon dioxide. The formation of both products reflects the mitochondrial ß-oxidation rate. Acid soluble β-oxidation products mainly represent ketone bodies and, to a small extent, citric acid cycle intermediates. [^{14}C] Carbon dioxide represents the activity of the following TCA cycle (Geneve et al., 1987). In this respect, the compounds interfere with different steps of the oxidation pathway. The compounds consist of a reactive functional group, which binds reversibly or irreversibly to the active sites of target enzymes of the ß-oxidation process, and inhibit the latter (Schulz, 1987). Rather than severely decreasing the

overall oxidation flux, the inhibition may act mainly by increasing precursor free fatty acid concentrations in the liver. This may contribute to their increased esterification and accumulation in form of triglycerides, leading to an increase in ketone bodies. The increased levels of triglycerides result in microvesicular hepatic steatosis, seen after administration of valproic acid, hypoglycin, 4-pentenoic acid, amineptine and pirprofen (Spaniol et al., 2003; Billington et al., 1978; Sherratt, 1986).

The ß-oxidation of fatty acids is also known to be a pathway of metabolic bioactivation as for 5,6-dichloro-4-thia-5-hexenoic acid (DCTH) and other 4-thiaalkanoic acids. DCTH has been shown to be cytotoxic in rat hepatocytes resulting in decreased cellular ATP concentrations and leading to mitochondrial dysfunction and loss in cell viability (Fitzsimmons et al., 1993). Herewith it is shown, that the fatty acid oxidation can be involved in mitochondrial cellular toxicity (Penzo et al., 2002).

3 TASKS AND SCOPE

Within this thesis, possible modes of action were investigated which might account for the cardiotoxicity of 1,1,1,3,3-pentafluoropropane and 1,1,1,3-tetrachloropropane. The work focuses on the hypothesis that the cardiotoxicity of 1,1,1,3,3-pentafluoro-propane is based on the formation of the stable metabolite 3,3,3-trifluoropropionic acid. Therefore, the experimental work involved a detailed characterization of the acute and repeat dose toxicity and possible biotransformation of 3,3,3-trifluoro-propionic acid as well as mechanistic work in suitable model systems. Moreover, the role of biotransformation to a stable, but toxic metabolite was also addressed, using 1,1,1,3-tetrachloropropane as another model compound.

4 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Chemicals and Reagents

1,1,1,3,3-Pentafluoropropane, with a purity of 99.8 % (based on FID GC analysis) was supplied by Honeywell (Morristown, NJ, USA). Uniformly labeled [U-^{14}C] palmitic acid (1.85MBq, 50µCi, 400-870 mCi/mmol) and Percoll® were purchased from Amersham Bioscience (Piscataway, NJ, USA). 1,1,1,3-Tetrachloropropane was supplied by Lancester Synthesis GmbH/ Clariant (Mühlheim a.d.R., Germany). 3,3,3-Trifluoropropanoic acid, trifluoroacetic acid, difluoroacetic acid, 4-pentenoic acid, 2,2,2-trifluoroacetophenone, phenazine methosulfate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), triton-X 100, trypane blue, phosphate buffered saline and other chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany) in the highest purity available. Thiamine pyrophosphate (TSP) was purchased from Merck (Darmstadt, Germany). Eco Plus RotiSzint Cocktail was purchased from Roth (Karlsruhe, Germany). Sodium 3,3,3-tri-fluoropyruvate was kindly provided by M.W. Anders (University of Rochester, NY, USA).

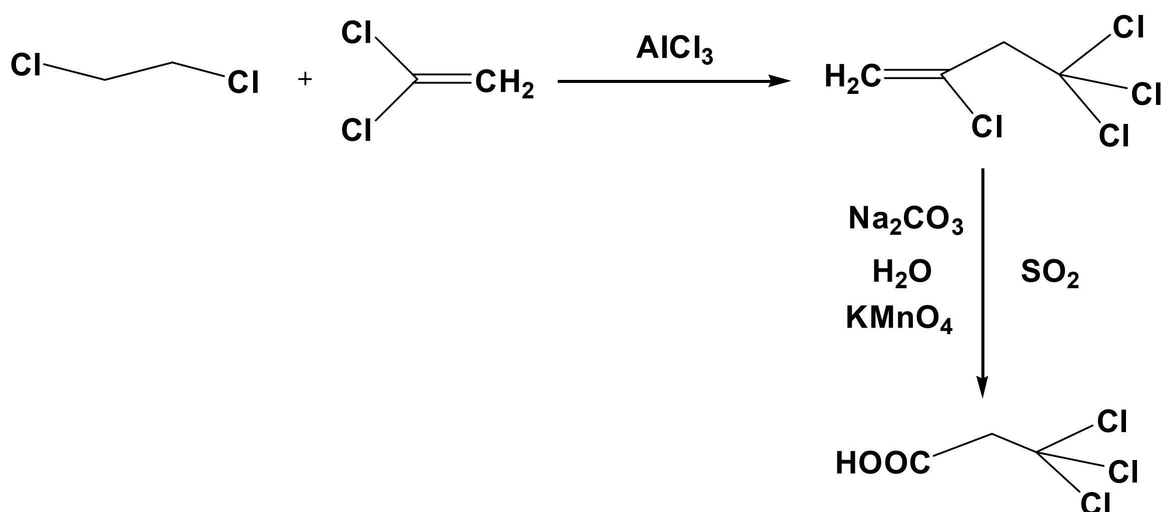
4.1.2 Diagnostic Kits and Consumables

The lactate dehydrogenase kit was obtained from Promega (Madison, WI, USA) and the rat insulin ELISA kit from Mercodia (Uppsala, Sweden). The different media for cell culture (DMEM with high/ low/ no glucose, with and without phenol red), trypsin and fetal bovine serum were purchased from PAA (Pasching, Austria). Enzymes and cofactors were obtained from Sigma-Aldrich (Deisenhofen, Germany). The manual enzymatic assays kits for determining glucose and L-lactate were purchased from Rolf Greiner Biochemica (Flacht, Germany). The keto-diastix® test sticks were supplied by Bayer (Leverkusen, Germany). The BioRad D_c Protein assay was purchased from BioRad, (Heidelberg, Germany).

The antibodies mouse-anti-rat CD3 and CD45 were purchased from DPC Biermann Acris (Bad Nauheim, Germany) and the Anti-Ig horseradish peroxidase kit was purchased by BD bioscience (Heidelberg, Germany). For the determination of rat insulin in serum, the Mercodia Rat Insulin ELISA assay (Mercodia, Uppsala, Sweden), a solid phase two-site enzyme immunoassay was used.
Monovetten® (EDTA and sodium fluoride) were obtained from Sarstedt (Nuernbrecht, Germany). The Microtainer™ tubes for serum preparation were purchased from BD bioscience (Heidelberg, Germany).

4.2 SYNTHESIS

4.2.1 Synthesis of 3,3,3-Trichloropropionic Acid



Scheme 4: Synthesis of 3,3,3-trichloropropionic acid [CAS 32765-68-7]

The two-step synthesis started with the dimerization of 1,2-dichloroethane (200 g) and 1,1-dichloroethene (100 g). Under cooling, aluminum chloride was added to the flask. The intermediate 2,4,4,4-tetrachlorobut-1-ene was identified by GC/MS and with a boiling point of 45°C/ 5 mm Hg. The specific gravity was determined to be 1.34 mg/µL.
2,4,4,4-Tetrachlorobut-1-ene (6 g), sodium carbonate (1 g), water (250 mL) and potassium permanganate (14 g) were stirred until the reaction mixture reached room temperature. After precipitation of brown manganese dioxide, sulphur dioxide was

passed through the flask until the manganese dioxide dissolved. 3,3,3-Trichloro-propionic acid was extracted with diethyl ether and purified by distillation and recrystallization in ethanol (Gough et al., 1971).

Characterization of 1,1,1-trichloropropionic acid:

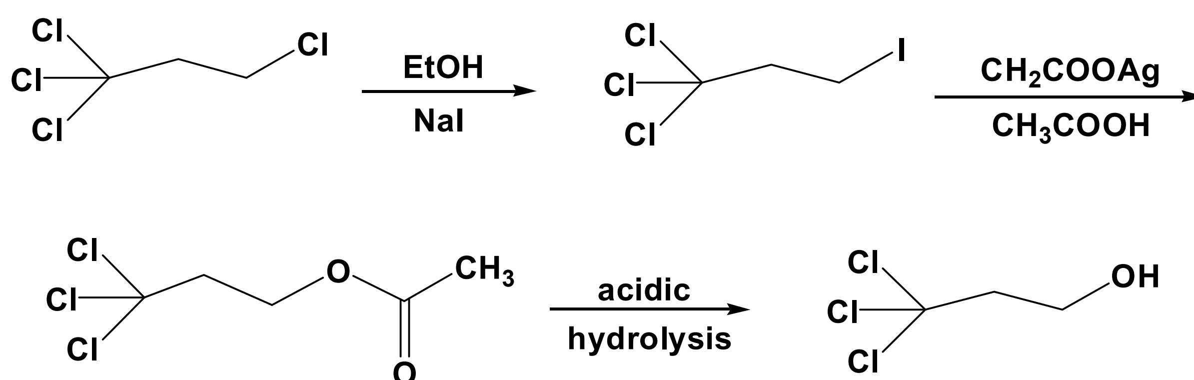
m.p. 73-75°C (literature 77°C)

MS (electron impact ionization 70 eV): m/z [^{35}Cl] = 141 [2 Cl], 117 [3 Cl], 105 [1 Cl], 96 [2 Cl], 61 [1 Cl]

^{1}H (250 MHz, $CDCl_3$): δ [ppm] = 1.3 [s; CCl_3-CH_2-COOH], 3.9 [s; CCl_3-CH_2-COOH]

^{13}C DEPT (250 MHz, $CDCl_3$): δ [ppm] =171.0 [s; CCl_3 -CH_2 -COOH], 92.3 [s; CCl_3 -CH_2 -COOH] 57.6 [s; CCl_3-CH_2-COOH]

4.2.2 Synthesis of 1,1,1-Trichloropropan-3-ol



Scheme 5: Synthesis of 1,1,1-trichloropropan-3-ol [CAS 64667-32-9].

1,1,1,3-Tetrachloropropane (1 mol) was heated at flux with sodium iodide (1 mol) in absolute ethanol (60 mL) for 2 days. The resulting 3,3,3-trichloro-1-iodo-propane was isolated by distillation at 1 mbar. Furthermore, 3,3,3-trichloro-1-iodo-propane (2 mmol) was stirred with glacial acetic acid (10 mL) containing 3 mmol silver acetate, and heated at flux for 2 hours. Precipitated AgI was filtered off, the supernatant was washed with water and extracted with diethylether (Singh et al., 1976). The diethylether-extract was concentrated and 3,3,3-trichloropropyl ethanoate isolated. The acidic hydrolysis as the last step occurred after stirring 3,3,3-trichloropropyl ethanoate in water with hydrochloric acid and the cation exchanger, amberlyst-15, under reflux for 24 hours. After purification by extraction with diethylether, 1,1,1-tri-chloropropan-3-ol was isolated by distillation (Bernhard et al., 1952).

Characterization of 3,3,3-trichloro-1-iod-propane:

MS (electron impact ionization 70 eV): m/z [^{35}Cl] = 127 [2 Cl], 96, [2 Cl], 91 [2 Cl], 61 [2 Cl], 61 [1 Cl]

Characterization of 3,3,3-trichloroacetate:

MS (electron impact ionization 70 eV): m/z [^{35}Cl] = 127 [2 Cl], 96, [2 Cl], 91 [2 Cl], 61 [2 Cl], 61 [1 Cl]

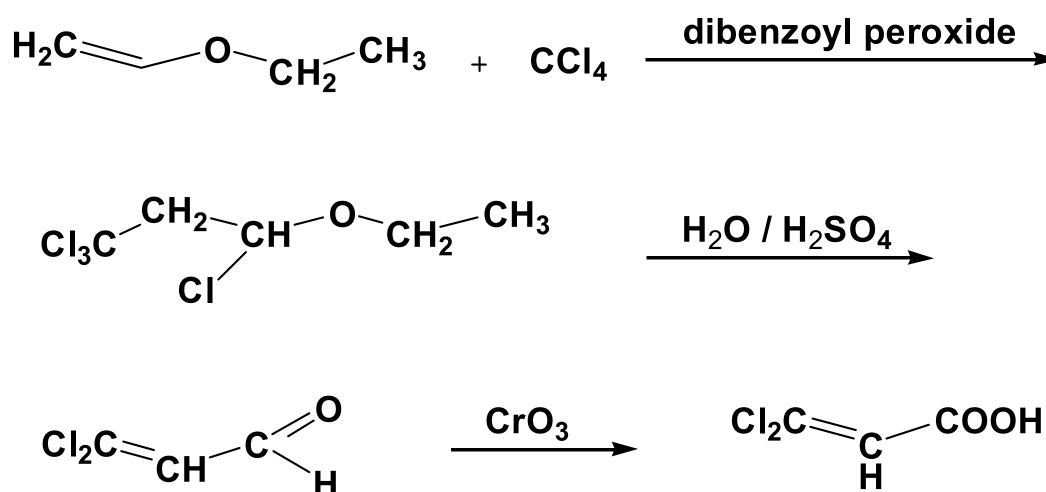
Characterization of 1,1,1-trichloropropan-3-ol:

Density : 1.348 g/mL

MS (electron impact ionization 70 eV): m/z [^{35}Cl] = 127 [2 Cl], 96, [2 Cl], 91 [2 Cl], 61 [2 Cl], 61 [1 Cl]

13C DEPT (63 MHz, CDCl3): δ [ppm] =92.3 [s; -CCl3), 171.0 [s; -COOH] 57.6 [s; -CH2]

4.2.3 Synthesis of Dichloroacrylic Acid



Scheme 6: Synthesis of dichloroacrylic acid [CAS 1561-20-2]

Tetrachloromethane and ethoxyethene react to the intermediate 1,3,3,3-tetrachloro-3-ethoxy-propane [CAS 1561-41-7] in the presence of dibenzoyl peroxide. After acidolysis of the ether, dichloroacrylic acid was obtained by oxidation with chromosulphonic acid. The end product was isolated by ether extraction (Schroth et al., 1989).

Characterization of ethyl-1,3,3,3-tetrachloropropyl ether:

b.p. 72-73°C /1,46kPa

MS (electron impact ionization 70 eV): m/z [^{35}Cl] = 127 [2 Cl], 96, [2 Cl], 91 [2 Cl], 61 [2 Cl], 61 [1 Cl]

Characterization of dichloroacrylic acid:

m.p. 77°C

MS (electron impact ionization 70 eV): m/z [^{35}Cl] = 123 [2 Cl], 95 [2 Cl], 60 [1 Cl]

^{1}H-NMR (250 MHz; CCl_3): δ = 6.5 [Cl_2C=CH-COOH], 1.3 [Cl_2C=CH-COOH]

^{13}C-NMR (63 MHz; CCl_3): δ = 235.0 [s;Cl_2C=CH-COOH], 119.5 [s; Cl_2C=CH-COOH]

4.3 INSTRUMENTAL ANALYSIS

4.3.1 Fluoride Electrode

To quantify the excretion of inorganic fluoride in rat urine, 5 mL of urine were centrifuged and combined with an equal volume of total-ionic-strength-adjustment buffer (TISAB: 1 M acetic acid, 1 M sodium chloride and 0.012 M (±)-trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate in deionized water, pH 5.5). Samples were constantly stirred during analysis, and the response (mV) was read at room temperature after 10 minutes with a fluoride-selective electrode and a reference electrode (type ISE, Metrohm, Herisau, CH). The fluoride-selective electrode was calibrated daily with freshly prepared solutions containing 0.1, 0.2, 0.5, 0.75, 1 and 10 ppm sodium fluoride. For the measurement procedure no glass ware was used. The calculation of fluoride in samples and calibration curves was performed by plotting the response vs. the fluoride concentration (Dekant et al., 1995).

4.3.2 Nuclear Magnetic Resonance Spectrometry (NMR)

4.3.2.1 ^{19}F-NMR

^{19}F-NMR spectra were recorded with a Bruker Avance DRX 300 NMR spectrometer operating at 282.4 MHz at the Institute for Inorganic Chemistry at the University of Wuerzburg. ^{19}F chemical shifts were referenced to external trichlorofluoromethane

($CFCl_3$). ^{19}F-NMR spectra were recorded with a 90° pulse length of 11 µs and a recycle delay of 1 second. The acquisition time was 1.5 seconds and 2 000 up to 5 000 scans were recorded to obtain a satisfactory signal to noise (S/N) ratio. For comparison purposes, the ^{19}F-NMR spectra were acquired with and without proton decoupling. Before the Fourier transformation a line broaderning of 1 Hz, was applied. Urine samples were stored at -20°C. Before analysis, urine samples were thawed and centrifuged at 13 000 rpm. To record NMR spectra, 720 µL of rat urine supernatant were diluted with 80 µL of deuterium oxide (D_2O). To analyze *in vitro* incubation mixtures, proteins were sedimented by centrifugation at 100 000 x g for 20 minutes, and 80 µL of D_2O were added to the supernatant (720 µL). The samples were analyzed by ^{19}F-NMR spectroscopy without further workup in a 5-mm o.d. NMR tube.

Table 4: Resonances of fluorine containing compounds used, by ^{19}F-NMR (proton coupled) ^{19}F chemical shifts were referenced to external trichlorofluoromethane ($CFCl_3$).

Fluorine Containing Compounds	**δ [ppm]**
1,1,1,3,3-pentafluoropropane	-63.2 (m), -117.5 (m)
3,3,3-trifluoropropionic acid	-63.92 (t)
trifluoroacetic acid	-75.92 (s)
metabolite A	-63.46 (t)
hexafluoroacetone trihydrate	-82.9 (s)
sodium trifluoropyruvate	-83.2 (s)
methyl trifluoropyruvate	-83.6 (s)
3,3,3-trifluorolactate	-75.85 (d)
trifluoroacetaldeyde monohydrate	-85.36 (d)
trifluoroacetaldeyde	-85.35 (d)
1,1,1-trifluoroacetone	- 86.9 (s)
2,2,2-trifloroacetophenone	- 63.32 (m)
inorganic fluoride	-119.2 (s)

4.3.2.2 ^{1}H-NMR

^{1}H-NMR spectra were measured at 600.13 MHz on a Bruker DRX 600 spectrometer at the Institute of Organic Chemistry, University of Wuerzburg. The water resonance was suppressed using the first increment of a NOESY pulse sequence with irradiation during a 2-second relaxation delay and during the 150 ms mixing time. Between 64 and 192 free induction decays (FIDs) were collected into 64k data points using a spectral width of 7164 Hz, an acquisition time of 4.57 seconds and a total pulse cycle delay of 6.72 seconds. Prior to Fourier transformation (FT), the FIDs were zero-fitted to 128k and an exponential line-broadening factor of 0.1 Hz was applied. The addition of 3-trimethylsilyl-(2,2,3,3,-$^2H_4$)-1-proprionate (TSP), (2 mM; δ = 0 ppm) in D_2O provided a chemical shift and a deuterium lock signal. To minimize variations in the pH of the urine samples, 400 µL of 0.2 M phosphate buffer, pH 7.4, were mixed with 800 µL of rat urine. The resulting solution was left to stand for 10 minutes and then centrifuged at 13 000 rpm for 10 minutes to remove any precipitates. The supernatant or the serum samples (700 µL) were placed into a 5 mm o.d. NMR tube and 70 µL of 10 mM TSP are added. The samples were prepared immediately prior to measurement. Urine samples were stored at -20°C until analysis (Nicholls et al., 2001).

4.3.2.3 ^{1}H-NMR and ^{13}C-NMR Cosy

^{1}H-NMR and ^{13}C-NMR were recorded at a Bruker AC 250 spectrometer, operating at 250 MHz for ^{1}H and 63 MHz for ^{13}C at the Institute of Organic Chemistry, University of Wuerzburg. One-dimensional NMR spectra (^{1}H and ^{13}C COSY) were recorded with 16 000 to 32 000 scans. Chemical shifts (δ- values) were referenced to the solvent deuterated chloroform ($CDCl_3$) or D_2O. The coupling constants J were denoted in Hz and the multiplicity was indicated with singlet (s), duplet (d), triplet (t) and multiplet (m). About 10 mg of the compound were analyzed in a 5 mm o.d. NMR tube with 800µL D_2O respectively $CDCl_3$.

4.3.3 Mass Spectrometry, coupled with Gas Chromatography (GC/MS)

GC/MS analyzes of *in vitro* incubations, urine and blood samples was performed on an Agilent 5973 mass spectrometer coupled to an Agilent 6890 gas chromatograph

(Agilent, Heidelberg, Germany) equipped with a CTC Combi-PAL autoinjector (Chromtech, Idstein, Germany) with capability for head-space injections. Analysis was performed in the electron impact modus (EI) with an ionization energy of 70 eV.

4.3.3.1 Quantification of Trifluoroacetic Acid and 3,3,3-Trifluoropropanoic Acid as Methyl Esters

Urine samples (200 µL) or supernatants of microsomal incubations (1 mL) were mixed with 80 µL 0.1 M sodium hydroxide and 100 nmol difluoroacetic acid in 50 µL H_2O as an internal standard. Samples were then taken to dryness in an evacuated desiccator containing anhydrous phosphor pentoxide. The organic acids in the dried residues were converted to methyl esters by the addition of 100 µL of methanol and 100 µL of concentrated (97 %) sulphuric acid and heated for 1 hour at 80°C in gas-tight reaction vials. Head-space samples (250 µL) were removed with a warmed (85°C) gas-tight syringe, and the concentrations of methyl esters were quantified by GC/MS. Compounds were separated on Agilent Q-Plot fused-silica capillary column (30 m x 0.32 mm i.d.; film thickness, 20 µm) with the following conditions: the linear temperature program started from 100°C to 220°C with a heating rate of 15°C/minutes; the injector and detector temperature was set at 250°C; helium was used as the carrier gas at a flow rate of 2 mL/minutes, and split injection was set to a split ratio of 5:1. During the chromatographic separation, the intensities of m/z 51, 59, 60, 69, 83, 111, and 142 were monitored with a dwell time of 100 ms. Retention times were 4.3 minutes for trifluoroacetic acid, 5.8 minutes for difluoroacetic acid, and 7.0 minutes for 3,3,3-trifluoropropanoic acid. Quantification was performed relative to the content of difluoroacetic acid as internal standard (m/z 51). It was referenced to calibration curves prepared with urine samples of animals not exposed to the analytes or microsomal supernatant samples, containing 0–350 nmol/mL trifluoroacetic acid (intensity quantified at m/z 69) or 0–15 nmol/mL 3,3,3-trifluoro-propanoic acid (intensity quantified at m/z 111). This method permitted the quantification of 0.5 nmol/mL trifluoroacetic acid and 0.5 nmol/mL of 3,3,3-trifluoropropanoic acid with a signal to noise ratio of 10:1. Deviations between repeatedly analyzed reference samples were <10% and the response of the detector was linear in the concentration ranges used. Samples were analyzed in triplicate and calibration curves were measured before every sample sequence.

Characterization of 3,3,3- trifluoropropionic acid:

MS (electron impact ionization 70 eV, methyl ester): m/z = 142 [M^+], 111, 83, 59; rt = 7.0 min

Characterization of the trifluoroacetic acid:

MS (electron impact ionization 70 eV, methyl ester): m/z = 128 [M^+], 69, 59; rt = 4.3 min

4.3.3.2 Quantification of the Ketones, Acetone, 2-Butanone and 2-Pentanone

Excretion of ketones in the urine was analyzed by headspace gas chromatography. Samples of urine were heated for 30 minutes at 80°C in gas-tight reaction vials and 100 µL of the headspace sample were separated on a DB-WAX capillary column (30 m x 0.25 mm i.d.; film thickness, 25 µm). The temperature was kept at 40°C for 8 minutes with a solvent delay of 1 minute. Helium was used as the carrier gas at a flow rate of 1 mL/min and a split ratio of 5:1. The inlet and auxiliary temperature were set to 250°C. For acetone, the intensities of m/z 43 and 58, for 2-butanone the intensities of m/z 43, 57 and 72 and for 2-pentanone the intensities of m/z 43, 58 and 86 were monitored with a dwell time of 100 ms. The retention times were 2.27 minutes for acetone, 3.03 minutes for 2-butanone and 4.33 minutes for 2-pentanone. Quantification was performed for all compounds at the intensities of m/z 43. Calibration curves were monitored with 100 µL of the compound in water in a concentration range from 1 nmol/100 µL to 100 nmol/100 µL for acetone, 1 nmol/100 µL to 150 nmol/100 µL for 2-butanone, 1 nmol/100 µL to 250 nmol/100 µL for 2-pentanone. All samples were analyzed in duplicate.

4.3.3.3 Analysis of Hexafluoroacetone Trihydrate, 2,2,2-Trifluoroacetophenone, Hexafluoropropan-2-ol, 1,1,1-Trifluoroacetone and 2,2,2-Trifluoro-ethanol

Samples were heated for 60 minutes at 80°C in gas-tight reaction vials. From the vial, a headspace volume of 250 µL was removed by the autosampler and was separated without dramatization on a DB-WAX capillary column with following conditions: A linear temperature program from 45°C to 240°C with a heating rate of 10°C/min was used. The injector and detector temperature were set at 220°C. As carrier gas, helium was used at a flow rate of 1 mL/min with split injection and a split

ratio of 20:1. During the chromatographic separation, the intensities of m/z 35 to m/z 400 were monitored.

4.3.3.4 Search for possible Glucuronidation Products

Fragments of compounds in urine, conjugated with glucuronic acid, were analyzed by GC/MS after cleavage of the glucuronide moiety with the enzyme ß-glucuronidase. A urine volume of 500 µL (after 1,1,1,3,3-pentafluoropropane or 3,3,3-trifluoropropionic acid exposure) was combined with 500 µL of 0.2 M acetate buffer and 500 µL of ß-glucuronidase, 1100 Units/mL (type HP-2 from helix pomatia). The solution was incubated for 18 hours at 37°C in a sealed vial. After incubation, the reaction volume was centrifuged at 10 000 rpm and extracted with 1 mL chloroform. The two phases were separated by centrifugation, the chloroform phase was dried with sodium sulphate, and the supernatant was analyzed by GC/MS for chlorinated respectively fluorinated compounds (alcohols, ketones).

4.3.3.5 Quantification of 1,1,1,3-Tetrachloropropane

Urine samples were heated for 15 minutes at 100°C in gas-tight reaction vials. 100 µL of the headspace sample were removed with a warmed (100°C) gas-tight syringe of the auto sampler and were separated on a DB-WAX capillary column. For chromatography, a linear temperature program from 50°C to 150°C with a heating rate of 15°C/min was used. The injector and detector temperature were set at 220°C. Helium was used as carrier gas at a flow rate of 1 mL/min and split injection with a split ratio of 20:1. During the chromatographic separation, the intensities of m/z 109 and 145 for 1,1,1,3-tetrachloropropane and the intensity for m/z 20 for neon as the internal standard were monitored with a dwell time of 100 ms. Retention times were 1.31 minutes for neon and 4.70 minutes for 1,1,1,3-tetrachloropropane. Quantification was performed using calibration curves from 50 to 250 ppm 1,1,1,3-tetrachloropropane in matrix.

Characterization of 1,1,1,3-tetrachloropropane:

MS (electron impact ionization 70 eV) m/z [^{35}Cl] = 145 [3 Cl], 117 [3 Cl], 109 [2 Cl], 63 [1 Cl]

4.3.3.6 Quantification of 1,1,1-Trichloropropane-3-ol, 3,3,3-Trichloropropanoic Acid and Dichloroacetic Acid

Urine samples (150 µL) were added to a GC vial to 250 µL of the following mix: 1 mL methanol, 6 mL water and 5 mL concentrated sulphuric acid (Muralidhara et al., 1999). Trichloroacetic acid was used as an internal standard. After incubation for 1 hour at 60°C the samples were extracted with 200 µL of chloroform and the organic layer was analyzed by GC/MS. Compounds were separated on a DB-5 capillary column. The chromatography conditions were set as a gradient temperature program from 50°C to 130°C with a rate of 7°C/min, 1-minute hold, and final temperature increase to 230°C with a heating rate of 35°C/min. The injector and detector temperature were 250°C. Helium was used as the carrier gas with a flow rate of 1 mL/min and split injection with a split ratio of 5:1. Quantification was performed relative to the content of trichloroacetic acid and referenced to calibration curves prepared with samples of control urine. During the separation, the following intensities of m/z were monitored; starting at 7 minutes, the intensities of m/z 117, 119, 123, 125; at 11 minutes, the intensities of m/z 155, 157; and at 14 minutes, the intensities of m/z 96, 97. Dwell times of 30 milli seconds were used.

4.4 *IN VITRO* INCUBATION

4.4.1 Preparation of Human and Rat Microsomes and Protein Determination

Human liver microsomes were obtained from the Keystone Skin Bank (Exton, PA; USA). Rat liver microsomes were prepared freshly from untreated rats and from rats treated with pyridine (c.f. 4.6.2). Preparation was performed by standard protocols (Dekant et al., 1995). Protein concentrations were determined with the BioRad D_C Protein assay kit and bovine serum albumin as standard (Lowry et al., 1951; Peterson, 1977).

4.4.2 Microsomes Incubated with the Volatile 1,1,1,3,3-Pentafluoropropane (HFC-245fa)

Reaction mixtures with 1,1,1,3,3-pentafluoropropane contained microsomes, NADPH generating system (7,6 mg/mL $NADP^+$, 61 mg/mL glucose-6-phosphate and 100 Units/mL glucose-6-phosphate dehydrogenase) and substrate, respectively inhibitor, in a total volume of 1.1 mL 0.1 M phosphate buffer containing 1 mM EDTA (pH 7.4). Microsomes (2 mg protein/mL), the NADPH generating system and the corresponding amount of buffer were placed in a sealed 2-mL GC vial and were preincubated for 5 minutes at 37°C. The substrate 1,1,1,3,3-pentafluoropropane (10 µL of liquid at 4°C) was added through the septum with a micro liter syringe at -4°C. Reaction mixtures were incubated at 37°C in a shaking water bath for 20 minutes. The vials were submerged in water to ensure constant temperatures in the vials. The reactions were stopped by placing the vials on ice. Each reaction was repeated four times. Under same conditions the fluorine containing substrates 3,3,3-trifluoropropionic acid, trifluoroacetone, trifluoroacetophenone and hexafluoroacetone trihydrate (10 µM) were incubated with rat or human microsomes.

4.4.3 p-Nitrophenol Assay and Inhibitors

p-Nitrophenol was used as a model substrate to determine the activity of cytochrome P-450 2E1 enzyme in microsomes. As described by Urban *et al*, the absorbance of the formed product 4-nitrocatechol was measured spectrophotometrically at 510 nm with a molar extinction coefficient ε, determined to be $\varepsilon = 14.6\ mmol^{-1}\ cm^{-1}$. The final protein concentrations were 1 and 2 mg/mL for rat and human liver microsomes, respectively. The source and characterization of the human liver samples has been described previously (Dekant et al., 1995).

Diethyldithiocarbamate, a selective inhibitor of the cytochrome P-450 isoenzyme 2E1, was used in final concentrations of 100 or 300 µM. For all reaction mixtures, microsomes, a NADPH-generating system, and diethyldithiocarbamate were incubated for 5 minutes at 37°C before addition of substrate (Guengerich et al., 1991; Harris et al., 1991; Koop, 1986; Koop, 1992).

Microsomal incubations under anaerobic conditions were maintained with a constant flux of 50 mL/minute nitrogen gas through the reaction vial under pressure compensation. To differentiate the cytochrome P-450 dependent monooxygenase

from the flavine monooxygenase, the reaction vials were flushed with an air/carbon monoxide-volatile mixture (20/80%).

4.4.4 Incubation of Liver Homogenate with 3,3,3-Trifluoropropionic Acid and Sodium Trifluoropyruvate (α-Oxidation)

Dialyzed rat liver homogenates were incubated with 3,3,3-trifluoropropanoic acid or sodium trifluoropyruvate (0.1–1 mM) and the cofactors required for the several steps in the fatty acid α-oxidation pathway. Some samples were incubated in the absence of 2-oxoglutarate, thiamine pyrophosphate (TPP) or NAD^+. After incubation for 20 to 40 minutes, the samples were placed on ice, protein was sedimented by centrifugation at 100 000 x g, and samples were analyzed using ^{19}F-NMR spectroscopy and GC/MS (Croes et al., 1996a; Croes et al., 1996b; Foulon et al., 1999).

4.4.5 Preparation of Liver Mitochondria

The isolation of rat liver mitochondria was performed according to the method of G. Beutner, University of Rochester, USA. Mitochondria were freshly prepared for experiments and were active for 4 to 6 hours after preparation. To induce the activity of ß-oxidation in mitochondria, control rats were deprived of feed for 42 hours (night/day/night) before the preparation of mitochondria. All procedures were performed on ice to maintain the activity of mitochondria. The liver was removed immediately after sacrifice, weighed, put in ice-cold buffer A (250 mM sucrose, 1 mM EGTA, 1 mM glutathione, 10 mM HEPES; pH 7.4) and subsequently minced with scissors. During this procedure, the buffer was changed 3 to 5 times to remove blood cells and glycogen. A glas-on-teflon potter was used to homogenize the tissue for 10 minutes in 60 mL buffer A. After centrifugation for 10 minutes at 2 000 rpm at 4°C (using a Kendro Sorvall cooling centrifuge, SS-34), the supernatant was transferred to fresh tubes and centrifuged again for 10 minutes at 10 000 rpm. The sediment was resuspended in 50 mL buffer B (250 mM sucrose, 1 mM glutathione, 10 mM HEPES; pH 7.4) and centrifuged for 10 minutes at 10 000 rpm. Finally, the supernatant was removed and the pellet of liver mitochondria was resuspended in 2 to 4 mL of buffer B and kept on ice. An aliquot was taken and the protein content was

determined. Before use, mitochondria were checked for their activity by measurement of the oxygen consumption (c.f. 4.4.7).

4.4.6 Preparation of Heart Mitochondria

For the isolation of rat heart mitochondria, a slightly modified method of Rehncrona *et al* (1979) was used. As with the liver mitochondria, rats were fasted for 42 hours before preparation. Heart mitochondria were prepared freshly for experiments and kept on ice. The whole heart was isolated, carefully minced and suspended in ice-cold isolation buffer A (225 mM mannitol, 75 mM sucrose, 0.5 mM EGTA, 1 mM glutathione, 10 mM HEPES; pH 7.4) and cut in small pieces. During this procedure, the buffer was changed three to five times to remove blood cells and tissue from arteries and veins. The tissue was transferred to a glas-on-teflon potter and homogenized for 15 minutes in 10 mL buffer A. The homogenate was centrifuged for 10 minutes at 2 000 rpm at 4°C (using a Kendro Sorvall cooling centrifuge, SS-34) to sediment nuclei and cell membranes. The supernatant was transferred to fresh tubes and centrifuged again for 10 minutes at 10 000 rpm to separate the mitochondria from cytosolic parts. The sediment was resuspended in 15 mL buffer A and divided into two tubes. To each tube 10 mL Percoll® solution (8.6 g sucrose, 64.6 mL Percoll®, 25.4 mL water) was added and the tubes were filled completely for centrifugation at 22 000 rpm for 45 minutes at 4°C in a ultracentrifuge (Beckmann Ti 70) with a deceleration rate at the end. A Percoll® gradient was performed by centrifugation and the mitochondria built a dense layer at the interface between Percoll® and the buffer. The layer was transferred into 10 mL buffer B (225 mM mannitol, 75 mM sucrose, 1 mM glutathione, 10 mM HEPES; pH 7.4) and centrifuged again for 10 minutes at 10 000 rpm at 4°C. The sediment was re-suspended in buffer B and centrifuged as before. Finally, the pellet of heart mitochondria was resuspended in 1 mL of buffer B and kept on ice. An aliquot was used to determine the protein content. Before use, mitochondria were checked for their activity by measurement of the oxygen consumption (c.f. 4.4.7).

4.4.7 Oxygen Consumption in Liver and Heart Mitochondria

The mitochondrial function was determined by measuring the respiration rate of freshly prepared mitochondria with a Clark oxygen electrode. The buffer, as used for resuspended mitochondria (buffer B), was air-equilibrated at 37°C, in a 2-ml final

volume chamber containing a small magnetic bar that was connected to a circulating water bath at 37°C. The oxygen concentration was calibrated daily with air-saturated water and sodium dithionite, assuming 0.438 µmol O_2 at 37°C. After recording the endogenous respiration rate and stabilization, the chamber was opened and mitochondria (2 mg/mL of protein) were added. Protein content was measured by the method of BioRad D_c. The designations state 3, for ADP-stimulated respiration, and state 4, for ADP-limited respiration, are those used by Villani *et al* (2001) for the respiration control ratio (RCR), ADP/O_2-ratio (P/O), and oxygen consumption rate and were measured according to Estabrook (1967). The reaction was started by addition of succinate (Gregersen, 1979). For quality assurance, used mitochondria were always coupled (RCR>5) and used within 4 hours after preparation (Villani et al., 2001).

The uncoupled respiratory rate was measured and expressed as percentage of the starting coupled endogenous respiration rate. For data analysis, the software Mac Lab® was used.

4.4.8 *In Vitro* ß-Oxidation of [U-^{14}C] Palmitic Acid in Mitochondria and Incubation with 3,3,3-Trifluoropropionic Acid and 4-Pentenoic Acid

The influence of 3,3,3-trifluoropropionic acid and 4-pentenoic acid on the β-oxidation of [U-^{14}C] palmitic acid was studied *in vitro* in liver and heart mitochondria. Studies were performed in the presence or absence of 3,3,3-trifluoropropionic acid and 4-pentenoic acid. The protocol was assessed as described by Freneaux *et al.*, 1988. The degree of ß-oxidation was measured by the determination of resulting [^{14}C] acid-soluble ß-oxidation products. These mainly represent ketone bodies and to a lesser extent TCA cycle intermediates. The amount of formed [^{14}C] CO_2 represented less 1 % regarding to Freneaux, and were negligible. In a plastic screw-vial, 4 mg of mitochondrial protein were added to the incubation medium (70 mM sucrose, 43 mM KCl, 3.6 mM $MgCl_2$, 7.2 mM KH_2PO4, 36 mM Tris-HCl buffer; pH 7,4) with a final incubation volume of 2 mL. 100 µL of the β-oxidation buffer (4 mM ATP, 1 mM Carnitine, 300 µM CoA) and the substrates were added and the mixture was preincubated for 5 minutes at 30°C. In addition 100 µL of incubation medium were added and incubated for 5 to 30 minutes (optimal time was determined to be 20 minutes) at 30°C while shaking slowly. The incubation medium consisted of 25 mg

BSA, 250 µL of 16 mM palmitic acid and [U-^{14}C] palmitic acid at a final concentration of 0.05 µCi in 5 mL incubation medium. To stop the reaction, 400 µL of 5% perchloric acid were added. Before analysis, the protein was separated by centrifugation for 10 minutes at 4 000 g. An aliquot of 300 µL of the supernatant was transferred to a vial with 4.7 mL of scintillation cocktail. The [^{14}C]-activity was counted, which represented the *in vitro* formation of perchloric acid-soluble β-oxidation products. Non-reacting [U-^{14}C] palmitic acid precipitated (Fromenty et al., 1989).

4.4.9 Determination of Radioactivity in Incubation Samples

An aliquot (100 µL) of the incubation supernatant was transferred into a liquid scintillation vial with an excess (4.9 mL) of scintillation cocktail (Rotiszint EcoPlus). [^{14}C] Radioactivity was determined in a liquid scintillation counter (Tricarb 4000, Packard Instruments, Downers Grover, IL, USA). Each sample was counted for 5 minutes and the mean of three samples was taken.

4.5 CYTOTOXICITY

4.5.1 Cell Culture of the Cell Lines H9C2 and HepG2

The cell line H9C2 (ATCC CRL-1446) was grown in Dubelcco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The HepG2 human hepatocellular carcinoma cell line (ATCC HB-8065) was cultured in DMEM, low glucose and with 10% FBS. The cells were grown at 37°C, 95% air and 5% CO_2 in a humidified incubator. The cell line H9C2 (ATCC CRL-1446) is a subclone of the original clonal cell line derived from rat heart tissue and exhibits many of the properties of skeletal muscle. Myoblastic cells in this line fuse to form multinucleated myotubes and respond to acetylcholine stimulation. This cell line was supposed to mimic the heart as target organ. The HepG2 human hepatoma cell line expresses 3-hydroxy-3-methylglutaryl-CoA reductase and hepatic triglyceride lipase activities. Expressed receptors are insulin and insulin-like growth factor II (IGF II).

The experimental design was set up in 96-well plates. HepG2, respectively H9C2 cells were plated with a density of 10 000 cells/well in 200 µL of medium. The plates were incubated at 37°C for 24 hours to allow cells to attach and grow on the plate.

Before the assay was conducted, cells were visibly inspected, the growth media was removed and the cells were exposed to medium without FBS and with the target concentration of the test item.

For the lactate dehydrogenase (LDH) assay, the cells were incubated again for 6 hours at 37°C. The MTT assay was conducted on the same plate after incubation of the cells with the MTT solution for 2 hours.

4.5.2 Experimental Design

For testing the cytotoxicity of the compounds the LDH and MTT assay were performed. The cells were treated in triplicate with different concentrations of various test items. For each 96 well plate the following assay controls were performed: blank, i.e. no cells with media only, positive control, i.e. cells with triton X in the media and negative control, i.e. cells with media only. The negative control represented the 100% viability value and the endogenous LDH release. The positive control represented no viable cells and therefore the maximum LDH release of the cells into the media. For treated cells, for every different concentration and time point a separate "total LDH release" was performed by adding triton X. Therefore, effects like reduced cell proliferation were taken into account. The optical density (OD) was recorded by the Spectramax® software program and transferred to Microsoft Excel® sheets. Statistical analysis with mean and standard deviation were performed.

4.5.3 LDH – Assay

The LDH assay was performed measuring the oxidation of lactate to pyruvate, induced by LDH, and resulting in generation of NADH. The reduced NADH drived the conversion of a tetrazolium dye to a soluble colored formazan derivative. The absorbance of resulting formazan was measured at a wavelength of 340 nm. Total LDH was determined as a 100% release of LDH. The cells were treated with a 6% triton X-solution in media, with a final concentration of 0.4% in the well at 45 minutes before the end of the incubation. The assay was performed following the protocol of Promega (Promega GmbH, Mannheim, Germany). 50 µL of the media were mixed with 50 µL 'substrate mix' and incubated for 30 minutes in the dark at room temperature. After adding 50 µL of the 'stop solution', the absorbance was read at a wavelength of 490 nm. LDH release was defined as "% cytotoxicity" by the percentage of LDH in the culture medium with respect to total LDH (LDH in the

culture medium and inside the cell, treated with triton X). The endogenous LDH release was subtracted as backround absorption. As a blank control, complete medium without cells was used (Korzeniewski et al., 1983; Decker et al., 1988). LDH release was higher in a medium deprived of serum than in a medium supplemented with serum, also found in literature.

4.5.4 MTT – Assay

The number of viable cells was determined by a colorimetric assay in 96-well plates. One such assay, developed by Mosmann, depends on the reduction of the tetrazolium salt MTT by living cells respectively various mitochondrial dehydrogenases, to form a blue formazan product (Mosmann, 1983). The blue formazan crystals were dissolved in acid-isopropanol (111,25 mL isopropanol, 1,25 mL concentrated hydrochloric acid, 12,5 mL triton X-100). The MTT assay was measured at the absorption at a wavelength of 570 nm with a backround absorption at a wavelength of 630 nm. The cell viability was calculated as the percentage of the absorbance difference (*A* 570–630) of the sample divided by the absorbance difference of the control. The negative control value was set for 100% viability and represents non treated cells with medium only. For the MTT solution, 15 mg of MTT were dissolved in 3 mL of phosphate buffered solution (PBS) and added as a 10% final concentration to each well. The incubation time was 2 hours.

4.5.5 Crystal Violet – Assay

The assay was used for counting viable cells in 96-well plates and setting up growing studies. The crystal violet solution was prepared as 0.5% of crystal violet (N-hexa-methyl-pararosanilin), dissolved in water and 20% of methanol. To commence the assay, the medium in the wells was removed, 150 µL of the staining solution were added and incubated at room temperature for 15 minutes. The staining solution was then removed and the wells were washed 5 times with water. Before measurement of the crystal violet dye, which was bound to the DNA of viable cells, it was dissolved in 150 µL of methanol. The OD was measured at a wavelength of 550 nm, which represents the number of viable cells. The stimulation or inhibition of cell growth was calculated as percentage of control cells.

4.6 *IN VIVO* EXPERIMENTS

4.6.1 Animals and Treatment

Sprague-Dawley rats were used for all studies and obtained from Charles-River Wiga, Sulzfeld, Germany. The age of the animals at the start of treatment was between 8 and 12 weeks. The body weight was 220–280 g. Animals were kept at constant humidity and temperature (21°C) in the animal facility of the department with a controlled 12 hour light/dark cycle. Before the start of the experiments, animals were held for an acclimatization period for at least one week.

4.6.2 Treatment of Rats with Pyridine to Induce the Cytochrome P-450 2E1 Activity

To induce the activity of the cytochrome P-450 2E1 enzyme in liver microsomes, rats were treated intraperitoneally with 100 mg/kg b.w. pyridine, dissolved in isotonic sodium chloride solution, for five consecutive days. All animals were fasted 18 hours before sacrifice and preparation of the microsomal fraction (Herbst et al., 1994; Koster et al., 1994; Urban et al., 1994b).

4.6.3 Exposure of Rats to Sodium Trifluoropyruvate, Methyl Trifluoropyruvate and Hexafluoroacetone Trihydrate

To study the fate of possible metabolites of 1,1,1,3,3-pentafluoropropane, three fluorine-containing components were given to rats. Sodium trifluoropyruvate, methyl trifluoropyruvate and hexafluoroacetone trihydrate were orally administered to two male rats by gavage, each at a dose level of 10 mg/kg b.w.. The animals were transferred to metabolic cages, and urine was collected over a 24-hour period. Urine samples were analyzed for metabolites by ^{19}F-NMR spectroscopy and GC/MS.

4.6.4 Exposure of Rats to a Single Dose of 3,3,3-Trifluoropropionic Acid

Effects of single dose administration of 3,3,3-trifluoropropionic acid were studied in rats with free access to feed or rats deprived of feed for 24 hours. To study acute toxicity effects, rats were exposed to dose levels in a range of 2 to 80 mg/kg b.w. by gavage.

For monitoring insulin and blood glucose concentrations after drug administration, five male rats were exposed each to 80 mg/kg b.w. 3,3,3-trifluoropropionic acid by gavage. Blood was taken from the anaesthetized capped tail after every 7 minutes within the first hour and every 30 minutes thereafter. Blood glucose was analyzed by an enzymatic assay and insulin by an ELISA assay.

4.6.5 Exposure of Rats to a Repeated Dose of 3,3,3-Trifluoropropionic Acid for 14 Days via Drinking Water

One control group and two dose groups (target dose level of 1 mg/kg b.w. and 2 mg/kg b.w.) of each four male Sprague-Dawley rats were exposed to 3,3,3-trifluoro-propionic acid in drinking water for 14 days. During exposure, the animals were kept in metabolic cages and had access to food and water *ad libitum*. Food and water consumption were monitored on a daily basis. Urine was collected in 24-hour intervals on ice, the urine volume was recorded and samples were stored at -20°C until analysis. For acclimatization, the animals were kept in metabolic cages for three days in advance of commencement of the experiment. During this period, urine was collected as control urine.

4.6.6 Exposure of Rats to a Repeated Dose of 3,3,3-Trifluoropropionic Acid by Oral Intubation (Gavage) for 28 Days

Male and female Sprague Dawley rats (8 weeks-old) were allocated to groups of five animals per sex and dose level, and kept in gang housing. Dose levels were in addition to one control group per sex, 2.5 mg/kg b.w., 5 mg/kg b.w., 10 mg/kg b.w. and 30 mg/kg b.w. for male rats and 1 mg/kg b.w., 2.5 mg/kg b.w., 10 mg/kg b.w. and 30 mg/kg b.w. for female rats. During the study, the animals were provided with food and water *ad libitum*. The compound 3,3,3-trifluoropropanoic acid was administered daily by oral intubation (gavage) in 0.5 mL phosphate buffered solution

at pH 7.4. The formulation of 3,3,3-trifluoropropionic acid, administered by gavage was analyzed for its chemical composition for purity and stability by ^{19}F-NMR. The spectrum showed a triplet at δ = -63.9 ppm. The administered dose formulations were considered as pure and stable. Animals were observed daily for clinical signs during treatment. Body weight, food consumption and water consumption were recorded daily. Urine was collected on ice for 24 hours, using metabolic cages, once during the study and on day 28, before necropsy. During the study, urine was collected on the following days: on day 15 for male animals dosed with 2.5 and 10 mg/kg b.w. and for female animals dosed with 1 mg/kg b.w.. On day 16 for male control rats and male animals dosed with 5 mg/kg b.w.. On day 17 for female control animals and on day 18 for female animals dosed with 2.5 mg/kg b.w.. For necropsy, all animals were killed by exsanguination under ether anesthesia and the organs liver, heart, kidney, lung and spleen were collected and fixed for histopathological evaluation. Urine and blood samples were processed for clinical chemistry and quantification of fluorine containing compounds.

4.6.7 Whole Body Inhalation Exposure of Rats to 100 and 200 ppm 1,1,1,3-Tetrachloropropane for 6 Hours

Five male (320-350g, 12-weeks-old) Sprague Dawley rats were exposed to targeted atmosphere concentrations of 100 and 200 ppm 1,1,1,3-tetrachloropropane in a dynamic exposure chamber. The inhalation system consisted of a separate heated mixing chamber, which ensured the transfer of the test item as volatile into a 20.6-litre desiccator as exposure chamber. Metered amounts of 1,1,1,3-tetrachloropropane were processed by perfuser to the mixing chamber which is flushed with compressed air, fitted with flow meters (c.f. Figure 2). Test item concentrations in the exposure chamber were monitored at 15-minute intervals by taking 1-mL samples of the chamber atmosphere with a gas-tight syringe. The concentration of 1,1,1,3-tetrachloropropane was determined by GC/MS (c.f. 4.3.3.5). Exposure concentrations were measured against standard calibration curves with headspace samples containing known concentrations of 1,1,1,3-tetrachloropropane. After the termination of the exposure, the animals were transferred to metabolic cages, and urine was collected on ice for four consecutive days in 12-hour intervals. The estimated achieved inhaled dose of 1,1,1,3-tetrachloropropane for each rat was calculated to be 0.126 mmoles for the 100 ppm dose group and 0.25 mmoles for the 200 ppm

dose group (using an alveolar ventilation rate of 0.169 L/min and an assumed retention factor of 0.5; Nihlen et al., 1998). The possible oral exposure of 1,1,1,3-tetrachloropropane due to whole body inhalation procedure and the dermal absorption of the body were considered as negligible and were not taken into account.

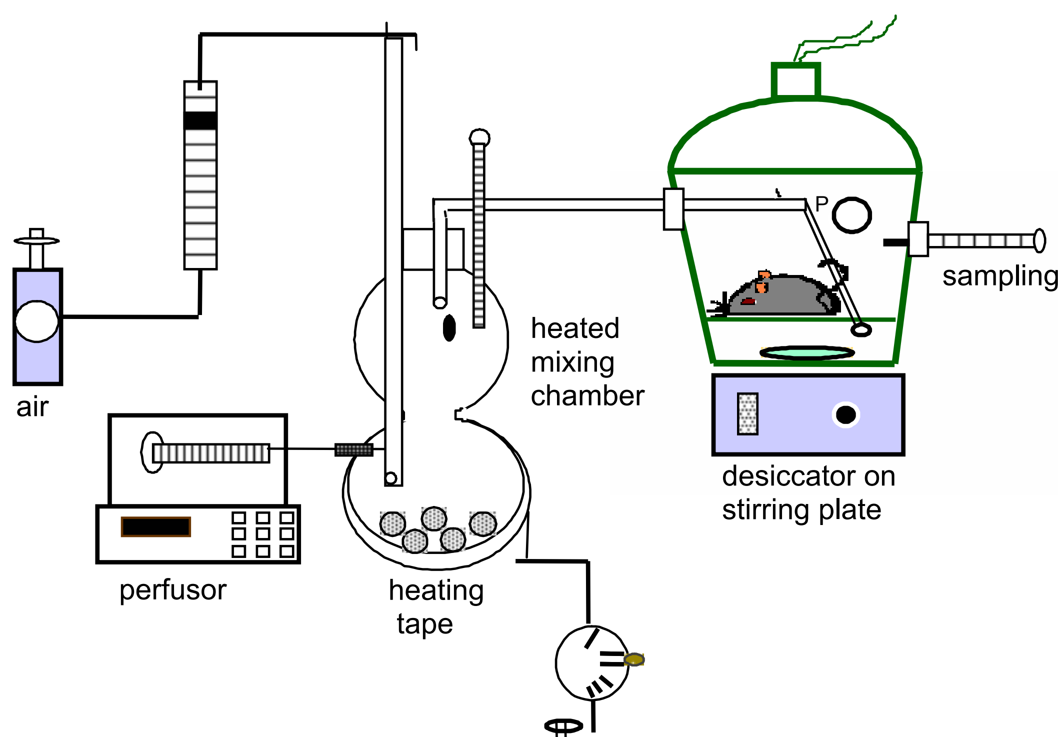


Figure 2: Dynamic exposure chamber for whole body inhalation exposition of rats to 1,1,1,3-tetrachloropropane.

4.6.8 Exposure of Rats to a Single Dose of 3,3,3-Trichloropropionic Acid

A metabolic study was performed by administering a single dose of 3,3,3-trichloro-propionic acid. Three male rats were exposed to 10 mg/kg b.w. by gavage and were housed individually in metabolic cages with free access to feed and water for 72 hours. Urine samples were collected in 6-hour intervals on ice.

4.7 CLINICAL CHEMISTRY

Clinical chemistry parameters of urine and serum samples were analyzed in the clinical chemistry laboratory (University of Wuerzburg). Analysis of blood chemistry and urinalysis was set up following the OECD Guideline 407. Parameters of urine analysis were glutamate oxalacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline aminotransferase (ALP), γ-glutamyl transferase (GGT), urea, osmolarity, total protein, sodium, potassium, calcium, chloride, creatinine and triglycerides. Serum samples were analyzed for GOT, GPT, ALP, GGT, blood urea nitrogen (UBUN), osmolarity, creatine kinase (CK), creatine kinase isoenzyme (CKMB), LDH, lactate, total protein, sodium, potassium, calcium, chloride, triglyceride and creatinine. Blood samples were taken at necropsy from the liver aorta and transferred into Microtainer™ with clot activator and gel for serum separation. The blood was allowed to clot at room temperature for at least 30 minutes and centrifuged. Urine samples were collected within defined time periods, using metabolic cages. Urine and serum samples were transferred in chilled, insulated containers shortly after sampling or if necessary immediately frozen and stored at -80°C until analysis. They were forwarded to the clinical chemistry laboratory without any sample preparation. For glucose and L-lactate analysis, sodium fluoride was used as inhibitor of glycolytic enzymes in serum, in combination with an anticoagulant. The Monovetten® contained 2-3 mg fluoride per mL of blood. Glucose and L-lactate levels were analyzed by manual enzymatic assays from Rolf Greiner Biochemica, Flacht, Germany. Glucose levels were determined by the glucose hexokinase method. The endpoint reaction was based on the conversion of glucose by glucose-6-phosphate. Contained NAD^+ was converted to NADH, which was measured at the absorption maximum at a wavelength of 340 nm. L-Lactate was determined after oxidation with lactate oxidase to pyruvate and hydrogen peroxide by using a colorimetric method. The reaction of hydrogen peroxide with 4-aminoantipyrine and peroxidase to a red chinoline dye was quantified at a wavelength of 546 nm. The test procedures were assessed according to the manuals. Ketone bodies were semi-quantified with keto-diastix® test sticks.

The rat insulin level was determined by ELISA, provided as a ready to use kit from Mercodia, Uppsala, Sweden. It was based on the direct sandwich technique in which two monoclonal antibodies were directed against separate antigenic determinants on the insulin molecule. During incubation, insulin reacted with peroxidase-conjugated anti-insulin antibodies, and anti-insulin antibodies bound the well of the 96-well plate. A simple washing step removed unbound enzyme labeled antibody. The bound conjugate was detected by reaction with 3,3',5,5'-tetramethylbenzidine. The reaction was stopped with the addition of acid and resulted in a colorimetric endpoint, read by spectrophotometer at a wavelength of 530 nm. The test procedure was following the standard manual instructions.

4.8 HISTOPATHOLOGY

For subsequent histopathology examination, tissue samples of the organs liver, heart, kidney, lung and spleen were fixed in neutral phosphate-buffered 4% formaldehyde solution. The organs were weighed before fixation and relative organ weights were calculated based on the body weight. The tissues were processed and embedded in paraffin in the Department of Pathology, University of Wuerzburg. The paraffin sections were cut on a Leica® microtome in the Department of Pharmacology, University of Wuerzburg, at a nominal thickness of 4 µm. The slides were processed for hematoxylin and eosin staining as well as sirus red staining following standard protocols. Slides of the tissues were examined by light microscopy by the pathologist S. Cazsch, Merck KGaA (Boor et al., 1982; Robertson et al., 1993; Bhattacharya et al., 2002).

4.9 IMMUNOHISTOCHEMISTRY

The immunohistochemistry was performed with the frozen organs heart, liver and kidney. Sectioning was done in the Department of Pharmacology, University of Wuerzburg, using a Leica® CM3050 S -cryostat (Fa. Leica®, Nussloch, Germany). The immunohistochemical staining procedure for frozen sections was performed, using the Anti-mouse Ig HRP detection kit from BD Biosciences (San Diego, CA,

USA). The diaminobenzidine (DAB) system visualized the immunostaining pattern via the horseradish peroxidase (HRP) enzyme. The primary antibodies used were mouse-anti-rat CD3 and mouse-anti-rat CD45 from DPC Biermann (Bad Nauheim, Germany).

4.10 STATISTICAL METHODS

Statistical analysis of all data was performed using the calculation for mean values, standard deviations and Student's t-test of Excel® spreadsheets, Microsoft® Office professional for Windows XP®. *P*-values of less than 0.05 were considered as significant. Quantifications were based on calibration curves, plotted in Excel® graphs. Samples were calculated based on the linear regression formula. Half-lives were calculated using exponential regression in Microsoft Excel® spreadsheets. The curve fitting function of the program was used and curves were stripped based on correlation coefficients. R^2-values of > 0.98 were considered for validation.

4.11 QUALITY ASSURANCE

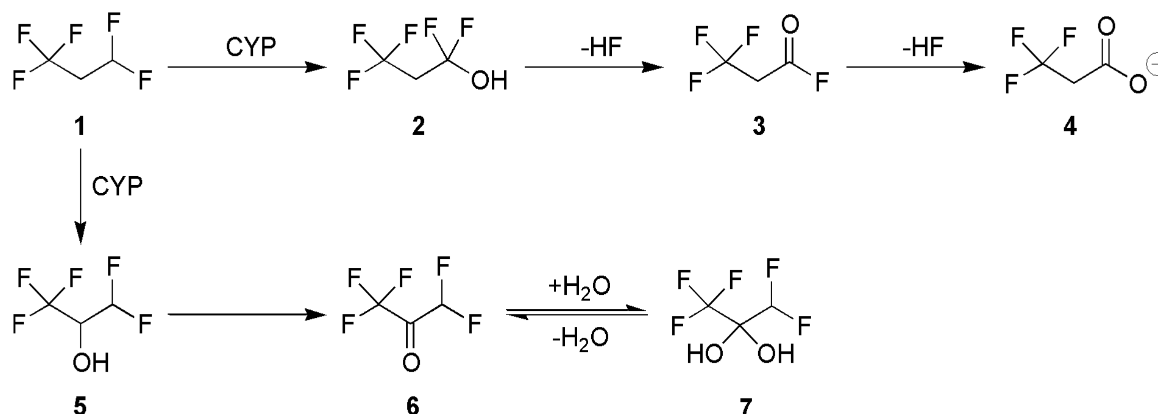
All study designs and experimental work were conducted in the spirit of Good Laboratory Practice (GLP). All analytical methods were validated. Standard operational procedures were generated for analytical methods and for various assays. All animal studies were in compliance with the policy of animal ethics to avoid pain, suffering and harming. They were performed according to the German Animal Welfare Law and correlated Guidelines.

5 RESULTS

5.1 BIOTRANSFORMATION OF 1,1,1,3,3-PENTAFLUORO-PROPANE *IN VITRO*

The studies on the biotransformation of 1,1,1,3,3-pentafluoropropane in rats resulted in the formation of trifluoroacetic acid as major metabolite, excreted in urine. Trifluoroacetic acid was considered as an unexpected metabolite. In addition to trifluoroacetic acid, the minor metabolites 3,3,3-trifluoropropionic acid and 1,1,1,3,3-pentafluoroacetone were formed (c.f. Scheme 7). The oxidative metabolism of 1,1,1,3,3-pentafluoropropane was suggested to be mediated by cytochrome P-450 but the formation of trifluoroacetic acid could not be rationalized by a known mechanism of cytochrome P-450 oxidation of 1,1,1,3,3-pentafluoropropane. Therefore, a series of experiments were conducted to elucidate possible mechanisms of formation of trifluoroacetic acid from 1,1,1,3,3-pentafluoropropane.

To characterize the biotransformation of 1,1,1,3,3-pentafluoropropane *in vitro*, 1,1,1,3,3-pentafluoropropane was incubated with rat and human liver microsomes. The purpose was to test alternative pathways accounting for the formation of trifluoroacetic acid from 1,1,1,3,3-pentafluoropropane, as seen *in vivo*. This reaction involved the cytochrome P-450-catalyzed oxidation, resulting directly in the formation of trifluoroacetic acid or in the formation of an intermediate that is converted to trifluoroacetic acid. These studies revealed an unusual biotransformation reaction, *i.e.*, the cleavage of a carbon-carbon bond in 1,1,1,3,3-pentafluoropropane to give trifluoroacetic acid as the major metabolite; the biotransformation, which involves C-C bond cleavage, has not been previously described for halogenated hydrocarbons.



Scheme 7: Proposed biotransformation of 1,1,1,3,3-pentafluoropropane in rats: [1] 1,1,1,3,3-pentafluoropropane, [2] 1,1,3,3,3-pentafluoropropan-1-ol, [3] 3,3,3-trifluoropropanoyl fluoride, [4] 3,3,3-trifluoropropionic acid, [5] 1,1,1,3,3-pentafluoropropan-2-ol, [6] 1,1,1,3,3-penta-fluoroaceton, [7] 1,1,1,3,3-pentafluoropropane hydrate.

5.1.1 Cytochrome P-450 mediated Reactions

Compared to *in vivo* studies, *in vitro* incubations of 1,1,1,3,3-pentafluoropropane did not result in the formation of detectable amounts of 3,3,3-trifluoropropanoic acid in the presence of microsomal protein from non treated rats (c.f. Table 5). Based on the detection limit of the used GC/MS-assay, rates of formation of 3,3,3-trifluoro-propanoic acid were less than 0.75 pmol per mg protein per minute. Quality and activity of microsomal preparations were based on *p*-nitrophenol oxidase activity, as described previously (Koop, 1986). The *p*-nitrophenol oxidase activity is a marker of the activity of the cytochrome P-450 isoenzyme 2E1, which is involved in the biotransformation of several other hydrochlorofluorocarbons and hydrofluorocarbons (Dekant, 1996a). The main metabolite, trifluoroacetic acid was present as a contaminant in small concentrations in reaction mixtures that contained buffer, microsomal protein, and 1,1,1,3,3-pentafluoropropane, but lacked of NADPH. This was shown in the chromatogram of GC/MS analysis of microsomal incubations (c.f. Figure 3). In reaction mixtures containing NADPH, the concentration of trifluoroacetic acid was significantly increased ($p < 0.05$), compared with reaction mixtures, lacking NADPH after an incubation time of 20 minutes, but not seen after 10 minutes. The metabolites 3,3,3-trifluoropropionic acid and trifluoroacetic acid were identified in the single ion monitoring mode with m/z 59 and m/z 69 for trifluoroacetic acid and m/z 111 for 3,3,3-trifluoropropionic acid (c.f. Figure 4). GC/MS analysis of

the gas phase from the reaction mixtures did not show the formation of any other volatile metabolite.

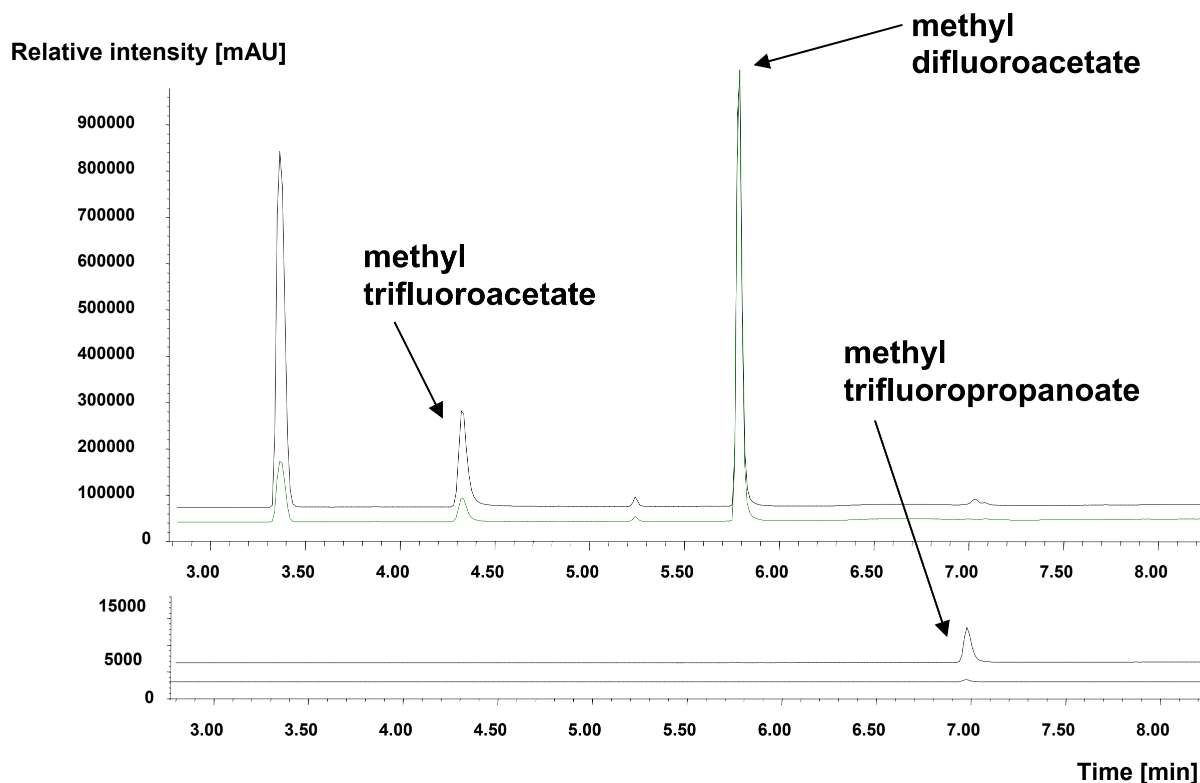


Figure 3: GC/MS analysis of reaction mixtures containing 1,1,1,3,3-pentafluoropropane and rat liver microsomes in the absence or presence of a NADPH-generating system.
The upper panel shows the combined intensities of m/z 51, 59, 60, 69, 83, 111, and 142 during the chromatographic separation; the lower panel shows the intensity of m/z 111. The peak at a retention time of 4.3 minutes (upper panel) represents methyl trifluoroacetate, and the peak at 5.6 minutes (upper panel) represents the internal standard methyl difluoroacetate. The peak at a retention time of 7.0 minutes (lower panel) represents methyl 3,3,3-trifluoropropanoate. The upper traces in each panel were from reaction mixtures containing NADPH, and the lower traces were from reaction mixtures lacking NADPH. All reaction mixtures were incubated for 20 minutes.

In addition, the microsomal incubation samples were analyzed using ^{19}F-NMR spectroscopy. In all samples the concentration of 3,3,3-trifluoropropanoic acid was below the sensitivity of the ^{19}F-NMR spectroscopic method, but besides the peak for the parent, an increase in concentrations of trifluoroacetic acid could be observed using ^{19}F-NMR spectroscopy (c.f. Figure 5). A time- and protein-concentration-dependent increase in trifluoroacetic acid and 3,3,3-trifluoropropanoic acid formation was observed using GC/MS analysis in microsomes from rats given the cytochrome P-450 2E1-inducer pyridine (c.f. Figure 3).

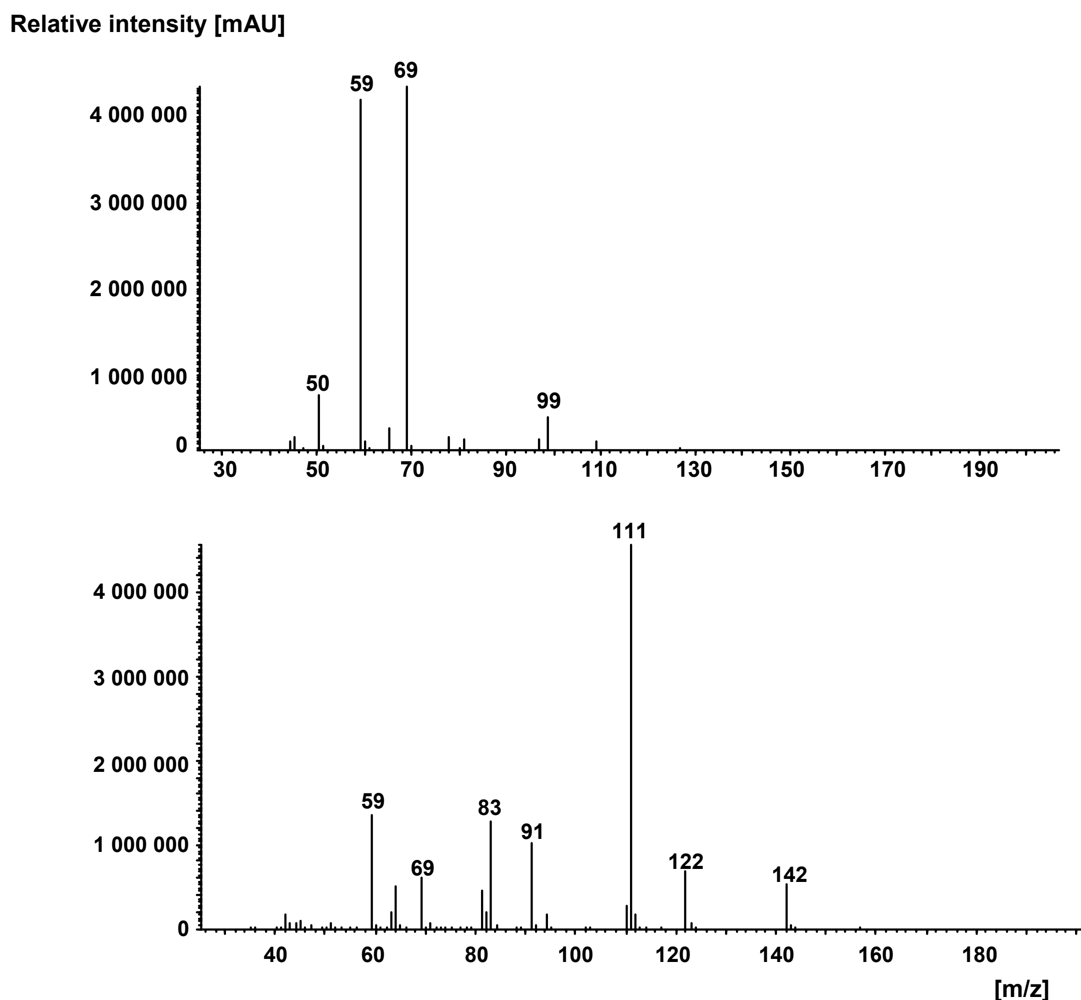


Figure 4: GC/MS full scan mass spectra of methyl trifluoroacetate (upper spectra) and methyl 3,3,3-trifluoropropanoate (lower spectra) after *in vitro* incubation of 1,1,1,3,3-pentafluoropropane.

Based on the measured concentration of trifluoroacetic acid, rates of formation of trifluoroacetic acid were calculated. The rates of oxidation of 1,1,1,3,3-pentafluoropropane to trifluoroacetic acid were slightly decreased in the microsomal fraction from pyridine-treated male rats and increased in the microsomal fraction from pyridine treated female rats, compared with control rats. The p-nitrophenol oxidase activity was markedly increased in the microsomal fractions from male and female rats (c.f. Table 5).

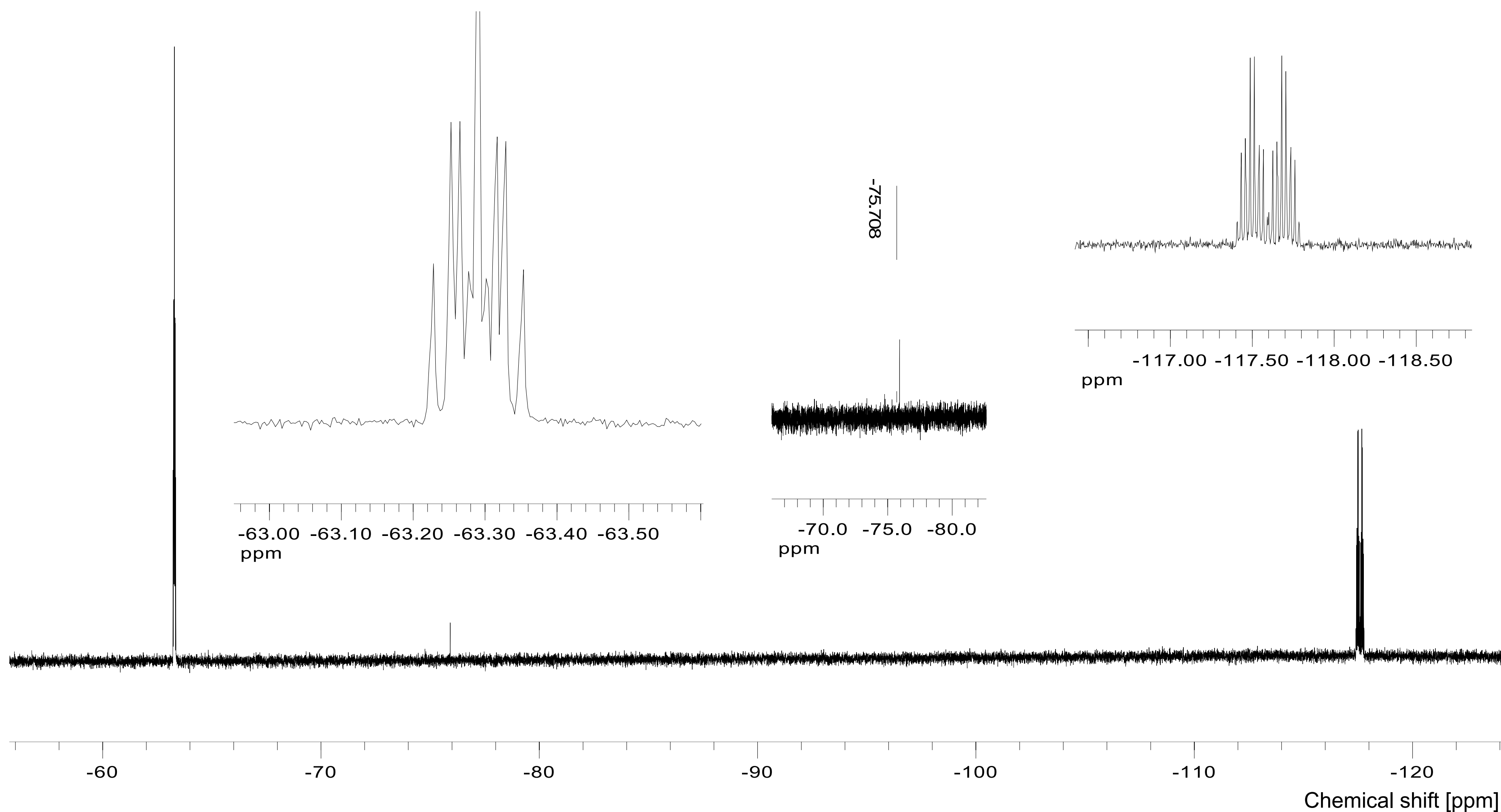


Figure 5: ^{19}F-NMR spectrum (proton coupled) of microsomal incubation sample exposed to 1,1,1,3,3-pentafluoropropane. The resonance at δ = –63.29 (m) and the multiplet at the resonance δ = –117.6 (m) were identical in chemical shift with the resonance obtained with authentic 1,1,1,3,3-pentafluoropropane. The signal at δ = –75.708 (s) represents trifluoroacetic acid.

Table 5: Biotransformation of 1,1,1,3,3-pentafluoropropane in liver microsomes from non-treated rats and those, pre-treated with pyridine. Incubations were performed at 37°C for 20 minutes.

Microsome source	Concentration (nmol/mL)	Rate of formation (pmol x mg^{-1} protein min^{-1})	p-Nitrophenol oxidation (nmol x mg^{-1} protein min^{-1})
	Trifluoroacetic acid		
Male rats			
- NADPH	1.99 ± 0.26		
+ NADPH	5.96 ± 0.73*	99.2 ± 20.5	0.18 ± 0.05
Male rats, pyridine			
- NADPH	1.3 ± 0.5		
+ NADPH	5.0 ± 1.3**	92.8 ± 32	3.22 ± 1.2
Female rats			
- NADPH	1.65 ± 0.24		
+ NADPH	4.80 ± 0.76*	80.1 ± 19.8	0.11 ± 0.02
Female rats, pyridine			
- NADPH	1.6 ± 0.16		
+ NADPH	7.7 ± 2.1**	151 ± 53	7.14 ± 1.67
	3,3,3-Trifluoropropanoic acid #		
Male rats, pyridine	No background	17.5 ± 4.0	
Female rats, pyridine		26.7 ± 4.8	

Concentrations of trifluoroacetic acid in samples with NADPH (n = 5) were significantly different when compared with samples lacking NADPH (*p <0.05, **p <0.001). Rates were calculated after subtraction of the background. # 3,3,3-Trifluoropropanoic acid was not detected after incubation of 1,1,1,3,3-pentafluoropropane with liver microsomes from control rats. Incubations with supersomes did not show the formation of 3,3,3-trifluoro-propionic acid.

The oxidation of p-nitrophenol and 1,1,3,3,3-pentafluoropropane was further studied in six human liver microsome samples (c.f. Table 6). The human liver microsome samples showed similar p-nitrophenol oxidase activities to those, reported previously (Urban et al., 1994b). In some of the liver microsome samples, increased concentrations of trifluoroacetic acid were formed in reaction mixtures, incubated in the presence of NADPH, as compared with controls, lacking NADPH.

Enzymatic formation of 3,3,3-trifluoropropanoic acid from 1,1,1,3,3-pentafluoro-propane was observed at low rates in all samples of human liver microsomes. As with rat liver microsomes, trifluoroacetic acid was the predominant metabolite, formed after the oxidation of 1,1,1,3,3-pentafluoropropane.

Table 6: Oxidation of 1,1,1,3,3-pentafluoropropane in human liver microsomes.

Samples	**Rate of oxidation of *p*-nitrophenol (nmol x mg^{-1} protein min^{-1})**	**Rate of oxidation of 1,1,1,3,3-pentafluoropropane to**	
		Trifluoroacetic acid (pmol x mg^{-1} protein min^{-1})	**3,3,3-Trifluoropropanoic acid (pmol x mg^{-1} protein min^{-1}) #**
HL11	2.17 ± 0.06	n.d.	2.9 ± 0.5
HL19	1.19 ± 0.27	11.5 ± 3.5*	4.1 ± 1.0
HL2a	1.23 ± 0.21	n.d.	7.6 ± 2.0
HL13	1.6 ± 0.57	n.d.	2.8 ± 0.7
HL15	0.76 ± 0.06	11.6 ± 5.3*	3.2 ± 0.3
HL1b	0.49 ± 0.03	7.7 ± 0.9*	1.2 ± 0.3

*Trifluoroacetic acid concentrations in incubation mixtures with and without NADPH were significantly different ($p < 0.05$); n.d.: no significant difference in trifluoroacetic acid concentrations between samples with or without NADPH. Rates were calculated after subtraction of the background. #Absence of background for 3,3,3-trifluoropropanoic acid permitted quantification of low rates of biotransformation.

To quantify the amount of inorganic fluoride, released after microsomal incubation with 1,1,1,3,3-pentafluoropropane, the incubation mixture was measured with a fluorine electrode. The sensitivity of inorganic fluoride was not sufficient for quantification in *in vitro* experiments.

To clarify the metabolic pathway of formation of these metabolites, experiments were designed to inhibit specific enzymes, responsible for biotransformation. The cytochrome P-450 isoenzyme 2E1 was assumed to be involved in the formation of 3,3,3-trifluoropropionic acid and trifluoroacetic acid (Guengerich et al., 1991; Koop, 1992). In the following, the activity of this enzyme was investigated. Diethyldithiocarbamate and carbon monoxide are known as cytochrome P-450 inhibitors and were therefore added to the incubation mixtures.

Diethyldithiocarbamate is a specific inhibitor of cytochrome P-450 2E1. After adding 100 µM of diethyldithiocarbamate to liver microsomes incubated with 1,1,1,3,3-pentafluoropropane, no conversion to 3,3,3-trifluoropropionic acid or trifluoroacetic acid was observed. In presence of diethyldithiocarbamate, the p-nitrophenol oxidation was reduced by about 95%.

Carbon monoxide inhibits the activity of cytochromes P-450 in general by binding with the same affinity as oxygen to the bivalent iron-ion of the cytochrome. The rate of formation of trifluoroacetic acid was decreased by 67% in microsomes from male rats and by 69% in microsomes from female rats, compared with incubations in the absence of carbon monoxide. Equally, the formation of 3,3,3-trifluoropropionic acid was decreased by 77% and 51% respectively in incubations in the presence of carbon monoxide.

These experiments confirmed that both, the formation of 3,3,3-trifluoropropionic acid and trifluoroacetic acid from 1,1,1,3,3-pentafluoropropane were catalyzed by cytochrome P-450, but the exact molecular mechanisms of the transformation could not be defined. To get a further insight into possible mechanisms and to investigate, whether similar carbon cleavage reactions may occur with other trifluoromethyl compounds, the biotransformation by cytochrome P-450 of a number of trifluoromethyl-containing ketones was studied *in vitro*.

The compounds selected were hexafluoroacetone trihydrate, 1,1,1-trifluoroacetone, 2,2,2-trifluoroacetophenone (c.f. Scheme 8) and 3,3,3-trifluoropropionic acid. These were incubated with pyridine induced rat liver microsomes, following the standard procedure as used above. Analyzes of the incubations with hexafluoroacetone trihydrate and trifluoroacetone by ^{19}F-NMR did not show the presence of metabolites. No ^{19}F-NMR signals other than those of the parent compounds were observed. However, metabolites were formed after incubation of liver microsomes with 2,2,2-trifluoroacetophenone as seen in the ^{19}F-NMR (c.f. Figure 6).

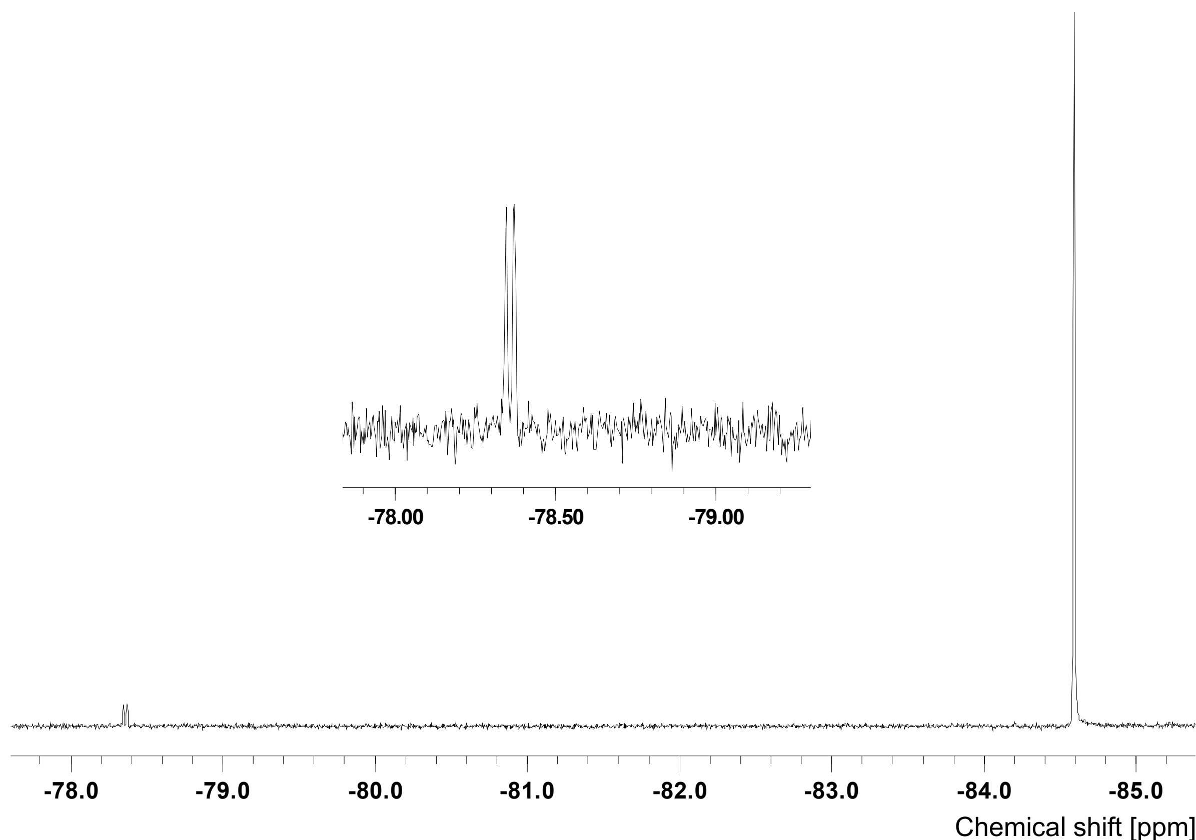
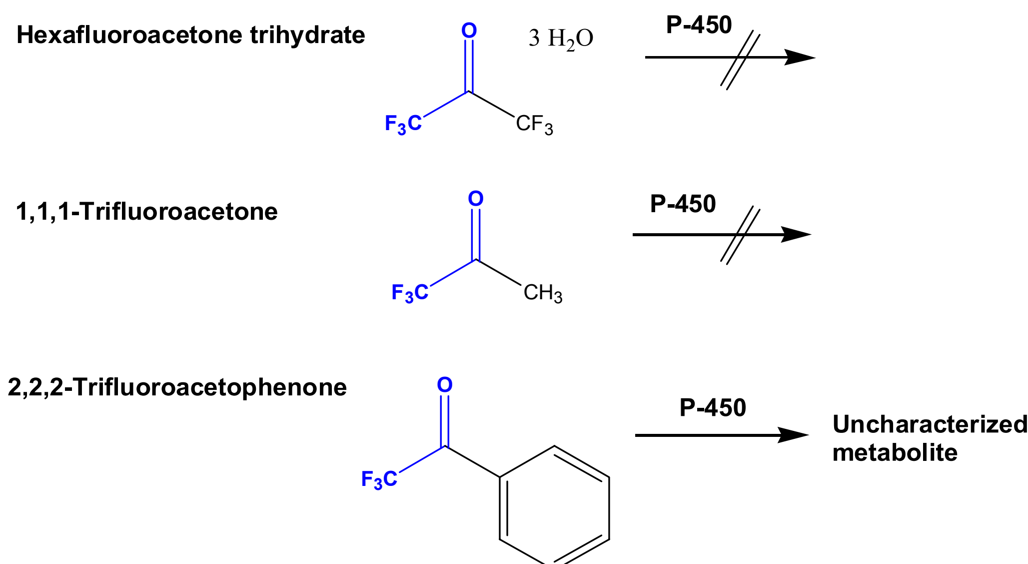


Figure 6: ^{19}F-NMR spectrum (proton coupled) of the incubation medium after incubation of liver microsomes with 2,2,2-trifluoroacetophenone. One metabolite was formed at a resonance of δ = -78.35 (d). The resonance at δ = -84.59 (s) represents the parent compound 2,2,2-trifluoroacetophenone.

The resulting peaks were not identified, but based on the chemical shifts, no formation of trifluoroacetic acid (c.f. Figure 6) was observed. Further on, trifluoroacetic acid was not present in concentrations above the backround level, after analyzing the samples by GC/MS.

Biotransformation of the substrate 3,3,3-trifluoropropanoic acid to possible 2-hydroxy-3,3,3-trifluoropropanoic acid or other fluorine-containing metabolites, was not observed by ^{19}F-NMR spectroscopy in rat liver microsomes, incubated in the presence of the NADPH-generating system. This indicated that 3,3,3-trifluoro-propanoic acid is not a substrate for cytochrome P-450.



Scheme 8: Cytochrome P-450 metabolism of hexafluoroaceton trihydrate, 1,1,1-trifluoro-acetone and 2,2,2-trifluoroacetophenone in rat liver microsomes.

5.1.2 Biotransformation of Hexafluoroacetone Trihydrate, Sodium Trifluoropyruvate and Methyl Trifluoropyruvate *In Vivo*

An alternative mechanism to explain the formation of trifluoroacetic acid from 3,3,3-trifluoropropionic acid may be the degradation of a stable metabolite by pathways other than cytochrome P-450. The hypothesis was investigated with the administration of possible intermediates of 1,1,1,3,3-pentafluoropropane biotrans-formation to rats. Single doses of hexafluoroacetone trihydrate, sodium trifluoro-pyruvate and methyl trifluoropyruvate were administered to rats (n=2) by gavage in a phosphate buffered solution at pH 7.4. The animals were kept in metabolic cages and urine was collected for 24 hours. The urine samples were analyzed for metabolites by ^{19}F-NMR. When comparing the spectra of the urine samples collected, with spectra of reference compounds, formation of metabolites was observed after dosing the substrates sodium trifluoropyruvate and methyl trifluoropyruvate. Both compounds were transformed to inorganic fluoride and trifluorolactate (c.f. Figure 7 and Scheme 9).

Hexafluoroaceton trihydrate was recovered as not metabolized in the urine, and no indication for formation of trifluoroacetic acid was obtained by ^{19}F-NMR.

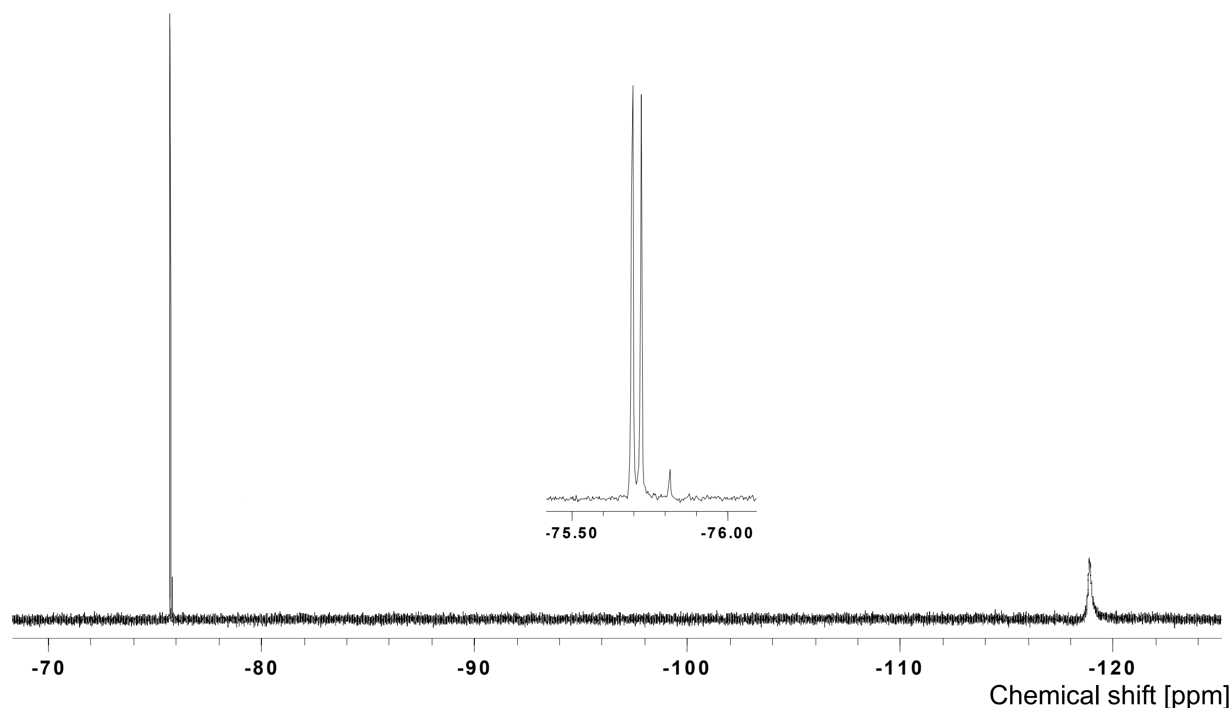
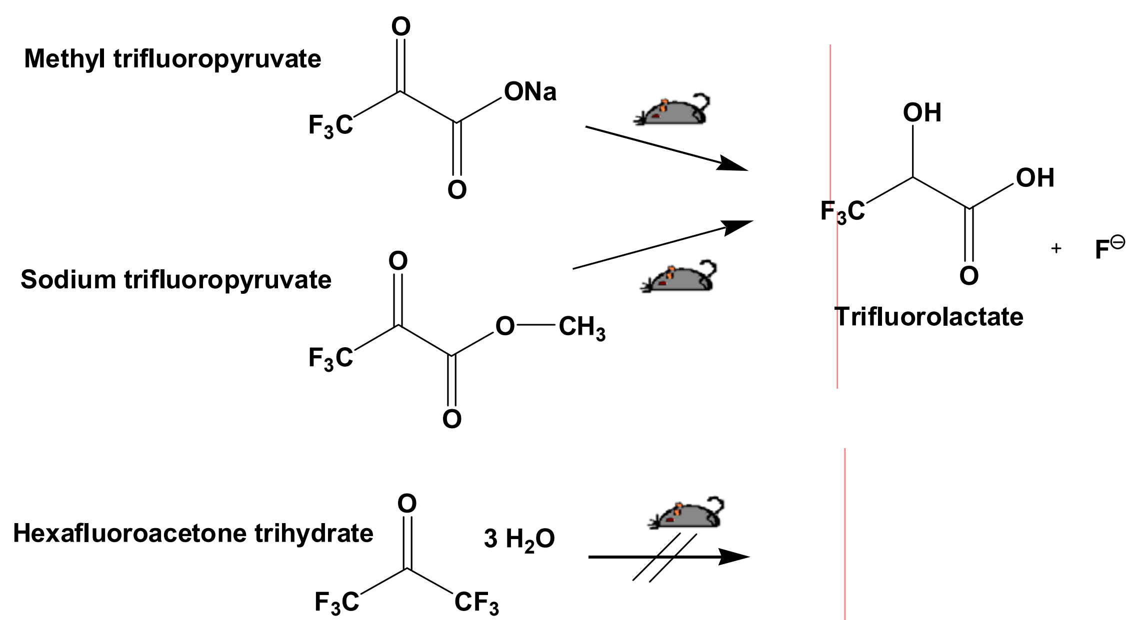


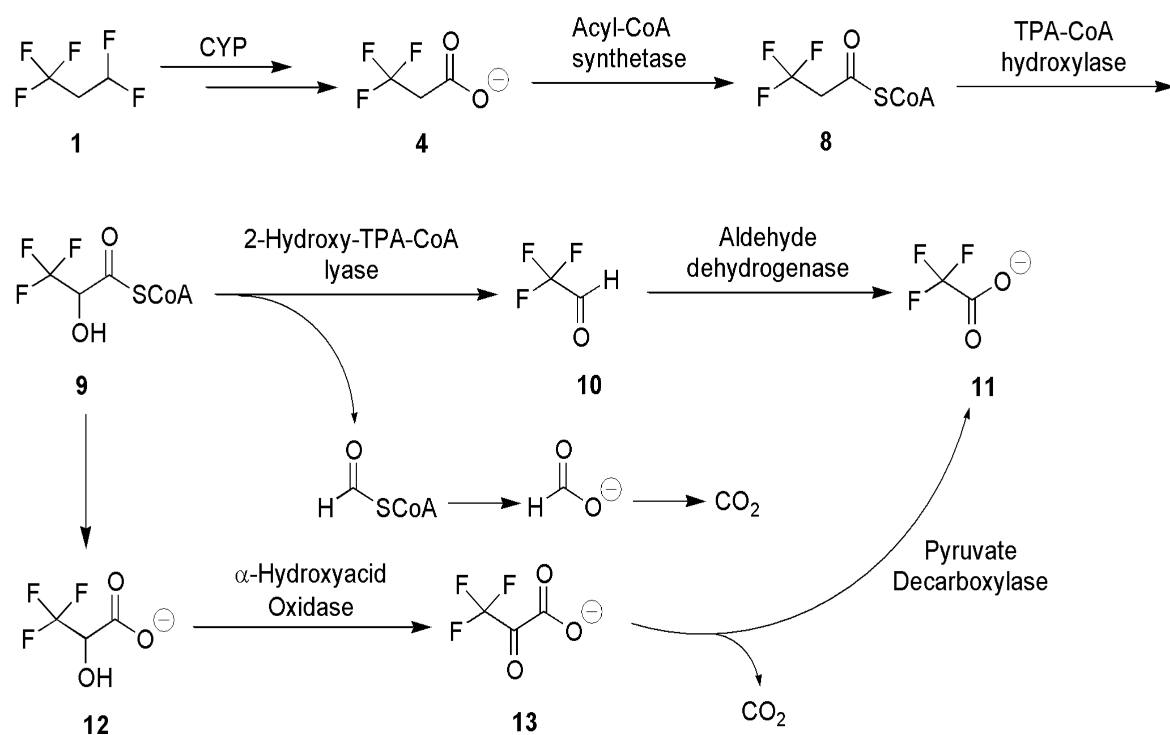
Figure 7: ^{19}F-NMR spectrum (proton coupled) of a urine sample (collected for 24 hours) from a male rat after oral administration of 10 mg/kg sodium 3,3,3-trifluoropyruvate. Trifluoro-lactate was formed and showed a resonance at δ = -75.55 (d) and in addition inorganic fluoride, as the degradation product with the resonance shift at δ = -119.52 (s).



Scheme 9: Metabolites of the substrates sodium trifluoropyruvate, methyl trifluoropyruvate and hexafluoroacetone trihydrate in rats, detected using ^{19}F-NMR.

5.1.3 α-Oxidation of 1,1,1,3,3-Pentafluoropropane, 3,3,3-Trifluoropropionic Acid and 3,3,3-Trifluoropyruvate in Liver Homogenate

The α-oxidation of 1,1,1,3,3-pentafluoropropane and 3,3,3-trifluoropropionic acid, followed by decarboxylation, was considered to be a possible mechanism other than the involvement of cytochrome P-450, to explain the formation of trifluoroacetic acid from 1,1,1,3,3-pentafluoropropane (c.f. Scheme 10). Reactions by α-oxidation have been described for a number of fatty acids. The α-oxidation takes place in mitochondria, microsomes and peroxisomes and describes a sequence of reactions particularly for 3-methyl-branched fatty acids. These are inhibited in a stereochemical process for undergoing ß-oxidation. The 3-methyl group blocks the dehydrogenation of the hydroxyl group by the hydroxyacyl-CoA dehydrogenase.



Scheme 10: Potential pathway for the biotransformation of 1,1,1,3,3-pentafluoropropane and 3,3,3-trifluoropropionic acid to trifluoroacetic acid. [1] 1,1,1,3,3-pentafluoropropane, [4] 3,3,3-trifluoropropionic acid, [8] 3,3,3-trifluoropropanoyl-CoA, [9] α-hydroxy-3,3,3-trifluoropropanoyl-CoA, [10] trifluoroacetaldehyde, [11] trifluoroacetic acid, [12] α-hydroxy-3,3,3-trifluoropropanoic acid, [13] 3,3,3-trifluoropyruvic acid.

The first steps would involve the ligation of CoA and the fatty acid [8], for activation, and the hydroxylation. The α-hydroxl-3,3,3,-trifluoropropionyl-CoA [9] would be converted by a thioesterase to the α-hydroxyacid [12], followed by oxidation to the α-ketoacid [13]. This might be a substrate for the oxalyl-CoA decarboxylase, which is TPP dependent, and the resulting fatty acid [11], shortened by one C-atom, would be formed after cleavage of carbon dioxide. Activated α-hydroxl-3,3,3-trifluoropropionyl-CoA may represent also a substrate for the 2-hydroxy-TPA-CoA lyase. Cleavage of acetyl-CoA results in trifluoroacetaldehyde [10], which might be transformed to trifluoroacetic acid by the enzyme aldehyde dehydrogenase.

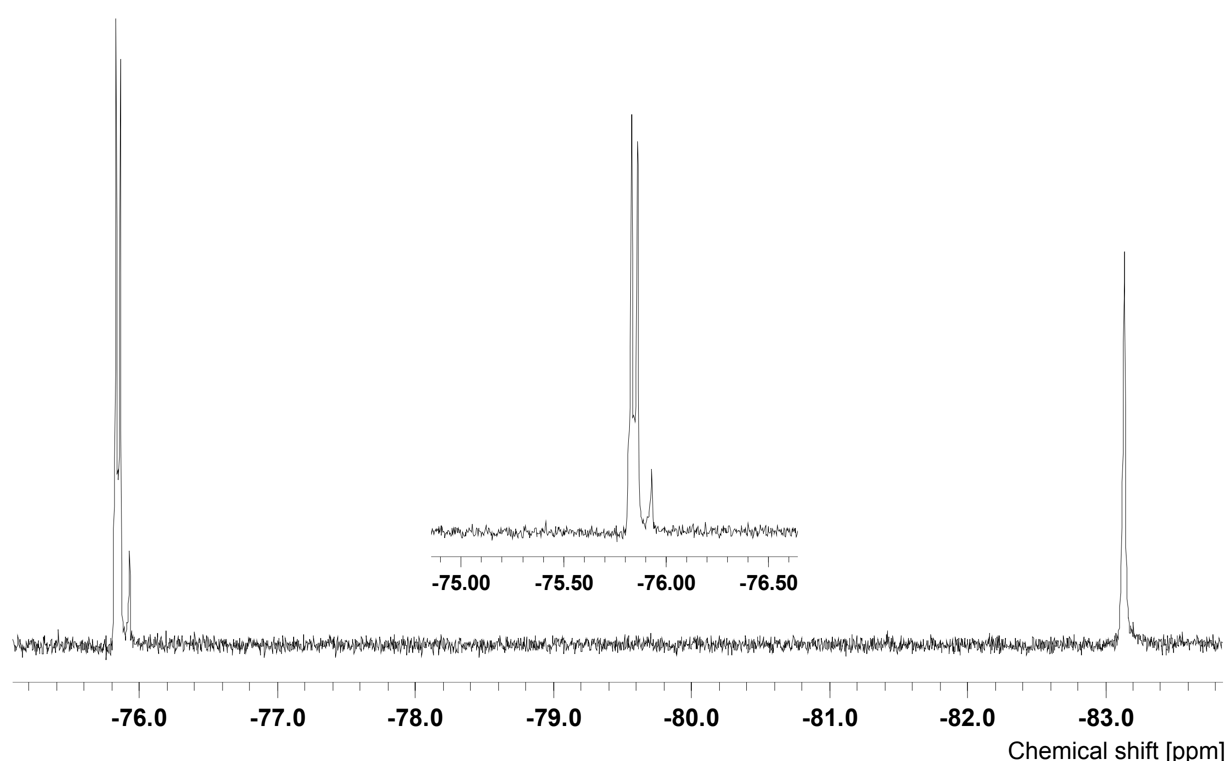


Figure 8: ^{19}F-NMR spectrum (proton coupled) after α-oxidation of sodium 3,3,3-trifluoro-pyruvate at δ = -83.15 ppm (s) in liver homogenate. The metabolite trifluorolactate showed a resonance shift at δ = -75.83 ppm (d).

No signals for metabolites were detected by ^{19}F-NMR after incubations under conditions to support α-oxidation of 1,1,1,3,3-pentafluoropropane and 3,3,3-trifluoro-propionic acid in rat liver homogenates. Signals with very small intensities of trifluoroacetic acid and inorganic fluoride were present in all samples, but no signals for fluorine-containing products were formed, as indicated by ^{19}F-NMR spectroscopy. Moreover, the concentrations of trifluoroacetic acid, as determined by GC/MS analysis, were identical in all samples irrespective of cofactors present, indicating

that 3,3,3-trifluoropropanoic acid was not biotransformed to trifluoroacetic acid by the α-hydroxylation pathways *in vitro.*

In comparison, sodium trifluoropyruvate, added as substrate for α-oxidation to liver homogenate, resulted in a signal for the metabolite trifluorolactate, detected by ^{19}F-NMR (c.f. Figure 8). This result was in consent with the experiments *in vivo* (c.f. Scheme 9).

5.2 CHARACTERIZATION OF THE TOXICITY OF 3,3,3-TRI-FLUOROPROPIONIC ACID IN RATS

Preliminary work resulted in high lethality after single administration of 3,3,3-trifluoro-propionic acid at doses of 80, 50, 25 and 5 mg/kg b.w.. The high potency of 3,3,3-tri-fluoropropionic acid prompted a number of studies to characterize the toxic effect and to delineate possible mechanisms of action, explaining the high potency of this compound. In a first series the acute lethality of 3,3,3-trifluoropropionic acid was assessed. As *in vivo* model the outbred rat strain Sprague Dawley was used, corresponding to the previous *in vivo* studies with 1,1,1,3,3-pentafluoropropane.

5.2.1 Toxicity after Single Dose Exposure

3,3,3-Trifluoropropionic acid was shown to be highly toxic in young male rats, with eight weeks of age and a body weight of approximately 220 g, resulting in death within twelve hours in most animals. Based on the results of various experiments the no observed adverse effect level (NOAEL) was 2.5 mg/kg b.w. of 3,3,3-trifluoro-propionic acid. The lowest dose, lethality was observed for (LOAEL), was 5 mg/kg b.w of 3,3,3-trifluoropropionic acid. The lethal dose, quoted as LD_{50}, was estimated to 10 mg/kg b.w. for rats of the age of eight weeks, considered as young animals. Signs of acute oral toxicity observed were ataxia, coma, cramps, recumbency, convulsions and stupor.

In contrast to young rats, adult rats with 12 to 16 weeks of age were more resistant to the lethal effect of 3,3,3-trifluoropropionic acid. Effects such as clinical signs and lethality occurred after oral doses greater than 25 mg/kg b.w. 3,3,3-trifluoropropionic acid. Signs of toxicity observed, were similar to those seen in young rats. The course for appearance of observed toxicological signs was very similar for all treated rats.

Clinical signs were recorded about two to three hours after administration. In the stage of extremis, samples of biofluids were collected and moribund animals were sacrificed. For histopathological and immunohistochemical investigations the main organs were collected and stored. Urine and serum samples were analyzed by ^{19}F-NMR, ^{1}H-NMR and for clinical chemistry parameters.

5.2.1.1 ^{19}F-NMR and ^{1}H-NMR Analysis

^{19}F-NMR-analysis (proton coupled) of urine samples from rats treated with 3,3,3-trifluoropropionic acid showed increased intensities for signals of inorganic fluoride at a resonance of δ = -119.62 ppm (s) and a resonance attributed to 3,3,3-trifluoropropionic acid at -63.9 ppm (t). The smaller signal at -63.4 ppm (t) likely represented a glycine-conjugate of 3,3,3-trifluoropropionic acid (c.f.
Figure 9).

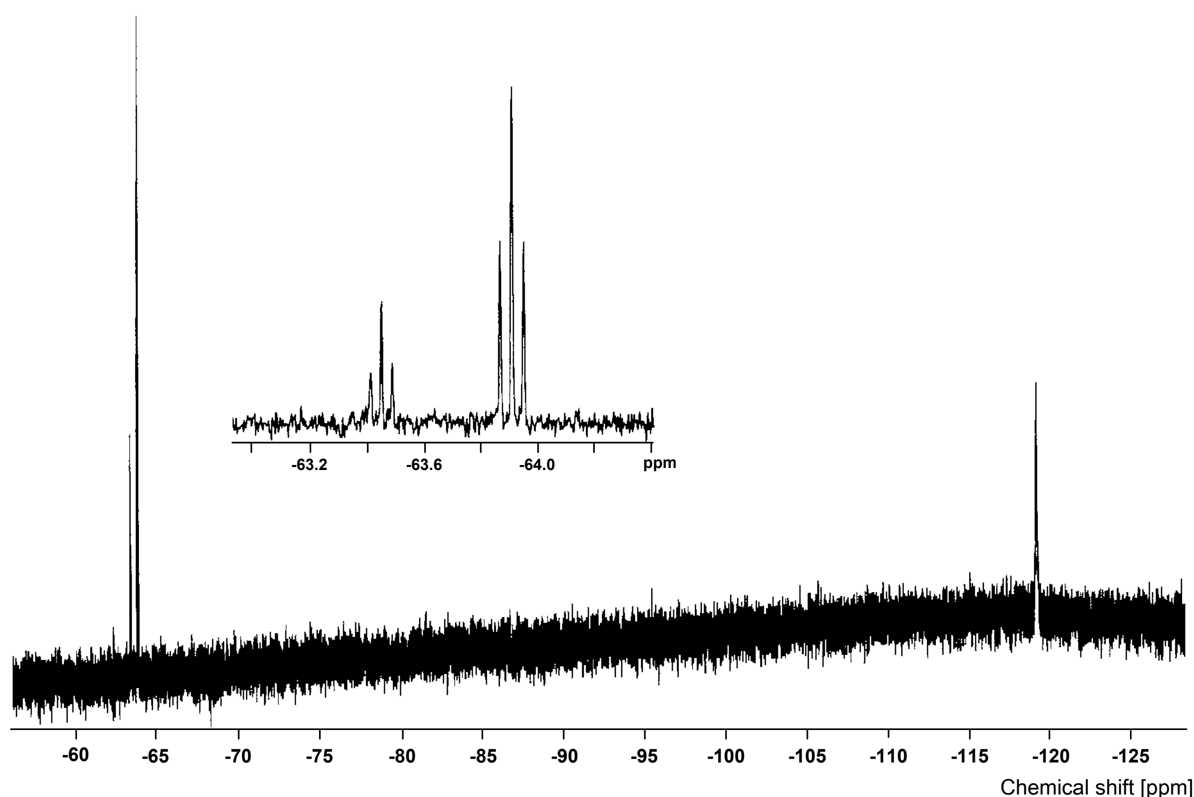


Figure 9: ^{19}F-NMR spectrum (proton coupled) of a urine sample (collected for 24 hours) from one male rat after oral administration of 5 mg/kg b.w. 3,3,3-trifluoropropanoic acid. The resonance at δ = -63.90 ppm (t) was identical in chemical shift and H-F coupling ($1J_{H\text{-}F}$ = 11.4 Hz) with the resonance, obtained with authentic 3,3,3-trifluoropropanoic acid. The smaller resonance, also a triplet with a H-F coupling constant of 11.4 Hz was not identified (metabolite A). The chemical shift at δ = -119.61 ppm (s) showed inorganic fluoride.

In ^{19}F-NMR spectra of serum samples from animals treated with 3,3,3-trifluoro-propionic acid, the same signals were detected: parent compound at a resonance of δ = -63.90 ppm (t) and inorganic fluoride at δ = -119.61 ppm (s). Inorganic fluoride was also detected in serum samples from control animals.

The ^{1}H-NMR spectra of urine from rats, treated with a high dose of 3,3,3-trifluoro-propionic acid showed significant differences to the spectra of control animals (c.f. Figure 10 and Figure 11). ^{1}H-NMR spectroscopy of urine is a non-invasive method which investigates time-related biochemical changes to create a metabolic pattern. The principle of metabonomics enables the simultaneous measurement of changes in the levels of a wide range of endogenous metabolites in biofluids (Beckwith-Hall et al., 1998). In the past years, it had been possible to assign signals as biomarkers of organ-specific toxicity. The urine composition is very variable, due to individual and diurnal variations, and the spectra interpretation has to be considered carefully. For reconstruction of the metabolic action, following a toxic insult, the signals of the NMR spectra need to be transferred by statistical methods. Data reduction and multivariate analysis as the principal component analysis (PCA) are used to visualize metabolic trajectories. In this thesis, statistical analysis was performed for the multidosing experiment and the data are shown in PCA plots (c.f. Figure 12 and Figure 13). For data evaluation, spectra of control and treated fluids were compared, and peaks were identified based on chemical shifts listed (Lindon, 1999). The spectra were referenced to the chemical shift and peak intensity of creatine at δ = 4.06 ppm. For the metabolic profile of samples, it is noteworthy to consider the fact that animals were fasted overnight. This led to an increase in the toxic response observed in changes in the ^{1}H-NMR, as well as in clinical signs.

In urine samples, major changes were observed in the range of δ = 0.75 to 1.75 ppm (c.f. Figure 11). In urine samples from rats treated with 3,3,3-trifluoropropionic acid, signal intensity increased for amino acids and their derivates: valine, *iso*-leucine, hydroxyvalerate and *iso*-butyrate; increased for the intermediates of glycolysis: lactate, *trans*-aconitate - and for adipate, as a metabolite of fatty oxidation. Additional signals appeared for ketone bodies or were increased in their intensity i.e. α-hydroxy-butyrate, ß-hydroxy-butyrate, acetone and acetoacetate. Further on, increased signal intensity was observed for proline, creatine, creatinine, arginine and/or taurine, malate and/or methionine. Decreased signal intensity was observed for

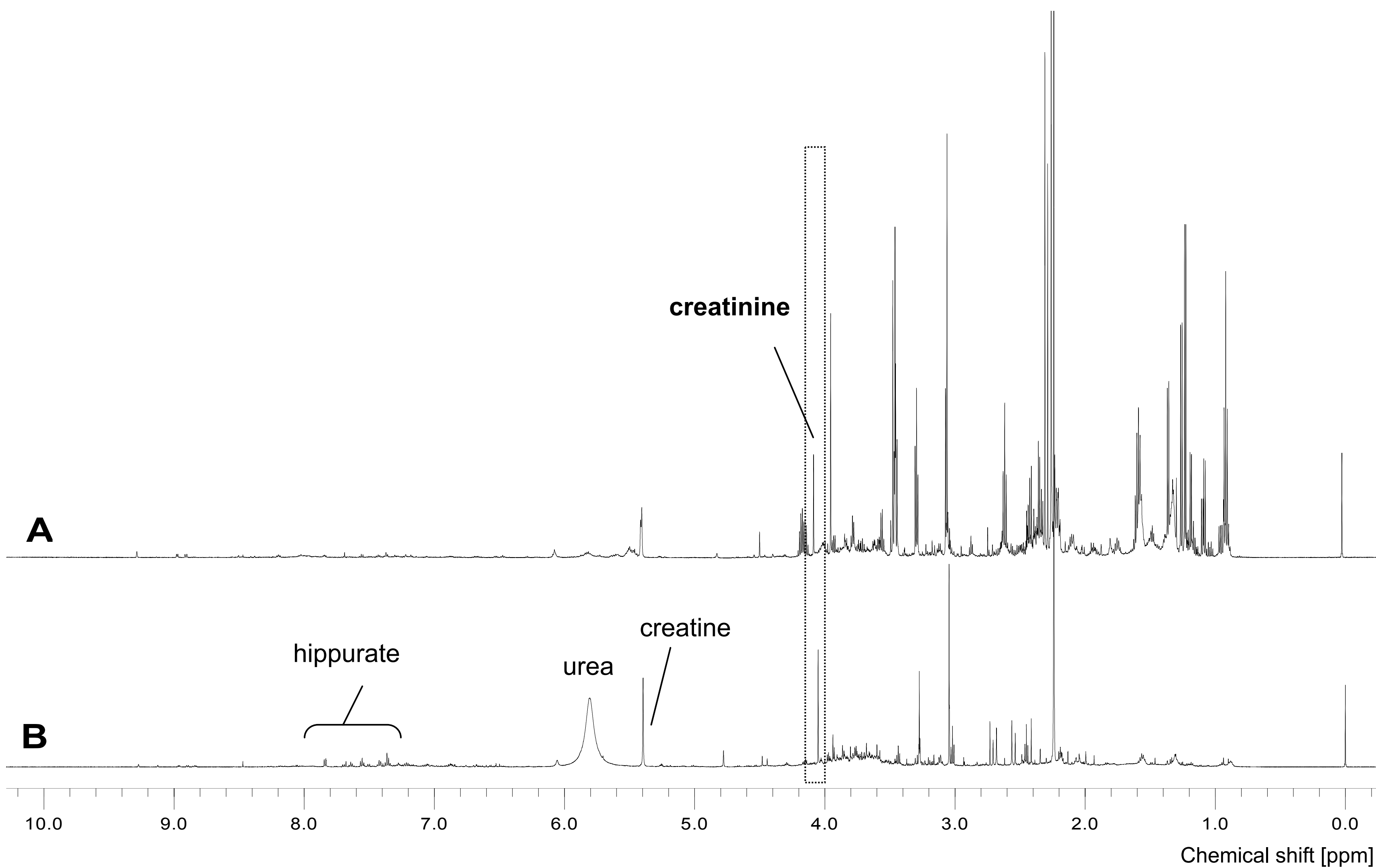


Figure 10: Representative ^{1}H-NMR spectra from urine samples of animals, fasted over night. The traces are: (A) after a single oral administration of 50 mg/kg b.w. of 3,3,3-trifluoropropionic acid. The urine samples were collected for 6 hours after administration. (B) of non treated animals.

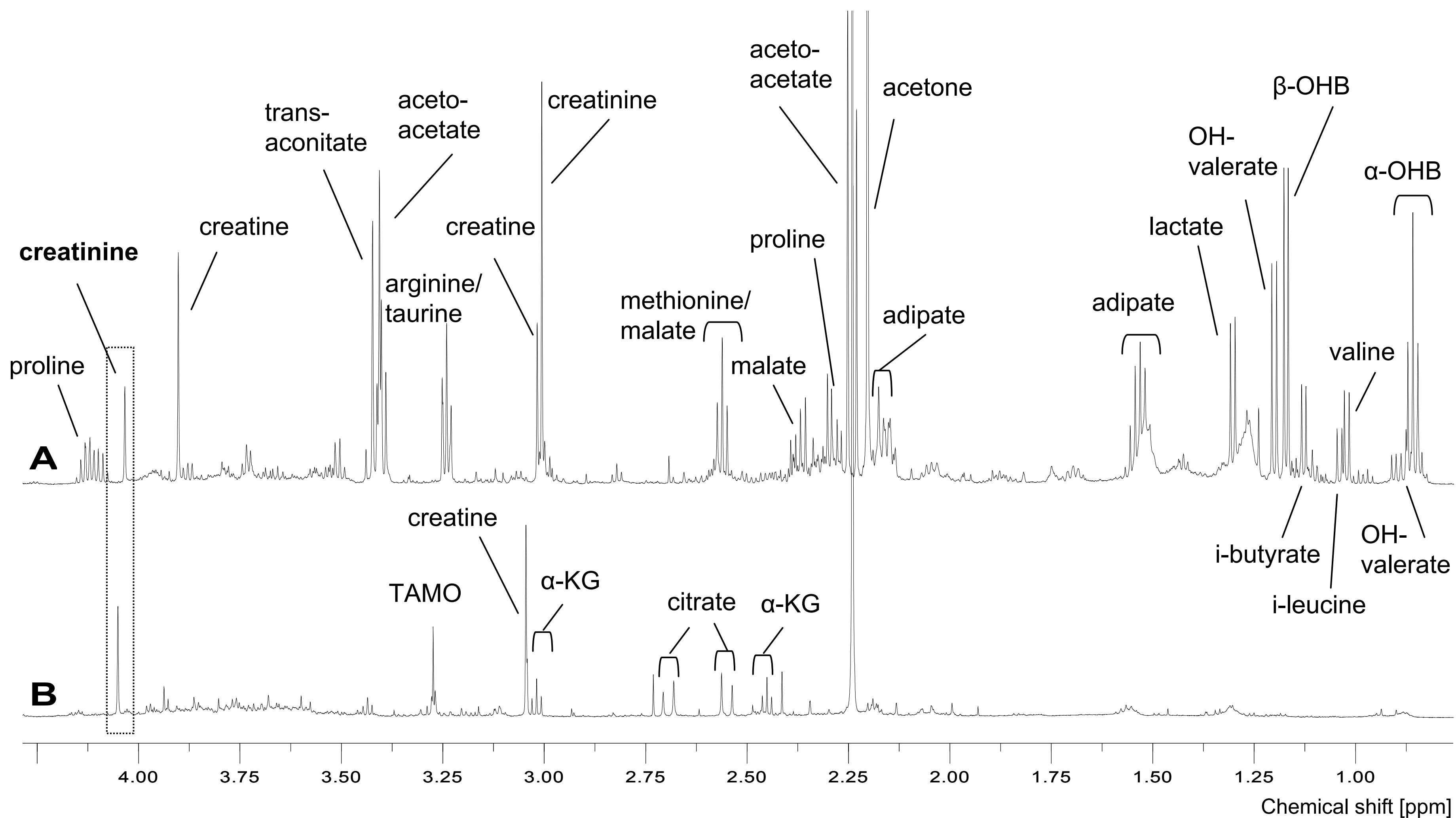


Figure 11: Representative ^{1}H-NMR spectra from urine samples of animals, fasted over night, plotted with high resolution (chemical shift from δ = 0.75 to 4.25 ppm) The traces are: (A) after a single oral administration of 50 mg/kg b.w. of 3,3,3-trifluoropropionic acid. The urine samples were collected 6 hours after drug administration. (B) of non treated animals (TAMO: trimethylamine-N-oxide, α-KG: alpha-ketoglutarate).

α-ketoglutarate, citrate and trimetylamine-N-oxide (TMAO). In the range of δ = 5.5 to 10.0 ppm of ^{1}H-NMR spectra of rats, treated with 3,3,3-trifluorpropionic acid, a signal was no longer detected for urea, and the signal intensity for creatine, hippurates and aminohippurate was decreased.

In ^{1}H-NMR spectra of serum samples, the vertical scale and chemical shift was referenced to the lactate doublet at δ = 1.33 ppm. Spectra of serum samples revealed a similar picture (c.f. Figure 23). In serum samples from rats treated with 3,3,3-trifluoropropionic acid, the intensity of peaks for acetone, acetoacetate and n-butyrate were increased. Additionally signals were seen for methylamine, dimethylamine (DMA), myo-inositol and glycerol. Peaks, observed in serum samples of control animals for α-glucose, ß-glucose and hippurates, showed a decreased intensity after a single high dose of 3,3,3-trifluoropropionic acid.

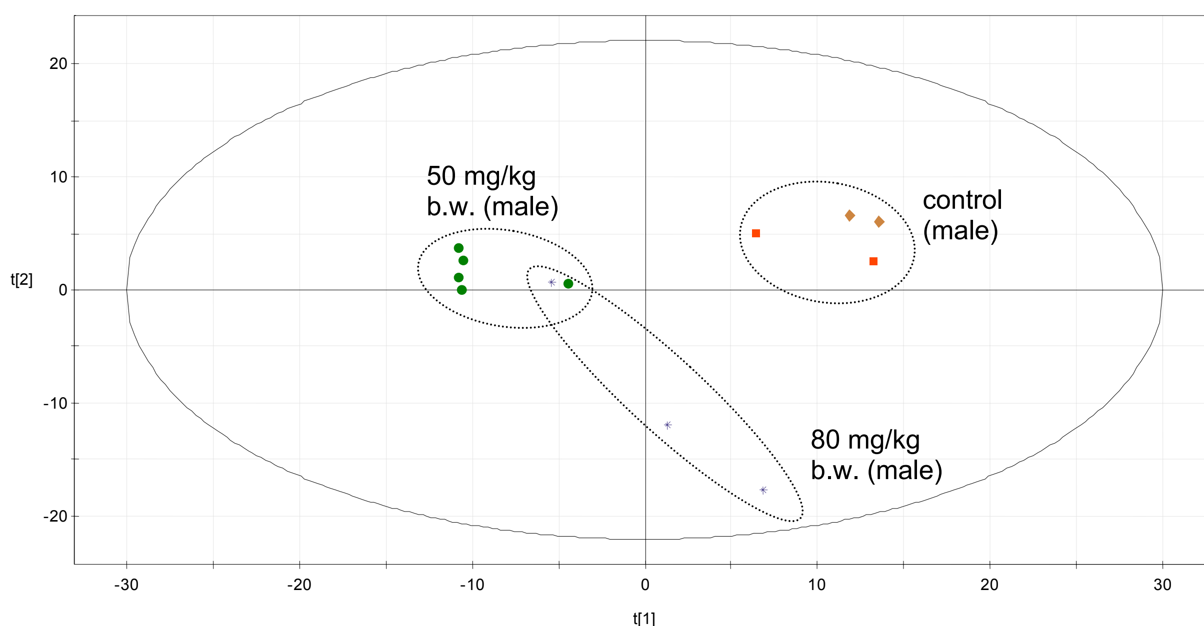


Figure 12: PCA plot of urine samples from non-treated rats and rats, treated with a single dose of 50 and 80 mg/kg b.w. 3,3,3-trifluoropropionic acid.

The statistical evaluation of ^{1}H-NMR spectra by PCA was performed on spectra of urine samples from male control rats and rats treated with a single dose of 50 and 80 mg/kg b.w. 3,3,3-trifluoropropionic acid. Samples from treated animals were found to be grouped together and could be differentiated from the non-treated male animals

(c.f. Figure 12). A similar plot was observed for serum samples, besides one outlier (c.f. Figure 13).

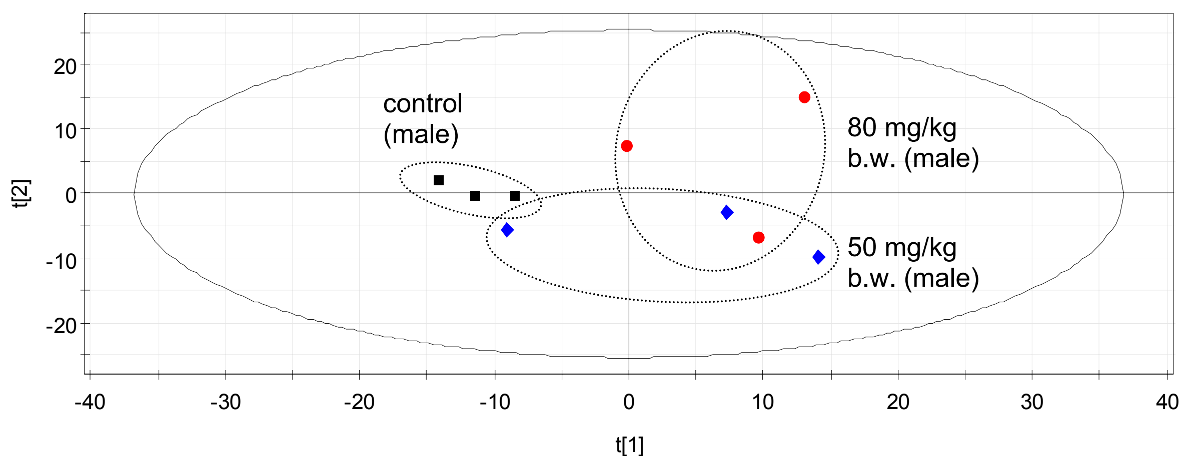


Figure 13: PCA plot of serum samples from non-treated rats and rats, treated with a single dose of 50 and 80 mg/kg b.w. 3,3,3-trifluoropropionic acid.

5.2.1.2 Clinical Chemistry

Major effects were observed for various clinical chemistry parameters in urine and serum samples. The sampling time point after administration of the compound was based on moribund signs of the individual animal. The major finding in urine samples was a decrease in glucose and the evidence of formation of ketone bodies. Decreased levels were measured for creatinine, protein, GGT and urea (c.f. Table 7). Similar findings were also seen in serum samples with decreased levels of blood glucose and increased levels of ketone bodies. The levels of GGT were decreased and the levels of triglycerides were reduced to below the limit of detection in serum samples after treatment. Moreover, the key enzymes GOT and GPT were significantly increased. The parameters creatine kinase and the isoenzyme CKMB, mainly present in the heart muscle, were also increased in serum samples from animals treated with 3,3,3-trifluoropropionic acid (c.f. Table 8).

Table 7: Clinical chemistry parameters in urine after oral (gavage) administration of 50 and 80 mg/kg b.w. of 3,3,3-trifluoropropanoic acid to male rats. Urine samples were taken 3 hours after administration.

	Control	**3,3,3-Trifluoropropionic acid mg/kg b.w.**
	n = 3	n = 9
Creatinine [mg/dL]	**144** ± 60	**48** ± 38
Protein [mg/L]	**159** ± 61	**122** ± 64
Glucose [mg/dL]	**89** ± 37	**33** ± 41
GGT [U/L]	**1417** ± 1079	**198** ± 220
Urea [mg/dL]	**4223** ± 631	**1491** ± 851 **
Ketone bodies [mg/ dL]	**< LOD**	**> 160 * °**

Values are mean ± standard deviation. LOD: limit of detection. ° Analyzed by test stick. *Significantly different from control values (p<0.05).

Table 8: Clinical chemistry parameters in serum after single oral (gavage) administration of 50 and 80 mg/kg b.w. 3,3,3-trifluoropropanoic acid to male rats. Serum samples were taken 3 hours after administration.

	Control	**3,3,3-Trifluoropropionic acid mg/kg b.w.**
	n = 23	**n = 7**
GOT [U/L]	**60** ± 25	**152** ± 39 *
GPT [U/L]	**49** ± 16	**94** ± 23 *
GOT / GPT	**1,2** ± 0,4	**1,7** ± 0,4 *
ALP [U/L]	**211** ± 55	**167** ± 25 *
CK [U/L]	**213** ± 142	**928** ± 478*
CKMB [U/L]	**323** ± 237	**1037** ± 571 *
Glucose [mg/dL]	**370** ± 109	**52** ± 20
GGT [U/L]	**1,8** ± 2,2	**0,6** ± 0,4 *
Triglycerides [mg/dL]	**127** ± 109	< LOD
Ketone bodies [mg/dL]	< LOD	**> 160** * °

Values are mean ± standard deviation. LOD: limit of detection. °Analyzed by test stick. *Significantly different from control values (p<0.05). The parameter lactate dehydrogenase was measured in single occasions and showed an increase after treatment with 3,3,3-trifluoropropionic acid.

5.2.1.3 Histopathology

The histopathological evaluation of heart, liver and kidney slices did not reveal changes, considered to be related to treatment with 3,3,3-trifluoropropionic acid.

5.2.2 Toxicokinetics of 3,3,3-Trifluoropropionic Acid after Single Administration

For further characterization of the toxicity of 3,3,3-trifluoropropionic acid, biotransformation and the kinetic profile of urine excretion were studied.
The excretion of 3,3,3-trifluoropropionic acid in urine was quantified after administration of a single dose at 5 mg/kg b.w. 3,3,3-trifluoropropionic acid to male rats (n=6), eight weeks of age. Urine samples were analyzed for the parent compound by GC/MS. The concentration of 3,3,3-trifluoropropionic acid reached the limit of detection at 48 hours after treatment. The mean value for the total excretion of 3,3,3-trifluoro-propionic acid within 60 hours was 584 ± 173 nmol. Based on the mean body weight of 220 g, the administered dose of 3,3,3-trifluoropropionic acid was calculated to be 8.67 µmol. Therefore, the recovery of unmetabolized and not catabolized 3,3,3-trifluoropropionic acid was calculated to be 6.7% of total 3,3,3-trifluoropropionic acid in urine. The half-life was calculated to be 4.5 hours (c.f. Figure 14).
In serum, six hours after administration of 3,3,3-trifluoropropionic acid, mean concentrations were 13.34 ± 3.67 nmol/mL. The limit of detection of 3,3,3-trifluoro-propionic acid in serum was reached 24 hours after administration (c.f. Figure 15).

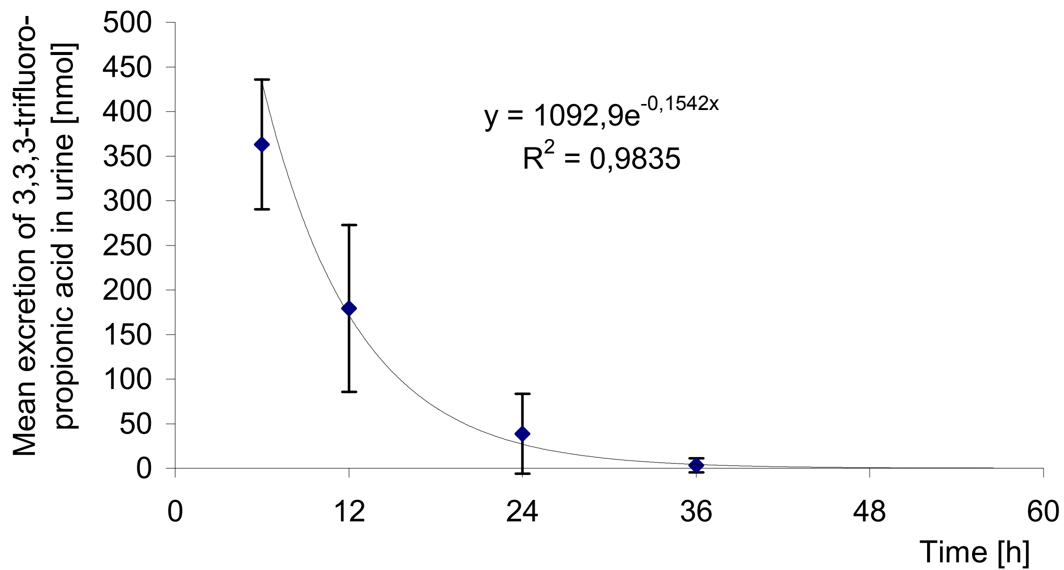


Figure 14: Excretion of 3,3,3-trifluoropropionic acid in urine after a single dose of 5 mg/kg b.w. 3,3,3-trifluoropropionic acid (n=6). The half-life was calculated to be 4.5 hours.

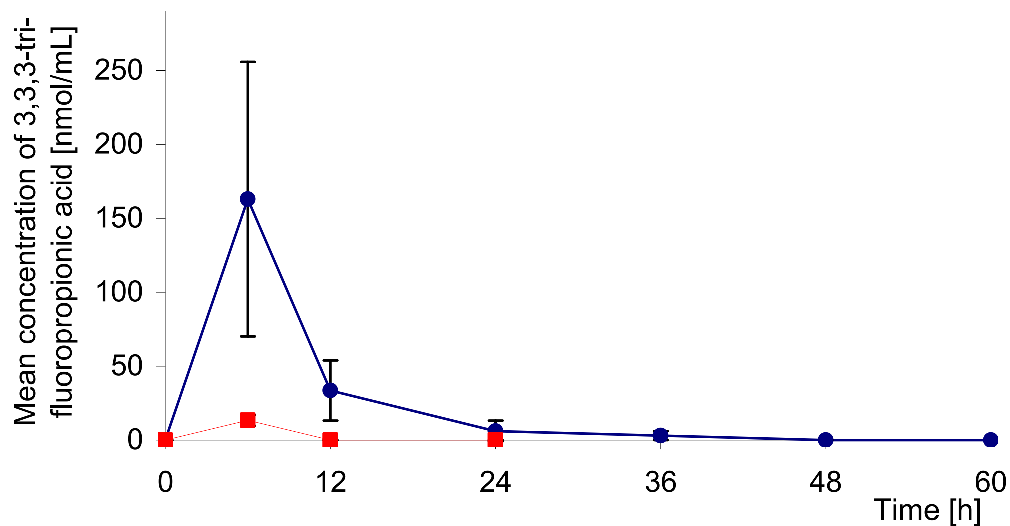


Figure 15: Comparison of the mean concentration of 3,3,3-trifluoropropionic acid, excreted in urine (--•--) and serum (--■--) after exposure to a single dose of 5 mg/kg b.w. 3,3,3-tri-fluoropropionic acid (n=6).

Serum levels were also determined after administration of a high dose level of 80 mg/kg b.w. 3,3,3-trifluoropropionic acid. The data was not evaluated as separate kinetic curve but it showed, that the concentration in the serum increased for high dose levels in a time span of three to nine hours after administration.

5.2.3 Effects on Body Weight, Food Intake and Water Consumption after Single Exposure of 3,3,3-Trifluoropropionic Acid

Body weight, food intake and water consumption were monitored following a single exposure at 5 mg/kg b.w. 3,3,3-trifluoropropionic acid. Twelve hours after treatment, the body weight was reduced by 7.7% and a gain in body weight was observed there onwards. Compared to before treatment, the food intake was decreased by 55% and the water consumption was decreased by 11% on the day of administration. Thereafter both parameters recovered. Polyuria was observed during the first 24 hours after administration. The urine volume was increased by 100% and recovered on the following day.

5.3 REPEATED DOSE TOXICITY OF 3,3,3-TRIFLUORO-PROPIONIC ACID

5.3.1 Toxicity after 14 Days of Repeated Dosing

A subchronic toxicity study was performed by administering 3,3,3-trifluoropropionic acid daily via drinking water for the time period of 14 days to rats. This route of administration was chosen to simplify the experiment with a view to longer term studies. Dose levels were estimated taking into account the results of acute toxicity. In preliminary multidosing experiments via drinking water, dose range findings were started at 8 mg/kg b.w. 3,3,3-trifluoropropionic acid. Because of the problem that animals drank very little due to poor palatability of the water solutions, the concentration was decreased. In the final study protocol, with the calculated dose levels of 1 mg/kg b.w. and 2 mg/kg b.w. daily via drinking water, no restriction in water consumption was seen. Rats (n=12) were kept in metabolic cages during the conduction of the study and urine samples were collected in 24-hour intervals for investigations. The urine was analyzed by ^{19}F-NMR and ^{1}H-NMR for the identification of the parent compound and in particular of additional fluorine containing metabolites.

The compounds 3,3,3-trifluoropropionic acid, ketones and inorganic fluoride were quantified by GC/MS in urine.

In the ^{19}F-NMR urine spectra of treated animals an increase in the intensity of inorganic fluoride was seen with a chemical shift at δ = -119.63 ppm. Two triplet signals (proton coupled) were observed. The signal at a resonance of δ = -63.92 ppm corresponded to the parent compound 3,3,3-trifluoropropionic acid. The signal with a chemical shift at δ = -63.46 ppm was considered to be a metabolite (metabolite A). The spectra corresponded to the ^{19}F-NMR of urine after a single dose administration (c.f. Figure 9).

The excretion of non metabolized 3,3,3-trifluoropropionic acid in urine is monitored by GC/MS and is shown in Figure 16. The concentration of the parent compound remained elevated over the 14-day period of treatment, but there was no evidence of accumulation.

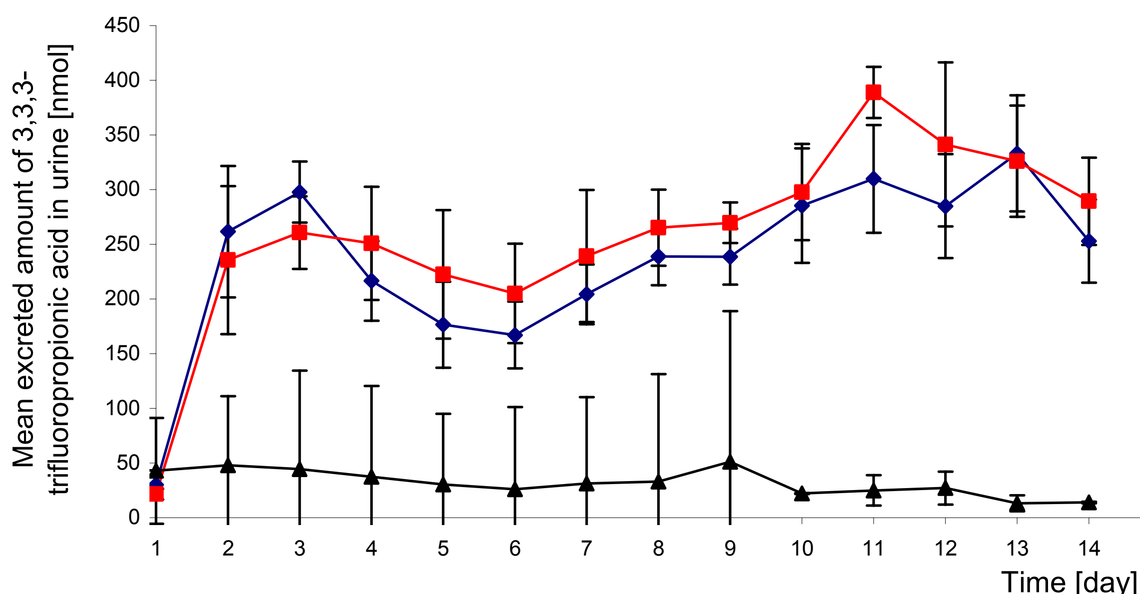


Figure 16: Excretion of 3,3,3-trifluoropropionic acid in urine over 14 days: (▲) control group (n=4) (◆), treated group with 1 mg/kg b.w. (n=4) and (■) treated group with 2 mg/kg b.w. (n=4) of 3,3,3-trifluoropropionic acid.

The concentration of the dosing solution was calculated based on the assumption of a daily water consumption of 30 mL and on the mean body weight of 240 g for all dose levels. For the target dose of 1 mg/kg b.w. daily, the concentration of 3,3,3-trifluoropropionic acid in drinking water was calculated to 62.3 nmol/mL and 124.9 nmol/mL for the target dose of 2 mg/kg b.w.. The mean water consumption

over the 14-day period was measured to be 295 mL for animals dosed with 1 mg/kg b.w.. Therefore a total amount of administered 3,3,3-trifluoropropionic acid was calculated to be 18.35 µmol. For all animals, dosed with 2 mg/kg b.w. daily, the mean of the total amount of administered 3,3,3-trifluoropropionic acid was calculated to be 36.09 µmol with the mean water consumption of 289 mL, measured over the 14-day period. The mean amount of 3,3,3-trifluoropropionic acid excreted in urine, was 3.51 µmol for animals dosed with 1 mg/kg b.w., respectively 3.94 µmol for animals dosed with 2 mg/kg b.w. over the 14-day period of treatment. Therefore, a dose dependency was not considered at these dose levels. The mean recovery of the parent compound in urine was calculated to 19 % for animals dosed with the target of 1 mg/kg b.w. and 11% for animals, dosed with the target of 2 mg/kg b.w. over the 14-day period of treatment (c.f. Table 9).

However, the excretion of inorganic fluoride in urine increased dose dependently after exposure to 3,3,3-trifluoropropionic acid. The level of inorganic fluoride in the urine was about 2.5- and 3.5-fold increased during the time course of treatment, respectively (c.f. Figure 17).

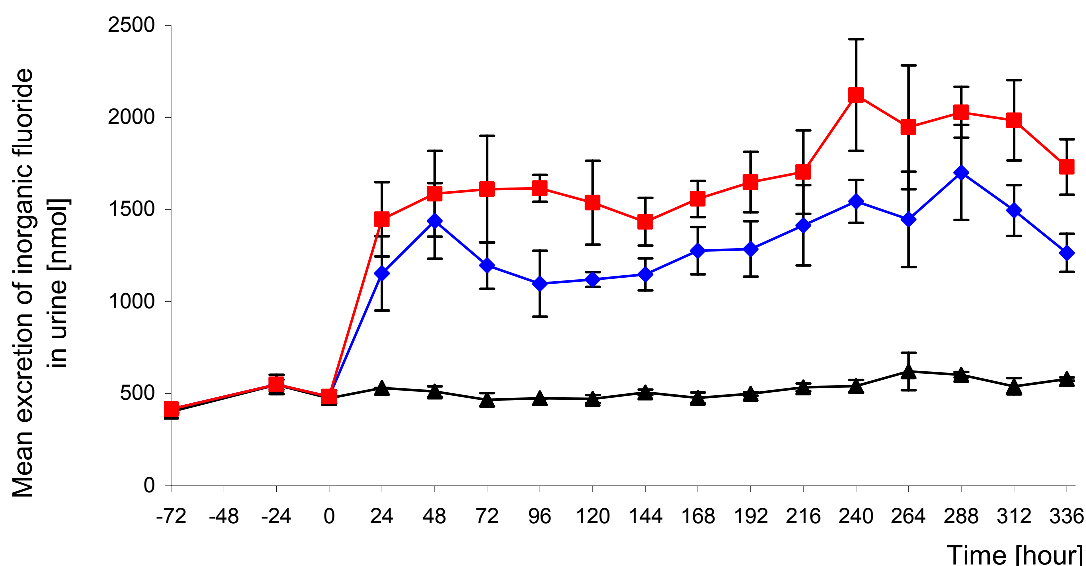


Figure 17: Mean excretion of inorganic fluoride in rat urine after administration of 3,3,3-trifluoropropionic acid via drinking water for 14 consecutive days; (▲) control group (n=4), (◆) dose group with 1 mg/kg b.w. (n=4) and (■) dose group with 2 mg/kg b.w. (n=4).

Table 9: Mean amount of 3,3,3-trifluoropropionic acid, administered and excreted in urine over a 14-day period. Administered amount of 3,3,3-trifluoropropionic acid was based on the overall water consumption over 14 days at each dose level, i.e. control group (n=4), treated group with the target dose of 1 mg/kg b.w. (n=4) daily and treated group with the target dose of 2 mg/kg b.w. (n=4) daily of 3,3,3-trifluoropropionic acid.

		Control	**Group 1**	**Group 2**
Target dose level, daily	[mg/kg b.w.]	-	1	2
Concentration of 3,3,3-trifluoropropionic acid in drinking water	[nmol/mL]	-	62.3	124.9
Mean amount of water consumption	[mL]	422	295	289
Mean administered dose level, daily	[mg/kg b.w.]	-	0.70	1.38
Administered total amount of 3,3,3-trifluoro-propionic acid	[µmol]	-	18.35	36.09
Mean of excreted amount of 3,3,3-trifluoro-propionic acid in urine	[µmol]	-	3.51	3.94
% of administered 3,3,3-trifluoropropionic acid, recovered in urine	[%]	-	19	11

After the exposure to 1 or 2 mg/kg b.w. of 3,3,3-trifluoropropionic acid over the 14-day period, ketone levels were increased in the first 24-hour urine and stayed elevated during the experiment (c.f. Figure 18 and Table 10).

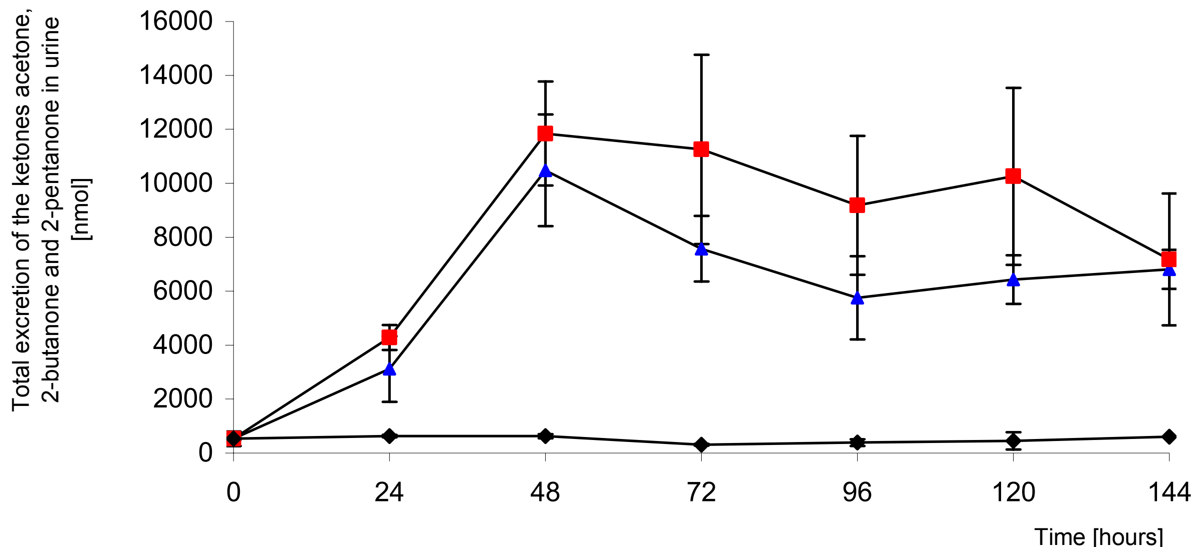


Figure 18: Excretion of ketones (sum of acetone, 2-butanone and 2-pentanone) after repeated dosing of 3,3,3-trifluoropropionic acid to rats: (◆) control group (n=4), (▲) dose group of 1 mg/kg b.w. (n=4) and (■) dose group of 2 mg/kg b.w. (n=4).

Ketone levels were quantified from days zero to six. As products of the ß-oxidation and intermediates in the energy metabolism, the ketone bodies ß-hydroxybutyrate, acetoacetate and acetone are important indicators of metabolic imbalance. Acetone was quantified in urine as a representative for the ketone bodies. The short chain ketones, 2-butanone and 2-pentanone, were quantified exemplary for possible intermediates after ß-oxidation of free fatty acids. The ketones were analyzed by GC/MS in rat urine also after exposure to 1,1,1,3,3-pentafluoropropane and 1,1,1,3-tetrachloropropane (c.f. Table 18).

Table 10: Quantification of the ketones, acetone, 2-butanone and 2-pentanone in rat urine after exposure to 3,3,3-trifluoropropionic acid.

Compound	**Hours**	**Mean amount in urine [nmol]**			**Mean [nmol]**	**St.Dev. [nmol]**
		Acetone	2-Butanone	2-Pentanone	Ketones, sum	
Mean of all	**0**	61	73	338	472	± 183
3,3,3-Trifluoropropionic acid						
Non treated	**0-144**	66	126	309	526	± 206
	0	121	171	239	531	± 264
1 mg/kg b.w. (14 days)	**24**	1014	1269	1109	3114	± 1218
	48	2582	3178	4719	10478	± 2073
	72	1424	2284	3862	7570	± 1214
	96	1032	1903	2814	5749	± 1540
	120	1027	1904	3499	6429	± 898
	144	1060	1929	3823	6811	± 725
2 mg/kg b.w. (14 days)	**24**	928	1461	1892	4280	± 465
	48	2281	2856	6705	11841	± 1929
	72	1592	2980	6681	11252	± 3509
	96	1295	2461	5423	9179	± 2573
	120	1443	2698	6115	10255	± 3281
	144	1052	2371	5005	7176	± 2444
5 mg/kg b.w. (single dose)	**12**	16930	14574	24011	55515	± 22985

For histopathologic evaluation of the heart, hypertrophy was measured by comparison of the size of the nucleus of the cells with non-treated heart slices after staining with hemalaun-eosin. Fibrosis was assayed by sirius red staining. The heart was examined for signs of mild myocarditis, such as hypertrophy, infiltration of inflammatory cells or areas of fibrosis. There were no significant effects on the

parameters hypertrophy and fibrosis, following a 14-day treatment with 3,3,3-trifluoro-propionic acid (c.f. Figure 19).

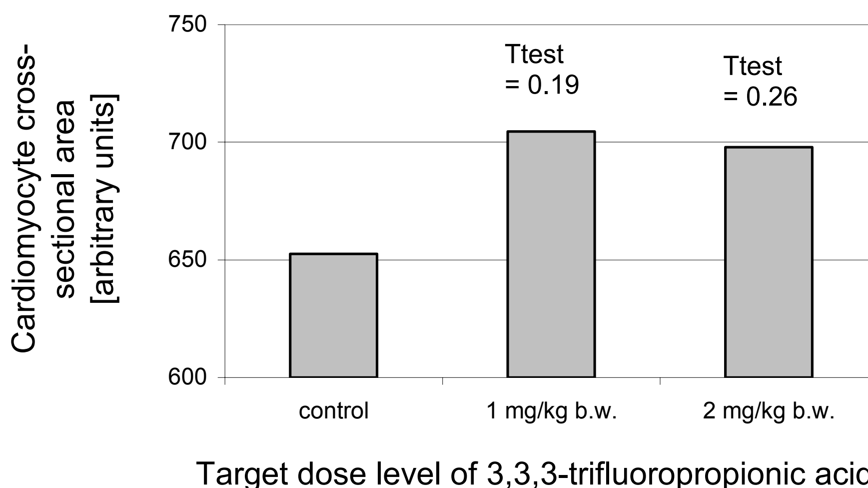


Figure 19: Cardiomyocyte cross-sectional areas in hearts of rats after treatment with the target dose level of 1 mg/kg b.w. or 2 mg/kg b.w. 3,3,3-trifluoropropionic acid for 14 days in drinking water.

5.3.2 Toxicity after 28-Day Repeated Dosing

The animal experiment was performed according to the "OECD Guideline For The Testing Of Chemicals: Repeated Dose 28-day Oral Toxicity Study in Rodents" (No. 407 Adopted by the Council on 27th July 1995). The study was designed with the use of five animals per gender and per dose level as well as three dose groups, one control group and one satellite group. All animals were observed daily for clinical signs of toxicity. Body weight, food intake and water consumption were also monitored daily.

Test item related mortalities occurred in four male rats, dosed with 30 mg/kg b.w. (4/5) within six hours on the first day of application. Further on, mortality was observed in one animal dosed with 10 mg/kg b.w. (1/5) over night and in four male animals, dosed with 5 mg/kg b.w. (4/5) on day 2 of dosing. Test item related mortalities in female rats occurred for all animals at a dose level of 30 mg/kg b.w. (5/5) and for four rats dosed with 10 mg/kg b.w. (4/5) or 2.5 mg/kg b.w. (4/5) within six to eight hours on day 1 of exposure. At the end of the experiment the number of animals was reduced due to mortality to three groups: 10 mg/kg b.w. or 2.5 mg/kg

b.w., males and 1 mg/kg b.w., females, each with four to five animals which were dosed repeatedly with 3,3,3-trifluoropropionic acid for 28 days.

5.3.2.1 Body Weight and Clinical Signs

A treatment related reduction was observed in the group mean body weight in male rats, dosed with 10 mg/kg b.w. 3,3,3-trifluoropropionic acid during week 1 of treatment to 75% of control animals. The body weight increased again beginning with day 8. The final body weight was 130% of the initial body weight. The body weight was monitored daily over the time (c.f. Figure 20). Control rats gained body weight continuously for up to approximately 140% of day 1. For animals, dosed with 10 mg/kg b.w. 3,3,3-trifluoropropionic acid, the decrease in body weight observed on day 25 was because of additional blood sampling. The initial loss in body weight was corresponding to the reduced intakes of food and water during the first week, which was determined to be approximately 20% less than the control group. The food and water intake recovered and remained stable from the beginning of week 2. Male animals, dosed with 2.5 mg/kg b.w. 3,3,3-trifluoropropionic acid showed an initial decrease in body weight to 90-95% and lasted for four days. The final body weight was 135% of the initial body weight.

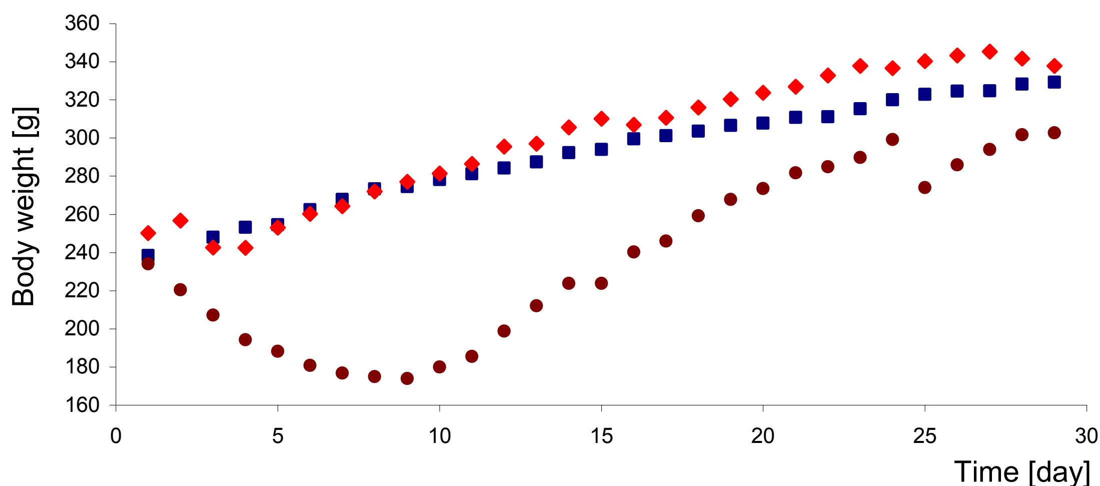


Figure 20: Body weight development in male rats, treated with 3,3,3-trifluoropropionic acid over a 28-day period: (■) control group (n=4), (●) 10 mg/kg b.w. (n=4) and (◆) 2.5 mg/kg b.w. (n=4).

5.3.2.2 ^{19}F-NMR and ^{1}H-NMR Analysis

The biofluids urine (day 14 and 28) and serum (day 28) were analyzed by ^{19}F-NMR. The ^{19}F-NMR spectra of urine of day 14 and 28 of treatment of male rats, showed increased intensities for inorganic fluoride, the presence of a triplet at δ = -63.9 ppm representing 3,3,3-trifluoropropionic acid, and of a triplet at δ = -63.4 ppm of unknown identity. In average the triplet was about double the intensity of 3,3,3-trifluoropropionic acid (equivalent to c.f. Figure 9). In most of the spectra of male control urines, a singlet of small intensity was detected at δ = -119.2 ppm, indicated as inorganic fluoride. ^{19}F-NMR spectra of urine from female rats, treated with 1 mg/kg b.w. 3,3,3-trifluoropropionic acid, showed a peak for inorganic fluoride, a small triplet at δ = -63.46 ppm in the 14-day urine but only inorganic fluoride in the 28-day urine. In ^{19}F-NMR analysis of serum samples, a signal representing 3,3,3-trifluoropropionic acid was only seen for male animals, treated with 10 mg/kg b.w. 3,3,3-trifluoropropionic acid.

Further investigations of urine and serum samples were performed by ^{1}H-NMR spectroscopy. The spectra of urine and serum samples of control rats were compared with those of rats treated with a single high dose, i.e. 80 mg/kg b.w of 3,3,3-trifluoropropionic acid (c.f. Figure 10 and Figure 11) - and rats, treated for 28 days with 10 mg/kg b.w. 3,3,3-trifluoropropionic acid (c.f. Figure 21, Figure 22 and Figure 23). The analysis of the spectra showed that hippurates, aminohippurate and urea tended to be increased in intensity in urine after exposure to 3,3,3-trifluoropropionic acid for 28 days. In addition, the intensity of the singlet with a resonance of δ = 8.46 ppm, representing formate, was clearly increased (c.f. Figure 21, trace B). As seen in the magnification (c.f. Figure 22) the intensity of amino acids isoleucine and valine, as well as α- and ß-hydroxybutyrate, hydroxylvalerate and adipate were less elevated after 28 days of exposure, than after a single high dose, but were visibly increased compared to the control spectra. The intermediates acetone and acetoacetate showed increased intensities but again not as much as that seen after a single high dose exposure. The intensity of the peaks were adjusted to the intensity of the signal of creatinine at δ = 4.06 ppm. Several peaks were more intense in relation to control urine, e.g. succinate, α-ketoglutarate, citrate and trimethylamine-N-oxide (TMAO). For serum samples no difference was observed in rats treated with 3,3,3-trifluoropropionic acid and control animals (c.f. Figure 23).

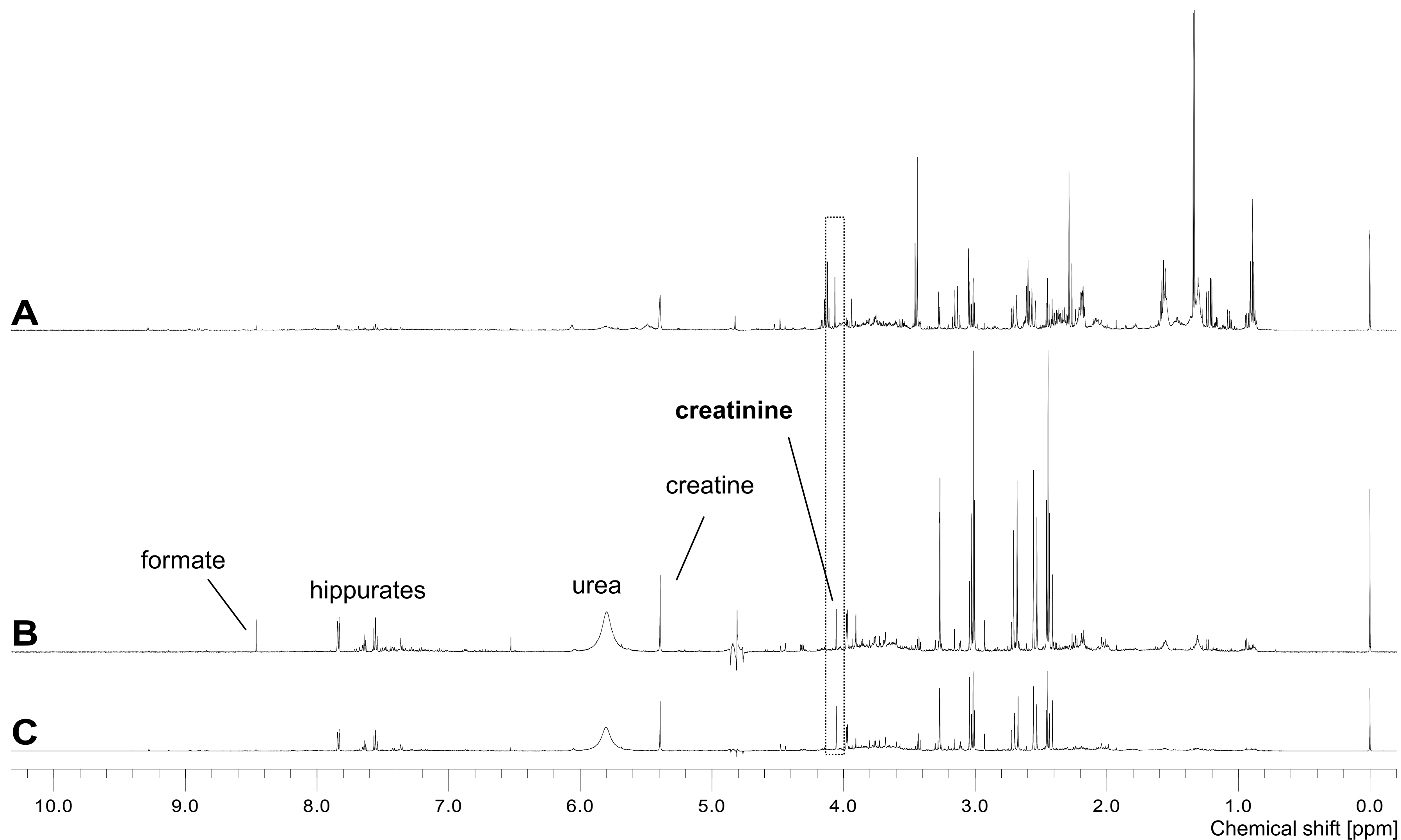


Figure 21: Representative ^{1}H-NMR spectrum from urine of male Sprague-Dawley rats. The vertical scales were manually adjusted to a constant intensity of creatinine at δ = 4.06 ppm. The traces are: (A) 3 hours after a single oral administration of 80 mg/kg b.w. of 3,3,3-trifluoropropionic acid; (B) 24 hours after a 28 day exposure of 10 mg/kg b.w. of 3,3,3-trifluoropropionic acid by gavage; (C) non treated animals as vehicle control to the 28-day exposure.

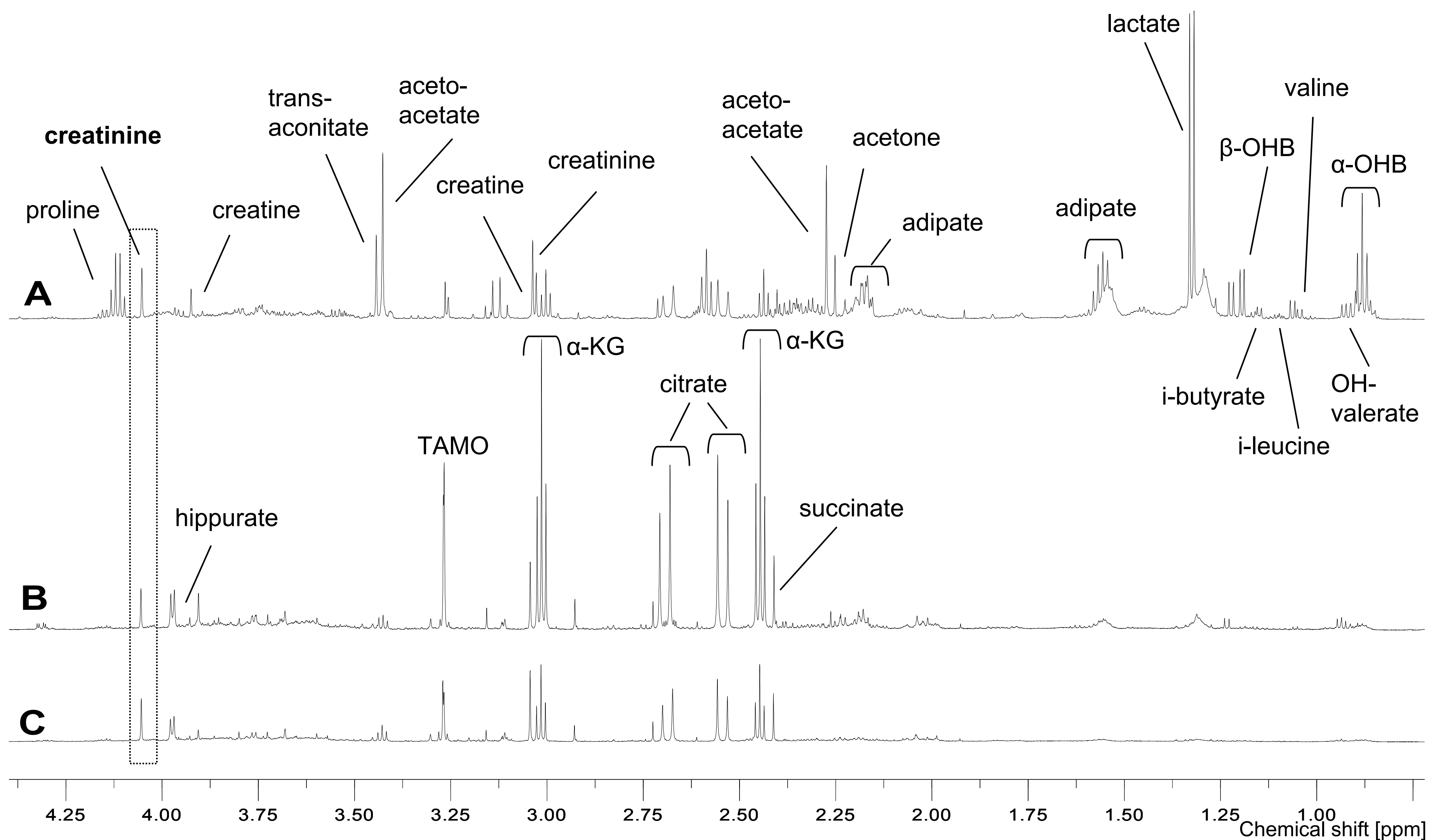


Figure 22: Representative ^{1}H-NMR spectrum from urine of male Sprague-Dawley rats. All spectra are calibrated to the intensity of creatinine at δ = 4.06 ppm and plotted with high resolution (chemical shift from δ = 0.75 to 4.25 ppm). The traces are: (A) 3 hours after a single oral administration of 80 mg/kg b.w. of 3,3,3-trifluoropropionic acid by gavage; (B) 24 hours after a 28-day exposure of 10 mg/kg b.w. of 3,3,3-trifluoropropionic acid; (C) non treated animals as vehicle control to the 28 day exposure.

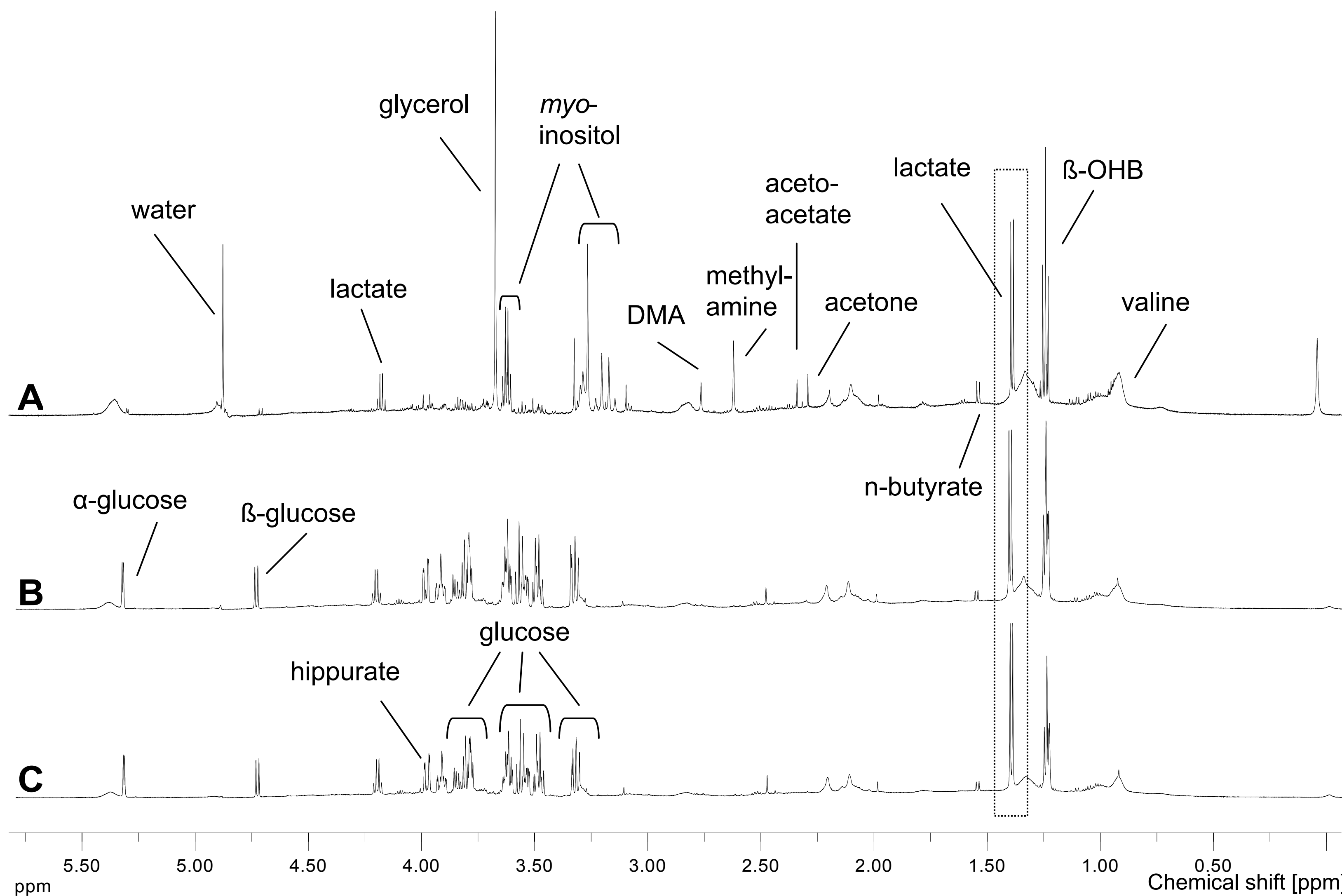


Figure 23: Representative ^{1}H NMR spectrum from serum of male Sprague-Dawley rats. All spectra are calibrated to the intensity of lactate at δ = 4.06 ppm. The spectrum is plotted in the range of chemical shifts from δ = 0 to 5.5 ppm (no signals were detected afterwards, up to δ = 10 ppm). The traces are: (A) 3 hours after a single oral administration of 80 mg/kg b.w. of 3,3,3-trifluoropropionic acid; (B) 24 hours after a 28-day exposure of 10 mg/kg b.w. of 3,3,3-trifluoropropionic acid by gavage; (C) non treated animals as vehicle control to the 28-day exposure.

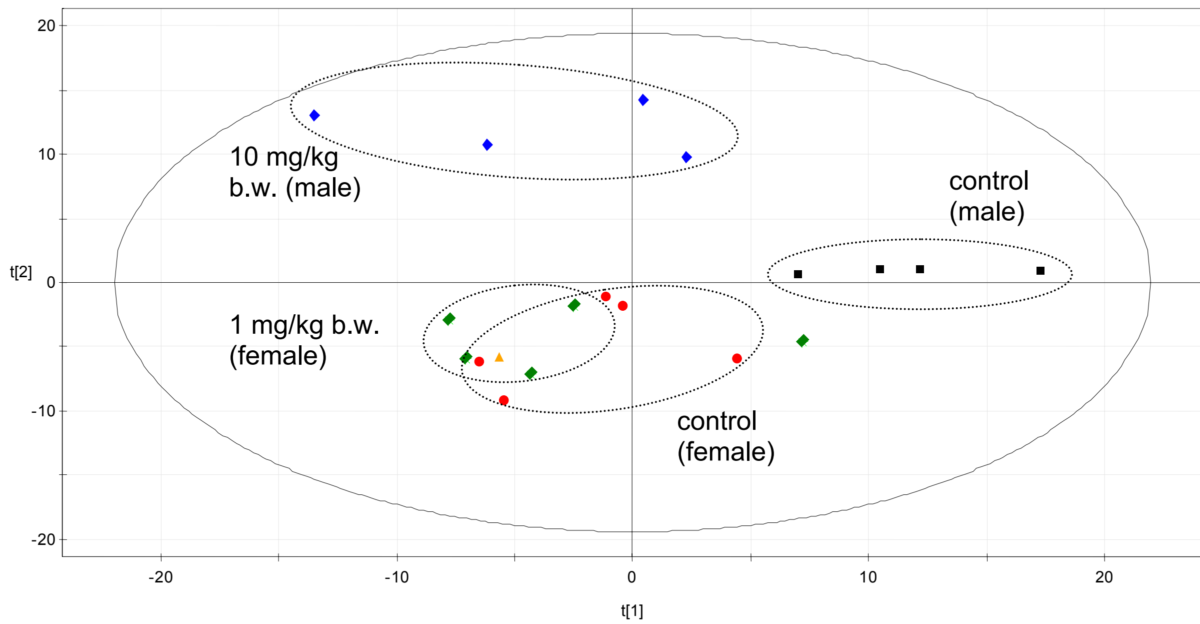


Figure 24: PCA plot of urine samples from control animals and rats treated with 3,3,3-tri-fluoropropionic acid for 28 days. Grouping of male and female control animals and treated animals.

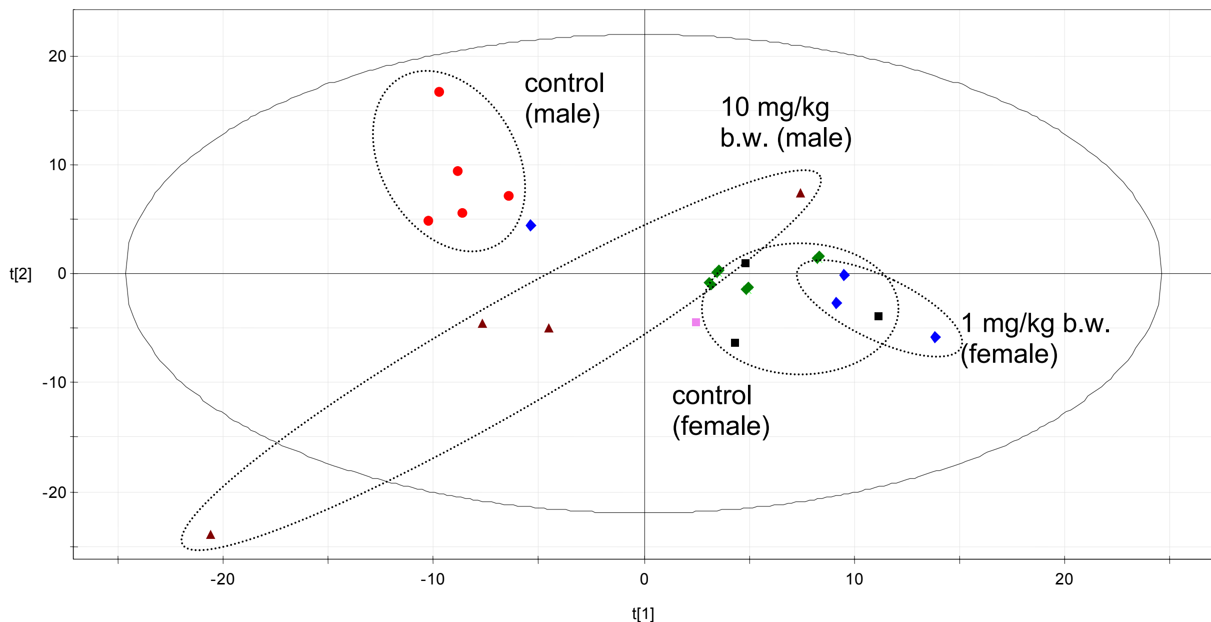


Figure 25: PCA plot of serum samples from control animals and rats treated with 3,3,3-tri-fluoropropionic acid for 28 days. Grouping of male and female control animals and treated animals.

The statistically evaluation of these ^{1}H-NMR spectra was performed by PCA, representing the comparison of the pattern of urine spectra from vehicle control animals and rats treated for 28 days with 3,3,3-trifluoropropionic acid. The plot in Figure 24 shows the grouping of urine samples from treated male and female rats and control animals. While a clear difference could be seen between treated male and control rats, the grouping of the treated female rats does not differ to the controls. A similar picture was seen in the processed plot of serum samples (c.f. Figure 25).

5.3.2.3 Clinical Chemistry

The assessment of clinical pathology in toxicology studies is an important indicator of toxic effects. The clinical chemistry of urine after 14 and 28 days is summarized in Table 11 (male animals) and Table 12 (female animals). The data of male and female serum samples is shown in Table 13. Serum samples were taken on day 29 before necropsy.
Changes in urine samples of male and female rats were observed after 14 and 28 days, when compared to urine samples from control animals. Herewith, a clear decrease in protein and creatinine excretion was observed in male and female animals over time. The observed decrease in urea was less pronounced and only on day 14 for female animals. No changes were observed for glucose, uric acid and potassium. Phosphate was decreased in female animals treated with 1 mg/kg b.w. and in male animals treated with 10 mg/kg b.w. 3,3,3-trifluoropropionic acid on day 28.
Sodium was not affected in female rats and was increased after treatment of male rats with 10 mg/kg b.w. 3,3,3-trifluoropropionic acid on day 14 and 28. Latter was also observed for calcium, whereas calcium was rather decreased in female animals on day 14 and 28. Absolute concentration values of GGT were not constant in controls animals and were therefore not evaluated.
For the parameters GOT and GPT no changes were observed in serum samples of female rats treated with 1 mg/kg b.w. and male rats treated with 2.5 mg/kg b.w.. These were marginal increased in male rats treated with 10 mg/kg b.w. 3,3,3-trifluoropropionic acid. ALP was not affected by treatment in male rats but marginal increased in female rats. The glucose level did decrease for both male groups treated with 3,3,3-trifluoropropionic acid but no change was observed for female

animals. Triglycerides were detected and were therefore elevated in the serum of males, treated with 10 mg/kg b.w. and females treated with 1 mg/kg b.w. 3,3,3-trifluoropropionic acid. GGT was slightly decreased in both sexes after treatment. No changes were observed for the parameters sodium, potassium, calcium, chloride, osmolarity, LDH, CK, CKMB and total protein (data not shown). Ketone bodies were not detected in serum samples.

Table 11: Clinical chemistry parameters of urine after exposure of male rats to 2.5 mg/kg b.w. or 10 mg/kg b.w. of 3,3,3-trifluoropropionic acid on day 14 and day 28.

	Day 14			Day 28		
	Control	**2,5 mg/kg b.w.**	**10 mg/kg b.w.**	**Control**	**2,5 mg/kg b.w.**	**10 mg/kg b.w.**
Creatinine mg/dL	**76** ± 13	**52 *** ± 17	**45 *** ± 4	**98** ± 21	**81** ± 12	**63 *** ± 11
Glucose mg/dL	**42** ± 5	n.d.	**41** ± 17	**41** ± 15	**60** ± 17	**59** ± 16
Protein mg/L	**2088** ± 1300	**1160** ± 630	**280 *** ± 142	**2492** ± 1601	**2108** ± 641	**930** ± 70
Uric acid mg/dL	**19** ± 3	**13** ± 5	**14 *** ± 2	**19** ± 3	**18** ± 4	**15** ± 3
Urea mg/dL	**3623** ± 469	**2923** ± 872	**3495** ± 315	**5057** ± 795	**4944** ± 912	**3998 *** ± 403
GGT U/L	**53** ± 37	**13** ± 10	**18** ± 16	**1037** ± 129	**164** ± 28 *	**506 *** ± 129
Sodium mmol/L	**130** ± 27	**114** ± 35	**172** ± 40	**124** ± 19	**144** ± 22	**170** ± 43
Calcium mmol/L	**4,1** ± 1,4	**4,5** ± 1,5	**16,9** ± 6,3	**2,6** ± 0,8	**3,0** ± 0,5	**8,3 *** ± 0,9
Potassium mmol/L	**230** ± 29	**178** ± 50	**244** ± 39	**253** ± 26	**252** ± 53	**238** ± 31
Phosphate mmol/L	**27** ± 6	**26** ± 10	**28** ± 11	**28** ± 3	**33** ± 6	**9 *** ± 7

Values represent mean ± standard deviation. *Significantly different from control values ($p<0,05$). Triglycerides and ketone bodies were not detected in urine. No changes were observed for the parameter osmolarity.

Table 12: Clinical chemistry parameters of urine after exposure of female rats to 1 mg/kg b.w. 3,3,3-trifluoropropionic acid on day 14 and 28.

	Day 14		**Day 28**	
	Control	**1 mg/kg bw**	**Control**	**1 mg/kg bw**
Creatinine mg/dL	**60** ± 18	**47** ± 8	**51** ± 17	**55** ± 11
Glucose mg/dL	**36** ± 11	**28** ± 5	**33**	**29**
Protein mg/L	**373** ± 250	**0** ± 0 **	**362** ± 468	**84** ± 43
Uric acid mg/dL	**14** ± 4	**9** ± 3	**11** ± 3	**11** ± 4
Urea mg/dL	**3322** ± 816	**2807** ± 393	**3722** ± 1183	**3836** ± 705
GGT U/L	**6** ± 8	**173** ± 57 **	**170** ± 90	**17** ± 13 **
Sodium mmol/L	**115** ± 31	**105** ± 27	**94** ± 14	**108** ± 22
Calcium mmol/L	**5,1** ± 2,1	**4,5** ± 1,0	**5,8** ± 2,3	**4,7** ± 0,9
Potassium mmol/L	**189** ± 32	**194** ± 23	**189** ± 52	**227** ± 35
Phosphate mmol/L	**24** ± 12	**18** ± 6	**19** ± 9	**13** ± 3

Values represent mean ± standard deviation. **Significantly different from control values ($p<0,01$). Triglycerides and ketone bodies were not detected in urine. No changes were observed for the parameter osmolarity.

Table 13: Clinical chemistry parameters in serum after exposure of female rats to 1 mg/kg b.w. 3,3,3-trifluoropropionic acid and male rats to 2.5 or 10 mg/kg b.w. 3,3,3-trifluoropropionic acid for 28 days.

	Male			**Female**	
	Control n = 5	**2,5 mg/kg b.w. n = 5**	**10 mg/kg b.w. n = 4**	**Control n = 5**	**1 mg/kg b.w. n = 5**
GOT U/L	**105** ± 15	**97** ± 9	**114** ± 16	**99** ± 12	**95** ± 13
GPT U/L	**105** ± 3	**97** ± 18	**115** * ± 3	**89** ± 4	**92** ± 14
GOT/ GPT	**1,00** ± 0,17	**1,02** ± 0,11	**0,99** ± 0,17	**1,11** ± 0,17	**1,04** ± 0,07
ALP U/L	**186** ± 37	**134** ± 21	**184** ± 27	**94** ± 14	**116** * ± 9
CK U/L	**272** ± 102	**230** ± 31	**311** ± 38	**229** ± 36	**166** * ± 15
CKMB U/L	**596** ± 183	**460** ± 17	**534** ± 44	**465** ± 21	n.d.
Glucose mg/dL	**445** ± 67	**391** ± 29	**344** ± 45	**245** ± 56	**283** ± 24
Triglycerides mg/dL	< LOD	< LOD	**29** ± 50	< LOD	**86** ± 100
GGT U/L	**2,2** ± 0,7	n.d.	**1,7** ± 1,1	**1,7** ± 0,8	**0,4** * ± 0,6
UBUN mg/dL	**18** ± 1	**19** ± 1	**16** ± 2	**20** ± 2	**20** ± 1
Ketone bodies	< LOD	< LOD	< LOD	< LOD	< LOD

Values represent mean ± standard deviation. n.d.: not determined. LOD: limit of detection. *Significantly different from controls ($p<0,05$). No changes were observed for the parameters sodium, potassium, calcium, chloride, osmolarity, LDH and total protein.

5.3.2.4 Organ Weights and Histopathology

The organ weights were determined on the day of necropsy, day 29 of the study. Relative liver and lung weights were significantly increased in male animals, treated with 10 mg/kg b.w.. In female animals, treated with 1 or 2.5 mg/kg b.w. the relative liver weight was also significantly increased. With a significance of $p<0.05$ the relative kidney weight was significantly increased in the male animals treated with 2.5, 5 or 10 mg/kg b.w. (c.f. Table 14). No changes in the organ weights were observed for heart and spleen in male and female animals and for the latter, in addition no changes were observed for kidneys and lungs.

Table 14: Organ weight to body weight ratios for rats, treated with 3,3,3-trifluoropropionic acid for 28 days. The organ weight to body weight ratios ae given in [g] organ weight to [g] body weight. Values represent mean ± standard deviation.

Dose of 3,3,3-trifluoropropionic acid [mg/kg b.w.]	Liver [g]	Heart [g]	Kidney [g]	Lung [g]	Spleen [g]
Control (m)	3,76 ± 0,46	0,34 ± 0,02	0,59 ± 0,04	0,38 ± 0,04	0,19 ± 0,01
2,5 (m)	3,79 ± 0,22	0,35 ± 0,01	**0,69 ** ± 0,02**	0,41 ± 0,03	0,20 ± 0,01
5 (m)	3,60	0,34	0,69	0,35	0,18
10 (m)	**4,87 * ± 0,56**	0,36 ± 0,02	**0,69 ** ± 0,01**	**0,43 * ± 0,01**	0,22 ± 0,03
Control (f)	3,41 ± 0,14	0,34 ± 0,03	0,58 ± 0,01	0,49 ± 0,02	0,26 ± 0,04
1 (f)	**3,85 * ± 0,25**	0,37 ± 0,02	0,60 ± 0,03	0,49 ± 0,02	0,26 ± 0,02
2,5 (f)	4,01	0,38	0,62	0,51	0,24

* p <0,05 and ** p <0,01 significantly different from controls.

Tissue samples were taken also for histopathological evaluation. Histological characteristics of myocarditis are lymphocyte and/or inflammatory infiltrations and myocyte necrosis. No pathological effects or inflammatory changes were observed which were considered to be related to treatment with 3,3,3-trifluoropropionic acid.

5.3.2.5 Immunohistopathology

Myocarditis is an inflammatory disease of the myocardium with a broad clinical picture. It is diagnosed by histological, immunological and immunohistochemical criteria. The acute myocarditis establishes ventricular dysfunction which may progress to dilated cardiomyopathy. The chronic myocarditis shows persistent histological infiltrates with foci of myocyte necrosis, possible after two weeks. This is mainly a secondary immune response with the expression of cytokines. Both pictures were assumed for the toxicity of 3,3,3-trifluoropropionic acid.

Half of each of the following organs heart, liver and kidney were shock frozen in liquid nitrogen and stored at -80°C. Cryostat sections were prepared and incubated with a B- and a T- cell marker. CD3 and CD45 are representatives for general inflammation. The cytokine CD3 represents a rat antigen with the function of T-cells. CD45 is a transmembrane protein which is required for the increase in the signal transduction through the antigen receptor complexes of T- and B- lymphocytes. CD45 is expressed on all cells of hematopoietic lineage except erythrocytes. A series of studies on CD45-deficient lymphocytes have demonstrated that CD45 is required for early activational responses such as calcium flux, phosphoinositide turnover, and protein tyrosine phosphorylation. The antibodies CD3 and CD45 were stained on the cryostat sections with an avidin-biotinylated peroxidase complex kit. A slight trend for increased appearance of CD45-marked cells was seen in sections of the heart of male animals treated with 10 mg/kg b.w. 3,3,3-trifluoropropionic acid, but was considered not to be an effect (Arendt et al., 1995; Hanawa et al., 1996; Kuhl et al., 1994; Kuhl et al., 1995; Smith et al., 1991; Staudt et al., 2001).

5.3.2.6 Excretion of 3,3,3-Trifluoropropionic Acid in Urine

The excretion of 3,3,3-trifluoropropionic acid in urine was monitored over 24 hours on two occasions, i.e. on day 14 and on day 28 (c.f. Table 15). A dose-dependent excretion of 3,3,3-trifluoropropionic acid was observed and differed between male and female animals. The concentration of 3,3,3-trifluoropropionic acid was similar after 14 and 28 days.

Table 15: Excretion of 3,3,3-trifluoropropionic acid in urine samples on day 14 and on day 28 of exposure.

Dose of 3,3,3-trifuoropropionic acid [mg/kg b.w.]	Sampling time point [days]	Amount of 3,3,3-trifluoropropionic acid	
		Mean [nmol]	St. Dev. [nmol]
Male animals			
0 (control)	14	0	0
0 (control)	28	0	0
2,5	14	614.1	± 251.3
2,5	28	381.2	± 117.0
5	14	328.2	- *
5	28	677.8	- *
10	14	1458.7	± 340.3
10	28	1494.4	± 333.9
Female animals			
0 (control)	14	0	0
0 (control)	28	0	0
1	14	24.1	± 3.8
1	28	35.0	± 7.8
2,5	14	43.6	- *
2,5	28	69.5	- *

*Statistics were not determined due the availability of only one sample.

5.3.2.7 Concentration of 3,3,3-Trifluoropropionic Acid in Serum

The steady state level of 3,3,3-trifluoropropionic acid in serum was quantified 24 hours after the last treatment for 28 consecutive days. The determined concentrations of 3,3,3-trifluoropropionic acid showed a high individual variation. (c.f. Table 16) .

Table 16: Concentration of 3,3,3-trifluoropropionic acid in serum samples, 24 hours after the last treatment for 28 consecutive days.

Dose of 3,3,3-trifuoropropionic acid [mg/kg b.w.]	Concentration of 3,3,3-trifluoropropionic acid in serum [nmol/mL]
Male animals	
0 (control)	0
2,5	19.4
5	193.6
10	*410,35*
10	*214,60*
10	*18,90*
Female animals	
0 (control)	*0*
1	86,15
1	*0*
1	83,17
1	116,29
1	0
2,5	0

5.4 CORRELATION OF GLUCOSE AND INSULIN FOLLOWING TREATMENT WITH 3,3,3-TRIFLUOROPROPIONIC ACID

5.4.1 Treatment with a Single High Dose of 3,3,3-Trifluoropropionic Acid

Insulin plays a key role in the regulation of the blood glucose level. Investigations were therefore performed to show if insulin release triggered a drop in the blood glucose concentrations and representing therefore the initial effect of toxicity.

The effect of 3,3,3-trifluoropropionic acid on insulin or the blood glucose level was monitored over time after exposure of three male Sprague Dawley rats to a high single dose of 80 mg/kg b.w. 3,3,3-trifluoropropionic acid. During the first 60 minutes the blood glucose levels were stable within the normal range. However, the glucose concentration dropped after 90 minutes to low levels in individual animals to 15-20 mg/dL (c.f. Figure 26).

High concentrations of insulin were detected after 14 to 21 minutes. A second insulin peak was found between 110 to 140 minutes (c.f. Figure 27). All animals showed severe clinical signs like drowsiness and apathy.

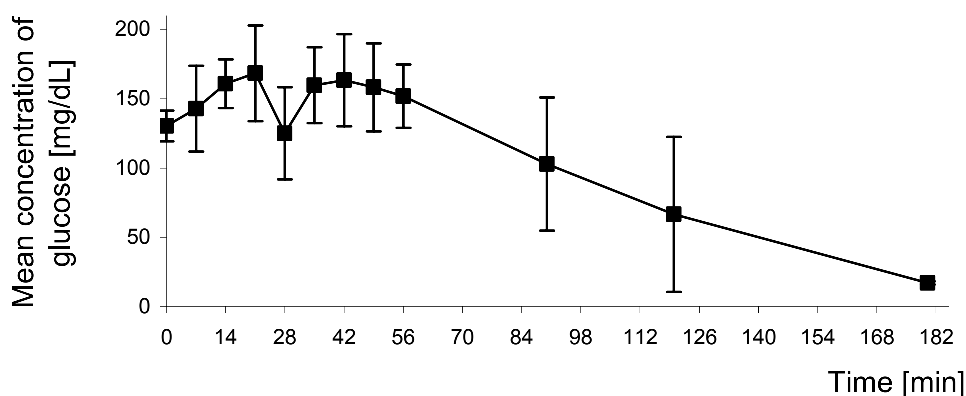


Figure 26: Glucose concentrations in serum samples after a single exposure of 80 mg/kg b.w. 3,3,3-tri-fluoropropionic acid to male rats up to 180 minutes after treatment (n=3).

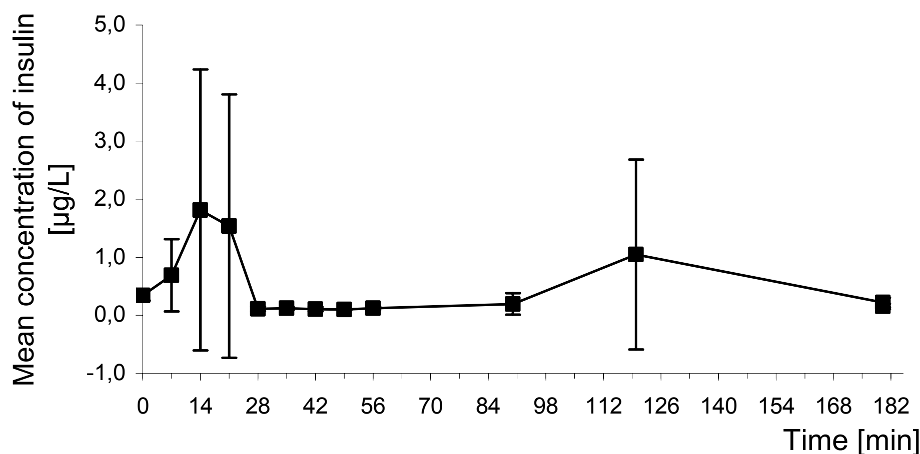


Figure 27: Insulin concentration in serum samples of male rats after a single exposure of 80 mg/kg b.w. of 3,3,3-trifluoropropionic acid (n=3).

5.4.2 Treatment with a Repeated Dose of 3,3,3-Trifluoropropionic Acid

After 28 days of exposure to 3,3,3-trifluoropropionic acid, insulin was determined in serum samples, 24 hours after the last gavage. For male animals treated with 10 mg/kg b.w. the insulin concentration was on average elevated compared to control animals. The glucose concentrations in the serum samples were shown in Table 13 and were within the normal range.

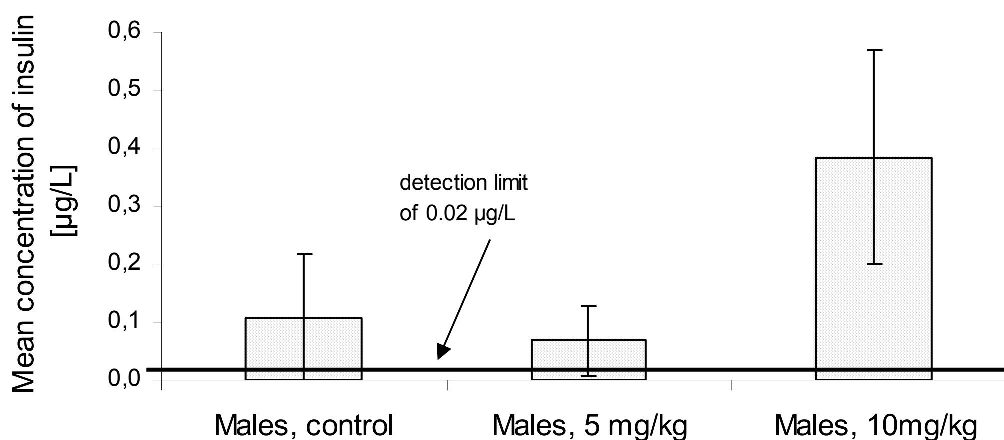
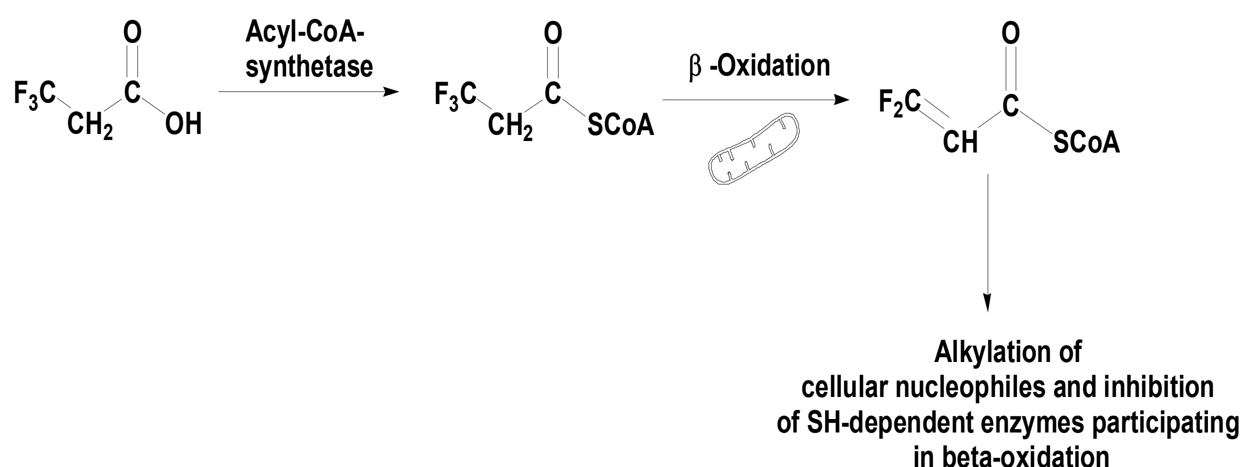


Figure 28: Quantification of insulin in serum after 28 days of exposure to 3,3,3-trifluoro-propionic acid in the individual animals.

5.5 IN VITRO INVESTIGATIONS ON THE TOXICITY OF 3,3,3-TRIFLUOROPROPIONIC ACID

5.5.1 Effect of 3,3,3-Trifluoropropionic Acid on the ß-Oxidation of Fatty Acids in Mitochondria

As one of its possible mechanisms of action, leading to toxicity, 3,3,3-trifluoropropionic acid may interfere with the mitochondrial ß-oxidation. This is outlined below in Scheme 11.



Scheme 11: Possible mechanism of inhibition of ß-oxidation by 3,3,3-trifluorpropanoic acid

Incubations of mitochondria with 3,3,3-trifluoropropionic acid or the positive control 4-pentenoic acid, a potent inhibitor of mitochondrial ß-oxidation (Freneaux et al., 1988) showed that 3,3,3-trifluoropropionic acid had a minor effect on the rate of formation of acid soluble products from [$U^{14}C$] palmitic acid. Equimolar concentrations of 4-pentenoic acid rapidly and almost completely inhibited the transformation of [$U^{14}C$] palmitic acid (Figure 29). An increase in the concentrations of 3,3,3-trifluoropropionic acid in the incubation media up to 3.75 mM also resulted in an inhibition of [$U^{14}C$] palmitic acid oxidation of less than 30%, suggesting that 3,3,3-trifluoropropionic acid was not a potent inhibitor of mitochondrial ß-oxidation

and that this mechanism is unlikely to account for the observed toxicity of 3,3,3-trifluoropropionic acid in rodents.

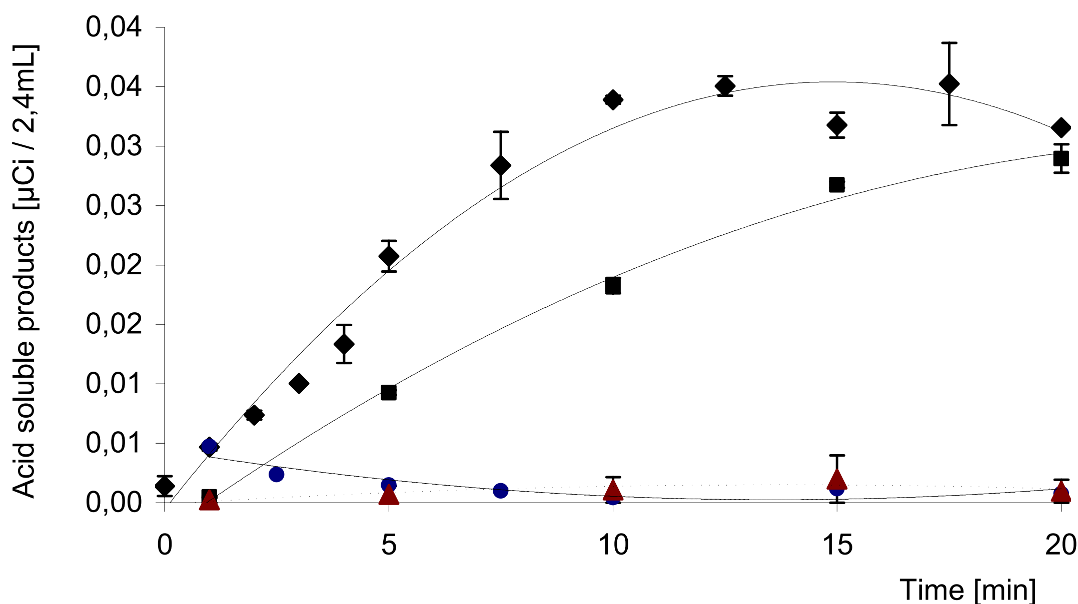


Figure 29: Effect of 3,3,3-trifluoropropionic acid (0.5 mM) (■) and 4-pentenoic acid (0.5 mM) (▲) on ß-oxidation of [U^{14}C] palmitic acid. Control incubations did not contain inhibitors (◆) or active mitochondria (●).

5.5.2 Cytotoxicity of 3,3,3-Trifluoropropionic Acid in Cell Culture

For *in vitro*- assays the cell lines H9C2 and HepG2 were used. Investigated endpoints were LDH and MTT. The cytoplasmic LDH is a parameter for membrane integrity as a function of the amount of cytoplasmic LDH, released into the culture medium. It also represents the cell biomass, i.e. the number of cells. The LDH assay is a classical endpoint for looking at cytotoxicity of a chemical compound. With affecting the membrane integrity, direct damage to the cell plasma membrane can be measured. LDH leakage was measured as an index of cytotoxicity and was determined as the ratio of triggered LDH release, to maximum LDH release after treatment with triton X, under consideration of endogenous LDH content (Chen et al., 1998a and 1998b).

The compounds 3,3,3-trifluoropropionic acid, 3,3,3-trichloropropionic acid, 4-pentenoic acid, monofluoroacetate or monochloroacetate were tested by the LDH and MTT assay.

The MTT assay was used to assess the cell viability and cell number (Mosmann, 1983). The assay detects only live cells and metabolically active cells, which cleave MTT. The amount of formazan generated depends therefore on the level of energy metabolism in the cell. The primarily metabolic capacity of the TCA cycle is determined by the mitochondrial dehydrogenase. The reduction of succinate to fumarate by the use of ATP is replaced here with the reduction of tetrazolium to formazan by the mitochondrial succinate oxidase.
For validation of the LDH-assay, hydrogen peroxide was used, which induced cell damage within a short time. For hydrogen peroxide a concentration dependent effect of the LDH release was seen from 1 µM to 200 µM H_2O_2 (c.f. Figure 30).
No effects were observed for cytotoxicity, measured by the LDH and MTT assay, after treatment with 3,3,3-trifluoropropionic acid.

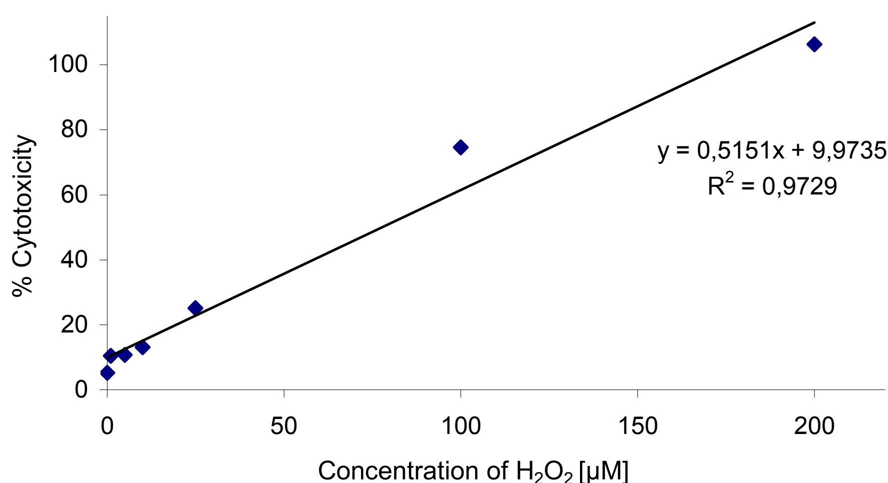


Figure 30: LDH release after treatment of H9C2 cells with different concentrations of hydrogen peroxide (1 µM to 200 µM).

The crystal violet cytotoxicity assay was based on the measurement of viable adherent cells. Therefore, the proliferation induced by the test compound was analyzed. For test viability purpose, a growing curve was standardized in medium without FBS. The curve was recorded for 72 hours without the addition of any drug. Because of the medium, lacking FBS, the growth was reduced in the beginning but constant for 48 hours. With the addition of 3,3,3-trifluoropropionic acid at concentrations of 0.01, 0.1 and 1 mM, the cell growth was monitored for initially 5 000, 10 000 and 25 000 cells per well (c.f. Figure 31 and Figure 32). For the H9C2

cell culture a decrease in cells was observed after 6 hours of incubation with 3,3,3-trifluoropropionic acid for 10 000 cells per well and stayed constant for 72 hours. In the assay with 25 000 cells per well the decrease continued up to 24 hours. In the HepG2 cell culture, a decrease in cell growth was observed up to 6 hours of incubation with 3,3,3-trifluoropropionic acid. Afterwards cell growth was observed up to 72 hours. Within the two cell culture systems used to assess the cytotoxicity of 3,3,3-trifluoropropionic acid, no consistent or concentration dependent effects of 3,3,3-trifluoropropionic acid were observed on cell viability and growth rate. This implicated that 3,3,3-trifluoropropionic acid does not induce lethality to the cells in cell culture, whereas the positive control hydrogen peroxide, induced massive loss of cell viability at low concentrations.

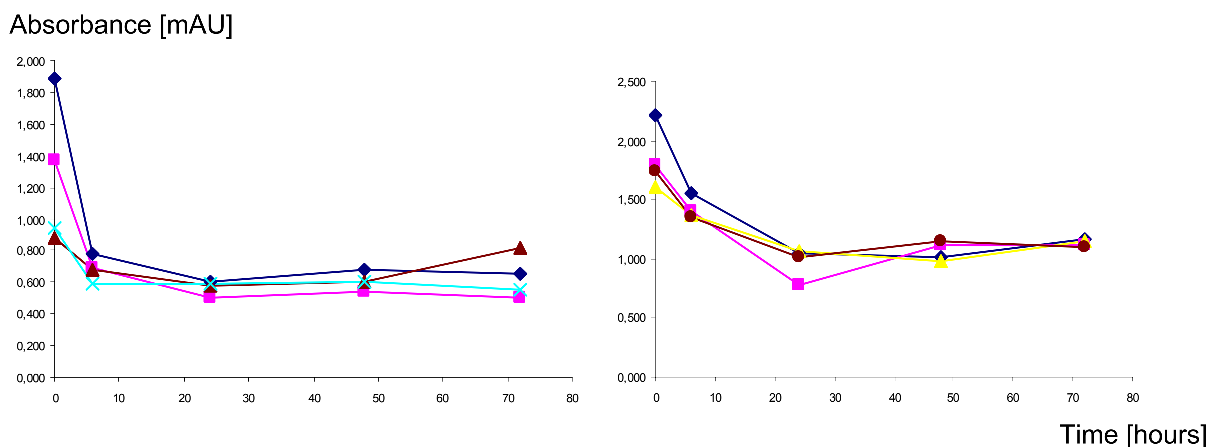


Figure 31: H9C2 cells (left: 10 000 cells per well, right: 25 000 cells per well), treated with 0.01, 0.1 and 1 mM 3,3,3-trifluoropropionic acid.

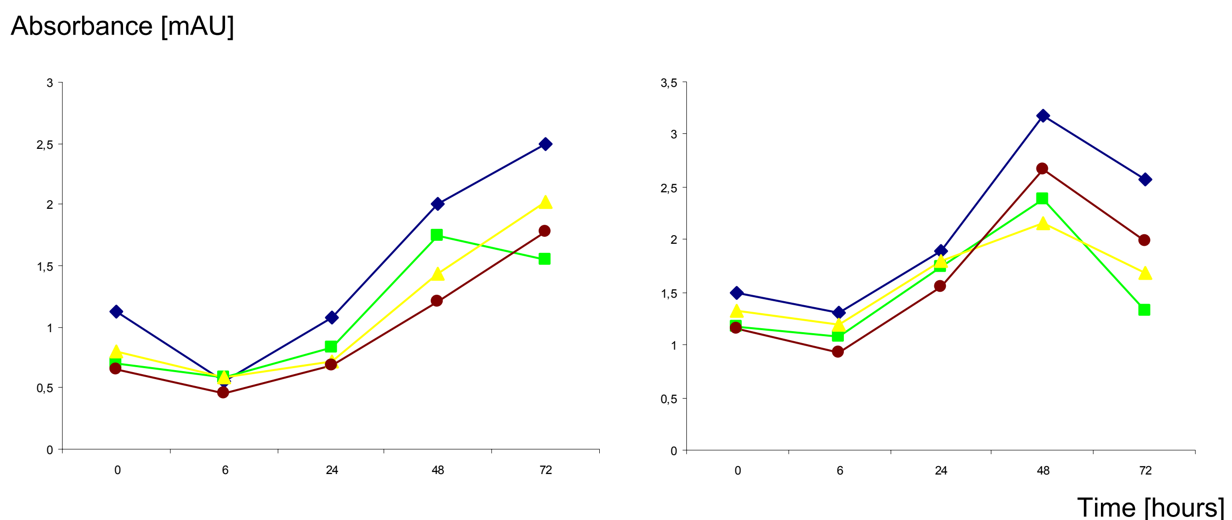


Figure 32: HepG2 cells (left: 10 000 cells per well, right: 25 000 cells per well), treated with 0.01, 0.1 and 1 mM 3,3,3-trifluoropropionic acid.

5.6 TOXICOKINETICS AND BIOTRANSFORMATION OF 1,1,1,3-TETRACHLOROPROPANE

1,1,1,3-Tetrachloropropane showed also an unusual toxicity profile for halogenated alkanes and induced a high incidence of cardiac myofiber degeneration in addition to liver and kidney toxicity. Toxicity of 1,1,1,3-tetrachloropropane may also be mediated by a trihalopropanoic acid, since 1,1,1,3-tetrachloropropane was metabolized to 3,3,3-trichloropropionic acid by cytochrome P-450. To assess similarities and possible differences in the toxicity of 3,3,3-trifluoropropionic acid and 1,1,1,3-tetrachloropropane, the biotransformation and toxicokinetics of 1,1,1,3-tetrachloropropane was studied in male rats after inhalative exposure to targeted concentrations of 100 ppm and 200 ppm 1,1,1,3-tetrachloropropane for 6 hours.

During all experiments, the deviation between the targeted concentrations and the actual concentrations was less than 16% of the targeted values. Actual concentrations were 95 ± 15 ppm and 206 ± 18 ppm 1,1,1,3-tetrachloropropane (mean ± standard deviation of determinations in 15-minute intervals over 6 hours).

5.6.1 Identification of Metabolites

Urine samples were collected for 96 hours after the end of the exposure in 12-hour intervals. Chlorine-containing metabolites in urine were analyzed using GC/MS. After esterification by sulphuric acid and methanol, headspace GC/MS analysis showed the presence of three peaks with the typical isotope clusters representative for the presence of three chlorines in the fragments. A peak with a typical isotope cluster of two chlorines in the fragment was also detected. A typical chromatogram is shown in Figure 33.

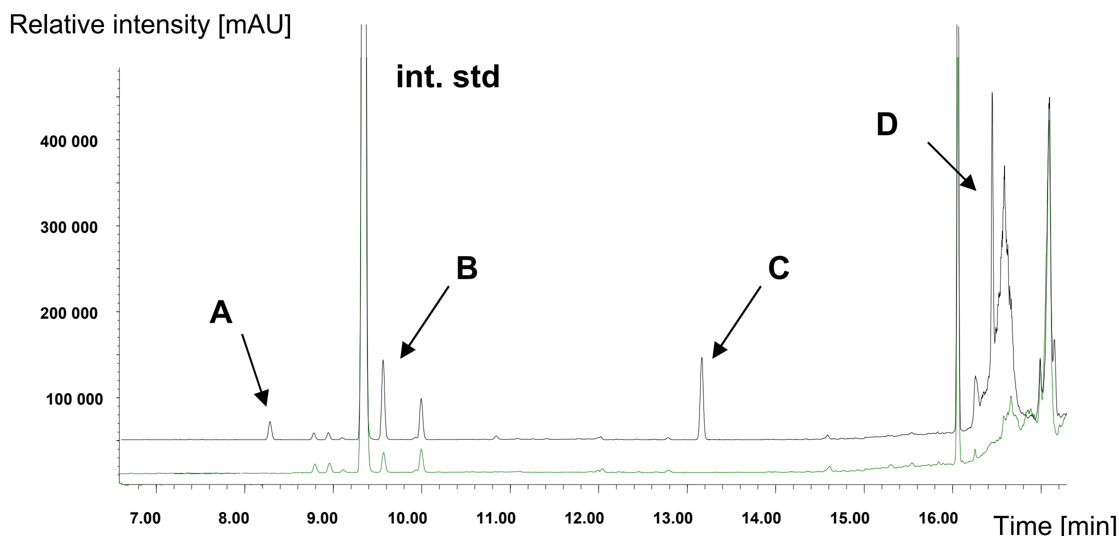
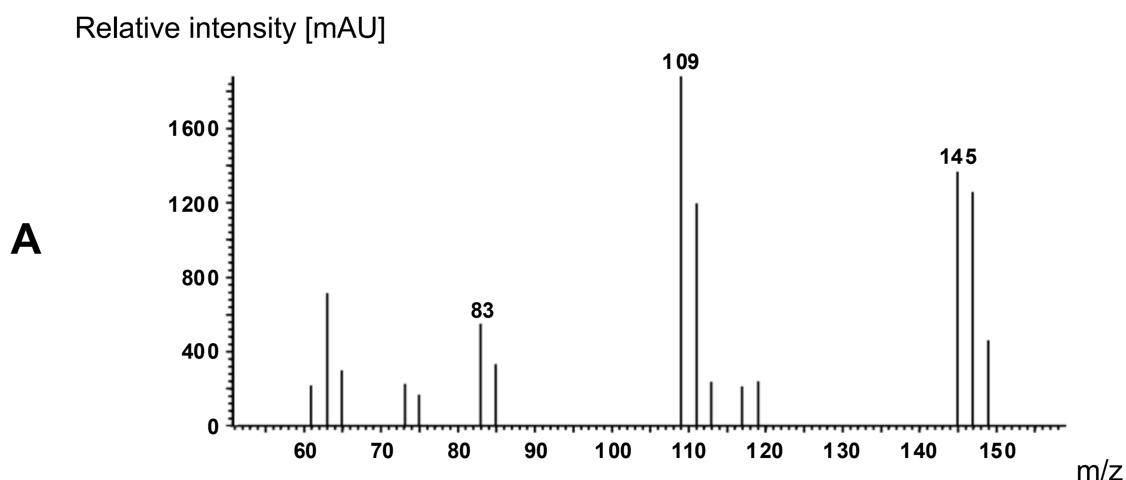


Figure 33: GC/MS analysis of urine samples, collected after 12 hours of inhalative exposure to 200 ppm 1,1,1,3-tetrachloropropane (upper trace) and control urine (lower trace). The spectra were recorded in the SIM mode. (A) 1,1,1,3-tetrachloropropane; (B) dichloroacrylic acid methyl ester; (C) 3,3,3-trichloropropionic acid methyl ester; (D) 1,1,1-trichloropropane-3-ol methyl ester.

The chlorine metabolites were identified by comparison of the mass spectra with those of synthesized reference compounds. The peak at a retention time of 8.29 minutes represented 1,1,1,3-tetrachloropropane (A) and at 9.60 minutes dichloroacrylic acid methyl ester (B). 3,3,3-Trichloropropionic acid methyl ester eluted at 13.17 minutes (C) and 1,1,1-trichloropropane-3-ol methyl ester (D) at 16.45 minutes. Metabolite mass spectra are shown in Figure 34.



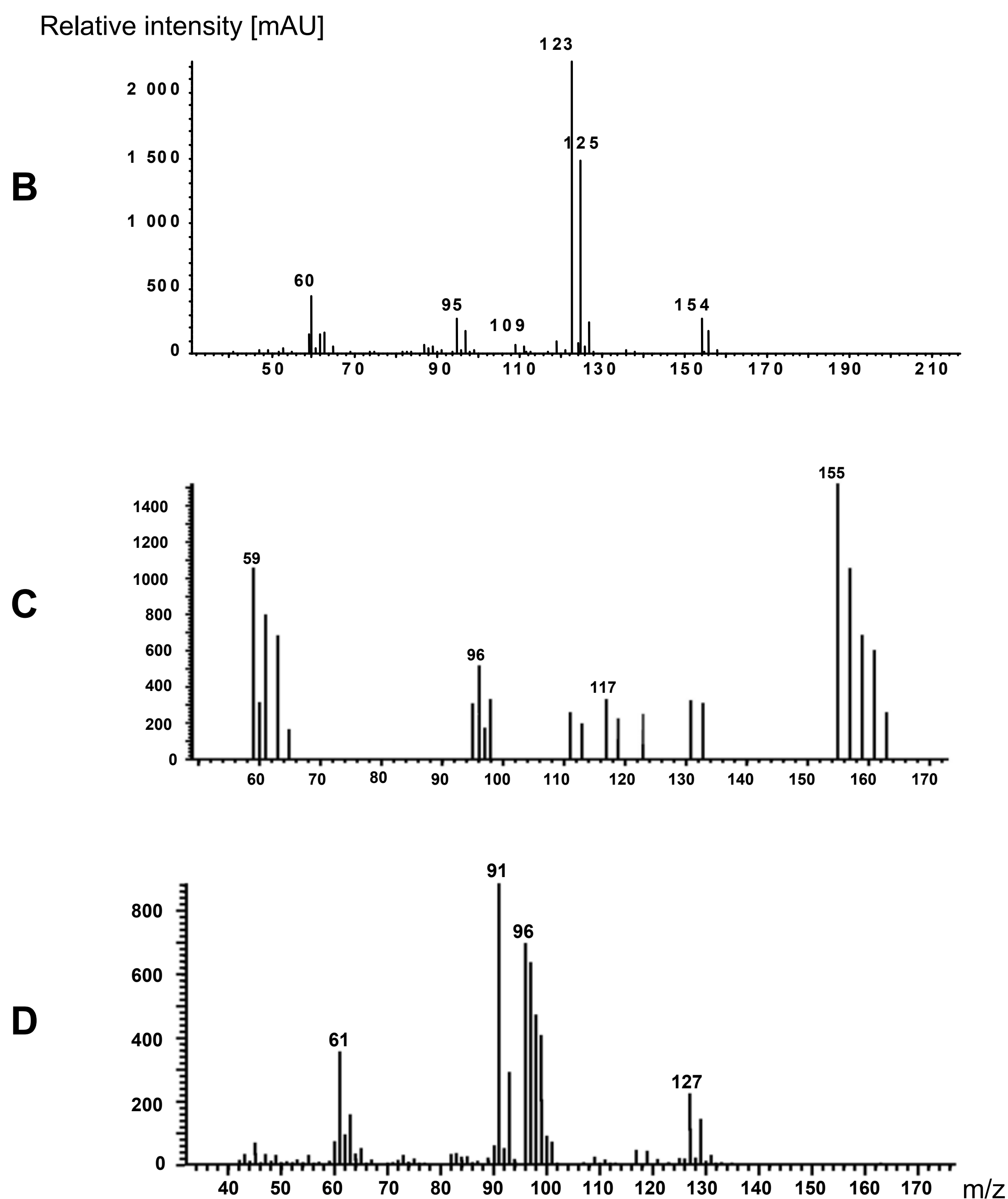
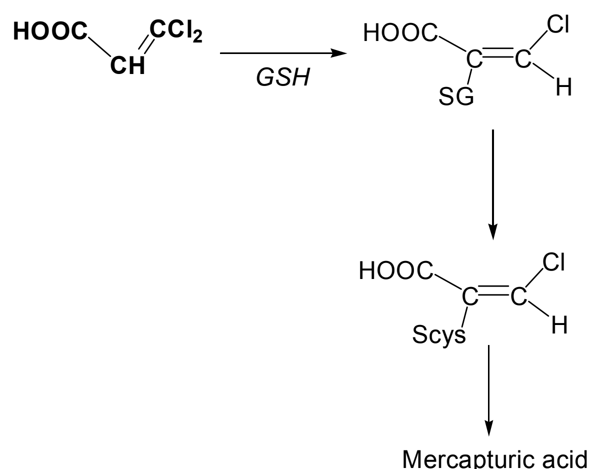


Figure 34: A typical full scan mass spectrum of 1,1,1,3-tetrachloropropane is shown in the upper panel (A), followed by the mass spectrum of dichloroacrylic acid methyl ester (B), the mass spectrum of 1,1,1-trichloropropanoic acid methyl ester (C) and the lower panel shows the mass spectrum of 1,1,1-trichloropropane-3-ol (D).

Dichloroacrylic acid is a highly reactive Michael-acceptor, which is rapidly conjugated with glutathione, the end products of which have been described in literature (James et al., 1998). A possible conjugation is shown in Scheme 12.

Urine samples were analyzed using GC/MS and LC/MS-MS. However, no peaks were observed with the expected fragments for these compounds, when using either GC/MS or LC/MS-MS in the multiple reaction monitoring (MRM) mode.



Scheme 12: Possible conjugation of metabolically formed dichloroacrylic acid with glutathione (GSH), catalyzed by glutathione-S-transferase to give excretable mercapturic acids.

5.6.2 Quantification of Metabolites after Inhalative Exposure to 1,1,1,3-Tetrachloropropane

The quantification of 3,3,3-trichloropropionic acid and 1,1,1-trichloropropan-3-ol was performed using GC/MS. Dichloroacrylic acid was quantified based on standard calibration curves, but it was also observed as a minor decomposition product formed from 3,3,3-trichloropropionic acid in the injection block of the gas chromatography system. However, in most samples, the concentrations of dichloroacrylic acid determined were significantly higher than those expected from the decomposition of 3,3,3-trichloropropionic acid. Therefore, dichloroacrylic acid represented a minor metabolite of 1,1,1,3-tetrachloropropane. As a major metabolite, 1,1,1-trichloro-propane-3-ol and 3,3,3-trichloropropionic acid were excreted in addition to the parent compound 1,1,1,3-tetrachloropropane. The amount of 3,3,3-trichloropropionic acid and 1,1,1-trichloropropan-3-ol recovered was dose dependent, whereas the excretion of the parent compound showed no dose dependency. Only a minor part of the calculated absorbed dose of 1,1,1,3-tetrachloropropane was recovered in urine in

form of chlorine containing metabolites (c.f. Table 17). Elimination of these metabolites in the urine was rapid, falling below the limit of detection in all urine samples taken more than 48 hours after the end of exposure (c.f. Figure 35).

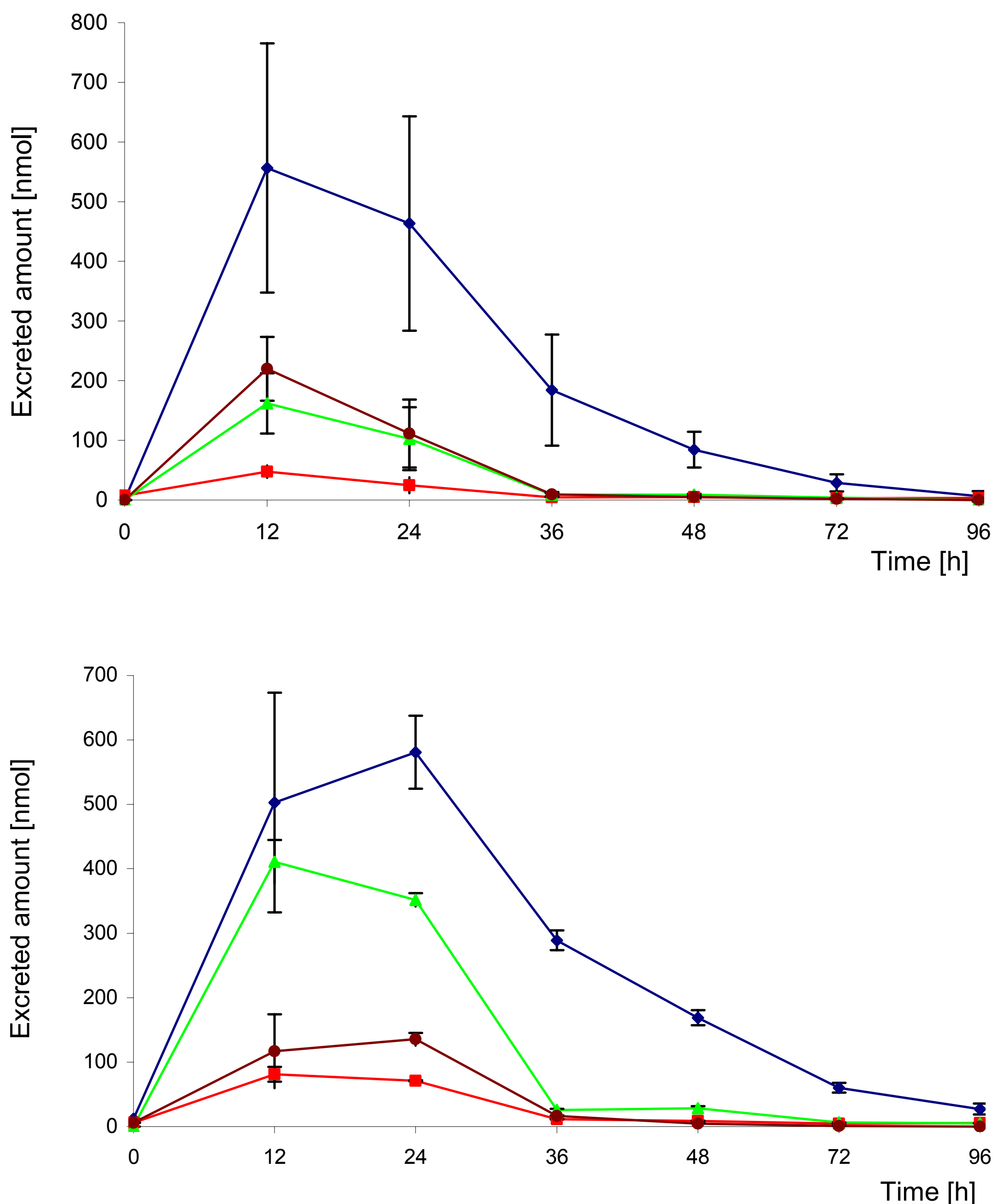


Figure 35: Excretion of 1,1,1,3-tetrachloropropane (◆) and of the metabolites 1,1,1-trichloro-propionic acid (▲), dichloroacrylic acid (■) and 1,1,1-trichloropropan-1-ol (●) after exposure of 100 ppm (upper trace) and 200 ppm (lower trace) of 1,1,1,3-tetrachloro-propane in urine.

Table 17: Biotransformation of 1,1,1,3-tetrachloropropane in rats after inhalative exposure to 100 ppm and 200 ppm for 6 hours. Urinary excretion of parent compound and identified metabolites.

1,1,1,3-Tetrachloro-propane (nmol)	3,3,3-Trichloro-propionic acid (nmol)	1,1,1-Trichloro-propan-1-ol (nmol)	Dichloro-acrylic acid (nmol)	Σ Excreted (µmol)	Dose administered *(µmol)*	% Excretion/ admin. dose (% of admin. dose)
100 ppm					***126***	
1311 ± 468	277 ± 74	347 ± 46	85 ± 11	**2,02** ± 0,54		***1,6***
200 ppm					***250***	
1629 ± 854	828 ± 381	274 ± 26	182 ± 96	**2,91** ± 0,85		***1,2***

Only a small part of the calculated administered dose was recovered as metabolites in urine. Since no other chlorine containing metabolites were indicated using both GC/MS and LC/MS-MS, the low recovery of metabolites may have been due to an intensive metabolism of stable metabolites formed from 1,1,1,3-tetrachloropropane.

Therefore, experiments were performed administering the metabolite 3,3,3-trichloro-propionic acid for checking the recovery of the administered dose. The recovery of 3,3,3-trichloropropionic acid was analyzed in urine after a single exposure of 10 mg/kg b.w. 3,3,3-trichloropropionic acid which is on average equal to 18.53 µmol per rat. The recovered amount in the 12-hour urine sample was in average 14.67 ± 2.20 nmol per rat. This amounted to less than 1% of the dose administered. Since in the urine samples collected from 24 to 72 hours 3,3,3-trichloropropionic acid concentrations were below the limit of detection and other potential chlorine containing metabolites were not present in any of the urine sample analyzed, this indicated intensive catabolism of 3,3,3-trichloropropionic acid and explained the low recovery of metabolites in urine after 1,1,1,3-tetrachloropropane exposure.

5.6.3 Biochemical Changes induced by 1,1,1,3-Tetrachloropropane

Since ketones were increased in urine after exposure to 100 ppm 3,3,3-trifluoro-propionic acid and a trihalopropanoic acid may be also a major metabolite of 1,1,1,3-tetrachloropropane, urine samples from rats exposed to 1,1,1,3-tetrachloropropane were analyzed for the presence of ketones using GC/MS. Figure 36 shows the chromatography with peaks at a retention time of 2.24 minutes representing acetone (A), at 3.04 minutes representing 2-butanone (B) and a peak at 4.44 minutes representing 2-pentanone (C).

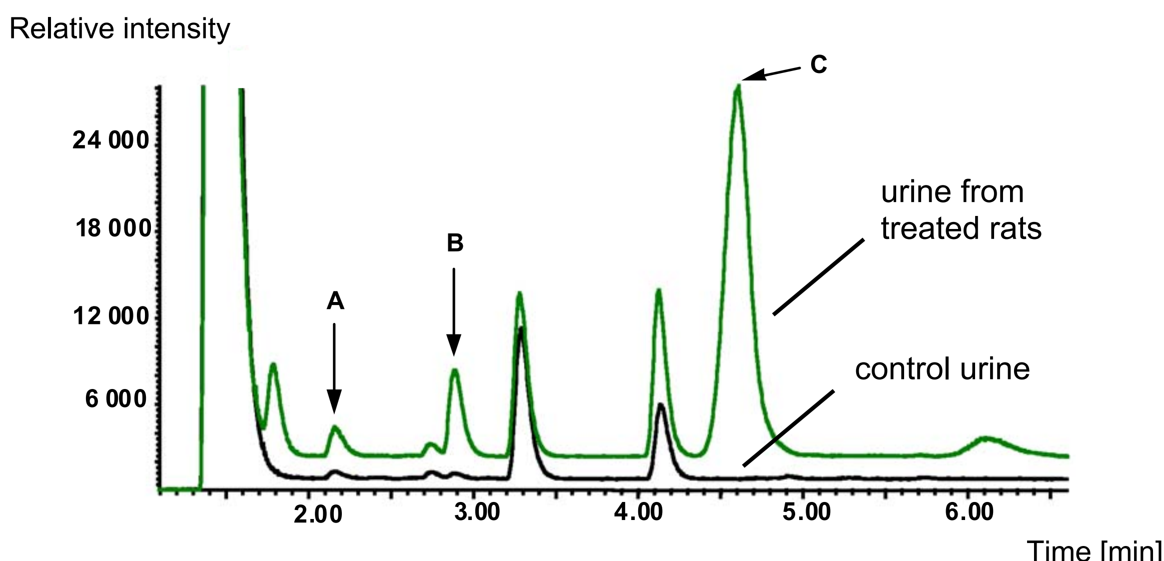


Figure 36: Quantification of ketones in urine by GC/MS. The upper trace shows urine samples, collected after 12 hours of 100 ppm inhalative exposure to 1,1,1,3-tetrachloro-propane and the lower trace shows urine from control rats.

An increased urinary excretion of the ketones, acetone, 2-butanone and 2-pentanone was observed up to 40-fold in the 12-hour rat urine, and thereafter decreased rapidly but remained elevated in the 24-hour urine samples (c.f. Table 18). In comparison, the increase in excretion of ketones was up to 20-fold in the 12-hour rat urine after exposure to 3,3,3-trifluoropropionic acid, and increased in the 24-hour urine. The increase in ketones was less pronounced after a single inhalation exposure to

10 000 ppm and 50 000 ppm 1,1,1,3,3-pentafluoropropane over 6 hours. The excretion of ketones increased dose dependently at 12 hours after administration.

Table 18: The amount of the ketones acetone, 2-butanone and 2-pentanone was increased at 12 hours after treatment with 100 ppm 1,1,1,3-tetrachloropropane and almost eliminated in 24 hours. A less pronounced effect was observed after exposure to 1,1,1,3,3-pentafluoropropane at 10 000 and 50 000 ppm.

Compound	Hours	Mean amount in urine [nmol]			Mean [nmol]	St.Dev. [nmol]
		acetone	2-butanone	2-pentanone	ketones, sum	
Mean of all non treated	**0**	61	73	338	472	± 183
1,1,1,3,3-Pentafluoropropane						
10 000 ppm (6 hours)	**12**	132	296	690	1118	± 332
	24	11	34	286	331	± 181
Total excretion in 24 h		143	330	976	1449	
50 000 ppm (6 hours)	**12**	432	538	1817	2787	± 833
	24	63	79	613	754	± 127
Total excretion in 24 h		495	617	2430	3541	
1,1,1,3-Tetrachloropropane						
100 ppm (6 hours)	**12**	3042	3753	2726	9521	± 3632
	24	489	815	1551	2855	± 1114
Total excretion in 24 h		**3.531**	**4.568**	**4.277**	**12.376**	

Metabonomic analysis of urine samples from rats exposed to 1,1,1,3-tetrachloropropane by ^{1}H-NMR did not show the typical changes in the recorded spectra, as seen in samples from rats exposed to 3,3,3-trifluoropropionic acid (c.f. Figure 36). In comparison to control rats, the ^{1}H-NMR spectra from animals treated with 1,1,1,3-tetrachloropropane, differed in the presence of formic acid at $\delta = 8.5$ ppm.

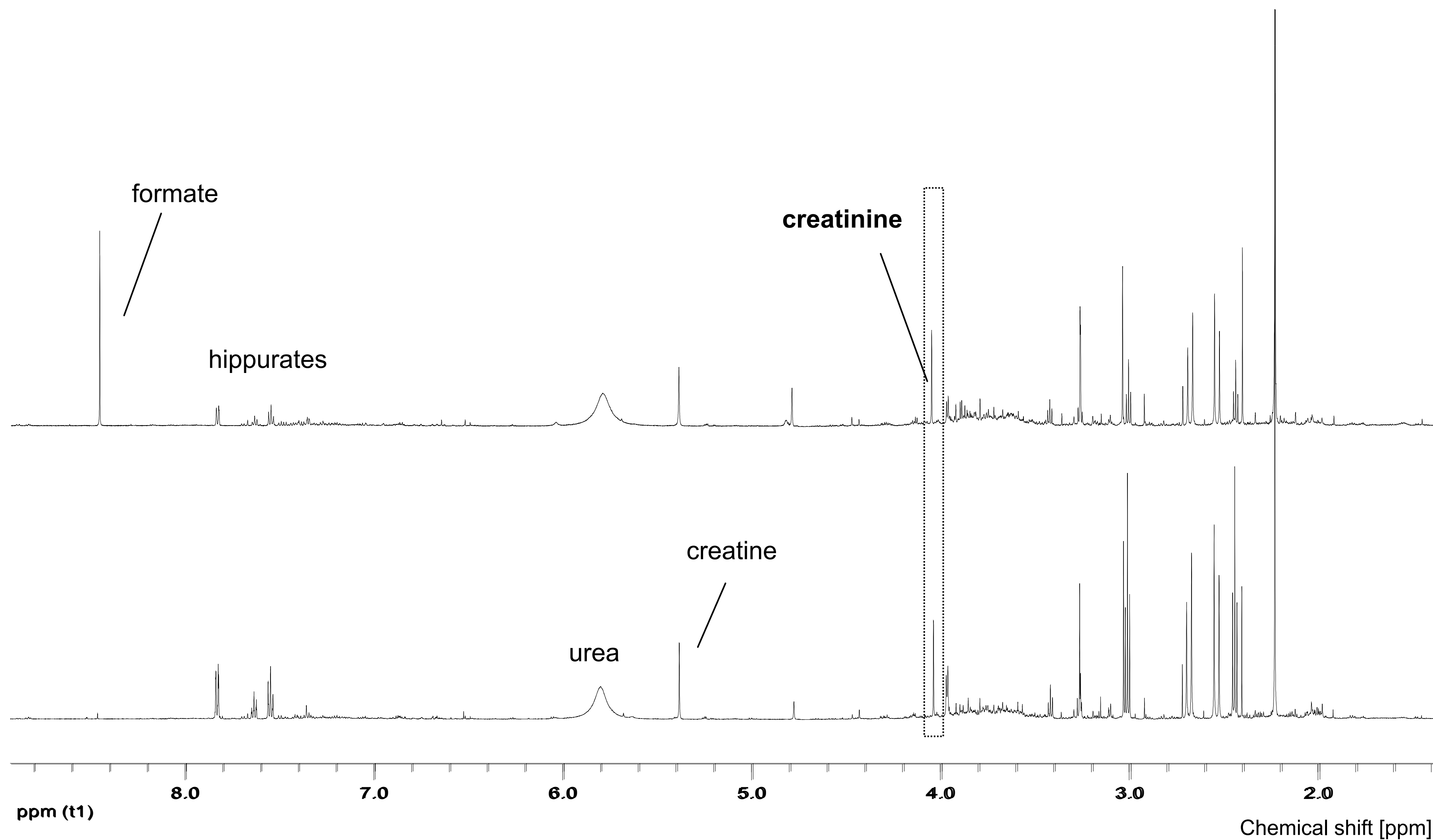


Figure 36 : Representative ^{1}H-NMR spectrum from urine of control rats (bottom) and rats treated with 1,1,1,3-tetrachloropropane (200 ppm for 6 hours by inhalation). The urine samples were collected between 6 and 18 hours after the end of the exposure. Control rats were kept in metabolic cages under identical conditions than exposed rats. The only consistent difference between the ^{1}H-NMR spectra from urine of control and 1,1,1,3-tetrachloropropane exposed rats was the presence of a resonance at δ = 8.5 ppm representing formic acid.

6 DISCUSSION

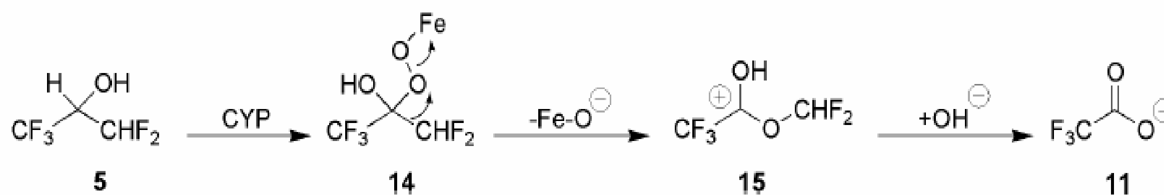
6.1 BIOTRANSFORMATION OF 1,1,1,3,3-PENTAFLUORO-PROPANE *IN VITRO*

The biotransformation of 1,1,1,3,3-pentafluoropropane *in vitro* was of interest because of its metabolism and mode of action seen in previous *in vivo* investigations. As indicated by the results, biotransformation of 1,1,1,3,3-pentafluoropropane in rats resulted in the formation of small amounts of 3,3,3-trifluoropropanoic acid and trifluoroacetic acid as the major metabolite (c.f. Scheme 7). However, the mechanism by which 1,1,1,3,3-pentafluoropropane is biotransformed to trifluoroacetic acid is not finally understood and experiments were designed to answer this question. The formation of 3,3,3-trifluoropropanoic acid [4] from 1,1,1,3,3-pentafluoropropane [1] may be rationalized by a cytochrome P-450-catalyzed oxidation of 1,1,1,3,3-pentafluoropropane [1] at C-3 to give the geminal fluorohydrine 1,1,3,3,3-pentafluoro-1-propanol [2], which may lose HF to give 3,3,3-trifluoropropanoyl fluoride [3] (c.f. Scheme 10). Hydrolysis of acyl fluoride [3] would give 3,3,3-trifluoropropanoic acid [4], which is excreted in the urine. The cytochrome P-450-catalyzed oxidation of 1,1,1,3,3-pentafluoropropane [1] at C-2 would give 1,1,1,3,3-pentafluoro-2-propanol [5], which may be conjugated with glucuronic acid; oxidation of alcohol [5] would give 1,1,1,3,3-pentafluoro-2-propanone [6], which may exist as the hydrate [7]. The quantification of metabolites indicated that 1,1,1,3,3-pentafluoropropane undergoes little biotransformation. The low extent of biotransformation was expected due to the high stability of the C-H bond in hydrofluorocarbons. For example, pentafluoroethane is also metabolized slowly in rats (Harris et al., 1992; Yin, 1995).

The formation of trifluoroacetic acid from 1,1,1,3,3-pentafluoropropane was an unexpected finding, and such reactions have apparently not been previously observed in the biotransformation of aliphatic halogenated hydrocarbons. Trifluoroacetic acid excretion was apparently slow, perhaps due to plasma protein binding; as described previously, trifluoroacetic acid is slowly excreted after animal

and human exposure to chemicals that are biotransformed to trifluoroacetic acid (Urban, 1994a).

The microsomal biotransformation experiments, indicated conclusively that trifluoroacetic acid [11] was formed by the cytochrome P-450-dependent oxidation of 1,1,1,3,3-pentafluoropropane [1] or a metabolite, which is formed by cytochrome P-450 from 1,1,1,3,3-pentafluoropropane (c.f. Scheme 10). Known mechanisms of cytochrome P-450-catalyzed oxidations do not suggest a pathway for the biotransformation of 1,1,1,3,3-pentafluoropropane to trifluoroacetic acid (Guengerich, 2001). The biotransformation of potential intermediates like sodium trifluoropyruvate, methyl trifluoropyruvate and hexafluoroaceton trihydrate, as tested *in vivo* (c.f. Scheme 9), and hexafluoroaceton trihydrate, 1,1,1-trifluoroacetone or 2,2,2-trifluoroacetophenon, as tested *in vitro* (c.f. Scheme 8), did not result in trifluoro-acetic acid as biotransformation product. A carbon-carbon bond cleavage reaction has been proposed for haloalkenes, such as trichloroethene, where the epoxide intermediate may undergo C-C fission (Miller, 1982; Henschler et al., 1979). With 1,1,1,3,3-pentafluoro-propane, however, this reaction would require prior olefin formation from 1,1,1,3,3-pentafluoropropane followed by oxidation and C-C bond cleavage. Neither 1,1,1,3-tetrafluoroprop-2-ene nor 1,1,1,3,3-pentafluoroprop-2-ene were observed in incubation mixtures by ^{19}F-NMR spectroscopy, or in the headspace of incubation mixtures in closed vials by GC/MS analysis. Carbon-carbon fission reactions have been observed in steroid biosynthesis, but this reaction might involve a Baeyer-Villiger rearrangement, which requires a carbonyl group that is converted to an intermediate peroxide which rearranges to give an ester (Swinney, 1994; Fischer, 1991). Cytochrome-catalyzed oxidation of the C-H bond in the putative metabolite 1,1,1-3,3-pentafluoro-2-propanol [5] may give an intermediate ferric peroxide intermediate [14] that is analogous to the peroxide intermediates in the Baeyer-Villiger rearrangement. The ferric peroxide intermediate [14] may rearrange via carbocation intermediate [15] to give trifluoroacetic acid [11] after reaction with water (c.f. Scheme 13). Attempts to prepare 1,1,1,3,3-pentafluoro-2-propanol [5] by synthesis were unsuccessful; hence, this proposed mechanism could not be tested.



Scheme 13: Baeyer-Villiger rearrangement of intermediates of the biotransformation of 1,1,1,3,3-pentafluoro-2-propanol [5] by cytochrome P-450, resulting in trifluoroacetic acid.

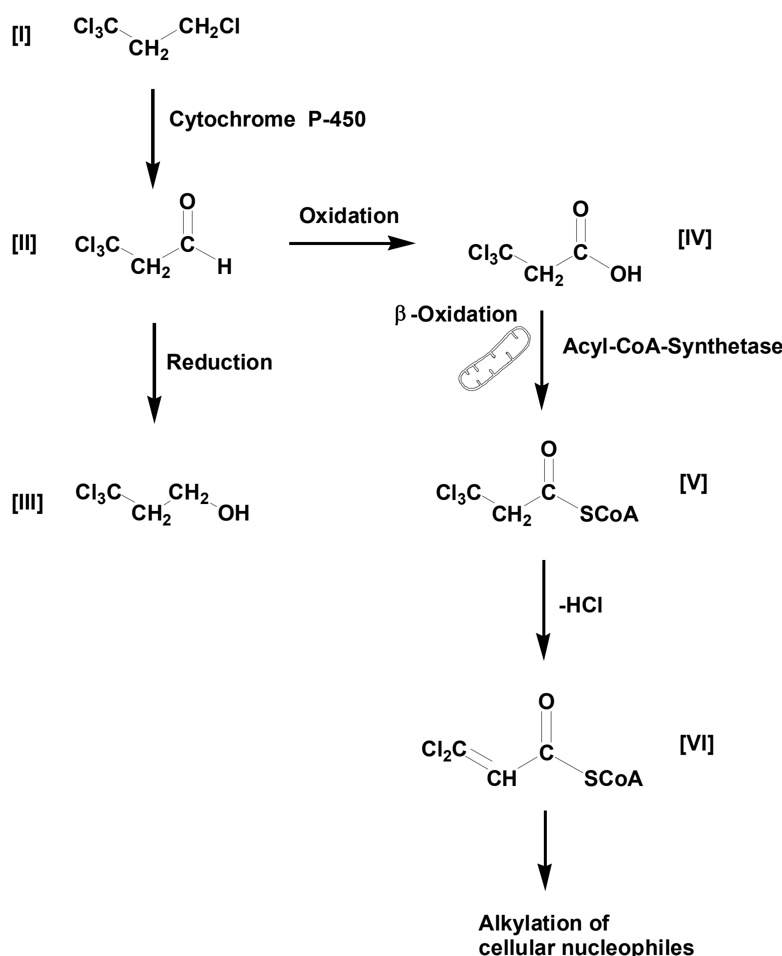
In human liver microsomes, the extent of biotransformation of 1,1,1,3,3-pentafluoropropane to trifluoroacetic acid was not correlated with cytochrome P-450 2E1 activity, as determined by *p*-nitrophenol oxidation. Rates of formation of trifluoroacetic acid in rat liver microsomes were increased proportional after enzymatic induction with pyridine, and were higher compared to rates of formation for 3,3,3-trifluoropropionic acid, which were not observed without cytochrome P-450 2E1 enzyme induction. Cytochrome P-450 2E1 is the major cytochrome P-450 involved in the biotransformation of hydrochlorofluorocarbons and hydrofluorocarbons and a number of other low molecular-weight compounds (Guengerich, 1991). Because the molecular size of 1,1,1,3,3-pentafluoropropane is similar to other cytochrome P-450 2E1 substrates, it is likely that 1,1,1,3,3-pentafluoropropane would also be a substrate for cytochrome P-450 2E1. The lack of correlation of trifluoroacetic acid formation and cytochrome P-450 2E1 activity may indicate the involvement of a sequential mechanism, as proposed above for the formation of trifluoroacetic acid from 1,1,1-3,3-pentafluoro-2-propanol. Such a sequential mechanism may involve more than one cytochrome.

The present investigations on the biotransformation of 1,1,1,3,3-pentafluoropropane were aimed at elucidating the role of metabolites and possible reactive intermediates in the myocarditis induced by 1,1,1,3,3-pentafluoropropane. The intermediate acyl fluoride that is likely to be formed during the oxidation of 1,1,1,3,3-pentafluoropropane may react with nucleophilic sites in proteins. Protein binding of reactive acyl fluorides has been implicated as a potential mechanism of toxicity for several halogenated aliphatic hydrocarbons (Pumford, 1997). With 1,1,1,3,3-pentafluoropropane, the role of protein acylation by 3,3,3-trifluoropropanoyl fluoride is uncertain, but its low rate of formation argues against a major role. Moreover, cardiac cytochrome P-450 activities are low, indicating limited formation of 3,3,3-pentafluoropropanoyl fluoride in the heart compared with other organs with higher cytochrome

P450 activity, such as the liver (Stee, 1982). Hepatotoxicity of 1,1,1,3,3-pentafluoropropane has, however, not been reported (Rusch, 1999). In addition, the probable short half-life of 3,3,3-pentafluoropropanoyl fluoride may limit its distribution to the heart after formation in other organs. Therefore, it is likely that other metabolites mediate the cardiotoxicity of 1,1,1,3,3-pentafluoropropane. Of the stable metabolites identified, trifluoroacetic acid shows little toxicity, but does induce peroxisome proliferation in the liver. Induction of myocarditis is not associated with other chemicals that are efficiently metabolized to trifluoroacetyl halides, such as halothane and 1,1,1-trifluoro-2,2-dichloroethane.

However, 3,3,3-trifluoropropanoic acid, even though it is a minor metabolite of 1,1,1,3,3-pentafluoropropane, may be a precursor of a toxic metabolite that induces myocarditis. The toxicity of 3,3,3-trifluoropropanoic acid may be associated with its biotransformation by mitochondrial ß-oxidation to 3,3-difluoroacryloyl-CoA, a highly reactive electrophilic-unsaturated carbonyl compound. Both, 3-fluoropropanoyl-CoA and 3,3-difluoropropanoyl-CoA, are substrates for butyryl-CoA dehydrogenase from *Megasphaera elsdenii* and give an α,ß-unsaturated thioester as a product. Reaction of 3,3-difluoroacryloyl-CoA with nucleophilic sites in mitochondria may induce mitochondrial dysfunction and myocarditis. The heart is highly dependent on mitochondrial fatty acid oxidation for energy production, and energy deprivation in the heart may be associated with the effect, described as focal and diffuse myocarditis with areas of inflammatory cell infiltration and degenerated myocardial fibers, seen after exposure to 10 000 and 50 000 ppm 1,1,1,3,3-pentafluoropropane. Support for this proposal is found in the observation that prolonged inhalation exposure of rats to 1,1,1,3-tetrachloropropane. In a 90-day toxicity study by Kolesar, inhalation of 1,1,1,3-tetrachloroporpane at concentrations up to 225 ppm resulted in an increased incidence of cardiotoxicity, represented by multifocal cardiac myocyte necrosis and areas of chronic myocarditis. In addition, mild diffuse hepatic necrosis and a random single-cell hepatocyte cytoplasmic vacuolation was observed in high-dose animals (Kolesar et al., 1995). 1,1,1,3-Tetrachloropropane [I] is expected to be metabolized by cytochromes P-450 and an aldehyde oxidase to 3,3,3-trichloro-propanoic acid [IV], which may be biotransformed by mitochondrial ß-oxidation to 3,3-dichloroacryloyl-CoA [V]. The analogous 3-chloropropanoic acid is a substrate for acetyl CoA synthetase. Indeed, 3-chloropropanoyl-CoA apparently inhibits fatty acid synthesis via the formation of acryloyl thioester, which reacts with an active site nucleophile.

The observed increased toxicity of 1,1,1,3-tetrachloropropane compared with 1,1,1,3,3-pentafluoropropane may be due to higher blood concentrations achieved during inhalation and to greater biotransformation to 1,1,1-trichloropropanoic acid. Experiments were designed to investigate the roles of 3,3-dihaloacrylic acids in the toxicity of 3,3,3-trihalopropanoic acids. In this work, the biotransformation of 1,1,1,3-tetrachloropropane was studied to identify formed metabolites and to obtain information on the mechanisms of action that induced cardiotoxicity. The observed biotransformation was explained by a cytochrome P-450 mediated oxidation of 1,1,1,3-tetrachloropropane [I] to give 3,3,3-trichloropropanal [II], which was reduced to 1,1,1-trichloropropane-3-ol [III] or oxidized to 3,3,3-trichloropropionic acid [IV], which were both excreted (c.f. Scheme 14).



Scheme 14: Biotransformation of 1,1,1,3-tetrachloropropane [I] by cytochrome P-450 to the identified metabolites 3,3,3-trichloropropionic acid [IV] and 1,1,1- trichloropropane-3-ol [III]. 3,3,3-Trichloropropionic acid is considered to undergo the ß-oxidation pathway and to impair mitochondrial function by alkylation of cellular nucleophiles.

However, 3,3,3-trichloropropionic acid was considered to give 3,3-dichloroacryloyl-CoA [VI] under mitochondrial ß-oxidation, which reacted with nucleophilic sites in mitochondria and impairs their function. Due to this mitochondrial dysfunction, myocarditis was possibly induced. 3,3-Dichloroacrylic acid was also identified in urine but not quantified. 3,3-Dichloroacrylic acid was considered to rapidly undergo conjugation with glutathione and be excreted as mercapturic acid. Whereas the excretion of 3,3,3-trichloropropionic acid [IV] and 1,1,1-trichloropropan-3-ol [III] was dose dependent, the excretion of the parent compound showed no dose dependency. Only a minor part of administered 1,1,1,3-tetrachloropropane was recovered in urine as chlorine containing metabolites. The elimination in the urine was rapid and below the limit of detection of 48 hours after the end of exposure.

6.2 BIOTRANSFORMATION OF 3,3,3-TRIFLUOROPROPIONIC ACID AND 3,3,3-TRICHLOROPROPIONIC ACID

In vivo investigations with the previously found minor metabolite 3,3,3-trifluoro-propionic acid were performed in rats to characterize its biotransformation. Analysis of urine samples using GC/MS and ^{19}F-NMR did not show the excretion of biotransformation products besides the cleavage product inorganic fluoride and a minor metabolite, likely to be a glycine conjugate, with one triplet by ^{19}F-NMR spectra. This triplet could not be identified but was considered as a possible biotransformation product. Both, ^{19}F-NMR and GC/MS analysis showed the major excretion product to be present as the unmetabolized parent compound. Quantification of 3,3,3-trifluoropropionic acid indicated that only a small part of the administered dose of the parent was recovered in urine, suggesting rapid catabolism to fluorine free products. The estimated half life of urinary excretion of 3,3,3-trifluoro-propionic acid in rats was determined to be 4.5 hours after single administration of 5 mg/kg b.w.. As a consequence, 3,3,3-trifluoropropionic acid was tested for its biotransformation *in vitro*, analogue to 1,1,1,3,3-pentafluoropropane. The result was that no metabolism by cytochrome P-450 enzymes of liver microsomes or by the α-oxidation pathway was observed.

For comparison, studies were performed on the biotransformation of the chlorine analogue metabolite 3,3,3-trichloropropionic acid. The recovery of 3,3,3-trichloro-

propionic acid was analyzed in urine after a single exposure of 10 mg/kg b.w. and was only found in the 12-hour urine. It was calculated to be 0.08% of the dose administered. Therefore, intensive catabolism of 3,3,3-trichloropropionic acid and of 1,1,1,3-tetrachloropropane was assumed, as no other potential chlorine containing metabolites were present.

6.3 CHARACTERIZATION OF THE TOXICITY OF 3,3,3-TRIFLUOROPROPIONIC ACID AND 3,3,3-TRICHLOROPROPIONIC ACID *IN VIVO*

Interactions of 3,3,3-trifluoropropionic acid with the generation of cellular energy in general were investigated. Major metabolic pathways are the ß-oxidation of fatty acids, the glycolysis, the TCA cycle, the respiratory chain and the oxidative phosphorylation (c.f. Figure 37).

In these studies acute toxicity was determined by two different routes of administration, by gavage and within the drinking water. Severe clinical signs, which resulted in individual occasions in lethality, were observed after single oral administration (gavage) of greater than 25 mg/kg b.w. 3,3,3-trifluoropropionic acid. The findings implicated possible effects on the energy supply of the organism. The course of clinical signs however was different for individual animals; it varied between the genders and was dependent on the age. Rats, eight weeks of age, were sensitive to 3,3,3-trifluoropropionic acid whereas 12-week old rats did not show effects after administration of up to 80 mg/kg b.w. 3,3,3-trifluoropropionic acid. Animals, which showed mild clinical signs, recovered, in the main, over night. The 28-day study was performed in rather young animals and therefore, toxic effects were observed at a dose level of 5 mg/kg b.w. in male animals and 2.5 mg/kg b.w. in female animals. These effects were recorded after the first dosing or, at the latest, on the third day of dosing. Thereafter, neither lethality nor severe impairment were observed for up to the end of the study. The metabolic activity after a single dose was considered as being different than after repeated dosing based on observed reactions. As repeated doses were more tolerated, it was assumed, that the organism stabilized by running adaptive mechanisms to compensate the interference of the compound. In

comparison to the toxic effects observed after treatment with 3,3,3-trifluoropropionic acid, no clinical signs were observed after treatment with the chlorine analogue.

Figure 37: Interactions of various metabolic pathways in the organism. These include the ß-oxidation of fatty acids, the glycolysis, the tricarboxylic acid cycle, the respiratory chain and the oxidative phosphorylation with the involvement of amino acids (AA) (Murray et al., 2003).

As seen in *in vitro,* no interference was observed for the ß-oxidation pathway of fatty acids. This was confirmed by the clinical pathology data. On the one hand a decrease of triglycerides in serum samples was observed down to not-detectable amounts. On the other hand levels of ketone bodies were increased in serum and

urine samples from non-detectable to maximum, using test stick analysis. This effect was also observed after exposure to 3,3,3-trichloropropionic acid. Therefore, the ß-oxidation pathway was assumed to be passed through to the interim end-product acetyl-CoA and the fatty acid oxidation was forced as a main source of energy. The decrease in triglycerides may have been triggered due to the lack of available ATP from inadequate carbohydrate utilization. However, the primary storage form of glucose is liver glycogen but supplies could quickly deplete during negative glucose balance. In this matter the breakdown of lipid stores may become activated. Consequently with lipolysis, elevated levels of glycerol were found in serum samples. The formation of ketone bodies can be rationalized by a surplus of acetyl-CoA, which was not utilized in the TCA cycle. This lead to the assumption that the TCA cycle is inhibited in one or several of its steps.

Increased levels of adipate, a short chain dicarboxylic fatty acid, indicate a possible alternate oxidation pathway, called omega-oxidation. The production of adipate is usually very low because of the predominant ß-oxidation pathway in the mitochondria. Transport of fatty acids into the mitochondria, however, require carnitine as a carrier and sub-optimal levels of carnitine cause inadequate transfer of fatty acids into the mitochondria. Subsequent compensation via omega oxidation results in excess amounts of adipate with symptoms like periodic mild weakness, nausea, fatigue or hypoglycemia.

Ketone bodies also provide the organism with energy. The noteworthy increase in ketone bodies was critical in the respect of leading to ketoacidosis. Ketone bodies are acids and therefore water soluble and were excreted in urine. With high elevated blood levels, the buffering system of the blood and the kidney become overloaded, followed by polyuria, reduced blood volume, hyperosmolarity and finally the so called “diabetic coma”. Measured urine volumes verified the presence of polyuria after a single exposure in addition to observed clinical signs such as apathy, drowsiness and decreased activity.

The overall dominating effect, however, was the depletion of blood glucose levels in urine and serum samples to values below the surviving range. In general, the symptoms for rapid decrease of blood glucose are restlessness, palpations, nausea and tremor. A slowly decrease of blood glucose concentration leads to weakness, dizziness and cramps. The rapid drop in blood glucose may implicate a possible enforcement of glycolysis with the interference of insulin. In addition, the glycolysis

pathway was assumed not to be inhibited as no interim substrates were elevated, resulting in the interim end-product acetyl CoA. However, acetyl CoA seemed to play a key role in the metabolic outcome. The assumed high concentrations of acetyl CoA could result in a decreased formation from pyruvate that alternatively reacts to lactate. Lactic acidosis occurs when there is a block in the final oxidative phosphorylation stage of energy production. Such block results in inactivation of the TCA cycle and therefore, the disturbances of the energy metabolism that lead to clinical signs as acute crisis and coma. However, lactic acidosis was not observed in serum samples after treatment with 3,3,3-trifluoropropionic acid, but ^{1}H-NMR spectra analysis indicated elevated lactate levels in urine.

As already discussed, the TCA cycle was assumed to be impaired in its overall pass through. This was based on the elevated substrate which enters the TCA cycle, acetyl CoA, and reduced levels of intermediate substrates which are in particular α-ketoglutarate and citrate, but only seen decreased in the ^{1}H-NMR spectra. Inhibited or impaired enzymes include citrate synthetase, aconitase and/or isocitrate dehydrogenase. This explains their reduced formation. In the case of α-ketoglutarate, its decrease could also be explained by the affection of the enzyme succinyl-CoA-synthetase leading to succinyl-CoA. The consequence would be an increased level of α-ketoglutarate but then depleted in formation of glutamate, which undergoes transaminase reactions resulting in the amino acids proline, arginine, glutamine and histidine. The amino acids proline and arginine were found elevated in urine.

In addition, general clinical biochemistry parameters were affected, i.e. elevated key enzymes GOT and GPT and elevated markers for skeletal or cardiac muscle damage, i.e. CK and the heart specific isoform CKMB. The parameters GGT, urea and creatinine were decreased. Changes in GGT levels are known to result from damage to the liver or myocardial infarction with secondary liver damage and brain lesions. Creatinine represents the glomerulus filtration and was decreased because of an increased urine volume and therefore insufficient function of the kidney. Urea is also a marker of metabolic function. A decrease would be observed during nephrosclerosis, impairment of liver function and diabetes insipidus. These parameters were considered as rather not specific for a direct metabolic answer to interferences of 3,3,3-trifluoropropionic acid with the organism and were considered as also being affected when adaptive mechanism occurred.

Finally, the involvement of messenger substrates like insulin was taken into account. In addition to impairment of the TCA cycle, the drop in blood glucose was considered to be caused by the impairment of glucose transporters or by the impairment of the insulin balance. Insulin is the principal hormone responsible for the control of blood glucose utilization in peripheral tissues via the glucose transporters, and is secreted from beta cells of the islets of Langerhans in the pancreas. ß-Cells are poised to adapt insulin secretion to the fluctuations in blood glucose concentrations. If a single dose of 3,3,3-trifluoropropionic acid had stimulated the insulin release, glycolysis would be activated, blood glucose would be decreased and high amounts of acetyl CoA would be produced. In case of subsequent impaired use in the TCA cycle, ketone body levels increase. Finally, the reduced production of ATP would not be sufficient to keep the functions of the organism alive. In addition, the rate of glycolysis is also determined by the formation of ATP. When ATP is exported into the cytosol, the increased ratio of ATP to ADP causes a depolarization of the plasma membrane by closure of the ATP-sensitive potassium channel. This allows the opening of the calcium channel and calcium concentrations increase in the cytosol, which is the main trigger for insulin secretion and exocytosis. Known substances that interfere with the insulin regulation include antidiabetica, which only effect if the endogen insulin production still functions or α-glucosidase-inhibitors like biguanid-derivatives or insulinotrope antidiabetica, which block the potassium channel. Furthermore, insulin-sensitizers induce the translocation of glucose receptors and increase the glucose uptake into the cell. The consequences, which occur after increased insulin release, are similar to the observed signs following treatment with 3,3,3-trifluoropropionic acid. Therefore, an interference of 3,3,3-trifluoropropionic acid with the potassium channel similar to antidiabetica was considered and needs further investigation. Experiments were performed *in vitro* with the design to characterize the direct effect of 3,3,3-trifluoropropionic acid on rat insulinoma-derived INS-1 cells. However, insulin is a very sensitive parameter to measure and rather instable. The plasma half-life period for example is less than 10 minutes. Due to the experimental and functional complexity of insulin release, no conclusion could be drawn from the small number of experiments.

After repeated exposure to 3,3,3-trifluoropropionic acid only a few changes were observed in clinical pathology, compared to control animals and to changes after a single dose. Decreased levels were observed in creatinine and protein, whereas

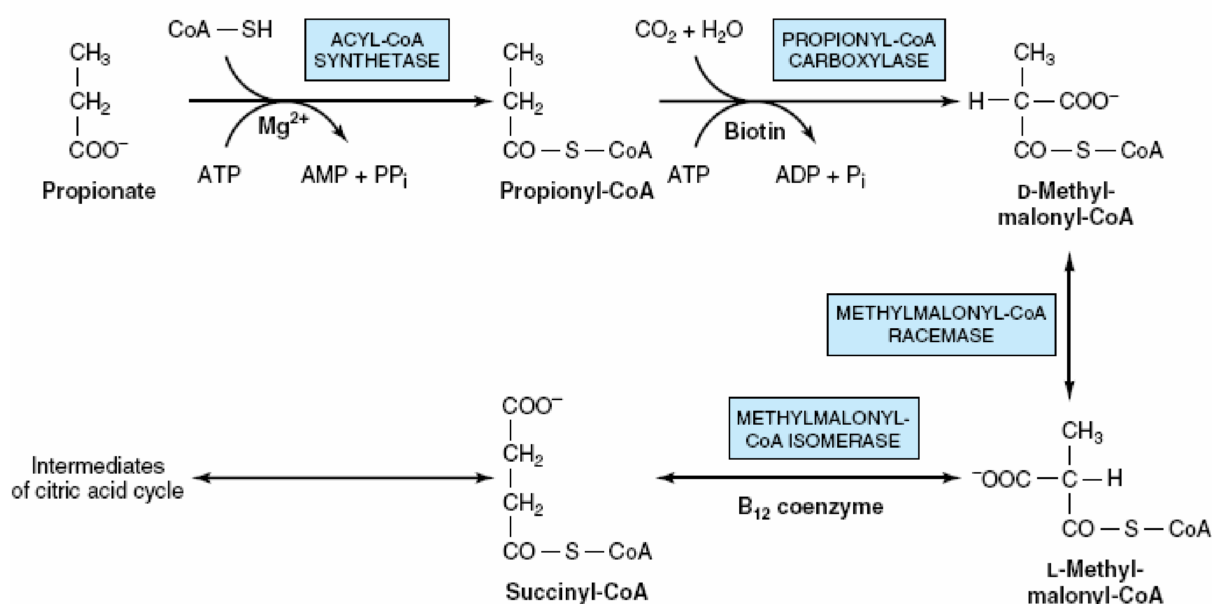
triglyceride levels were increased. This finding is in contrast to the effect after acute damage and is considered to be based on adaptive mechanistic reactions of the organism. If interactions with insulin are involved, e.g. an exaggerated release of insulin, increased gluconeogenesis would result, followed by a decreased uptake of glucose in the cells. Mechanisms like lipolysis would increase, resulting in increased levels of fatty acids in the plasma and increased amounts of ketone bodies. Furthermore, because of the elevated levels of fatty acids in the liver, increased levels of triglycerides appear in the blood, as well as an increased breakdown of proteins.

The metabolic profile seen in the ^{1}H-NMR investigations, varied only slightly from control spectra. The main difference was the appearance of a peak, identified as formate. Formic acid is not a specific metabolic product from formaldehyde, and arises as a metabolite from various endogenous substrates, e.g. food intake or after metabolism of amino acids. The excretion in urine is in general higher than the exogenous uptake.

No gross or macroscopic lesions or histological findings were observed in the heart, liver, kidney and lung. Inflammation was not diagnosed by histological or immuno-histochemical criteria, as expected with the assumed subchronic effect of myocarditis, an inflammatory disease of the myocardium. In general, the acute myocarditis leads to ventricular dysfunction, which progresses to dilated cardiomyopathy. The myocyte destruction could be a direct consequence of the exogenous agent, which causes cell-mediated cytotoxicity and cytokine release, contributing to myocardial damage or dysfunction. The chronic myocarditis, however, can be seen after two weeks of persistent histological infiltrates with foci of myocyte necrosis, also a secondary immune response and expression of cytokines. Both pictures were assumed for the toxicity of 3,3,3-trifluoropropionic acid but were not observed in the experiments. It has been observed following treatment with mono-chloroacetic acid. Histopathological evaluation of male and female rats showed cardiomyopathy after treatment of 60 mg/kg b.w. and higher for 13 weeks. The cardiomyopathy was indicated by degeneration of myocardial fibers and accumulation of mononuclear inflammatory cells. The reason for the effect was the inhibition of the TCA cycle with the inhibition of aconitase activity in the heart mitochondria (c.f. Scheme 3). Monofluoroacetic acid showed the same effect in liver mitochondria (Bryant et al., 1992). However, inhibition of aconitase was not

considered as mode of action for 3,3,3-trifluoropropionic acid because accumulation of citrate as intermediate was not evident.

Based on the interface of 3,3,3-trifluoropropionic acid in metabolic mechanism and its structural relationship to propionic acid, the following was considered (c.f. Scheme 15). The presence of 3,3,3-trifluoropropionic acid in the organism may result in the inhibition of the gluconeogenic pathway via propionic acid. Propionic acid, a non-substituted short-chain carboxylic acid, is together with lactate and glycerol, a substrate of the gluconeogenic pathway. Gluconeogenesis provides the organism with glucose if a lack of carbohydrates arises. This pathway prevents a failure in glucose supply to the brain and erythrocytes, preventing hypoglycemia, coma and death. The substrate propionate leads to glucose synthesis by entering the TCA cycle at the level of succinate and forms glucose via oxalacetate. This mechanism was considered as possible adaptive mechanism but also interacted by 3,3,3-tri-fluoropropionic acid in the TCA cycle. Elevated levels of propionic acid are a known inhibitor of the TCA cycle as shown by Gregersen (1979). Propionyl-CoA inhibited in a direct way the multi-enzyme complex of 2-ketoglutarate dehydrogenase, which was followed by a decrease of oxalacetate, an intermediate of the TCA cycle (Gregersen, 1979).



Scheme 15: Metabolism of propionic acid in humans (Murray et al., 2003).

6.4 CYTOTOXICITY OF 3,3,3-TRIFLUOROPROPIONIC ACID

Mechanistic studies *in vitro* have been performed to understand the cardiotoxic effect of 3,3,3-trifluoropropionic acid. The trifluoromethyl-group is among the most lipophilic groups of all substances and therefore the penetration across biological membranes is assumed to be enhanced and therefore interactions of specific binding to either, enzymes or receptors (Park et al., 2001), were considered. The mode of action of 3,3,3-trifluoropropionic acid was compared to known examples like the anthracycline doxorubicin, which is a known cardiotoxic compound. Anthracyclines in general induce acute clinical toxicity as well as cardiomyopathy after chronic administration. It has been observed, that doxorubicin induces oxidative stress and mitochondrial-mediated apoptosis, as well as adaptive responses by the mitochondria to protect cardiac myocytes *in vivo*. The *in vitro* test system in isolated rat cardiomyocytes, showed therefore an effect of doxorubicin on viability, morphology, oxygen consumption and ATP depletion by the collapse of the membrane integrity potential (Jung et al., 2001). Although the metabolism of anthracyclines is poorly understood, the lipophilicity seems to play an important role in the toxic endpoint. The more lipophilic the compound, the more cardiotoxicity is observed (Andersson et al., 1999). However, the *in vitro* assays with 3,3,3-trifluoropropionic acid showed no cytotoxic effects. The viability, proliferation of cells and the membrane activity were not affected in the experiments. A small number of initial experiments with primary cultured neonatal rat cardiomyocytes showed a reversible negative chronotrophic effect on the contractile function of cardiomyocytes, but were without an effect of cytotoxicity, tested with the release of LDH into the cytosol.

Furthermore, the ß-oxidation in particular was investigated, because of the heart being considered as the prospective target organ, and herewith the affection of mitochondria. Various disorders in mitochondria with far-reaching consequences are well known. The interferences of chemicals result either in the inhibition of ß-oxidation, the inhibition of the oxidative phosphorylation, the inhibition of the carnitine shuttle or in the decreased activity of the enzyme pyruvate carboxylase. All named cases lead to severe impairment of the organism. Known compounds that interfere with the ß-oxidation in mitochondria include hypoglycin (Sherratt, 1986),

4-pentenoic acid (Schulz, 1987; Holland et al., 1973), amineptine (Le Dinh et al., 1988), tianeptine (Fromenty et al., 1989) tetracycline (Freneaux et al., 1988), valproic acid (Tang et al., 1995) and pirprofen (Geneve et al., 1987). The inhibition of the ß-oxidation as observed in experiments with 4-pentenoic, was not confirmed for 3,3,3-trifluoropropionic acid. However, in common was the decrease in blood glucose, as described in literature. This was explained with the decreased hepatic gluconeogenesis which recovered after three hours. An additional effect of the above mentioned compounds was the high excretion of ketone bodies. The similar findings observed, indicate a similar mode of action for 3,3,3-trifluoropropionic acid. There are two mechanisms for increasing plasma levels of ketone bodies described in literature for these compounds. First, the increase of ketone bodies after treatment with amineptine, tianeptine and tetracycline was described as result of the inhibition of the TCA cycle. Secondly, in contrast, the increase of ketone bodies after treatment with hypoglycin and 4-pentenoic acid was rationalized by a decreased peripheral utilisation of ketone bodies. In this case, the decrease in the hepatic formation rate was less than the decrease in the peripheral utilization. The accumulation of ketone bodies resulted in ketoacidosis, as seen in the coma diabeticum of diabetic patients, which can not be excluded for the toxic effect of 3,3,3-trifluoropropionic acid.

6.5 CONCLUSION

Treatment related effects were differentiated from chance findings and individual biological variability and the issue of adaptive response was addressed. Criteria for the evaluation were a dose-response relationship, the magnitude of effects, and characterization of the endpoint. The mode of action was not understood but biological plausibility was given when considering the secondary effects. These were considered as an adaptive response, followed by different outcomes. Besides the severe effects a transient response was also observed in some animals. However, the demarcation for adverse effects, secondary effects and adaptive responses was not definable. The decreased level of blood glucose was considered as the main systemic effect being the consequence of the adverse effect on various points of biochemical pathways, rather than a direct effect of 3,3,3-trifluoropropionic acid to a single point of reaction.

7 SUMMARY

Investigations were performed *in vivo* and *in vitro* to elucidate the metabolic fate and the cardiotoxicity of the hydrofluorocarbon 1,1,1,3,3-pentafluoropropane. In addition, the identification and interactions of reactive intermediates were investigated. Hydrofluorocarbons are widely used in everyday life and are therefore among the most thoroughly tested chemicals. Characteristically they have a low toxicity, they are non-flammable, recyclable, highly energy efficient and are not capable of depleting the ozone layer.

The biotransformation of 1,1,1,3,3-pentafluoropropane was investigated in rats and in *in vitro* systems. First, the metabolites were identified *in vivo* using GC/MS and ^{19}F-NMR analysis. The main metabolite was identified as trifluoroacetic acid, the minor metabolite as 3,3,3-trifluoropropionic acid and as a cleavage product, inorganic fluoride was found. As the *in vitro* system, liver microsomes from rat and human samples and rat liver homogenates were used. Trifluoroacetic acid and 3,3,3-trifluoropropionic acid were confirmed *in vitro* as metabolic intermediates, following biotransformation of 1,1,1,3,3-pentafluoropropane by the cytochrome P-450-system.

Studies, designed for clarifying the cardiotoxicity of 1,1,1,3,3-pentafluoropropane were driven by the hypothesis that 3,3,3-trifluoropropionic acid is the toxic agent. This was based on the lethal toxicity of 3,3,3-trifluoropropionic acid, which was observed in previous *in vivo* experiments. In addition, the point of its structural similarity to toxic agents as for example monofluoroacetic acid or of possible metabolic intermediates like difluoroacrylic acid with known toxicity were considered to support this assumption. However, trifluoroacetic acid was neglected as the sought-after toxic agent because of its different toxic effects, known from literature.

In the following, investigations on the biotransformation of 3,3,3-trifluoropropionic acid were performed *in vivo* and *in vitro*. They resulted in no metabolic activity and in poor elimination of 3,3,3-trifluoropropionic acid *in vivo*. The histopathological effects on the heart, which were observed in the 90-day oral toxicity study of 1,1,1,3,3-pentafluoropropane in rats, namely mononuclear inflammatory cell infiltrations and degenerated myocardial fibers, were not observed after a 28-day repeated exposure of up to 10 mg/kg b.w. of 3,3,3-trifluoropropionic acid. In contrast, however, a single

high dose of 3,3,3-trifluoropropionic acid lead to severe toxicological effects, which were not observed in the 28-day repeated dose toxicity study. The difference in the observed toxic effects after a single and repeated administration may be due to adaptive mechanisms in rats. The toxicological effects seen included clinical signs like ataxia, coma and cramps. The conditions of the rats suggested possible inhibition of the energy supply to the organism. This lead to the assumption of impairment of either the glycolysis pathway, the ß-oxidation of fatty acids, the TCA cycle or the respiratory chain. Furthermore, the interference of 3,3,3-trifluoropropionic acid in the functionality of the organism was investigated. Experiments were performed *in vitro* in rat liver and heart mitochondria to investigate effects on the mitochondrial ß-oxidation. However, the transformation of the substrate [$U^{14}C$] palmitic acid in the ß-oxidation pathway was not inhibited by 3,3,3-trifluoropropionic acid. In addition, no cytotoxicity of 3,3,3-trifluoropropionic acid was observed in the cell culture systems HepG2, a human hepatocellular carcinoma cell line, and H9C2, a cardiac muscle like cell line. Assays with the endpoints for cytotoxicity were the leakage of LDH, a parameter for membrane integrity, the MTT reductase activity, representing metabolic active cells, and the crystal violet assay, representing viability of adherent cells.

The main effect however, after a single dose of 3,3,3-trifluoropropionic acid was seen in clinical pathology and metabonomic analysis. The noteworthy decrease in blood glucose is considered to have the most far-reaching consequences for the toxicity of 3,3,3-trifluoropropionic acid. If considering this change as the primary effect after a single dose, secondary effects, for example, the above-mentioned clinical signs could be explained. In addition, the observed high level of ketone bodies might have been responsible for life-threatening possible ketoacidosis. In general, ketoacidosis occurs after an imbalance between glycolysis, lipolysis, TCA cycle activity and respiratory function. Based on the results, ß-oxidation of fatty acids was not affected, and due to the decrease in glucose levels and the high levels of acetyl CoA, glycolysis was considered not to be impaired. Increased amounts of acetyl CoA might be a result of insufficient activity of the TCA cycle. However, the inhibition of the TCA cycle can be based on the impairment of specific enzymes and/or on the involvement of messenger substrates like insulin. Supporting the first mentioned aspect are decreased levels of TCA cycle intermediates, like α-ketoglutarate or citrate, as seen in ^{1}H-NMR spectra of urine. However, the second aspect would explain the drop in

blood glucose with the impairment of glucose transporters or the impairment of the insulin balance. If a single dose of 3,3,3-trifluoropropionic acid had stimulated the insulin release, glycolysis would be activated, and high amounts of acetyl CoA would be produced. In case of impaired use by the TCA cycle, levels of ketone bodies would be increased. Finally, less produced ATP would not be sufficient to keep the functions of the organism alive.

Experiments were designed to characterize the direct effect of 3,3,3-trifluoropropionic acid on rat insulinoma-derived INS-1 cells as possible increase in insulin release. Further investigations are necessary to answer in which step of the metabolic pathway 3,3,3-trifluoropropionic acid interferes or finally which specific enzyme is inhibited or activated by 3,3,3-trifluoropropionic acid, leading to the drop in blood glucose and finally in lethal toxicity.

8 ZUSAMMENFASSUNG

Mit dem Ziel, die Kardiotoxizität von 1,1,1,3,3-Pentafluorpropan, einem Hydrogenfluorkohlenwasserstoff zu erklären, wurden Untersuchungen zu dessen Metabolisierung *in vitro* und *in vivo* durchgeführt. Hierbei wurden die Metabolite identifiziert und ihre Rolle als mögliche reaktive Zwischenverbindungen untersucht. Die Gruppe der Hydrogenfluorkohlenwasserstoffe ist eine Chemikalienklasse die sehr ausgiebig in ihren Eigenschaften getestet worden ist, da sie in einem breiten und bedeutenden Bereich Anwendung findet, allem voran, im täglichen Gebrauch. Die vorteilhaften Eigenschaften der Hydrogenfluorkohlenwasserstoffe sind ihre nicht-Brennbarkeit, Wiederverwertbarkeit, das fehlende Ozonabbaupotential, der energieeffiziente Einsatz, und die dem gegenüberstehende geringe Toxizität.

Die Biotransformation von 1,1,1,3,3-Pentafluorpropan wurde in Ratten und in *in vitro* Systemen untersucht. Die in der Ratte mit dem Urin ausgeschiedenen Metabolite wurden per GC/MS und ^{19}F-NMR identifiziert. Als Hauptmetabolit entstand Trifluoressigsäure, als Nebenmetabolit 3,3,3-Trifluorpropionsäure und als Abspaltungsprodukt Fluorid. Als in *in vitro* Systeme wurden Ratten- und Humanlebermikrosomen, sowie Rattenleberhomogenate verwendet. Auch hier wurden Trifluoressigsäure und 3,3,3-Trifluorpropionsäure als metabolische Zwischenprodukte identifiziert.

Weitere *in vivo* Studien wurden durchgeführt um die beobachtete subchronische Kardiotoxizität von 1,1,1,3,3-Pentafluorpropan zu erklären. Da vorangegangene Experimente eine hohe letale Toxizität des Metaboliten 3,3,3-Trifluorpropionsäure zeigten, wurde dieser als das toxische Agens hypothetisiert. Diese Annahme unterstützend, ist dessen strukturelle Ähnlichkeit zu Substanzen mit bekannten toxischen Profilen wie Monofluoressigsäure oder Difluoracrylsäure, ein mögliches entstehendes Intermediat. Der Hauptmetabolit Trifluoressigsäure jedoch, wurde auf Grund seiner bekannten Toxizität als initiierendes Agens der Kardiotoxizität ausgeschlossen.

In vivo Untersuchungen mit 3,3,3-Trifluorpropionsäure zeigten jedoch keine weitere metabolische Aktivität und eine geringe renale Ausscheidung an 3,3,3-Trifluorpropionsäure. Nach einer einmalig hohen Dosis von 3,3,3-Trifluorpropionsäure traten

markante Symptome wie Ataxie, komatöse Zustände und Krämpfe auf. Dies deutete auf eine Beeinträchtigung des Energiezustandes des Organismus hin. Die histopathologischen Veränderungen des Herzens, mononukleäre Infiltrate von Entzündungszellen und degenerierte Myokard-Fasern, die für 1,1,1,3,3-Pentafluorpropan in einer 90-Tages Studie in Nagern beobachtet wurden, konnten jedoch nicht nach 28-tägiger Exposition mit 10 mg/kg Körpergewicht 3,3,3-Trifluorpropionsäure beobachtet werden. Die unterschiedlichen Effekte die nach einmaliger und wiederholter Gabe beobachtet wurden, lassen sich durch mögliche Adaptionsprozesse erklären, die initiale Schädigungen kompensieren. Die folgenden Experimente waren auf die funktionelle Beeinflussung des Organismus durch 3,3,3-Trifluorpropionsäure ausgerichtet. Ansatzpunkte hierbei waren die Beeinträchtigung der Glykolyse, der ß-Oxidation von Fettsäuren, des Zitronensäurezyklus oder der Atmungskette. *In vitro* Untersuchungen in Mitochondrien von Rattenleber und –herz mit dem Umsatz des Modellsubstrates [U^{14}C] Palmitinsäure zeigten jedoch keine Inhibierung der ß-Oxidation durch 3,3,3-Trifluorpropionsäure. Zytotoxizitätsassays wurden im Weiteren in der human-hepatonom Zell-Linie HepG2, und in der kardialen Muskelzell-Linie H9C2 mit folgenden Endpunkten durchgeführt: die Freisetzung von LDH, ein Parameter für die Membranintegrität, die MTT Reduktase Aktivität, ein Parameter für die metabolische Aktivität, und die Messung von Kristallviolett, ein Parameter für die Viabilität von Zellen. Für 3,3,3-Trifluorpropionsäure konnte jedoch keine Zytotoxizität beobachtet werden.

Deutliche Effekte wurden hingegen in der klinischen Pathologie und anhand Metabonomics beobachtet. Diese bestanden vor allem in der Abnahme der Glukosekonzentration im Blut, das weitreichende Konsequenzen mit sich führt, und als primärer Effekt die sekundären Effekte wie die beobachtenden klinischen Symptome erklären könnte. Ein weiterer Schlüsselparameter war die Erhöhung von Ketonkörpern in Urin und Serum, welche für eine lebensbedrohliche mögliche Ketoazidosis verantwortlich sein kann und durch ein Ungleichgewicht im Energiehaushalt ausgelöst werden kann. Da eine Beeinträchtigung der ß-Oxidation und der Glykolyse ausgeschlossen wurde, könnte das erhöhte Vorkommen von Acetyl-CoA auf eine limitierte Aktivität des Zitronensäurezyklus hindeuten. Grund hierfür kann die Inhibierung von beteiligten Enzymen sein oder auch der Einfluß von Messenger-Substraten wie Insulin. Ersteres wurde untermauert mit der Beobachtung in ^{1}H-NMR Spektren von Urin mit erhöhten Mengen an Intermediaten des

Zitronensäurezyklus, wie α-Ketoglutarate oder Zitrat. Letzteres würde den Abfall der Blutglukose, basierend auf Beeinflussung von Glukosetransportern oder des Insulinhaushaltes, erklären. Wenn 3,3,3-Trifluorpropionsäure die Freisetzung von Insulin stimulieren würde, würde der Abbau von Glukose aktiviert werden und erhöhte Mengen an Acetyl-CoA resultieren. Wenn gleichzeitig eine Beeinträchtigung des Zitronensäurezyklus vorliegt, kann dies zu einer Erhöhung an Ketonkörpern führen. Zusätzlich ergibt sich eine verminderte ATP-Bildung, die letztendlich nicht zur Aufrechterhaltung der Körperfunktionen ausreicht.

Experimente zur Messung des Insulin- und Glukosespiegels wurden *in vivo* und *in vitro* mit der Ratteninsulinoma Zell-Linie INS-1 durchgeführt, um den Effekt von 3,3,3-Trifluorpropionsäure auf die Freisetzung von Insulin zu charakterisieren. Basierend auf diesen Ergebnissen sind weitere Untersuchungen erforderlich um den Metabolismus von 3,3,3-Trifluorpropionsäure, sowie dessen mögliche Interaktion mit Enzymen oder Rezeptoren aufzuklären, und um den raschen Glukoseabfall im Blut zu erklären, der zu einer letalen Toxizität führen kann.

9 REFERENCES

AFEAS (1995). Report on production, sales and atmospheric release of Fluorocarbons, 1995. Alternative Fluorocarbon Environmental Acceptability Study.

American Society of Refrigerating Engineers (1957). Proposed ASRE standard 34. *Refrig Eng* **65**, 49-52.

Anders, M. W. (1991). Metabolism and toxicity of hydrochlorofluorocarbons: current knowledge and needs for the future. *Environ Health Perspect* **96**, 185-191.

Andersson, B. S., Eksborg, S., Vidal, R. F., Sundberg, M., and Carlberg, M. (1999). Anthraquinone-induced cell injury: acute toxicity of carminomycin, epirubicin, idarubicin and mitoxantrone in isolated cardiomyocytes. *Toxicology* **135**, 11-20.

Arendt, C. W., Hsi, G., and Ostergaard, H. L. (1995). Immobilized antibodies to CD45 induce rapid morphologic changes and increased tyrosine phosphorylation of p56lck-associated proteins in T cells. *J Immunol* **155**, 5095-5103.

Ballard, C. L., and Hyde, P. M. (1967). Effect of insulin on blood glucose and corticosterone levels in sodium fluoroacetate induced diabetes. *Proc Soc Exp Biol Med* **124**, 317-320.

Beckwith-Hall, B. M., Nicholson, J. K., Nicholls, A. W., Foxall, P. J., Lindon, J. C., Connor, S. C., Abdi, M., Connelly, J., and Holmes, E. (1998). Nuclear magnetic resonance spectroscopic and principal components analysis investigations into biochemical effects of three model hepatotoxins. *Chem Res Toxicol* **11**, 260-272.

Berends, A. G., Boutonnet, J. C., de Rooij, C. G., and Thompson, R. S. (1999). Toxicity of Trifluoroacetate to Aquatic Organisms. *Environmental Toxicology and Chemistry* **18**, 1053-1059.

Bernhard, S. A., and Hammett, L. P. (1952). Specific effects in acid catalysis by ion exchange resins. I. Hydrolysis of esters in 70% aqueous acetone. **75**, 1798-1800.

Bhattacharya, R., Rao, P. V., and Vijayaraghavan, R. (2002). In vitro and in vivo attenuation of experimental cyanide poisoning by alpha-ketoglutarate. *Toxicol Lett* **128**, 185-195.

Billington, D., Osmundsen, H., and Sherratt, H. S. (1978). Mechanisms of the metabolic disturbances caused by hypoglycin and by pent-4-enoic acid. In vitro studies. *Biochem Pharmacol* **27**, 2879-2890.

Boor, P. J., and Ferrans, V. J. (1982). Ultrastructural alterations in allylamine-induced cardiomyopathy: early lesions. *Lab Invest* **47**, 76-86.

Boutonnet, J. C. (1999). Environmental Risk Assessment of Trifluoroacetic Acid. *Human and Ecological Risk Assessment* **5**, 59.

Bryant, B. J., Jokinen, M. P., Eustis, S. L., Thompson, M. B., and Abdo, K. M. (1992). Toxicity of monochloroacetic acid administered by gavage to F344 rats and B6C3F1 mice for up to 13 weeks. *Toxicology* **72**, 77-87.

Canton, M., Gennari, F., Luvisetto, S., and Azzone, G. F. (1996). The nature of uncoupling by n-hexane, 1-hexanethiol and 1-hexanol in rat liver mitochondria. *Biochim Biophys Acta* **1274**, 39-47.

Chen, Q., and Cederbaum, A. I. (1998a). Cytotoxicity and apoptosis produced by cytochrome P450 2E1 in Hep G2 cells. *Mol Pharmacol* **53**, 638-648.

Chen, Q., Galleano, M., and Cederbaum, A. I. (1998b). Cytotoxicity and apoptosis produced by arachidonic acid in HepG2 cells overexpressing human cytochrome P-4502E1. *Alcohol Clin Exp Res* **22**, 782-784.

Childs, A. C., Phaneuf, S. L., Dirks, A. J., Phillips, T., and Leeuwenburgh, C. (2002). Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2:Bax ratio. *Cancer Res* **62**, 4592-4598.

Collins, M. A., Rusch, G. M., Sato, F., Hext, P. M., and Millischer, R. J. (1995). 1,1,1,2-Tetrafluoroethane: repeat exposure inhalation toxicity in the rat, developmental toxicity in the rabbit, and genotoxicity in vitro and in vivo. *Fundam Appl Toxicol* **25**, 271-280.

Croes, K., Casteels, M., De Hoffmann, E., Mannaerts, G. P., and Van Veldhoven, P. P. (1996a). alpha-Oxidation of 3-methyl-substituted fatty acids in rat liver. Production of formic acid instead of CO2, cofactor requirements, subcellular localization and formation of a 2-hydroxy-3-methylacyl-CoA intermediate. *Eur J Biochem* **240**, 674-683.

Croes, K., Casteels, M., Van Veldhoven, P. P., and Mannaerts, G. P. (1996b). Alpha-oxidation in intact and permeabilized rat hepatocytes. *Ann N Y Acad Sci* **804**, 682-683.

Decker, T., and Lohmann-Matthes, M. L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* **115**, 61-69.

Dekant, W., Assmann, M., and Urban, G. (1995). The role of cytochrome P450 2E1 in the species-dependent biotransformation of 1,2-dichloro-1,1,2-trifluoroethane in rats and mice. *Toxicol Appl Pharmacol* **135**, 200-207.

Dekant, W., and Vamvakas, S. (1995). *Toxikologie für Chemiker und Biologen*. Spektrum Akademischer Verlag, Heidelberg, Berlin, Oxford.

Dekant, W. (1996a). Biotransformation and renal processing of nephrotoxic agents. *Arch Toxicol Suppl* **18**, 163-172.

Dekant, W. (1996b). Toxicology of chlorofluorocarbon replacements. *Environ Health Perspect* **104 Suppl 1**, 75-83.

Ellis, M. K., Gowans, L. A., Green, T., and Tanner, R. J. (1993). Metabolic fate and disposition of 1,1,1,2-tetrafluoroethane (HFC134a) in rat following a single exposure by inhalation. *Xenobiotica* **23**, 719-729.

Engel, F. L., Fredericks, J., and Cole, B. T. (1957). Sodium fluoroacetate diabetes; insulin and carbohydrate tolerance and the antiketogenic effects of glucose and fructose. *Endocrinology* **60**, 446-459.

Estabrook, R. W. (1967). Mitochondrial Respiratory Control and the Polarographic Measurement of ADP:O Ratios. *Meth. Enzymol.* **10**, 41-47.

Feldwick, M. G., Mead, R. J., and Kostyniak, P. J. (1994). The biochemical toxicology of fluoroacetate and related pesticides: the potential of 4-methylpyrazole as an antidote. *Workshop* **28**, 74-81.

Feldwick, M. G., Noakes, P. S., Prause, U., Mead, R. J., and Kostyniak, P. J. (1998). The biochemical toxicology of 1,3-difluoro-2-propanol, the major ingredient of the pesticide gliftor: the potential of 4-methylpyrazole as an antidote. *J Biochem Mol Toxicol* **12**, 41-52.

Fischer, R. T., Trzaskos, J. M., Magolda, R. L., Ko, S. S., Brosz, C. S., and Larsen, B. (1991) Lanosterol 14-a-methyl demethylase. Isolation and characterization of the third metabolically generated oxidative demethylation intermediate. *J. Biol. Chem.* **266**, 6124-6132.

Fitzsimmons, M. E., and Anders, M. W. (1993). Fatty acid beta-oxidation-dependent bioactivation of halogenated thiaalkanoic acids in isolated rat hepatocytes. *Chem Res Toxicol* **6**, 662-668.

Forkert, P. G. (2001). Mechanisms of 1,1-dichloroethylene-induced cytotoxicity in lung and liver. *Drug Metab Rev* **33**, 49-80.

Foulon, V., Antonenkov, V. D., Croes, K., Waelkens, E., Mannaerts, G. P., Van Veldhoven, P. P., and Casteels, M. (1999). Purification, molecular cloning, and expression of 2-hydroxyphytanoyl- CoA lyase, a peroxisomal thiamine pyrophosphate-dependent enzyme that catalyzes the carbon-carbon bond cleavage during alpha-oxidation of 3- methyl-branched fatty acids. *Proc Natl Acad Sci U S A* **96**, 10039-10044.

Frank, H., Klein, A., and Renschen, D. (1996). Environmental Trifluoroacetate. *Nature* **382**, 34.

Freneaux, E., Labbe, G., Letteron, P., The Le, D., Degott, C., Geneve, J., Larrey, D., and Pessayre, D. (1988). Inhibition of the mitochondrial oxidation of fatty acids by tetracycline in mice and in man: possible role in microvesicular steatosis induced by this antibiotic. *Hepatology* **8**, 1056-1062.

Fromenty, B., Freneaux, E., Labbe, G., Deschamps, D., Larrey, D., Letteron, P., and Pessayre, D. (1989). Tianeptine, a new tricyclic antidepressant metabolized by beta-oxidation of its heptanoic side chain, inhibits the mitochondrial oxidation of medium and short chain fatty acids in mice. *Biochem Pharmacol* **38**, 3743-3751.

Geneve, J., Hayat-Bonan, B., Labbe, G., Degott, C., Letteron, P., Freneaux, E., Dinh, T. L., Larrey, D., and Pessayre, D. (1987). Inhibition of mitochondrial beta-oxidation of fatty acids by pirprofen. Role in microvesicular steatosis due to this nonsteroidal anti-inflammatory drug. *J Pharmacol Exp Ther* **242**, 1133-1137.

Gough, J. H., and Sutherland, M. D. (1971). Ethyl b,b,b-Trichloropropionate. *Aust J Chem* **24**, 1737-1738.

Gregersen, N. (1979). Studies on the effects of saturated and unsaturated short-chain monocarboxylic acids on the energy metabolism of rat liver mitochondria. *Pediatr Res* **13**, 1227-1230.

Gribble, G. W. (1973). Fluoroacetate toxicity. *J Chem Educ* **50**, 460-462.

Guengerich, F. P., Kim, D.-H., and Iwasai, M. (1991). Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* **4**, 168-179.

Guengerich (2001). Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity. *Chemical Research in Toxicology* **14**, 612-650.

Hanawa, H., Inomata, T., Sekikawa, H., Abo, T., Kodama, M., Izumi, T., and Shibata, A. (1996). Analysis of heart-infiltrating T-cell clonotypes in experimental autoimmune myocarditis in rats. *Circ Res* **78**, 118-125.

Harris J.W., A. M. W. (1991). Metabolism of the Hydrochlorofluorocarbon 1,2-Dichloro-1,1-difluoroethane. *Chem Res Toxicol* **4**, 180-186.

Harris, J. W., Jones, J. P., Martin, J. L., LaRosa, A. C., Olson, M. J., Pohl, L. R., and Anders, M. W. (1992). Pentahaloethane-based chlorofluorocarbon substitutes and halothane: correlation of in vivo hepatic protein trifluoroacetylation and urinary trifluoroacetic acid excretion with calculated enthalpies of activation. *Chem Res Toxicol* **5**, 720-725.

Henschler, D., Hoos, W. R., Fetz, H., Dallmeier, E., and Metzler, M. (1979) Reactions of trichloroethylene epoxide in aqueous systems. *Biochem. Pharmacol.* **28**, 543-548.

Herbst, J., Koster, U., Kerssebaum, R., and Dekant, W. (1994). Role of P4502E1 in the metabolism of 1,1,2,2-tetrafluoro-1-(2,2,2-trifluoroethoxy)-ethane. *Xenobiotica* **24**, 507-516.

Hoet, P., Graf, M. L., Bourdi, M., Pohl, L. R., Duray, P. H., Chen, W., Peter, R. M., Nelson, S. D., Verlinden, N., and Lison, D. (1997). Epidemic of liver disease caused by hydrochlorofluorocarbons used as ozone-sparing substitutes of chlorofluorocarbons. *Lancet* **350**, 556-559.

Hoet, P., Buchet, J. P., Sempoux, C., Nomiyama, T., Rahier, J., and Lison, D. (2001). Investigations on the liver toxicity of a blend of HCFC-123 (2,2-dichloro-1,1,1-trifluoroethane) and HCFC-124 (2-chloro-1,1,1,2-tetrafluoroethane) in guinea-pigs. *Arch Toxicol* **75**, 274-283.

Holland, P. C., Senior, A. E., and Sherratt, H. S. (1973). Biochemical effects of the hypoglycaemic compound pent-4-enoic acid and related non-hypoglycaemic fatty acids. Effects of their coenzyme A esters on enzymes of fatty acid oxidation. *Biochem J* **136**, 173-184.

Houghton, J., Filho, L. M., Callender, B., Harris, N., Kattenberg, A., and Maskell, K. (1996). *Climate Change 1995: The Science of Climate Change*. Cambridge University Press, Cambridge.

Hue, L., and Sherratt, H. S. (1986). Inhibition of gluconeogenesis by hypoglycin in the rat. Evidence for inhibition of glucose-6-phosphatase in vivo. *Biochem J* **240**, 765-769.

Jaeschke, H., Gores, G. J., Cederbaum, A. I., Hinson, J. A., Pessayre, D., and Lemasters, J. J. (2002). Mechanisms of hepatotoxicity. *Toxicol Sci* **65**, 166-176.

James, E. A., Gygi, S. P., Adams, M. L., Pierce, R. H., Fausto, N., Aebersold, R. H., Nelson, S. D., and Bruschi, S. A. (2002). Mitochondrial aconitase modification, functional inhibition, and evidence for a supramolecular complex of the TCA cycle by the renal toxicant S-(1,1,2,2-tetrafluoroethyl)-L-cysteine. *Biochemistry* **41**, 6789-6797.

James, M. O., Yan, Z., Cornett, R., Jayanti, V. M., Henderson, G. N., Davydova, N., Katovich, M. J., Pollock, B., and Stacpoole, P. W. (1998). Pharmacokinetics and metabolism of [14C]dichloroacetate in male Sprague- Dawley rats. Identification of glycine conjugates, including hippurate, as urinary metabolites of dichloroacetate. *Drug Metab Dispos* **26**, 1134-1143.

Jung, K., and Reszka, R. (2001). Mitochondria as subcellular targets for clinically useful anthracyclines. *Adv Drug Deliv Rev* **49**, 87-105.

Kawano, T., Trochimowicz, H. J., Malinverno, G., and Rusch, G. M. (1995). Toxicological evaluation of 1,1,1,2,2-pentafluoroethane (HFC-125). *Fundam Appl Toxicol* **28**, 223-231.

Kharasch, E. D., Hankins, D. C., Fenstamaker, K., and Cox, K. (2000). Human halothane metabolism, lipid peroxidation, and cytochromes P(450)2A6 and P(450)3A4. *Eur J Clin Pharmacol* **55**, 853-859.

Kolesar, G. B., Siddiqui, W. H., Crofoot, S. D., Evans, M. G., and Meeks, R. G. (1995). Subchronic inhalation toxicity of 1,1,1,3-tetrachloropropane in rats. *Fundam Appl Toxicol* **25**, 52-59.

Koop, D. R. (1986). Hydroxylation of p-nitrophenol by rabbit ethanol-inducible cytochrome P- 450 isozyme 3a. *Mol Pharmacol* **29**, 399-404.

Koop, D. R. (1992). Oxidative and reductive metabolism by cytochrome P450 2E1. *FASEB J* **6**, 724-730.

Korzeniewski, C., and Callewaert, D. M. (1983). An enzyme-release assay for natural cytotoxicity. *J Immunol Methods* **64**, 313-320.

Koster, U., Speerschneider, P., Kerssebaum, R., Wittmann, H., and Dekant, W. (1994). Role of cytochrome P450 2E1 in the metabolism of 1,1,2,3,3,3-hexafluoropropyl methyl ether. *Drug Metab Dispos* **22**, 667-672.

Kuhl, U., Seeberg, B., Schultheiss, H. P., and Strauer, B. E. (1994). Immunohistological characterization of infiltrating lymphocytes in biopsies of patients with clinically suspected dilated cardiomyopathy. *Eur Heart J* **15 Suppl C**, 62-67.

Kuhl, U., Noutsias, M., and Schultheiss, H. P. (1995). Immunohistochemistry in dilated cardiomyopathy. *Eur Heart J* **16 Suppl O**, 100-106.

Le Dinh, T., Freneaux, E., Labbe, G., Letteron, P., Degott, C., Geneve, J., Berson, A., Larrey, D., and Pessayre, D. (1988). Amineptine, a tricyclic antidepressant, inhibits the mitochondrial oxidation of fatty acids and produces microvesicular steatosis of the liver in mice. *J Pharmacol Exp Ther* **247**, 745-750.

Lieber, C. S. (1997). Cytochrome P-4502E1: its physiological and pathological role. *Physiol Rev* **77**, 517-544.

Lindon, J. C. (1999). *NMR Spectroscopy of Biofluids*. Academic Press.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265-275.

Malley, L. A., Carakostas, M. C., Hansen, J. F., Trochimowicz, H. J., and Rusch, G. M. (1991) Chronic toxicity of hydrochlorofluorocarbon HCFC-123. *Toxicologist* **11**, 103.

Malley, L. A., Carakostas, M., Hansen, J. F., Rusch, G. M., Kelly, D. P., and Trochimowicz, H. J. (1995). Two-year inhalation toxicity study in rats with hydrochlorofluorocarbon 123. *Fundam Appl Toxicol* **25**, 101-114.

Manno, M., Ferrara, R., Cazzaro, S., Rigotti, P., and Ancona, E. (1992). Suicidal inactivation of human cytochrome P-450 by carbon tetrachloride and halothane in vitro. *Pharmacology and Toxicology* **70**, 13-18.

Marquardt, H., and Schäfer, S. (2004). *Lehrbuch der Toxikologie.* Wissenschaftliche Verlagsgesellschaft mbH Stuttgart.

Miller, R. E., and Guengerich, F. P. (1982) Oxidation of trichloroethylene by liver microsomal cytochrome P-450: evidence for chlorine migration in a transition state not involving trichloroethylene oxide. *Biochemistry* **21**, 1090-1097.

Molina, M., and Rowland, F. S. (1974). Stratospheric sink for chlorofluoromethanes: chlorine atome-catalysed destruction of the ozone. *Nature* **249**, 810 – 812.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55-63.

Muralidhara, S., and Bruckner, J. V. (1999). Simple method for rapid measurement of trichloroethylene and its major metabolites in biological samples. *J Chromatogr B Biomed Sci Appl* **732**, 145-153.

Murray, R., D. Granner, P. Mayes, and V. Rodwell (2003). *Harper's Illustrated Biochemistry*. Janet Foltin, Jim Ransom, and Janene Matragrano Oransky.

Naito, E. (2002). [Mitochondrial aconitase]. *Nippon Rinsho* **60 Suppl 4**, 133-134.
Nicholls, A. W., Holmes, E., Lindon, J. C., Shockcor, J. P., Farrant, R. D., Haselden, J. N., Damment, S. J., Waterfield, C. J., and Nicholson, J. K. (2001). Metabonomic investigations into hydrazine toxicity in the rat. *Chem Res Toxicol* **14**, 975-987.
NICNAS (2001). National Industrial Chemical Notification And Assessment Scheme: HFC-245fa (NA/920). *http://www.nicnas.gov.au/PUBLICATIONS/car/new/NA/NAFULLR/NA0900FR/NA920FR.pdf*
Nihlén, A., Löf, A., and Johanson, G. (1998). Experimental Exposure to Methyl *tertiary*-Butyl Ether. I. Toxicokinetics in Humans. *Toxicol Appl Pharmacol* **148**, 74-280.
Olson, M. J., Kim, S. G., Reidy, C. A., Johnson, J. T., and Novak, R. F. (1991). Oxidation of 1,1,1,2-tetrafluoroethane in rat liver microsomes is catalyzed primarily by cytochrome P-450IIE1. *Drug Metab Dispos* **19**, 298-303.
Park, B. K., Kitteringham, N. R., and O'Neill, P. M. (2001). Metabolism of fluorine-containing drugs. *Annu Rev Pharmacol Toxicol* **41**, 443-470.
Penzo, D., Tagliapietra, C., Colonna, R., Petronilli, V., and Bernardi, P. (2002). Effects of fatty acids on mitochondria: implications for cell death. *Biochim Biophys Acta* **1555**, 160-165.
Peters, R. A. (1952). Lethal synthesis. *Proc R Soc Lond B Biol Sci* **139**, 143-170.
Peters, R. A., and Shorthouse, M. (1969). Aconitase, a complex with two active centres. *Nature* **221**, 774-775.
Peterson, G. L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* **83**, 346-356.
Pumford, N. R., and Halmes, N. C. (1997) Protein targets of xenobiotic reactive intermediates. *Annu. Rev. Pharmacol. Toxicol.* **37**, 91-117.
Raucy, J. L., Kraner, J. C., and Lasker, J. M. (1993). Bioactivation of halogenated hydrocarbons by cytochrome P4502E1. *Crit Rev Toxicol* **23**, 1-20.
Rehncrona, S., Mela, L., and Siesjo, B. K. (1979) *Stroke* **10**, 437-446
Reichelt, H. (1979). What is fluoroacetate diabetes? *Z Gesamte Inn Med* **34**, 401-404.
Reichl, F.-X. (2002). *Taschenatlas der Toxikologie*. Georg Thieme Verlag, Stuttgart, New York.
Rinaldo, P. (2001). Fatty acid transport and mitochondrial oxidation disorders. *Semin Liver Dis* **21**, 489-500.
Rissolo, S.B., and J.A.Zapp. (1967). Acute inhalation toxicity. *Report* No. 190–67. Haskell Laboratory Wilmington, USA. (Cited in NRC 1996).
Ritchie, G. D., Kimmel, E. C., Bowen, L. E., Reboulet, J. E., and Rossi, J., 3rd (2001). Acute neurobehavioral effects in rats from exposure to HFC 134a or CFC 12. *Neurotoxicology* **22**, 233-248.
Robertson, D. G., Loewen, G., Walsh, K. M., Dethloff, L. A., Sigler, R. S., Dominick, M. A., and Urda, E. R. (1993). Subacute and subchronic toxicology studies of CI-986, a novel anti- inflammatory compound. *Fundam Appl Toxicol* **20**, 446-455.
Rusch, G. M., Trochimowicz, H. J., Malley, L. J., Kelly, D. P., Peckham, J., Hansen, J., and Charm, J. B. (1994). Subchronic inhalation toxicity studies with hydrochlorofluorocarbon 123 (HCFC 123). *Fundam Appl Toxicol* **23**, 169-178.
Rusch, G. M. (1997). Liver abnormalities and hydrochlorofluorocarbons. *Lancet* **350**, 1248-1249; discussion 1249-1250.

Rusch, G. M., Coombs, D., and Hardy, C. (1999). The acute, genetic, developmental, and inhalation toxicology of 1,1,1,3,3-pentafluoropropane (HFC 245fa). *Toxicol Sci* **52**, 289-301.

Sabbagh, E., Cuebas, D., and Schulz, H. (1985). 3-Mercaptopropionic acid, a potent inhibitor of fatty acid oxidation in the rat heart mitochondria. *The journal of Biological chemistry* **260**, 7337-7342.

Salmon, A.G., Nash J.A., Oliver M.F.S., and Reeve A. (1980). Arcton-134a: Excretion, Tissue Distribution, and Metabolism in the Rat. *ICI Rep*. No. CTL/P/513. Central Toxicology Laboratory, Imperial Chemical Industries, Alderley Park, Macclesfield, Cheshire, U.K.

Schroth, W., Schmiedl, D., Jahn, U., and Spitzner, R. (1989). Eine vereinfachte Herstellung von Bis(2,2-dichlor-vinyl)-keton. *Z Chem* **29**, 419-420.

Schulz, H. (1987). Inhibitors of fatty acid oxidation. *Life Sci* **40**, 1443-1449.

Sherratt, H. S. (1986). Hypoglycin, the famous toxin of the unrie Jamaican ackee fruit. *TIPS*, 186-191.

Silber, L.S., and G.L.Kennedy. (1979). Acute inhalation toxicity study of tetrafluoroethane (FC 134a). *Report* No. 422–79, Haskell Laboratory, DuPont de Nemours and Company, Newark, USA.

Singh, P., and Weinreb, S. M. (1976). An efficient total synthesis of maleimycin. *Tetrahedron* **32**, 2379-2380.

Smith, S. C., and Allen, P. M. (1991). Myosin-induced acute myocarditis is a T cell-mediated disease. *J Immunol* **147**, 2141-2147.

Solomon, K. R., Tang, X., Wilson, S. R., Zanis, P., and Bais, A. F. (2003). Changes in tropospheric composition and air quality due to stratospheric ozone depletion. *Photochem Photobiol Sci* **2**, 62-67.

Spaniol, M., Kaufmann, P., Beier, K., Wuthrich, J., Torok, M., Scharnagl, H., Marz, W., and Krahenbuhl, S. (2003). Mechanisms of liver steatosis in rats with systemic carnitine deficiency due to treatment with trimethylhydrazinium-propionate. *J Lipid Res* **44**, 144-153.

Spracklin, D. K., Hankins, D. C., Fisher, J. M., Thummel, K. E., and Kharasch, E. D. (1997). Cytochrome P450 2E1 is the principal catalyst of human oxidative halothane metabolism in vitro. *J Pharmacol Exp Ther* **281**, 400-411.

Stacpoole, P. W., Henderson, G. N., Yan, Z., Cornett, R., and James, M. O. (1998a). Pharmacokinetics, metabolism and toxicology of dichloroacetate. *Drug Metab Rev* **30**, 499-539.

Stacpoole, P. W., Henderson, G. N., Yan, Z., and James, M. O. (1998b). Clinical pharmacology and toxicology of dichloroacetate. *Environ Health Perspect* **106 Suppl 4**, 989-994.

Staudt, A., Schaper, F., Stangl, V., Plagemann, A., Bohm, M., Merkel, K., Wallukat, G., Wernecke, K. D., Stangl, K., Baumann, G., and Felix, S. B. (2001). Immunohistological changes in dilated cardiomyopathy induced by immunoadsorption therapy and subsequent immunoglobulin substitution. *Circulation* **103**, 2681-2686.

Stee, E. W. V. (1982). *Cardiovascular Toxicology*. Raven Press, NY.

Swinney, D. C., and Mak, A. Y. (1994) Androgen formation by cytochrome P450 CYP17. Solvent isotope effect and pL studies suggest a role for protons in the regulation of oxene versus peroxide chemistry. *Biochemistry* **33**, 2185-2190.

Tang, W., Borel, A. G., Fujimiya, T., and Abbott, F. S. (1995). Fluorinated analogues as mechanistic probes in valproic acid hepatotoxicity: hepatic microvesicular steatosis and glutathione status. *Chem Res Toxicol* **8**, 671-682.

Urban, G., and Dekant, W. (1994a). Metabolism of 1,1-dichloro-2,2,2-trifluoroethane in rats. *Xenobiotica* **24**, 881-892.

Urban G., and Dekant, W. (1994b). Metabolism of the Chlorofluorocarbon Substitute 1,1-Dichloro-2,2,2-trifluoroethane by Rat and Human Liver Microsomes: The Role of Cytochrome P450 2E1. *Chem Res Toxicol* **7**, 170-176.

Villani, G., and Attardi, G. (2001). In vivo measurements of respiration control by cytochrome c oxidase and in situ analysis of oxidative phosphorylation. *Methods Cell Biol* **65**, 119-131.

Yin, H., Anders, M. W., Korzekwa, K. R., Higgins, L., Thummel, K. E., Kharasch, E. D., and Jones, J. P. (1995). Designing safer chemicals: predicting the rates of metabolism of halogenated alkanes. *Proc Natl Acad Sci U S A* **92**, 11076-11080.

Yuan, C., and Acosta, D., Jr. (1996). Cocaine-induced mitochondrial dysfunction in primary cultures of rat cardiomyocytes. *Toxicology* **112**, 1-10.

Zehavi, D., and Seiber, J. N. (1996). An Analytical Method for Trifluoroacetic Acid in Water and Air Samples Using Headspace Gas Chromatographic Determination of the Methyl Ester. *Analytical Chemistry* **68**, 3450-3459.

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Erklärung

Hiermit erkläre an Eides statt, dass ich die Dissertation „Toxicity and Biotransformation of 1,1,1,3,3-Pentafluoropropane, 3,3,3-Trifluoropropionic acid and 1,1,1,3-Tetrachloropropane" selbstständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ich erkläre außerdem, dass diese Dissertation weder in gleicher oder anderer Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ich habe früher außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Gelterkinden, den 11.06.2005