Evaluation of the short interfering RNA method as a tool in mechanistic Toxicology

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1 Indices

Abbreviations

ADP	Adenosine-5'-diphosphate
ALT	Alanineaminotransferase (=GPT)
AMC	7-Amino-4-methylcoumarin
Ampicillin	6-[*D*-(-)-α-Amino-α-phenylacetamido]-penicillic acid
ANOVA	Analysis of Variance (between groups)
AST	Aspartateanimotransferase (=GOT)
ATP	Adenosine-5'-triphosphate
BrdU	5-Bromo-2'-deoxyuridine
BSA	Bovine Serum Albumine
BW	Body Weight
CDU	Collagenase Digestion Units
CFP	Cyan Fluorescent Protein
CZX	Chlorzoxazone
6-OH-CZX	6-Hydroxychlorzoxazone
$\Delta\Psi_m$	Mitochondrial Membrane Potential
DAPI	4´,6-Diamidino-2-phenylindole
DEVD	asparagine-gluatmine-valine- asparagine
Diclofenac	2-[(2,6-Dichlorophenyl)amino] benzeneacetic acid
DMEM	Dulbecco's modified Eagle-medium
DMSO	Dimethylsulfoxide
ds	double-stranded
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
eGFP	enhanced Green Fluorescent Protein
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
F.U.	Fluorescence Units
GFP	Green Fluorescent Protein

HBSS	Hank`s balanced salt solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
Hoechst 33342	2'-(4-Ethoxyphenyl)-5-(4-methyl-1- piperazinyl)-2,5'-Bi-1H-benzimidazoletrichlorid
HPLC	High Pressure Liquid Chromatography
HRPO	Horseradish Peroxidase
IEPD	isoleucine-glutamine-proline-asparagine
IFIT	Interferon induced protein with tetratricopeptide repeats 1
IFN	Interferon
IHC	Immunohistochemistry
ISG	Interferon-stimulated Gene
iST	internal standard
I.U.	International Units
JAK	Janus Kinase
LEHD	leucine-glutamine-histidine-asparagine
LDH	Lactate Dehydrogenase
L.U.	Light Units
MEM	Minimun Essential Medium
miRNA	Micro Ribonucleic Acid
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NAD^+	β-Nicotinamide Adenine Dinucleotide
NADH	β-Nicotinamide Adenine Dinucleotide, reduced
ncRNA	Non-coding Ribonucleic Acid
NF-κB	Nuclear Transcription Factor κB
NSAID	Non-steroidal Antiinflammtory Drug
OAS	2`,5`-Oligoadenylatsynthetase
PBS	Phosphate-buffered Saline
PKR	dsRNA-dependent protein kinase
PMS	Phenazine methosulfate
PMSF	Phenylmethanesulfonylfluorid
pNA	p-Nitroaniline
RdRP	RNA-dependent RNA Polymerase
Rhodamine 123	Xanthylium, 3,6-diamino-9-(2-(methoxycarbonyl) phenylchloride

RISC	RNAi-induced Silencing Complex
RNAi	Ribonucleic Acid Interference
SDS	Sodium dodecyl sulfate
SDZ IMM125	*o*-Hydroxyethyl-(D-Ser)^{8}-cyclosporine
siRNA	Short Interfering Ribonucleic Acid
shRNA	Short Hairpin Ribonucleic Acid
SNK	Student-Newman-Keuls (post-hoc test)
stRNA	Small Temporal Ribonucleic Acid
STAT	Signal Transducers and Activators of Transcription
Tris	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
VDVAD	valine-asparagine-valine-alanine-asparagine
YFP	Yellow Fluorescent Protein
Zoledronic Acid	[1-Hydroxy-2-(3H-imidazol-1-yl)-1-phosphonoethyl]-phosphonic acid

Raw data

All raw data acquired was documented in the Laboratory Notebooks E-15094, E-33670, E-34380, E-36041 and E-36578 at Novartis Pharma AG, Switzerland. The laboratory notebooks have been sent to the Novartis Archive for permanent and safe storage.

Figures

Tables

2 Introduction

2.1 The history of RNA interference

Ribonucleic acid interference (RNAi) is a phenomenon first described by van der Krol and Jorgensen [*Napoli et al., 1990; van der Krol, 1990; Jorgensen, 1990*], when the search for a transgenic petunia expected to be more purple resulted in many of the flowers being white. The underlying mechanism of RNAi was discovered in the nematode worm *Caenorhabditis elegans* by Fire and Mello [*Fire et al., 1998*] eight years later. This research group could demonstrate that injection of double-stranded RNA into worms led to inhibition of gene expression. In mammals RNAi was first described by Wianny and Zernicka-Goetz [*Wianny et al., 2000*].

RNAi is a cellular mechanism applying RNA-fragments to inhibit protein expression from complementary messenger RNA. The effect is called posttranscriptional gene silencing. The mechanism is in charge for two tasks in the cell: endogenous regulation of mRNA-translation and protection of the cell towards invasion of viruses and transposons. Endogenous regulation is mediated by micro RNAs (miRNAs, see 2.3) who lead to blockage of target mRNA translation. Viral defense is mediated by short interfering RNAs (siRNAs) who lead to degradation of the cytoplasmatic target mRNA. Invading double-stranded RNA is recognised by the cell as foreign genetic information and used as a template to eliminate this information before translation into protein and alteration of cellular functions. The foreign double-stranded RNA is processed to short fragments, the siRNAs. These fragments serve as a template for a complex being able to degrade homologous RNA molecules.

The mechanism of RNAi requires a set of proteins present in all eukaryotes (except the baker's yeast *saccharomyces cerevisiae*) but lacking in *archae* and prokaryotes, thus being probably a eukaryotic innovation [*Brummelkamp et al., 2003; Zamore, 2002*].

2.2 Mechanism of short interfering RNA-mediated gene silencing

Mammalian cells and non-mammalian cells differ in their reaction to the presence of double stranded RNA molecules in the cytosole. Cells in higher developed organisms more likely undergo apoptosis due to activation of the interferon-pathway upon entrance of long double-stranded RNAs (see 2.4.5.1) while lower developed organisms like worms or flies try to eliminate the foreign genetic information by extensive activation of the RNAi-machinery [*Skipper, 2003*]. While in *Drosophila* and *C. elegans* the complete silencing of a gene is possible, only ~90% silencing could be achieved in mammals.

Long double stranded RNA can trigger posttranscriptional gene silencing in non-mammalian cells by processing of the long double stranded RNAs to siRNAs [*Nykanen et al., 2001; Bernstein et al., 2003*]. In mammalian cells double stranded RNAs can silence gene expression [*Elbashir et al., 2001*], but the dsRNA-molecules are limited to either siRNAs or short hairpin RNAs (see 2.4.1).

SiRNAs are RNA-duplexes <30 nt in length with a double-stranded core sequence and two 3'-overhanging nucleotides per strand [*Elbashir et al., 2001*].

Figure 1: Schematic representation of a siRNA molecule
OH= 3'-hydroxyl, Ⓟ= 5'-phosphate

There are three milestones in the mechanism of siRNA-mediated gene silencing.

1. In non-mammalian cells long double-stranded RNA is processed to siRNAs by a highly conserved cytoplasmatic ribonuclease called Dicer.
2. In all eukaryotic cells, siRNAs are implemented into a cytoplasmatic RNAi-induced silencing complex (RISC).
3. The siRNA-RISC complex binds the homologous target mRNA and initiates degradation of the target.

Step 1: processing of long double stranded RNAs

When double-stranded RNA-molecules longer than 30 nucleotides enter a cell of non-mammalian origin, Dicer processes the molecules ATP-dependent to siRNAs [*Nykanen et al., 2001; Bernstein et al., 2003*].

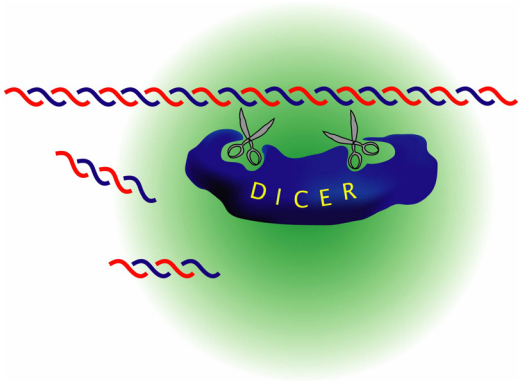


Figure 2: Processing of long ds RNA by Dicer
[http://www.web.mit.edu/mmcmanus/www/ home1.2files/Dicer.html]

Step 2: binding to RISC

The siRNA-molecule binds to the cytoplasmatic multi protein-complex RISC. RISC contains helicase for ATP-dependent unwinding of the two siRNA-strands starting from the less-thermo stabile end of the oligonucleotide. The lead strand stays implemented in the complex and the other strand is released [*Caudy et al., 2002; Ishizuka et al., 2002; Nykanen et al., 2001*], forming the active RISC*-complex [*Martinez et al., 2002*].
The complex persists within the cytoplasm presenting the sequence of the oligonucleotide to the outside.

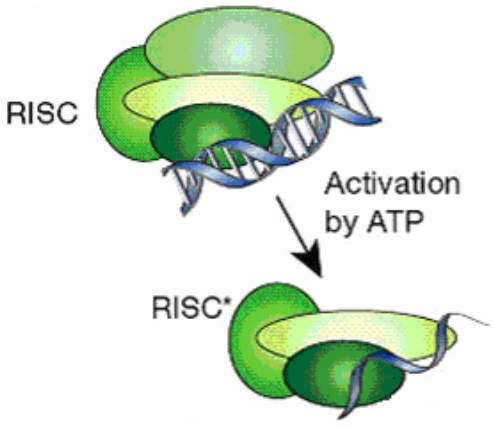


Figure 3: Binding of siRNA to RISC and unwinding
[Hannon, 2002]

Step 3: degradation of target mRNA

The active RISC*-complex contains homology searching domains for identification of the target mRNA. If a homologous sequence is identified, the complex binds to the target mRNA-molecule and cuts the mRNA in the middle of the presented sequence by intrinsic RNAse-activity [*Caudy et al., 2003*].

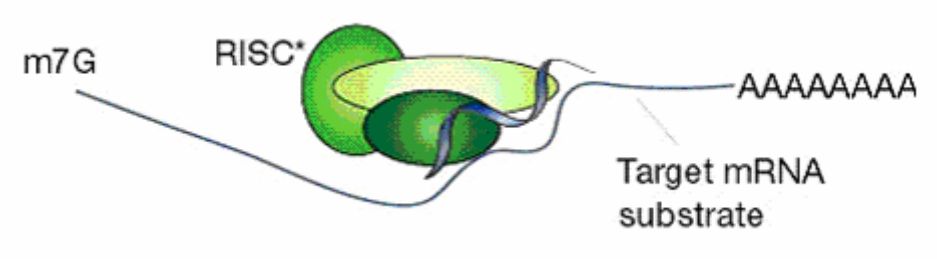


Figure 4: Degradation of target mRNA
[*Hannon, 2002*]

Thus instabilised, the mRNA is degraded quickly by cytoplasmatic RNAses and cannot lead to the expression of its encoded protein. Although the gene is still being read by polymerases, the protein is not expressed.

In nematode worms, there are hints that the initially up taken siRNAs are copied by RNA-dependent RNA polymerases (RdRP) to reach sufficient concentration for cleavage of the targets [*Sijen et al., 2001; Zamore, 2002*]. This is not true for mammalian cells, as RdRPs are absent from the mammalian genomes [*Lehner et al., 2004; Stein et al., 2003*], the siRNA-mediated suppression of a gene's function is transient and no systemic effect (spreading of silencing through most of the cells of the organism) has been observed [*Holen et al., 2002*].

2.3 Micro-RNA mediated gene silencing

Another mechanism of RNAi is the blockade of mRNA-translation, which is triggered by micro RNAs (miRNAs). In contrast to siRNAs miRNAs are single-stranded RNA molecules. The mechanism of miRNA-mediated gene silencing is a component of normal cell function. Although its impact on cell function is not fully understood, it has been demonstrated that there is a relation to developmental processes. Some of the endogenous miRNAs are temporally expressed to silence developmentally important genes as small temporal RNAs (stRNAs) [*McManus et al., 2002*]. Key roles of miRNAs in proliferation, haematopoesis and apoptosis have been postulated [*Rana et al., 2007*].

MiRNAs are expressed as long RNA-sequences with hairpin-motifs. The hairpin-structures are liberated from the original molecule by a multi protein-complex containing the endonucleases Drosha and the double-stranded RNA binding protein Pasha to give pre-miRNAs [*Denli et al., 2004*]. The hairpin structures that are about 70 nt in length are further processed by Dicer to single-stranded RNA molecules of about 21-23 nt in length, the mature miRNA miRNAs [*Dykxhoorn et al., 2003; McManus et al., 2002; Zamore, 2002*].

siRNAs and miRNAs might be exchangeable in regard to their effect on gene-silencing and share much of the same RNAi machinery (for example Dicer is not only capable of cleaving long double-stranded RNAs to siRNAs, but can also cut the hairpin precursors to produce miRNAs). The used pathway (si- or miRNA) might be solely determined by the degree of complementarity between the small RNA and the target mRNA [*Hutvagner et al., 2002*].

In fact ~98% of the total transcriptional output of human cells was formerly thought to be non-coding useless RNA (ncRNA). Part of this ncRNA is now recognised as precursor of miRNAs and thus a major regulator of gene expression [*Zhang et al., 2006*].

2.4 Short interfering RNA-mediated gene silencing as an investigative tool

The mechanism of siRNA-mediated gene silencing can be used in biological investigations as a method for specific inhibition of gene expression [*Fire et al., 1998*]. Since its discovery, the method has successfully been applied in many disciplines of investigative science. The method has been used in determination of gene function, pathway analysis and the identification and validation of drug targets [*Zhou et al., 2006*]. The applicability of the siRNA method in toxicological investigations will be described separately (see 2.5).

2.4.1 Suitable small RNA molecules for gene silencing

Before starting RNAi-experiments a careful consideration about the oligonucleotide to be applied and the strategy for delivery to the target cell should be done to circumvent setbacks. Two types of double stranded RNA-oligonucleotides can basically be applied: siRNAs (see figure 1) or short hairpin RNAs (shRNAs).

ShRNAs are RNAs with a hairpin-structure. The loop-motif is efficiently cleaved from the molecule by Dicer and the resulting molecule is a functional siRNA (see figure 1).

Figure 5: Schematic representation of a shRNA molecule

ShRNAs can experimentally be expressed from plasmids containing a sequence that is efficiently cut out of the vector, folded back to the hairpin-structure and cleaved to its functional siRNA-form by Dicer. ShRNAs can be endogenously amplified within the cells.

The application of both types of molecule has its strengths and weaknesses.

ShRNAs can be used to generate stable knockdown as the siRNA-molecules are amplified endogenously, leading to a permanent concentration of siRNA in the cytoplasm. The production of the siRNA from the vector can be induced at a desired time point by choosing a chemo sensitive promoter like the tetracycline-inducible system [*Yu et al., 2006*]. However, the application of shRNAs has to be well considered due to disadvantages. The screening is time consuming as for each tested siRNA a complete vector has to be produced. Several vectors have been reported to trigger interferon-response (see 2.4.5.1) of the cells. The endogenous production of the siRNA by vectors can lead to high intracellular concentrations

which can overload the RNAi-machinery. The consequence of an overload is down regulation of naturally occurring miRNAs and finally fatality for the cell and the whole organism [*Grimm et al., 2006*].

The use of chemically synthesised siRNAs can only lead to transient knockdown and the regimes for delivery to the target cells *in vivo* are to be optimised, but siRNAs have clear advantages over shRNAs. SiRNAs are quickly designed for screening purposes, and a complete dosing control is possible, making the method applicable *in vivo* and acceptable for authorities as therapeutics. Sequence-independent interferon-response has in no case been reported.

Transfectability of the cell type, growth speed of the cell system and desired duration of the silencing are important factors contributing to the decision which type of oligonucleotide should be applied. The general considerations have recently been reviewed in an excellent way [*Sandy et al., 2005*] and are summarised graphically below.

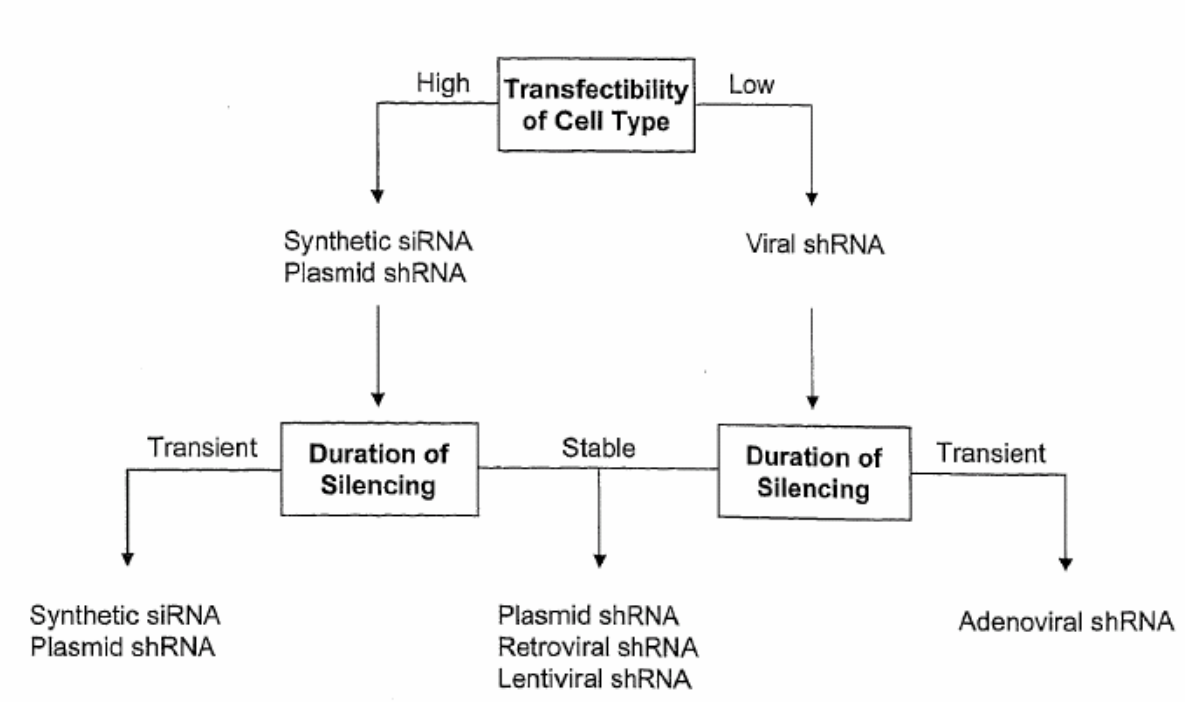


Figure 6: Decision tree for siRNA-experiments
[*Sandy et al., 2005*]

2.4.2 Techniques to deliver short interfering RNAs to target cells

For effective gene silencing, the siRNAs have to reach the cytosole of the target cell. SiRNAs are charged polycations and thus do not cross the lipid bilayer of the cell membrane. The methods of transfection (chemical or physical) or infection can be applied to achieve cellular uptake of siRNAs.

Chemical transfection is either based on cellular uptake of mixed sediments of calcium phosphate and the nucleic acid (Calcium phosphate precipitation) or on fusion of lipid- or polycation-complexed nucleic acid with the membrane of the target cell (lipofection). Attempts for targeted delivery to a special cell type are made by designing complex-forming transfection reagents that contain a receptor-substrate and are taken up via endocytosis.

These methods are suitable *in vitro* for easy-to-transfect adherent cells. Suspension cells are a harder target, as the complexes settle quickly at the bottom of the culture vessels. The methods provide a good throughput, but the concentration of the complex-forming reagents has to be titrated carefully to avoid unspecific toxicity [*Chollet et al., 2002*]. Chemical transfection efficiency can be raised by gentle centrifugation of the culture plates after addition of the transfection reagent [*Boussif et al., 1996*]. Complex-forming reagents can be used for delivery to the target cell *in vivo* as well. Whether to apply complex-forming lipids *in vivo* is a critical decision. The penetration of the target cells by complexed siRNAs is much higher and lower doses of siRNA can be used. Nevertheless, reports from *in vitro*- and *in vivo*-applications demonstrated triggering of interferon-response by complex-forming reagents [*Ma et al., 2005*]. If the use of a formulation could be avoided, an additional toxic substance [*Chollet et al., 2002*] could be eliminated from the system.

Physical transfection is either based on penetration of the cellular membrane by force and thereby delivery of the nucleic acid to the cell (microinjection, biolistic), or permeabilisation of the membrane with a short electrical impulse (electroporation). Microinjection has very limited throughput and is thus not suitable for application in standard studies with chemically synthesised siRNAs. SiRNAs are not endogenously amplified and the daughter generations of the cell do not have the siRNAs present in their cytoplasm. Thus the effect is transient and only the amount of originally transfected cells is available for characterisation. It is impossible to achieve the cell numbers needed for biochemical endpoints. Biolistic (shooting of gold-covered RNA into the cell) is very seldom used as the delivery of siRNA to the target cell can only be applied to exposed tissues.

Especially for primary cells and hard-to-transfect cell lines electroporation is more fertile than chemical transfection and – in contrast to lipofection – cell-cycle independent [*Ovcharenko,*

2003; Brunner et al., 2002]. Electroporation leads to high transfection efficiencies and no case of siRNA-independent interferon response has been reported. Despite reduced throughput, the method is the gold standard for gene delivery to the target cell and can be even successfully applied *in vivo* [*Liu et al., 2002; Prud'homme et al., 2006; Sakai et al., 2005*].

Infection (viral delivery) can also be used for delivery of shRNAs to the target cells. The cells are infected with adeno-, retro- or lentivirusses containing the demanded shRNA. The shRNAs are amplified in the cell with the virus and liberated. Viral delivery leads to high transfection efficiencies but has the disadvantages discussed for shRNAs (see 2.4.1).

Modifications of the siRNA-molecule for delivery to a specific cell type have been investigated. The active uptake of siRNA into hepatocytes by covalent binding of the siRNA to cholesterol [*Soutschek et al., 2005*] and the target-directed delivery of aptamer-connected siRNAs to prostate cancer cells [*McNamara et al., 2006*] were demonstrated and could if reproducible be used as another possibility for siRNA-delivery to the intended cell type.

2.4.3 Time-dependence of short-interfering RNA-mediated gene silencing

It is not enough to identify an efficient siRNA and gain high transfection efficiency to effectively silence a gene's expression. The doubling time of the cell system as well as the half-life of the target mRNA and its protein have to be considered.

In rapidly dividing cell systems (*in vitro* as well as *in vivo*) the silencing effect of an siRNA lasts < 1 week due to dilution of the transfected cells by division, while in non-dividing systems the effect can stay stable up to 3 weeks [*Bartlett et al., 2006*]. To keep protein expression at a low level for prolonged periods repeated dosing or endogenous production of the siRNA (shRNA) is inevitable. Mathematical models are under construction [*Bartlett et al., 2006*].

MRNA- and protein half-lives can vary between minutes and days. Both half-lives have severe impact on the expectable knock-down efficiency by siRNA-application. For most of the targets the recommended time course ranges are 12 to 72 hours to deplete target mRNA and 24 to 96 hours to adequately knockdown target proteins and assess phenotypic outcomes [*Amaxa, 2007*].

2.4.4 Chemical modification of siRNAs for *in vivo* application

SiRNAs as double-strands are more stable than RNA single-strands like antisense-oligonucleotides but are still a target for nuclease-attacks.

Although plain unmodified siRNAs are able to satisfy most of the *in vitro*-applications they are in most of the cases not suitable for *in vivo*-delivery of siRNA to the target cell. Non-modified siRNAs have very short half-lives in blood and serum, as they are a target for circulating exo- and endoribonucleases. Additionally, they are as low-mass molecules within minutes renally excreted, as demonstrated by the application of radio labelled siRNAs [*van de Waters et al., 2006*]. Excretion was identified to have more influence on siRNA-performance than serum stability. Furthermore, successful siRNA delivery to the target cells is impeded by unspecific off-target distribution to unintended body compartments, and decreased accessibility to the target by tissue barricades [*Xie et al., 2006*].

To circumvent these facts, several chemical modifications have been introduced into the molecules, leading to improved pharmacokinetics, increased stability, enhanced specificity (using the sense-strand as the guide) and improved cellular uptake [*Zhang et al., 2006*].

The most common chemical modification is the phosphorothioate-modification of the sugar backbone where one of the non-connective oxygen atoms is replaced by sulfur.

Chemical modification can lower the silencing-capacity of the siRNA. Blocking of the termini of the sense strand or the 3'-terminus of the antisense-strand does not lead to decreased silencing potential, whereas 5'-terminal modifications of the antisense strand result in huge loss of function. 2'-O-methyl-modifications at specific positions were shown to increase stability without lowering the silencing-capacity [*Czauderna et al., 2003*].

Locked nucleic acids have recently been used for stabilisation of siRNAs [*Elmén et al., 2005*]. Locked nucleic acids are modified RNA nucleotides, where the ribose moiety of nucleotides is modified by an extra bridge connecting the 2' and 4' carbons, thus increasing thermal stability. In combination with locked nucleic acids DNA-nucleotides have been introduced into the siRNA-molecules (chimera siRNAs) [*Braasch et al., 2002*] to further improve stability.

2.4.5 Off-target effects of short interfering RNAs

2.4.5.1 Interferon-response

Interferons (IFN) are a class of cytokines (mostly glycoproteins) that are produced by cells in response to external stimuli. IFN-α and -β lead to a hindering of virus replication via destruction of the cell-own initiation factor eIF2α by a protein kinase and indirectly to removal of viral RNAs by a 2`,5`-oligoadenylatsynthetase (OAS). IFN-γ, an acid-instable dimer, leads to activation of macrophages (cellular immune response) and reduction of antibody-producing cells (humoral immune response). All interferons bind to a receptor and transduce their signal via the JAK/STAT-pathway into the nucleus [*Roempp online*].

Feeding of long dsRNA (~500 nt) to worms leads to specific gene silencing, but delivery of such long dsRNA into cells of higher eukaryotic organisms leads to an extensive interferon response resulting in a transcriptional shut down of the cells [*Skipper, 2003*]. Interferon-response becomes evident by activation of the JAK/STAT transcription factor signaling pathway and stimulation of a battery of ISGs (interferon-stimulated genes) [*Sledz et al., 2003*]. The culmination of this mechanism is general suppression of protein production and apoptosis [*Williams et al., 1997; Stark et al., 1998; Frantz, 2003*]. Observable milestones of interferon-response are activation of IFN- α and IFN-β production, induction of RNaseL (antiviral gene coding for Ribonuclease L) and broad degradation of cellular RNA [*Dillin, 2003*], activation of NF-κB nuclear translocation [*Robbins et al., 2003*] and dsRNA-dependent protein kinase (PKR) with subsequent eIF2α-phosphorylation and OAS-1-or IFIT-induction [*Sledz et al, 2003; Patzwahl et al., 2001; Bridge et al., 2003*].

Ds RNAs shorter than 30 base pairs, best between 21 and 23 base pairs, are commonly considered as safe in regard to interferon response [*Elbashir et al., 2001*], but studies applying siRNAs at "considered-as-safe" conditions revealed an increase in interferon-levels [*Sledz et al., 2003*].

The expression of shRNAs can also lead to the induction of ISGs, dependent on the vector used and the starting sequence of the transcript [*Bridge et al., 2003; Pebernard et al., 2004*].

As the RNAi machinery is saturable, the risk of interferon-response increases with the concentration of siRNAs [*Lehner et al., 2004; Sledz et al., 2003*].

2.4.5.2 Off-target silencing

SiRNA-mediated gene-silencing is in general a specific method as confirmed by gene expression profiling [*Semizarov et al., 2003*]. In rare cases off-target genes can be influenced either due to homologies in the mRNA-sequences (which is seldom the case and can be countered by using longer siRNAs) or to activation of yet unknown pathways triggered by the knockdown of the target gene.

Experiments in *C. elegans* showed that regions 5` to the target were affected by siRNAs, which was called transitive RNAi [*Sijen et al., 2001*]. This does not happen in human cells as investigated with an artificial GFP-luciferase-construct [*Chi et al., 2003*].

Studies investigating non-targeted genes in mammalian cells (western blot, micro arrays) revealed changes in protein levels after use of siRNAs in cell lines [*Scacheri et al., 2003; Jackson et al., 2003; Jackson et al., 2006*]. Although another micro array study did not confirm this finding [*Chi et al., 2003*] this phenomenon should in general be observed carefully when working with these techniques. Backbone-modifications have been shown to result in efficient reduction of off-target silencing [*Jackson et al., 2006a*].

2.5 The role of gene silencing in Mechanistic Toxicology

Toxicology is not yet applying siRNA techniques routinely and little is discussed in the literature [*Jason et al., 2004*].

For a long time toxicology was mainly focused on the observation and interpretation of adverse effects to develop safety profiles for the tested compounds. Within the last 15 years toxicology has developed rapidly. The insight into mechanisms has gained more and more attention and can be helpful for the development of follow-up compounds and for notification or registration of new substances. Pure safety margins are not sufficient any longer and mechanistic investigations are requested by the authorities, leading to specialisation of subgroups in the field of investigative or exploratory toxicology.

For exploratory Toxicology there is a clear need for an easy-to-apply and reliable gene-silencing method in the area of functional validation. Functional validation is the investigation of functional consequences resulting from alteration of a single gene's expression in the cell. Rapid inhibition of gene expression is made possible by RNAi and can be applied as a method for functional validation.

Tools for functional validation include over expression, generation of knock-in or knock-out animals and the antisense-technologies (direct antisense-structures targeting DNA/protein/RNA or ribozymes and RNAi). Each technique has its strengths and limits.

Over expression might be helpful when kinetics of a reaction is to be investigated, but its application is very limited with regard to pathway analysis.

Gene knock-out and -in are stable and reliable systems for *in vivo*-investigations. The gene, the mRNA and the resulting protein are absent from the generated organism. Thus the models are delivering the most predictive data but their production is time-consuming and not possible for genes whose absence is lethal in development. Another limitation of the knock-out model is the compensatory effect which may confound the phenotype.

Antisense-oligonucleotides (ASO) targeting RNA are similar to siRNAs in their mode of action. An advantage of ASOs is the more advanced stage of development due to the earlier discovery and the low costs of production. A lot of ASOs have already reached clinical trials as therapeutics while only little siRNAs have so. Nevertheless, ASOs as single-strands are more labile to nuclease-attack [*Bertrand et al., 2002*] and they have to be used in higher concentrations (one molecule for one target-molecule) compared to siRNAs (one molecule can cleave several mRNA-target molecules). Neither ASOs nor siRNAs do perform well *in vivo* if they are not modified. Modification of ASOs is more flexible in its chemistry and

much more experience has been gained in this field, but siRNA modification has benefited a lot from the ASO-experience and is thus gaining on quickly.

For the application of ribozymes (catalytic RNA-RNA enzymes) conformational information about the active sites of the target has to be available. This is rarely the case and thus limits the application of the method. Problematic delivery of ribozymes to the cell is another limitation.

Many hopes of exploratory toxicology and pharmacology are set on the use of genomics. Genomics has been highly promoted within industrial development over the last ten years. A lot of efforts have been made in this field, but the link of an identified mRNA-signature to protein expression and the translation into functional consequences or toxicity is still missing. The RNAi technology can provide a solution as the method can be applied universally for each identified target without extensive developmental work. The siRNA-method can be used for specific knockdown of a single mRNA while in genomics analysis multi- mRNA regulations are observed and functional consequences are the sum of these diverse events and cannot be linked exclusively to the alteration of a distinct gene.

Target validation is the verification of a proposed protein function. It plays a great role in a lot of mechanistic toxicological studies to identify the trigger for toxicity. Usually investigations are carried out applying chemical inhibitors for the proposed target protein. The siRNA-method can be applied to specifically down regulate the target. If the chemical inhibitor is mediating toxicity due to the inhibition of the target, this should result in either direct toxicity of the siRNA-treatment or sensitisation to a chemical inhibitor.

Another clear advantage of siRNA is the specificity provided by the method. In mechanistic toxicology and pharmacology the use of chemical inhibitors is a routine operation. A common problem of using inhibitors is the specificity which is rarely investigated to a satisfactory level and design and screening is labor-intensive. Inhibitors often lead to unexpected cross-specificities which result in hardly interpretable experimental data and thus slow down the verification or rejection of theories. RNAi may help at this point due to the superior specificity of the method.

Although the explanatory power of experimental results is highest in *in vivo*-models, the resolution is best in minimalistic models (*in vitro*-culture of one specific established cell type). Therefore, this thesis work was performed to evaluate the applicability of RNAi-techniques in different *in vitro* model systems and subsequently start with first applications *in vivo*.

2.6 Purpose of the Study

The aim of this thesis was to establish selected RNAi-methods in the laboratory and evaluate the feasibility for toxicological mechanistic studies. The primary goal was to investigate the link between specific gene silencing and its biological function in the respective cellular model. The work will focus mainly on the following areas:

1. Establishment of robust and reproducible methods for siRNA-delivery to various cell types and identification of strategies for selection of siRNAs for *in vitro*- and *in vivo*-applications.
2. Silencing of mitochondrial F_1-ATP synthase (β-subunit) in HepG2-cells and evaluation of functional cellular consequences. Establish a possible link of F_1-ATP synthase silencing and key genes found after Diclofenac treatment *in vivo*.
3. Evaluation of the function of farnesylpyrophosphate synthase (FPPS) as the target of Zoledronic Acid in HK-2-cells. Investigation of the FPPS silencing effects on Zoledronic Acid-induced cytotoxicity and prenylation-dependent sub cellular GTPase-dislocalisation.
4. Evaluation of the caspase-3/-7 specificity in SDZ IMM-125-induced caspase signaling by means of specific knock-down of caspase-3 and caspase-7 in rat primary hepatocytes.
5. *In vivo*-silencing of cytochrome P450 2E1 (CYP2E1) as a mechanistic tool to investigate xenobiotica metabolism-induced liver toxicity. Evaluation of siRNA delivery to hepatic cells comparing the intraperitoneal and hydrodynamic intravenous application routes.

3 Materials and methods

3.1 Materials

All chemicals were obtained from Sigma-Aldrich, USA, unless otherwise indicated. All culture media and supplements were obtained from Invitrogen, USA. Foetal bovine serum (FBS) was from HyClone, USA. All cell lines were provided by ATCC, USA, and human primary hepatocytes by Cytonet, Germany. Cell culture flasks and culture plates were from BD Biosciences, USA.

3.1.1 SiRNAs

All siRNAs were manufactured at Qiagen, USA, unless otherwise indicated. SiRNAs 3 & 4 targeting Hepcidin were from Ambion, USA. The siRNAs targeting CYP2E1 and the corresponding mismatch were in-house produced at Novartis Research.

Gene name	NM-No.	sense	antisense
F_1-ATP synthase, β polypeptide	NM_001686 (homo sapiens)	r(CCG UGA AGG CAA UGA UUU A)dTdT	r(UAA AUC AUU GCC UUC ACG G)dTdT
Green fluorescent protein / mismatch	(artificial)	r(GCA AGC UGA CCC UGA AGU UCAU); 5`-Phosphate	r(GAA CUU CAG GGU CAG CUU GCCG); 5`-phosphate
Hepcidin	NM_021175 (homo sapiens)	Pool (1:1:1) 1. 5'-GUG GCU CUG UUU UCC CAC A tt-3' 2. r(GAA CAU AG UCU UGG AAU A)dTdT 3. r(GGC UGC UGU CAU CGA UCA A)dTdT	Pool (1:1:1) 1. 5'- UGU UUU AAA ACA GAG CCA C tg-3' 2. r(UAU UCC AAG ACC UAU GUU C)dTdT 3. r(UUG AUC GAU GAC AGC AGC C)dGdC
AKR1B10	NM_020299 (homo sapiens)	r(GGA UGU AAA GAU CAA UAA A)dTdT	r(UUU AUU GAU CUU UAC AUC C)dTdT
BiP	NM_005347 (homo sapiens)	r(GGG UGU GUG UUC ACC UUC A)dTdT	r(UGA AGG UGA ACA CAC ACC C)dTdT

Gene name	NM-No.	sense	antisense
FPPS	NM_002004 (homo sapiens)	Pool (1:1:1:1) 1. r(GAC AGU UAC AGC CAC AUU A)d(TT) 2. r(CGG AGA CCA GAA UUC AGA U)d(TT) 3. r(UGC CAU UGG AGG CAA GUA U)d(TT) 4. r(GCG GAG AAA GUG ACC UAG A)d(TT)	Pool (1:1:1:1) 1. r(UAA UGU GGC UGU AAC UGU C)d(TT) 2. r(AUC UGA AUU CUG GUC UCC G)d(TT) 3. r(AUA CUU GCC UCC AAU GGC A)d(TT) 4. r(UCU AGG UCA CUU UCU CCG C)d(TT)
Caspase-3	NM_012922 (rattus norvegicus)	r(GCC ACA AUA CAA UAC CUC A)dTdT	r(UGA GGU AUU GUA UUG UGG C)dTdT
CYP2E1-mismatch	(artificial)	5'-CAA TGT AAT GGC GGC TAT T-3'(NAS-114928)	5'-AAT AGC CGC CAT TAC ATT G-3'(NAS-114927)
Cytochrome P450 2E1	NM_021282 (Mus musculus)	5'-CCA TGT CAT TGC GGA TAT T-3' (NAS-114924)	5'-AAT ATC CGC AAT GAC ATT G-3' (NAS-114923)

Table 1: Tested siRNAs

3.2 Cell culture

3.2.1 Isolation of primary rat/mouse hepatocytes

Hepatocytes were isolated applying a modified two-step *in situ* collagenase perfusion technique [*Berry et al., 1969; Seglen 1976*].

Pre-perfusion solution	100 mM NaCl, 4 mM KCl, 1 mM $MgSO_4$, 150 mg/l KH_2PO_4, 20 mM $NaHCO_3$, 15 mM HEPES, 7.5 mM Glucose, 0.1 mM EGTA, 2 µM Insulin mice: 4 ml/min after addition of 21 µg/ml Liberase [Roche, Switzerland, #1814176], total 30 ml; rats: 25 ml/min, total 150 ml
Perfusion solution	DMEM:F12 1:1, containing 10 mM HEPES, 100 u/ml Penicillin, 0.1 mM Streptomycin, 2 mM L-Glutamine, 3 mM $CaCl_2$, pH 7.4, 2 µM Insulin rats: +100 CDU/ml collagenase [Sigma, USA, # C-5138], 20 ml/min, 4.5 min mice: +21 µg/ml Liberase, 4 ml/min until 30 ml have passed Keratinocyte-SFM containing 10% of FBS
Wash buffer	DMEM:F12 1:1, containing 10 mM HEPES, 100 u/ml Penicillin, 0.1 mM Streptomycin, 2 mM L-glutamine, 20% FBS

Animals were anesthetised by intraperitoneal injection of 75 mg/kg BW Pentobarbital. The liver was perfused with pre-perfusion solution for exsanguination and pre-dissociation (mice: via vena cava; rats: from portal vein). Perfusion solution containing active collagenase was introduced through the same route afterwards to digest the collagen-matrix. The liver was excised, transferred to wash buffer and the hepatocytes liberated by mechanical dissociation of the liver. Hepatocyte-suspension was filtered over a cell strainer (70 µm), and collagenase-activity was stopped by addition of ice-cold wash buffer. Cells were pelleted (50xg, 4°C; mice: 2 min, rats: 5 min) and the supernatant discarded. Cells were washed in wash buffer, then resuspended in attachment medium and viability determined by trypan blue exclusion [*Freshney, 1987*]. Preparations with viabilities below 90% (trypan blue-exclusion) were rejected.

3.2.2 Culture conditions

HepG2 culture medium	Minimum essential medium containing 0.1 mM Non-Essential Amino Acids, 100 u/ml Penicillin, 0.1 mM Streptomycin, 2 mM L-Glutamine, 1 mM Sodium Pyruvate and 10% FBS;	
HK-2 culture medium	Keratinocyte-SFM containing 10% of FBS	
Primary hepatocytes culture media	Attachment	Waymouth medium:DMEM: F12 2:1:1 containing 0.1 µM Dexamethasone, 100 u/ml Penicillin, 0.1 mM Streptomycin, 2 mM L-Glutamine, 1 µM Insulin, 5 µg/ml Transferrin, 30 nM Sodium Selenite and 10% FBS
	Maintenance	William's medium E containing 0.1 µM Dexamethasone, 100 u/ml Penicillin, 0.1 mM Streptomycin, 2 mM L-Glutamine, 1 µM Insulin, 5 µg/ml Transferrin, 30 nM Sodium Selenite and 0.1% Bovine Serum Albumin
Sterile coating solution	per ml: 100 µl 10 x Minimum Essential Medium, 10 µl 1 M HEPES, 385 µl 70 mM $NaHCO_3$ in ddH_2O (sterile-filtered), 5 µl 1 M NaOH (sterile-filtered); right before coating 0.5 ml 2 mg/ml rat tail collagen in 0.1% acetic acid [Menal GmbH, Germany, on demand] coating: 100 µl/well (24-well) / 200 µl/well (6-well) / 500 µl/well (6qcm-dish)	

All steps were performed under sterile conditions, and all solutions were brought to 37°C before coming in contact with cells. Cells were cultured in incubators at 37°C, 95% humidity and 5% CO_2. Cryopreserved cells were thawed quickly (~1 min) in a water bath at 37°C, transferred to 50 ml culture medium, collected by centrifugation and the supernatant (containing the cryopreservative) was discarded.

Dividing cells (HepG2, HK-2) were resuspended at the desired density in culture medium, and grown in filter-cap culture flasks. Cells were passaged (using TrypLE-Express for detachment) twice a week and thereby split~1:4.

Non-dividing cells (rat/mouse hepatocytes) were plated directly after isolation in attachment medium on collagen-gel [*Schuetz et al., 1986/1990*]. Therefore, culture plates were coated

with sterile coating solution, and collagen was allowed to jelly for 1 h in the incubator. For sandwich-culture cells were washed after 3 h of attachment and overlaid with the same amount coating-solution; maintenance medium was added after 1 h jellying in the incubator. For monolayer collagen-gel culture, cells were not overlaid with coating solution but the attachment medium was exchanged by maintenance medium after 3 h of attachment. Medium was daily replaced by fresh maintenance-medium for cultured hepatocytes.

3.2.3 Inquiries on cell culture quality and response to compound treatment

This thesis will not focus on the functional consequences of compound treatments or culture conditions but on the siRNA-mediated effects. The following section will reflect the key results of dose-finding and important control experiments, serving as a basis for further investigations and is thus placed uncommonly in the Cell Culture section of Material and Methods.

Diclofenac will be used to induce ATP-depletion, decrease in mitochondrial membrane potential and breakdown of membrane integrity in hepatocyte-derived cells, Zoledronic acid to induce kidney toxicity in HK-2-cells and SDZ IMM125 to induce caspase-activation and secondary necrosis in rat primary hepatocytes.

Inducing cytotoxicity with a low-toxic compound like Diclofenac requires high *in vitro*-concentrations. At very high concentrations, osmolaric substance-effects could have severe influences on cytotoxicity and were thus investigated first.

		[mOsm/kg H_2O]	
		vehicle	Vehicle + Diclofenac
Minimum essential medium, 10% FBS	0.1% DMSO (+100 µM Diclofenac)	307	305
	0.2% DMSO (+200µM Diclofenac)	320	321
	0.4% DMSO (+400µM Diclofenac)	361	350
	0.8% DMSO (+800µM Diclofenac)	410	410
William's medium E	1% DMSO (+50 µM SDZ IMM 125)	457	455
Keratinocyte-SFM, 10% FBS	1% water (+100 µM Zoledronic Acid)	269	271

Table 2: Osmolality of the incubation media
The experiment was executed once after careful calibration of the osmometer.

None of the test compounds had influence on the osmolarity of the incubation media, even not in high concentrations.

To identify dose and time point for ATP-depletion, decrease in mitochondrial membrane potential and breakdown of membrane integrity, the short- and long-term toxicity of Diclofenac was investigated in different hepatocyte-models. For this purpose, HepG2-cells were incubated with 100, 200, 400 and 800 µM Diclofenac for 1, 2 and 24 h. Rat primary hepatocytes were incubated with 100, 200 and 400 µM Diclofenac for the same periods. Short-time incubations (1 and 2 h) were executed in HBSS containing 2 g/l glucose, and 24 h-incubations in serum-free culture medium. ATP content was investigated after 1-2 h, LDH release and mitochondrial membrane potential after 24 h.

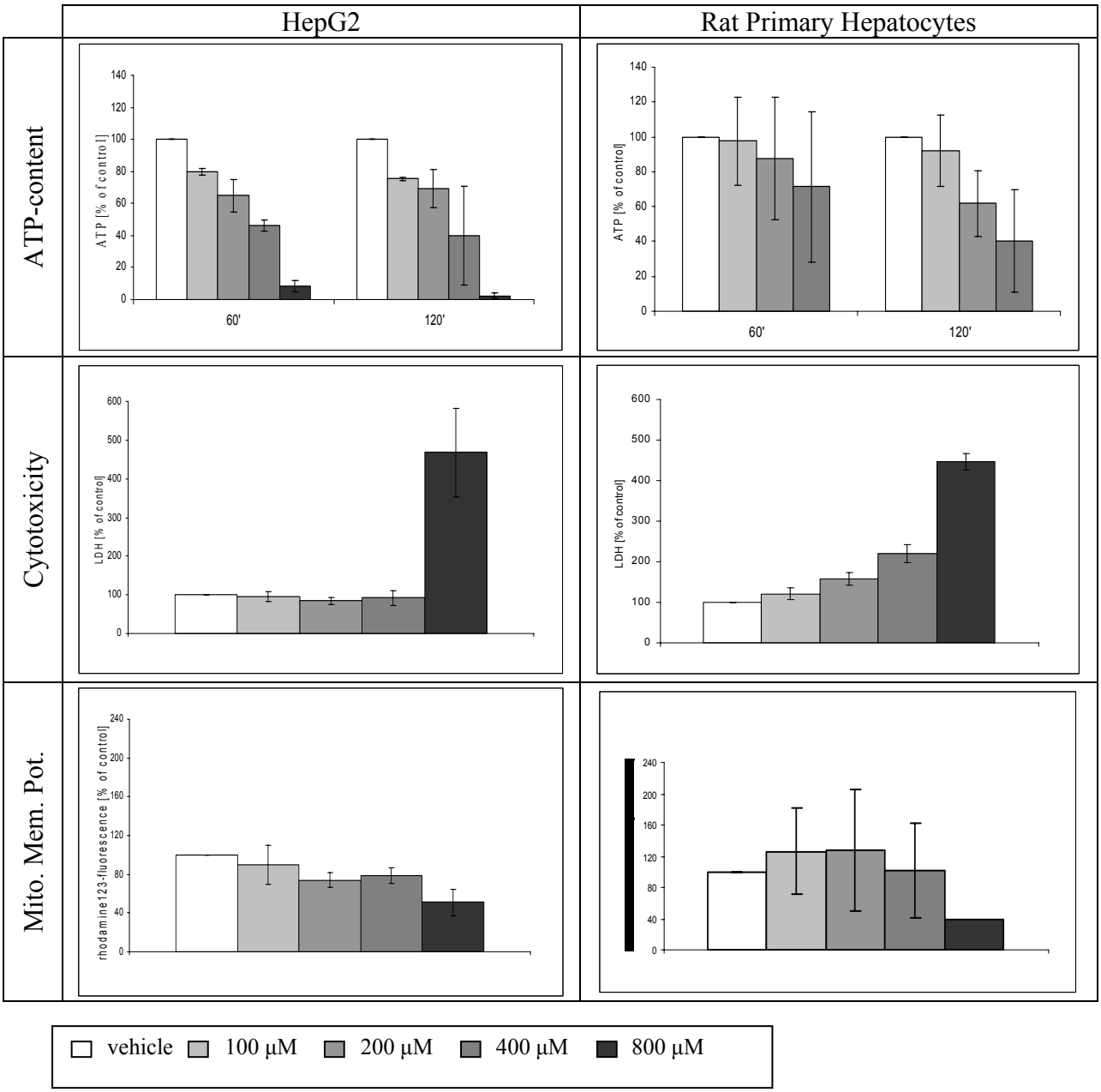


Figure 7: Cytotoxicity-parameters in HepG2-cells and primary hepatocytes
The experiments were performed 3-4 times in triplicate. Error bars indicate the standard deviation of the experiments.

ATP was dose- and time-dependently depleted in HepG2 and rat hepatocytes. Time dependence was observable by comparison of the maximal depletory effect after 1h and 2h of treatment, resulting in doubling of the effect in rat hepatocytes. After 2 h of Diclofenac-treatment a maximal depletion of 60% was reached in both cell types at 400 µM. Incubations with 800 µM in HepG2 led to a depletion of 95% after 120 min Integrity of the cellular membrane was disturbed by Diclofenac-treatment, resulting in 4-fold elevated LDH release to the cell culture supernatant at the highest applied dose. The mitochondrial membrane potential was dose-dependently compromised after 24 h, with a maximal reduction of 60% (HepG2) and 75% (rat hepatocytes), respectively.
These findings were used for the determination of the experimental conditions in later experiment where only selected doses of Diclofenac were applied for selected periods.

Hepcidin and BiP are discussed as stress-response genes in the literature. Hepcidin is sensitive to iron- and BiP to glucose-concentrations [*Mote et al., 1998*]. Thus, they can be heavily influenced by suboptimal cell culture conditions. In general, medium is replaced 2-3 times a week in the culture of permanent cell lines. HepG2-cells were cultured for 24, 48, 72 and 96 h, and stress was induced by not replacing culture media. The influence on the mRNA-expression of Hepcidin and the protein-expression of BiP were investigated by qPCR or western blot, respectively.

	Hepcidin mRNA-expression [ΔCt Hepcidin/ Δ Ct β-actin]	BiP protein-expression [% of 24 h]
24 h	6 ± 1.2	100
48 h	53 ± 0.4	349 ± 53
72 h	238 ± 2.4	689 ± 261
96 h	287 ± 9.9	777 ± 161

Table 3: Inducibility of stress-response genes
The housekeeping gene for PCR was β-actin. The experiment was performed three times in triplicate, ± values symbolise the standard deviation of the experiments

BiP protein expression was induced in a time-dependent manner, reaching 8-fold induction after 96h compared to the expression at 24 h. Hepcidin mRNA-expression was time-dependently induced reaching a maximum of 50-fold induction compared to the expression after 24 h after 96 h.
Therefore, in all mRNA-expression experiments in HepG2, the medium was renewed every day.

In summary, the mitochondrial membrane potential and cellular membrane integrity were demonstrated to be affected by Diclofenac-treatment after 24 h of incubation, while ATP depletion triggered by Diclofenac-treatment became evident at 1-2 h. High concentrations of Diclofenac were required to induce the observed effects. Hepcidin and BiP were very sensitive to cell culture conditions, and thus daily medium change and routine handling of the cells had to be applied for investigations on these targets.

SiRNA-mediated gene silencing needs longer periods of cell culture due to the kinetics of the knockdown. For application in primary hepatocytes this is critical due to the loss of metabolic activity of the cells with prolonged cultivation.

The extra cellular matrix has been shown to have heavy impact on metabolic activity and viability of primary hepatocytes [*Schuetz et al., 1986/1990*]. Collagen-coating of the culture dishes is essential to retain metabolic activity of hepatocytes, but could interfere with test compounds and endpoint-determinations. Sandwich culture between two layers of collagen would be ideal to retain metabolism, and uncoated culture material would be worst in this regard.

The impact of collagen-coating on caspase-activation in rat hepatocytes upon treatment with a fixed dose of SDZ IMM125 was compared under different conditions of collagen-coating. For this purpose, rat hepatocytes were either cultured for 96 h on uncoated cell culture material, on a monolayer of collagen, or between two layers of collagen (sandwich). The cells were incubated with 50 µM SDZ IMM125 for 4 h and caspase-3/-7 activity was determined.

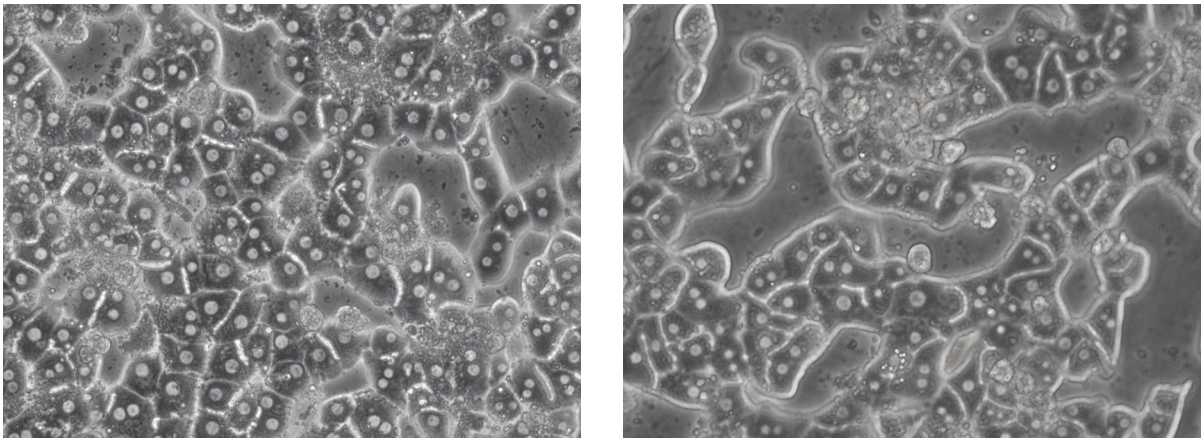
	Caspase-3/-7-activity [pmol AMC liberated/min at 30°C/mg protein]		
	uncoated	collagen-gel	collagen-sandwich
vehicle (0.5% DMSO)	6 ± 1	8 ± 1	5 ± 1
50 µM IMM 125	217 ± 5	134 ± 5	113 ± 30

Table 4: Influence of extra cellular Matrix on SDZ IMM125-induced caspase-activation
The experiment was performed in triplicate from one animal. ±-values symbolise the standard deviation of the different wells.

SDZ IMM125-mediated caspase-inducing effect was reduced dependent on the amount of collagen surrounding the cells. While SDZ IMM125-treatment led to 36-fold caspase-activation compared to the vehicle-treated control in cells without extracellular matrix, the effect was decreased by 38% on collagen monolayer and by 48% in collagen sandwich. Therefore, rat hepatocytes were cultured on single-layer collagen-gel in all later experiments

as a compromised to retain metabolic activity of the cell and allow substance-induce caspase-activation.

The quality of the hepatocytes-culture was evaluated by viability and morphology. Preparations with viabilities below 90% (trypan blue-exclusion) were rejected. Cellular attachment was monitored after 2 hrs. Formation of canaliculi and polygonal morphology were checked after 24 h and before substance-treatments.
To investigate the morphologic quality of the cell culture after electroporation and long-time treatment, rat hepatocytes were electroporated with 300 nM of mismatch siRNA and cultured for 48 and 96 h. The culture was photographed under a light microscope.



48h-old culture 96h-old culture

Figure 8: Morphology of rat hepatocytes 48 and 96 h after electroporation

Cells displayed normal polygonal hepatocyte morphology and formation of canaliculi after electroporation. Prolonged cultivation of hepatocytes led to detachment of some cells. The remaining attached cells were morphologically at fine quality.

As prolonged culture periods are suboptimal in regard to cytotoxicity in primary hepatocytes, the dependence of the SDZ IMM125-mediated caspase-induction on the age of the cell culture was investigated. For this purpose, rat hepatocytes were electroporated with 300 nM of either mismatch-siRNA or siRNAs vs. Caspase-3. Cells were cultured for 24, 48, 72 and 96 h, and subsequently treated with 50 µM SDZ IMM125 for 4 h under serum-free conditions. Caspase-3/-7 activity was determined at the end of incubation.

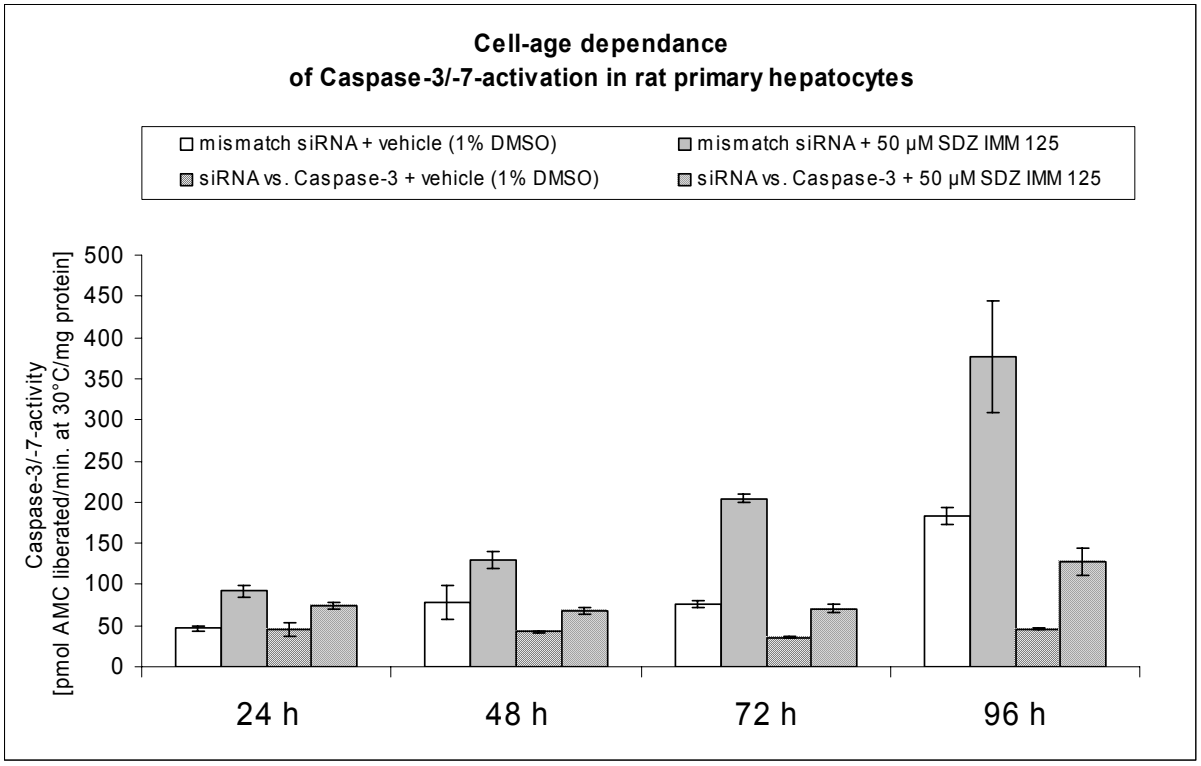
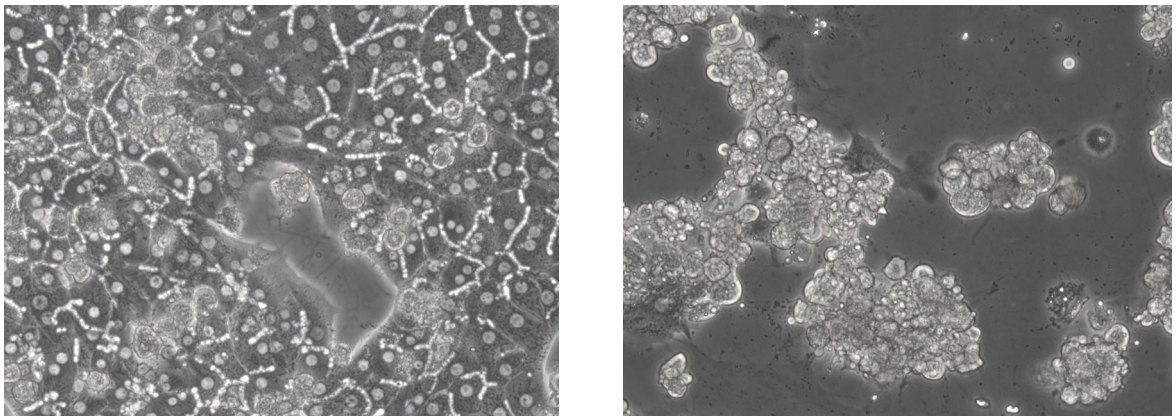


Figure 9: Cell-age dependence of caspase-activation in rat hepatocytes
The experiment was performed in triplicate from one animal. Error bars symbolise the standard deviation of the different wells.

SDZ IMM125-induced caspase-activity was at all time points 2-fold compared to the vehicle-treated control, but while caspase-activity in the vehicle treated controls increased over time (3-fold 96 h after transfection compared to the value of 24 h), the activity was constant in caspase-3-silenced hepatocytes. The maximal SDZ IMM125-induced caspase activity was observed in mismatch-transfected cells 96 h after silencing. The increasing background-activity of caspase-3/-7 indicated deterioration of the cells at later time points. Therefore, cultures were not kept longer than 96 h before treatment.

Protein depletion in response to silencing of a gene is always delayed (depending on the gene). For this reason, the SDZ IMM125-treatment was in all cases on 96 h-old cultures.
Beside caspase-activation, SDZ IMM125 could induce breakdown of cellular membrane integrity by secondary necrosis. For investigation of this effect, rat primary hepatocytes were electroporated with 300 nM mismatch-siRNA and cultured for 96 h. The cultures were treated 50 µM SDZ IMM 125 for 4 h and photographed under a light microscope.



vehicle SDZ IMM125

Figure 10: Morphology of rat hepatocytes upon SDZ IMM125-treatment

The SDZ IMM125-treated hepatocyte population was severely blebbed and close to complete breakdown of integrity but still attached.

To investigate cytoprotective activity the chemical caspase-inhibitor Ac-DEVD-CHO, rat hepatocytes were electroporated with 300 nM mismatch-siRNA and cultured for 96 h. The cultures were treated 50 µM SDZ IMM 125 and 100 µM Ac-DEVD-CHO for 5, 8 and 24 h. LDH was determined in the cell culture supernatant and the cell lysate, and the percentage of leaked LDH was determined as a measure of membrane damage.

	LDH-leakage		
time point	vehicle	50 µM SDZ IMM125	50 µM SDZ IMM125 + 100 µM Ac-DEVD-CHO
5 h	2.6 ± 0.5	23.8 ± 2.1	7.3 ± 1.4
8 h	1.7 ± 0.1	81.2 ± 1.0	24.5 ± 2.9
20 h	2.2 ± 0.7	93.0 ± 0.9	82.9 ± 3.4

Table 5: Cytotoxicity of SDZ IMM125 to rat hepatocytes at different time points
The experiment was executed in triplicate with hepatocytes from 1 animal ± values bars symbolise the standard deviation of replicates.

Cytotoxicity increased rapidly over time, reaching a value of ~80% damaged cells after 8 hrs of treatment. At 5h, the effect could be completely abolished by addition of Ac-DEVD-CHO. At later time points, the effect could only be reduced to a smaller extent (70% reduction at 8h and 11% reduction after 20h). Thus, the observed cytoprotective effect was investigated in short-time co incubations in all later experiments.

In summary, SDZ IMM125-treatment for induction of apoptotic and cytotoxic mechanisms could be fixed to 4h-treatment at 50 µM under serum-free conditions. Collagen-coating of the cell culture ware was demonstrated to have impact on the amplitude of caspase-activation and

was fixed to monolayer gel culture. 96h-old rat hepatocyte cultures were suitable for the planned applications. SDZ IMM125-induced cytotoxicity can be decreased by incubations with caspase-inhibitor, but only during the early time points of treatment.

3.3 Animal experimentation

All animal experiments were conducted with permission of the Kantonales Veterinäramt Basel-Landschaft. Experimental animals were obtained from Charles River, Germany, and housed under optimised hygienic conditions with a 12/12h fluorescent light-dark cycle in Macrolon type III cages (mice) or Macrolon type IV cages (rats) with sterilised softwood particle bedding. Animals were allowed to acclimatise for 10 days before entering a study. Routinely tested (RCC Ltd., Switzerland) pelleted standard diet (NAFAG, Switzerland) and water were available ad libitum. Optimal hygienic conditions were controlled by sentinel-animals, which were kept under a rotation system through the used cages of the study-animals, and were sent in for testing of microbiological/viral/parasitous contaminations after 3 months of housing (MicroBioS GmbH; Switzerland).
For the *in vivo*-application of siRNA, male CD-1-mice (5-6 weeks at start of dosing, 27-33g) were chosen as the model, as older animals showed higher symptomatic after hydrodynamic injection.

3.3.1 Compound treatment

Isotonic buffer for RNAi-preparation	100mM K-Acetate, 2 mM Mg-Acetate, 30 mM HEPES in RNAse-free water, pH 7.6

Intraperitoneal applications were conducted by injections of 5 ml/kg BW through 1 ml syringes and 27 gauge-needles. Hydrodynamic intravenous application into the tail vein was executed using a restraint-device, 27 gauge-needles and 3ml-Luer-Lok syringes [BD Biosciences, USA, #309585], injecting 100 ml/kg BW in 5-7sec.
Treatments were performed between 6:00 and 11:00 a.m. and each dose group was finished in 15 (i.p.) or 30 (i.v.) minutes to avoid influences of the circadian rhythm on gene expression.
Preparation for administration: siRNA-single strands were dissolved at 100 mg/ml in ice-cold RNAse-free Isotonic buffer for siRNA-preparation by vortexing. The corresponding strands were combined and incubated for 5 min at 90°C, followed by a 1 h-annealing phase at 37°C to give the stock solution.

All work with the siRNA-solution including filling of the syringes was under sterile and RNAse-free conditions, and injection was conducted within 30 min after dilution.

i.p.: The stock solution was diluted 1:10 with TransIT-QR to achieve 10mg / ml.
hydrodynamic i.v.: The stock solution was diluted 1:4'000 with TransIT-QR to achieve 0.025 mg / ml.

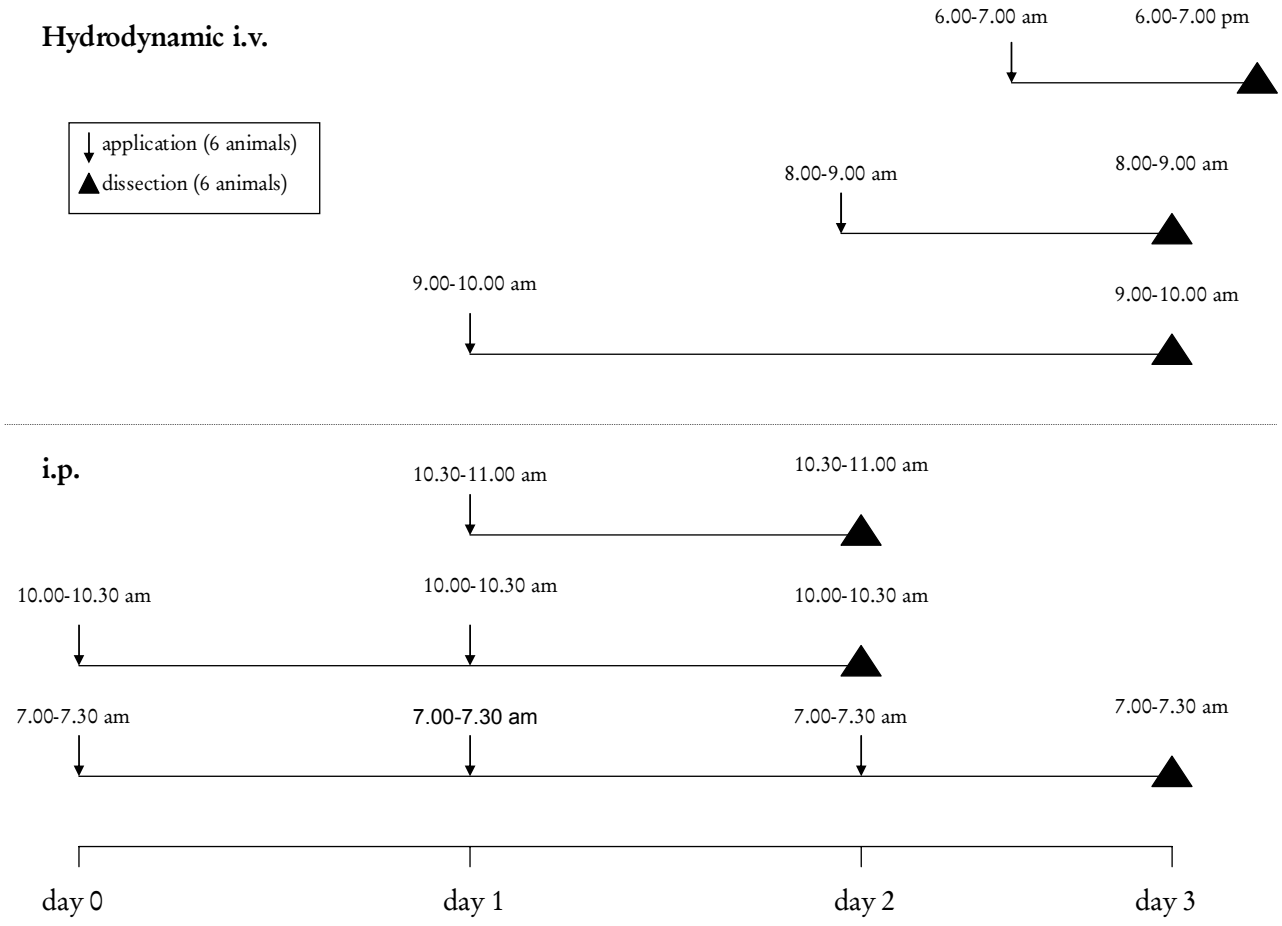


Figure 11: Application schedule for kinetic investigation of siRNA-treatment *in vivo*

3.3.2 Necropsy and sampling

All dissections were conducted between 6:00 and 11:00 am, except the 12h-groups after hydrodynamic injection, which were dissected between 18:00 and 19:00 pm. Blood was obtained by punctation of the orbital sinus and collection through 20 µl-glass capillaries in tubes containing coagulation factors [Sarstedt AG, Germany, #41.1500.005] under Isofluran-anesthesia [Abbott GmbH, Germany, #B506]. Blood was allowed to clot at RT for 30 min, and serum was separated by centrifugation (10'000 x *g*, 10 min, 4°C). Animals were sacrificed by decapitation while still under anesthesia. Organs were excised during dissection and either shock-frozen in plastic bags in liquid nitrogen (afterwards stored at -80°C), or fixed in 10% buffered formalin and embedded in Paraplast after exactly 48h for immunohistochemistry or eosin-hematoxylin-staining.

3.4 Transfection

3.4.1 Amplification of Plasmids

Competent bacteria [*Ito et al., 1983*] were transformed with plasmids containing an antibiotic-resistance, and the culture was expanded in antibiotic-containing systems to avoid mutation of the plasmid and to enrich the population amplifying the plasmid [*Raleigh et al., 2002*]. Plasmids were recovered by alkaline extraction and SDS-precipitation [*Birnboim et al., 1979*].

The original plasmid (pCMVSPORT6CYP2E1 [Invitrogen, USA, clonedatabase IRAK25_P17] or pHygeGFP [BD Biosciences, USA, #6014-1]) was transformed into competent E. coli [Invitrogen, USA, #C4040-06] according to the manufacturer's recommendations. Bacteria were grown on an orbital shaker (37°C, 225 rpm), the starter culture was spread on LB-Agar-plates containing 200 µg/ml Ampicillin and grown overnight at 37°C. A defined colony was picked and used for inoculation of 5 ml LB-medium 0.57 mM Ampicillin. The culture was expanded on the orbital shaker (37°C, 225 rpm) for 8 h, and this starter culture was diluted 1:500 in LB-medium 0.57 mM Ampicillin. The culture was grown overnight, the bacteria harvested by centrifugation (6'000 x *g*, 15 min, 4°C), and the plasmid isolated endotoxin-free using a Maxi Prep-kit according to the manufacturer's recommendations [Qiagen, Germany, #12362]. The concentration of the plasmid was determined by its absorption at 280 nm, and the identity was compared to the original plasmid by restriction digestion with a single-site cutting enzyme followed by horizontal agarose-gel electrophoresis and visualisation with ethidium bromide.

3.4.2 Electroporation of cells

Upon treatment with electric square-wave pulses, the cellular membrane is porated, and nucleic acids can diffuse into the cells [*Neumann et al., 1982*]. The exact mechanism of electroporation is unknown.

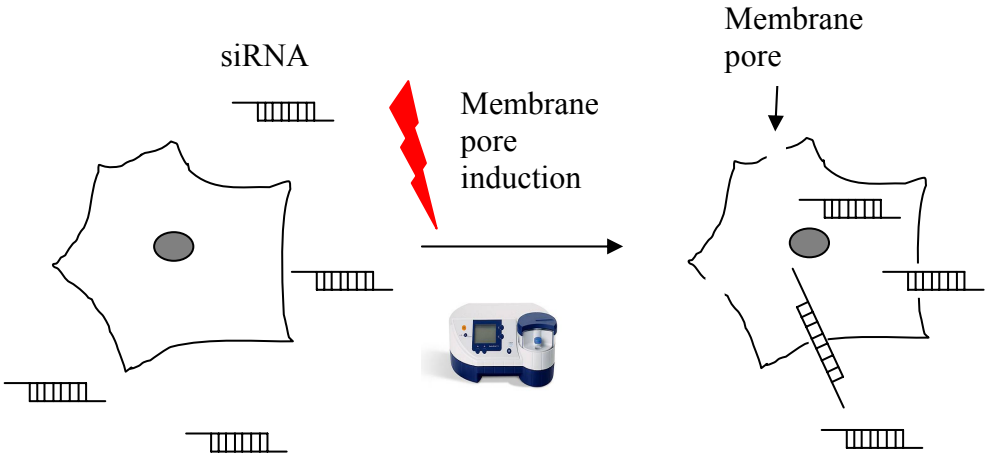


Figure 12: Mechanism of electroporation

Cell type	Cells/ electroporation	kit	program	AMAXA ref.
HepG2	1 x 10^6	Nucleofector Kit V	T-028	#VCA-1003
HK-2	1 x 10^6	Basic Nucleofector Kit for primary mammalian epithelial cells	U-017	#VPI-1005
Rat primary hepatocytes	2 x 10^6	Rat hepatocyte Nucleofector Kit	Q-025	#VPL-1003
Mouse primary hepatocytes	2 x 10^6	Mouse Hepatocyte Nucleofector Kit	T-028	#VPL-1002

For all electroporations, the Nucleofector II-device and respective kits were used [AMAXA GmbH, Germany]. Cells were collected after detachment with TrypLE Express and stopping of the dissociative activity with protein-containing culture medium. Primary hepatocytes were used immediately after isolation or thawing. The desired amount of cells was pelleted by centrifugation (HepG2, HK-2: 130 x *g*, 4°C, 5 min; mouse hepatocytes 50 x *g*, 4°C , 2 min; rat and human hepatocytes 50 x *g*, 4°C, 5 min), the supernatant discarded and the cells resuspended in 100 µl/cuvette of the corresponding nucleofection buffer. The suspension was mixed with the desired amount of pre-laid 20 µM siRNA-solution (in all cases 300 nM at the moment of electroporation), transferred to cuvettes and electroporated using the corresponding program. Cells were either immediately after electroporation (HepG2, HK-2), or after 15 min-incubation at RT in the cuvette (rat-/mouse and human hepatocytes) transferred to 2 ml/cuvette pre-warmed culture medium and plated in culture plates.

3.5 Analytical assays

3.5.1 Molecular biology

3.5.1.1 SiRNA-screen by reporter gene-assay

Designed sequences were tested in a reporter-system according to the method of Huesken [*Huesken et al., 2003; Huesken et al., 2006*].

Briefly, a mouse CYP2E1-plasmid was amplified in E. coli, isolated and sequence-verified. The mCYP2E1-sequence was cut out by restriction enzyme digestion and inserted into a CFP- and YFP-expressing vector as a fusion-construct with the YFP. In a fully automated process, COS-1-cells were cotransfected with the resulting vector and the test-siRNA (2 µl/ml Fugene, 1 ng/µl plasmid, 5 and 15 nM siRNA using 8 µl/ml Oligofectamin). The fluorescence of CFP and YFP was measured after 72 h. CFP-fluorescence served as a control for transfection-efficiency, and YFP-fluorescence was the reporter-fluorescence for the silencing-capacity of the siRNAs.
After amplification and isolation of the purified plasmid, sequencing and insertion to the vector was outsourced to Solvias AG, Switzerland. The vector system was handed over to Novartis Research/GPS, Switzerland, where the siRNAs were designed, produced and tested in the reporter system. The best 2 candidates and their corresponding mismatches were chemically modified, scaled up and provided for the *in vivo*-use.

3.5.1.2 qRT-PCR

RNA from tissue or cells was extracted by binding of the RNA to streptavidin-coated magnetic beads in the lysate [*Miyachi, 2000*]. The RNA was transcribed into cDNA by polymerase-chain reaction using random primers [*van Ness et al., 1980*]. PCR was quantified by applying the TaqMan-system [*Livak et al., 1995*] with detection of the first replication cycle with significantly elevated reporter-fluorescence for the amplicon of interest (CT-value) and comparison to the CT-value of a housekeeping gene.

For quantitative real-time PCR, cells were washed with PBS, lysed in Trizol and stored at -80°C until RNA extraction. For PCR from organ lysates, ~100 mg of tissue was lysed in 1.5 ml Trizol [Invitrogen, USA, #15596-026] on the Bio101 Fast Prep system (bead-filled tubes) [MP Biomedicals, USA, #6913-100] and frozen to -80°C until RNA extraction.
RNA was extracted using the MagnaPur system [Roche, Switzerland] according to the manufacturer's recommendation. RNA was transcribed into cDNA using the cDNA Arch. Kit [Applied Biosystems, USA, #P/N 4322171], and PCR was performed using the TaqMan Gene Expression Assay primer mix / TaqMan-Master mix on a ABIPRISM 7900HT real time-PCR-system [Applied Biosystems, USA]. 10 µl of the master mix, 1 µl of the primer/probe mix and 2 µl of the sample cDNA were filled up to a final volume of 20 µl with water. The PCR conditions consisted in one cycle at 50°C for 2 min, followed by one denaturation cycle at 95°C for 10 min and 40 cycles of amplification. An amplification cycle consisted of a denaturation step at 95°C for 15s and annealing/elongation at 60°C for 1 min.
Results for *in vitro*-experiments were calculated by the delta-delta CT-method. The CT value of the housekeeping gene was subtracted from the CT-value of the gene of interest (delta CT), and the delta CT values of the groups to be compared (for example mismatch/siRNA-treated) were subtracted from each other (delta-delta CT) and inserted in to the equation

$$\text{n-fold expression (group A to group B)} = 2^{-\text{delta-delta-CT}}$$

Results of *in vivo*-experiments were calculated with the help of a cDNA standard curve. The efficiency of the PCR was calculated from the slope of the standard curve with known amounts of cDNA.

$$\text{efficieny} = 10^{(-1/\text{slope})} \mathbf{-1}$$

Efficiency was in all cases ~99.5%.
As each dilution of standard cDNA had a known concentration, a quantity (copy number) of cDNA in each sample could be determinated according to the CT values. All expressions of the gene of interest were normalised to the expression of the housekeeping gene in the same sample

$$\text{Relative expression of gene of interest} = \frac{\text{copy number (gene of interest cDNA)}}{\text{copy number of (housekeeping gene cDNA)}}$$

Upon delivery of the Trizol-homogenate, extraction and qRT-PCR was executed by the PCR Lab-team at Novartis SP&A. As the sequence of the primers is not supplied by the manufacturer, the order reference of the commercially available assay is given.

TaqMan® Gene Expression Assays (Applied Biosystems)		
gene	species	ref.
β-actin	human	#Hs99999903_m1
β-actin	mouse	#Mm00607939_s1
GAPDH	human	#Hs99999905_m1
GAPDH	mouse	#Mm99999915_g1
GAPDH	rat	#Rn99999916_s1
AKR1B10	human	Hs00252524_m1
ALAS-1	human	Hs00167441_m1
ATP synthase	human	Hs00266077_m1
BiP	human	Hs00607129_gh
CYP1A1	mouse	Mm00487218_m1
CYP1A2	mouse	Mm00487224_m1
CYP2E1	mouse	Mm00491127_m1
CYP3A11	mouse	Mm00731567_m1
FDPS	human	Hs00266635_m1
Hepcidin	human	Hs00221723_m1
IFIT	human	Hs00356631_g1
IFIT	mouse	Mm00515153_m1
OAS-1	human	Hs00242943_m1
OAS-1	rat	Rn00594390_m1
PKR	human	Hs00169345_m1
PKR	mouse	Mm00440966_m1
QuantiTect Primer Assays (Qiagen)		
β-actin	rat	QT00193773
Caspase-3	rat	QT00186333
Caspase-7	rat	QT00189497

3.5.2 Biochemical Assays

3.5.2.1 Osmolarity

The determination of freezing point depression of a solution was applied to calculate osmolarity [*Dufour, 1993*]

Freezing point depression was determined in the cell culture media on a Microosmometer [Advanced Instruments, USA] with automated calculation of osmolarity.

3.5.2.2 Enzymatic methods

Lactate dehydrogenase (LDH)-leakage as an index of plasma membrane integrity [*Tyson et al., 1987; Welder et al., 1994; Amador et al., 1963*] was determined in the cell culture supernatant. Glucose and Lactate as markers of glycolysis were determined enzymatically in the cell culture supernatant [*Center for Disease Control, 1976*]. Aspartateanimotransferase (AST) and Alanineaminotransferase (ALT) as markers of liver damage [*Karmen et al., 1955*] were determined enzymatically in serum.
All determinations were conducted on a Beckman-Coulter Synchron-CX according to the manufacturer's recommendation by the Novartis SP&A Clinical Chemistry team.

Parameter	Enzymatic reaction	Ref.
LDH	LDH + Pyruvate + NADH + H^+ → L-lactate + NAD^+ assessment of the UV-absorption-change of NAD^+ / NADH	[Beckman Coulter, USA, #442660]
Glucose	Glucose + ATP + Hexokinase → glucose-6-phosphate + ADP Glucose-6-phosphate + NAD^+ +glucose-6-phosphate-dehydrogenase → 6-phosphogluconat + NADH + H^+ assessment of the UV-absorption-change of NAD^+ / NADH	[Beckman Coulter, USA, #442640]
Lactate	L-lactate + O_2 + lactateoxidase → pyruvat + H_2O_2 H_2O_2 + dichlorbenzolsufonic acid + 4-aminoantipyrin → chromophor measurement of absorption-change due to formation of the chromophor	[Beckman Coulter, USA, #445875]

Parameter	Enzymatic reaction	Ref.
AST	AST + L-aspartate + α-ketoglutarate → Oxalacetate + L-glutamate Oxalacetate + NADH + H^+ + malate dehydrogenase→ malate + NAD^+ measurement of the UV-absorption-change of NAD^+ / NADH	[Beckman Coulter, USA, #442665]
ALT	ALT + L-alanine + α-ketoglutarate → pyruvate + L-glutamate Pyruvate + NADH + H^+ + LDH→ latate + NAD^+ measurement of the UV-absorption-change of NAD^+ / NADH	[Beckman Coulter, USA, #442620]

3.5.2.3 MTS-bioreduction

MTS, a tetrazolium compound (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt), is bioreduced by mitochondrial dehydrogenase activity in metabolically active cells to a coloured soluble formazan. The absorbance of the formazan was measured in cell culture supernatant as a marker of cytotoxicity [*Malich et al., 1997*].

The CellTiter 96® AQueous One Solution [Promega, USA, #G3582] was added to the cell culture (5% of the culture medium) and incubated for 2-4 hrs, depending on the cell density and –type. 100 µl of the supernatant were transferred to a 96-well-plate and absorbance at 490 nm measured. All data was expressed relative to the value of the non-treated cells after subtraction of the blanc (medium + MTS).

3.5.2.4 Intracellular ATP

Luciferase requires ATP to convert luciferin to oxyluciferin, whereby energy is emitted as bioluminescence. The emitted light was used to calculate the amount of ATP present in the lysate against a defined ATP standard-curve [*Crouch et al., 1993*].

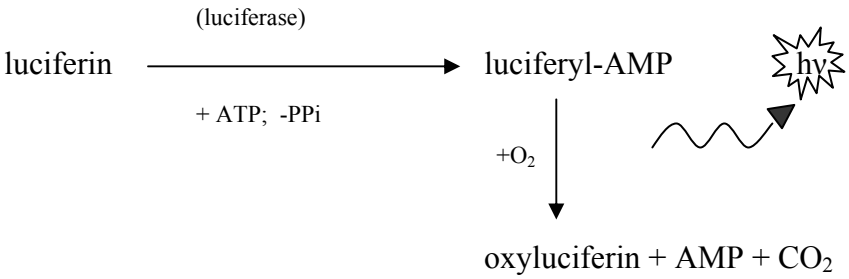


Figure 13: Principle of ATP-determination

For determination of ATP, cells were scraped into 300 µl of ice-cold ATP-buffer (100 mM Tris, 4 mM EDTA, pH 7.75), subsequently lysed by boiling for 4 min, and debris was removed by centrifugation (10`000 x *g*, 1 min). The clear supernatant was used with the ATP Bioluminescence Assay Kit CLC II [Roche, Switzerland, #1699695] according to the manufacturer`s recommendations. Protein content was determined using BioRad Protein Assay Kit [BioRad, USA, #500-0006; *Bradford et al., 1976*] with BSA as standard according to the manufacturer's recommendations from samples drawn before boiling.

3.5.2.5 Rhodamine 123-uptake

Rhodamine 123 uptake into intact mitochondria is dependent on the electrochemical gradient and was determined as a measure of the mitochondrial membrane potential [*Wu et al., 1990*].

Cultured cells were incubated with 1 µM rhodamine 123 [Invitrogen, USA, # R-302] in culture medium for 30 min, followed by washing cells on ice, lysis by ultrasonification in 50% ethanol and removal of debris (10`000 x *g*, 5 min). Rhodamin fluorescence was measured at λ_{ex} 485 nm / λ_{em} 530 nm on a fluorescence plate reader in 96-well format.

3.5.2.6 Caspase-activity

Liberation of a fluorescence-tag from a caspase-substrate was used to determine caspase-activity in cell lysates [*Thornberry, 1994*].

Buffers:

Collagenase-solution	DMEM:F12 1:1, containing 10 mM HEPES, 3 mM $CaCl_2$, pH 7.4, 365 CDU/ml Collagenase
stopping solution	DMEM:F12 1:1, containing 10 mM HEPES, 20% FBS, Complete protease inhibitory cocktail (Roche), 5 mM DTT, pH 7.
lysis buffer	25 mM HEPES, 5 mM $MgCl_2$, 5 mM EDTA, Complete protease inhibitory cocktail (Roche), 5 mM DTT , pH 7.5

Assay systems

caspase	inhibitor	substrate	Ref.
2	Ac-VDVAD-CHO	Ac-VDVAC-pNA	[Bachem, Switzerland, #N-1790 / L-2065]
3/7	Ac-DEVD-CHO	Ac-DEVD-AMC	[Promega, USA, #G3540]
8	Ac-IEPD-CHO	Ac-IEPD-AMC	[Bachem, Switzerland, #N-1740 / I-1835]
9	Ac-LEHD-CHO	Ac-LEHD-AMC	[Bachem, Switzerland, #N-1720 / N-1825]

Cells were liberated from collagen sandwich by 5 min incubation with collagenase-solution at 37°C. The activity was stopped by addition of an equal amount of stopping solution on ice. Cells were collected by centrifugation (100 x *g*, 4 min, 4°C), the supernatant discarded and the pellet resuspended in 250 µl lysis buffer and lysed by 4 repeated freeze-thaw-cycles. Debris was removed (16`000 x *g*, 4°C, 20 min), and the protein content of the clear supernatant was determined according to the method of Bradford [*Bradford et al., 1976*] using the BioRad Protein Assay Kit [BioRad, USA, #500-0006] with BSA as standard according to the manufacturer's recommendations.

All determinations of caspase-activities were developed parallel to the manufacturer's recommendations for the CaspACE Assay System [Promega, USA, # G3540].

For the following kinetic reactions, all samples were adjusted to contain the same amount of DMSO, assay buffer and DTT at the end. Per sample, 4x 75 µg of protein were pipetted into separate wells of a micro titer plate. To 2 portions, the respective caspase-inhibitor was added (2.5 mM). A standard curve of the respective label (0-20 µM AMC or 0-200 µM 4-nitroaniline [pNA]) was prepared under the same conditions as the other samples. Per sample, all wells (2x blanc without protein, 2x protein, 2x protein + inhibitor) were incubated with the

respective labelled caspase-substrate at 2.5 mM for exactly 60 min at 37°C. For AMC, fluorescence was measured at λ_{ex}=360 nm and λ_{em}=460 nm (gain=50); for pNA, absorption was measured at 405 nm. Caspase-activity was calculated: ΔFU1= (mean assay FU)-(mean blanc FU); ΔFU2= (mean negative control FU)-(mean blanc FU).

$$X\ [\text{pmol label liberated / min without inhibitor}] = \frac{\Delta FU1\text{-intercept}}{\text{time [min] x slope}}$$

$$Y\ [\text{pmol label liberated / min with inhibitor}] = \frac{\Delta FU2\text{-intercept}}{\text{time [min] x slope}}$$

$$\text{caspase-activity} = \frac{X-Y}{\text{protein [µg]}}\ [\text{pmol label liberated/min at 30°C / µg protein}]$$

3.5.2.7 Chlorzoxazone-6-hydroxylation

Cytochrome P450 2E1 protein activity was determined in the microsomal fraction of hepatocytes [*Mitoma et al., 1956*] by its capacity to hydroxylate Chlorzoxazone at the 6-position [*Abdulla et al., 2006; Frye et al., 1996; Kharasch et al., 1993; Yamazaki et al., 1995*].

Isolation of microsomes:

Buffers:

Mic I	80 mM K_2HPO_4, 20 mM KH_2PO_4, 250 mM sucrose, 1.3 mM EDTA, pH 7.4
Mic II	100 mM Tris-HCl, 100 mM KCl, pH 7.4
Storage buffer	25 mM K_2HPO_4, 15 mM KH_2PO_4, 20% glycerol, 50 mM KCl, pH 7.4

The frozen liver pieces (~1g/animal) were weighed and homogenised in ice-cold Mic I-buffer on a teflon-glass-potter, 10 strokes at 870 rpm, 15 sec/passage. Debris was removed by two centrifugation steps (9'000 x *g*, 20 min, 4°C), and the microsomal fraction was pelleted (100'000 x *g*, 1h, 4°C). Microsomes were washed once in Mic II-buffer, pelleted (100'000 x *g*, 1h, 4°C) and resuspended in either 2 volumes of the original wet weight storage buffer for

ex vivo-chlorzoxazone-6-hydroxylation or 1 volume of RIPA buffer [Santa Cruz, USA, # sc-24948] for western blotting. Microsomes were frozen to -80°C until further processing.

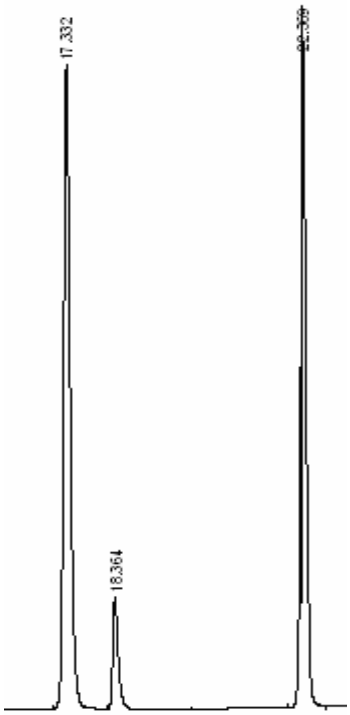
Ex-vivo-reaction and HPLC

HPLC conditions

Instrument	Hewlett-Packard 1100-series
Column	250 x 4 RP-C18e-column [Merck, Germany, #1.50957.0001/1.50838.0001]
Gradient	Acetonitrile: 0.5% H_3PO_4 10:90 isocratic for 10 min, gradient to 50:50 during 10 min, gradient to 10:90 during 3 min, 5 min isocratic
Detection	296 nm

The protein content of the individual samples was determined according to the method of Smith [*Smith et al., 1985*] using bovine serum albumin as a standard for the MicroBCA protein Kit [ThermoFischer Scientific, USA, #23235], according to the manufacturer's recommendation.

The kinetic reaction was conducted at 37°C in a total volume of 1 ml 50 mM KH_2PO_4, pH 7.4 and contained per sample 1 mg of microsomal protein, 100 µM Chlorzoxazone and 1 mM NADPH. The reaction was terminated after exactly 10 min by addition of 5 ml dichloromethane. 2 µg of pentoxifylline was added to each sample as internal standard. The organic phase was separated, and 4.5 ml were evaporated under a stream of nitrogen to complete dryness. Samples were taken up in Acetonitrile : H_3PO_4 22:78 and analyzed by HPLC/UV. Quantification was done vs. a defined mixture of Chlorzoxazone/6-OH-Chlorzoxazone and Pentoxifylline, which was run every tenth sample using the AUC/iST-value.



compound	retention time [min]
6-OH-CZX	17.366 ± 0.0042
pentoxifylline	18.389 ± 0.006
CZX	22.338 ± 0.0015

	Area [mAUC*s]					
	6-OH-CZX			CZX		
conc. [mg/l]	average	standard deviation	R^2 (linearity)	Average	standard deviation	R^2 (linearity)
5	537.6	12.8		57.4	1.3	
10	1110.0	23.1		107.0	1.6	
25	2793.3	11.9		271.1	1.1	
50	5350.8	199.5	0.9999	521.3	0.7	0.9979
100	10573.4	239.7		1077.8	4.0	
200	21991.7	336.1		2006.9	12.0	
400	43509.8	1382.5		3726.0	39.9	

typical chromatogram
obtained from a HPLC-run applying CZX, 6-OH-CZX and iST

validation of signal/concentration linearity:
Chlorzoxazone and the metabolite 6-hydroxychlorzoxazone in acetonitrile were run at different concentrations on the HPLC-system. The experiment was conducted three times.

3.5.3 Immunoassays

3.5.3.1 ELISA

3.5.3.1.1 BrdU-incorporation

BrdU is incorporated into the genomic DNA of proliferating cells alternatively to thymidin and can be used as a marker of cell proliferation [*Porstmann et al., 1985*]. BrdU-incorporation was detected by ELISA [*Engvall et al., 1971*] with tetramethylbenzidine-reaction [*Mesulam, 1978*] for detection.

In brief, cells were incubated during 24 h with BrdU and fixed on the culture plate.
BrdU-uptake was assessed by a commercially available ELISA kit [Roche, Switzerland, #1647229] according to the manufacturer's recommendation.

3.5.3.1.2 Interferon-β /-γ

The respective interferon of interest was bound to specific immobilised antibody [*Engvall et al., 1971*] and quantified by tetramethylbenzidine-reaction [*Mesulam, 1978*].

Interferon-γ [eBiosciences, USA, #88-8314-22] / Interferon-β [PBL Biomedical Laboratories, USA, #42400-1] from mouse serum was determined by ELISA according to the manufacturer's recommendations using non-diluted serum samples.

3.5.3.2 Western blot

Denaturized proteins were separated according to their mass/charge-ratio in an electric field by their migration pattern in a polyacrylamide-gel [*Schägger et al., 1987*]. The proteins were transferred to the surface of a polyvinylidenfluorid-membrane by electrochemical migration, where they were accessible for antibody-binding [*Renart et al., 1979*]; the protein band of interest was visualised by specific antibody binding and a chemoluminescent reaction [*Seitz, 1984*].

Buffers:

Cytosole-buffer	5 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, Complete protease inhibitory cocktail [Roche, Switzerland, #1697498], 5 mM DTT, pH 7.4
Membrane buffer	cytosole-buffer containing 2% Triton X-100
TBS-T	20 mM Tris base, 150 mM NaCl, 0.05 % Tween-20, pH 7.3

Assay-conditions:

protein	protein amount/lane	gel	blot
ATP synthase	50 µg of whole cell protein	Bis-Tris gel 4-12% (200 V, 35 min)	25 V, 1 h
BiP	10 µg of whole cell protein		
CYP2E1	10 µg of microsomal protein		
FPPS	25 µg of whole cell protein	Bis-Tris gel 12% (200 V, 45 min)	25 V, 1.5 h
RhoA/Rap1a	25 µg of protein from the desired fraction		

For western blotting from cultured cells, 2 x 10^6 cells/sample were scraped into 1 ml of HBSS, pelleted at 4°C by gentle centrifugation, the cells lysed by ultrasonification on ice in 25 µl of buffer (for whole cell lysate: RIPA-buffer completed with protease inhibitors (Santa Cruz), for fractionation cytosole-buffer). For whole cell lysate, the debris was removed by centrifugation (10'000 x *g*, 10 min, 4°C). For fractionation in cytosolic and membranous fraction, the lysate was centrifuged (1 h, 100'000 x *g* , 4°C), the supernatant collected as the cytosolic fraction, the pellet resuspended in 25 µl membrane buffer.

For western blotting from mouse liver microsomes, microsomes (see 3.5.2.7) were at the end of preparation resuspended in RIPA-buffer completed with protease inhibitors.

Protein was determined according to Smith [*Smith et al., 1985*] using the MicroBCA protein assay kit [ThermoFischer Scientific, USA, #23235] with BSA-protein standard according to the manufacturer's recommendations.

Protein was denaturised by boiling with 50 mM DTT (95°C, 5 min), subjected to each lane of the gels, followed by electrophoretic separation and wet blot [all material from Invitrogen, USA]. The membranes were blocked with 5% milk powder in TBS-T for 1 h at room temperature, incubated with primary antibody in TBS-T with 1% milk powder overnight at 4°C, and finally with the HRPO-linked secondary antibody in TBS-T with 1% milk powder for 1 h at room temperature.

Antibody [anti-]	from host	ref.
ATP synthase	goat	[Santa Cruz, USA, # sc-16689]
BiP	goat	[Santa Cruz, USA, # sc-1050]
CYP2E1	rabbit	[Stressgen, USA, # MFO-100]
FPPS	rabbit	[Novartis, Switzerland, # sz-2101]
RhoA	rabbit	[Santa Cruz, USA, # sc-179]
Rap1a	goat	[Santa Cruz, USA, # sc-1482]
Actin	goat	[Santa Cruz, USA, # sc-1615]
Calnexin	rabbit	[Sigma, USA, # C4731]
rabbit-HRPO	goat	[Sigma, USA, # A0545]
goat-HRPO	bovine	[Santa Cruz, USA,# sc-2378]

The membrane was covered with ECL-solution [Thermo Fisher Scientific, USA, #1856145/1856146], incubated for 5 min and analyzed on a FUJI LAS-3000 scanner applying AIDA-software [Raytest, France] for quantification of the specific protein band luminescence.

3.5.3.3 Immunohistochemistry

All stainings were performed by modified methods based on the principle technique of immunohistochemistry [*Coons et al., 1942*].

Cultured hepatocytes:

Buffers:

TB-T	0.05 M Tris, 0.15 M sodium chloride , 1% BSA, 0.05% Tween-20

Cells were cultured on collagen I-coated chamber-slides, after incubation washed with PBS, air-dried, sealed in plastic foil, and frozen to -80°C until staining. Slides were fixed with buffered paraformalin, washed twice with PBS and permeabilised (1% Triton X-100 in PBS, 4 min, RT). Slides were washed twice in PBS, unspecific binding sites were blocked (5% normal goat serum in TB-T, 10 min, RT) and incubated with the rabbit anti-CYP2E1-IgG (1:200 in TB-T, 4°C, overnight). The slides were washed twice in PBS and incubated with Alexa647-conjagated anti-rabbit IgG (1:200 in TB-T, 30 min, RT) [Invitrogen, USA, #A31634]. Slides were washed twice with PBS, the nuclei stained with DAPI (10 µg/ml in

TB-T, 10 min, RT), and mounted with Gel/Mount [Biomeda, USA, #M01], covered with a coverslip and analyzed on a confocal laser scanning microscope, iCys or Cellomics High Content reader.

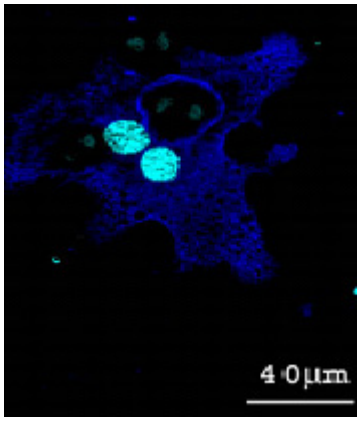


Figure 14: Intracellular distribution of CYP2E1 (IHC)
light-blue: nuclei; dark blue: CYP2E1

Paraffin-embedded organ slices:

Paraplast-blocks were sectioned at approximately 3 µm. Immunohistochemical staining for CYP 2E1 was performed using the fully automated instrument Discovery [Ventana Medical Systems SA, France]. Briefly, liver sections were deparaffinised and rehydrated under solvent-free conditions using EZprep solution for 8 minutes at 75°C followed by 8 more minutes at 42°C. Sections were then submitted to a heat induced epitope retrieval pretreatment by 8 successive incubations at 100°C for 4 minutes in Tris/Borate/EDTA cell conditioning™ solution (pH 8.00). Slides were incubated with primary antibody (anti-CYP2E1 [Stressgen, USA, # MFO-100] at 1/500) for 1 h at room temperature. Biotinylated secondary antibody [Jackson Immunoresearch, USA, #711065152] was applied at 1/500 for 16 minutes at room temperature. Finally, the immunologic reaction was revealed by a chromogenic detection with DAB Map™ kit. Slides were counterstained with hematoxylin, mounted with Crystal Mount and post-mounted with Pertex. Immunohistochemistry on paraffin-embedded organ slides was executed by the Molecular Pathology team of Novartis SP&A.

4 Results and Discussion

4.1 *In vitro*-Investigations

The following chapter will reflect the results obtained from *in vitro*-applications of the siRNA method. After development of the methods, results from three concrete applications of siRNA will be discussed. In these projects the siRNA method was used to silence specific gene expression of ATP synthase β subunit, AKR1B10, BiP and Hepcidin in HepG2-cells, the expression of farnesylpyrophosphate synthase in HK-2-cells and caspase-3 and -7 expression in rat primary hepatocytes.

4.1.1 Method development

4.1.1.1 Set-up of transfection conditions

The delivery of the siRNA to the target cells by transfection is a critical step to achieve successful gene silencing. There are different possibilities to establish transfection conditions. The delivery of fluorescence-labelled molecules or plasmids coding for fluorescent proteins to the target cells can be applied as they are easily detectable by eye under the fluorescence-microscope and give a quick overview about the amount of cells transfected. Nevertheless, there are some scientific concerns about the use of these techniques.

Fluorescent labeling of a siRNA increases its size and mass/charge-ratio, making it difficult to draw clear conclusions on the transfection efficiency of the target molecule. Whether the siRNA-molecules are implemented into RISC and functional, or degraded and the fluorescence-tag alone being detected is not clear. This can only be confirmed after transfection with siRNAs of known silencing capacities and monitoring of the knockdown on mRNA- or protein level. Furthermore, the real penetration of the cells is not clearly demonstrated as the fluorescent molecules can be associated with outer side of the cell membrane, especially when applying lipid-complexed siRNAs that are known to fuse with the membrane.

The application of plasmids coding for fluorescent proteins is not predictive for delivery of siRNAs to the target cell due to the high difference in size. The conventionally applied plasmids (~1.5 MDa) are much bigger than siRNAs (~15 kDa). From the point of delivery efficiency, the transfection of plasmids can thus only give a hint for the efficiency of the applied transfection method in regard to the intended siRNA-delivery to the target cell. Nevertheless, the method can demonstrate assured delivery of intact molecules to the target

cell as the fluorescence only becomes detectable upon expression of the protein which requires cellular uptake and intactness of the molecule.
The final target of the following investigations was to identify a robust and reproducible method to achieve siRNA-mediated knockdown. For this purpose, direct assessment of the knockdown on mRNA-level was chosen to be the most predictive marker of efficient siRNA delivery to the targeted cells.

Human primary hepatocytes with unknown transfection conditions were chosen to serve as a model for comparison of different transfection methods. A siRNA targeting ATP synthase with proven silencing-capacity (see table 9) was selected as the molecule to be delivered to the human hepatocytes by several chemical methods, chemical modification by tagging with cholesterol (active uptake into hepatocytes propagated [*Soutschek et al., 2004*]) or physical delivery of siRNAs by electroporation. Monitoring of direct silencing capacity by detection of specific target mRNA-depletion was chosen as the most predictive marker of successful delivery of siRNA to the target cells.
In all cases, silencing effects were compared to mismatch-transfected cells. The mismatch-siRNA was an oligonucleotide directed against Green Fluorescent Protein which is not expressed in any of the cells used in this work, and served here as a non-silencing control.
Human primary hepatocytes were either plated on collagen gel and transfected under different conditions or electroporated in suspension and afterwards plated.
For chemical transfection of 150'000 hepatocytes, siRNAs complexed with the transfection reagents were applied at 50 nM final concentration for 24 h. For each reagent, three siRNA/reagent-ratios were tested, covering the optimal complexation ranges recommended by the manufacturers. Lipofectamin [Invitrogen, USA, #11668-019] was used at 100, 50 and 33.3 pmol siRNA/µl, and jetPEI [Polyplus,USA, #10101N] at 100, 50 and 25 pmol/ µl.
For application of cholesterol-linked siRNA, cells were serum-starved overnight to trigger cholesterol-uptake by intracellular depletion. The siRNA was directly applied at 50 and 100 nM for 24 h to the attached hepatocytes without complexation.
For electroporation, $2x10^6$ hepatocytes were electroporated with 30 µmol siRNA (end concentration 300 nM, as suggested in the literature [*Jarvis et al., 2003*]) and plated on collagen gel. Two different electroporation media with each four conditions of electric square wave-pulsing recommended by the manufacturer were tested, as human primary hepatocytes had not been tested in this system.

Cells were covered afterwards by a second layer of collagen and cultured for mRNA-depletion. The mRNA-expression of the target gene ATP synthase β-subunit was investigated by qPCR 48h after start of transfection. The expression of the target mRNA was normalised to the corresponding expression in mismatch-transfected cells. The highest mRNA-knockdown achieved by the corresponding method is shown.

	ATP synthase mRNA-expression [% of control]	
	mismatch siRNA	siRNA vs. ATP synthase
Lipofectamin 2000	100	45 ± 79
jetPEI-gal	100	74 ± 75
Cholesterol-modification of the siRNA	100	61 ± 1
electroporation	100	37 ± 3

Table 6: Comparison of different transfection methods
The experiment was executed once with 3 replicates. ± values indicate the standard-deviation of replicates. GAPDH was used as the housekeeping gene for qPCR.

The achieved knockdown-efficiencies were very diverse, ranking from 25% to 63% knockdown. The chemical transfection methods resulted in scatter of the replicate data. Cholesterol-modification of the siRNA led to a minor knockdown of 39%. Electroporation achieved the highest efficiency with 63% knockdown, and little statistical scatter of the data was observed.

The mRNA-knockdown investigated in the previous experiment allows conclusions on overall delivery of the siRNA to the entire population but not on the percentage of transfected cells. Electroporation was investigated in this regard, and fluorescent systems were used for visualisation. A plasmid containing green fluorescent protein (pHygeGFP) was amplified in *E. coli*, isolated and applied for transfection. The intracellular expression of the expressed fluorescent protein was checked microscopically, and quantified using automated fluorescence microscopy.

HepG2-cells were transfected with the plasmid at 1, 5, 7.5, 10, 15 and 20 µg/µl using the electroporation conditions recommended by the manufacturer. The transfection efficiency was analyzed by means of GFP-fluorescence after counter-staining of the nuclei with Hoechst 33352 using a high-content screening system [Cellomics, USA]. The average fluorescence in cells electroporated without plasmid was subtracted from all experimental values and set to 0% transfection efficiency.

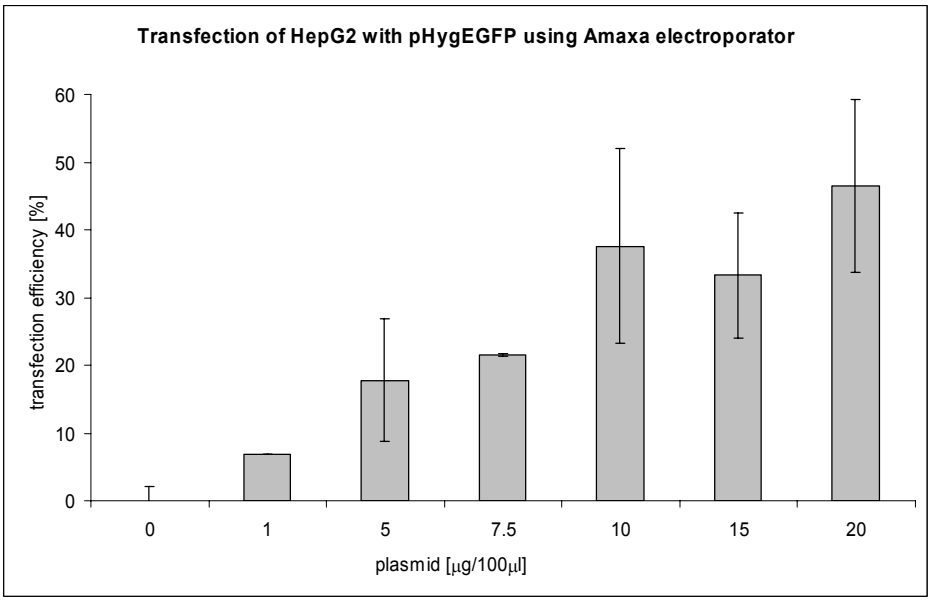
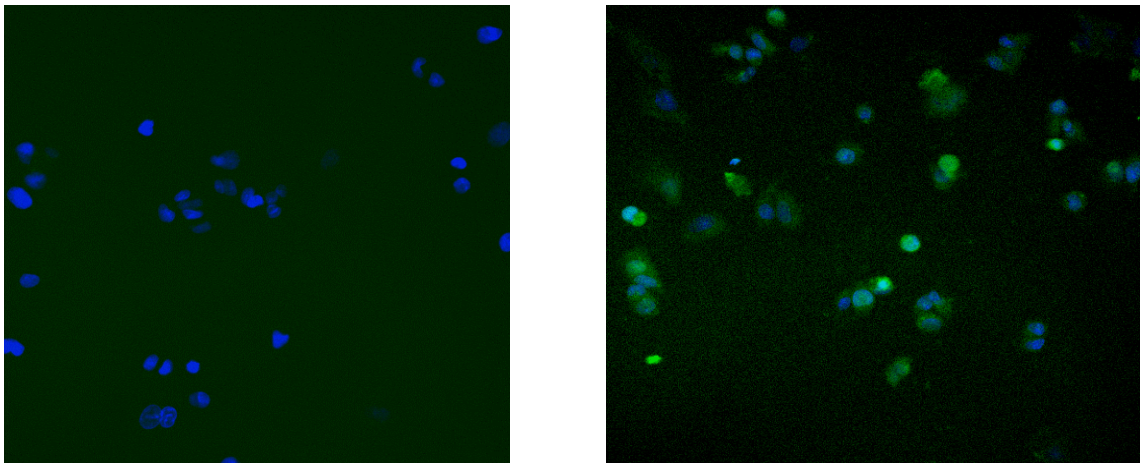


Figure 15: Assessment of transfection efficiency by a plasmid
The experiment was executed 2 times, the error bars symbolise the range of the experimental values.

Electroporation with different concentrations of plasmid led to a dose-dependent increase in the subpopulation of GFP-expressing cells. The percentage of HepG2-cells displaying GFP-fluorescence at the highest plasmid-concentration applied was 46%.

In order to more closely estimate the transfection efficiency with the small siRNA molecules, HepG2-cells were transfected with 200 nM fluoresceinisothiocyanate (FITC)-labelled mismatch-siRNA. Control cells were sham-electroporated without siRNA. After counterstaining of the nuclei with Hoechst 33352, cells were photographed under the fluorescence-microscope.



control labelled siRNA

Figure 16: Assessment of transfection efficiency by labelled siRNA

Nuclei appear blue, and FITC-fluorescence in the cytoplasm appears green. Upon transfection by electroporation, cytoplasmatic FITC-fluorescence became detectable in a huge population of HepG2-cells (estimated 85%).

The application of elevated concentrations of siRNA applied for electroporation could have impact on gene silencing.

HepG2-cells were electroporated with either 100, 200, 300 or 400 nM mismatch- or active siRNA targeting BiP (see table 9). qPCR on BiP mRNA-expression was performed 48 h after transfection. The mRNA-expression of the cells transfected with active siRNA was normalised to the expression in mismatch-transfected cells.

	BiP mRNA-expression [% of control]	
siRNA-concentration	mismatch siRNA	siRNA vs. BiP
100 nM	100	36
200 nM	100	33
300 nM	100	36
400 nM	100	34

Table 7: Dose-dependence of mRNA-knockdown at electroporation
GAPDH was the housekeeping gene for qPCR. The experiment was conducted once

None of the different concentrations of siRNA (100-400 nM) applied at the time point of electroporation had significant impact in the siRNA-mediated reduction of mRNA-expression, which was reduced by ~65% compared to the mismatch-transfected cells.

Electroporation was also applied to transfect HK-2-cells. Transfection of the cell-line was optimised applying the ATP synthase-siRNA (see table 9) at 300 nM. Cells were electroporated in an electroporation medium provided by the manufacturer for primary mammalian epithelial cells applying different sequences and strengths of electric pulses represented by different electroporation-programs. mRNA-expression as a marker of silencing efficiency was investigated 48 h after transfection. The ATP synthase-mRNA expression in silenced cells was normalised to the corresponding expression in mismatch-transfected cells.

	ATP synthase mRNA-expression [% of control]	
electroporation program	mismatch siRNA	siRNA vs. ATP synthase
S-05	100	41
T-13	100	69
T-20	100	39
U-17	100	8

Table 8: Optimisation of HK-2-transfection
GAPDH was the housekeeping gene for qPCR. The experiment was conducted once.

Application of the electroporation program T-13 led to a knockdown of 31%, S-05 and T-20 achieved ~60% silencing, while U-17 reached the most significant knockdown with 92%.

4.1.1.2 Selection of siRNAs

SiRNAs can be selected by determination of the silencing-capacity on different molecular levels. The functional consequence of gene silencing for the cell is the most predicative readout. Therefore, activity of the target protein is the most predictive endpoint for selection. If not accessible, protein expression would be the endpoint of choice, still reflecting posttranslational consequences of the gene silencing. The determination of gene silencing on mRNA-level is not predictive for cellular consequences, and the silencing capacity has to be proven by investigation on protein level.
Attack of a target mRNA from different sites could be considered to be more efficient than from one single site. Thus, pooling of different siRNAs while not increasing the overall siRNA-concentration could lead to over-additional silencing.

4.1.1.2.1 Selection on mRNA-level

Five genes were chosen as targets for siRNA (see table 16). Two siRNAs per gene were tested in HeLa cells as an easy-to-transfect target with known transfection-conditions. The candidates were identified by a computer-based in-house algorithm. 75% reduction of mRNA was set as the threshold for acceptance of the siRNA.
Cultured HeLa-Cells were transfected using 1 µl RNAifect [Qiagen, USA, #301605] and 113 nM siRNA vs. ATP synthase. mRNA-expression of ATP synthase was determined after 48 h by qPCR. The ATP synthase-mRNA expression in silenced cells was normalised to the corresponding expression in mismatch-transfected cells.

	mRNA-expression [% of mismatch]				
	ATP synthase	BiP	AKR1B10	Hepcidin	ALAS-1
mismatch	100	100	100	100	100
siRNA 1	20 ± 7.5	82 ± 88.6	25 ± 2.2	45 ± 2.3	147 ± 77.4
siRNA 2	28 ± 7.6	22 ± 7.8	40 ± 9.9	44 ±2.2	63 ±1.7

Table 9: siRNA selection on mRNA-level: target genes in HepG2
GAPDH was the housekeeping gene for qPCR. The experiment was executed 2 times, ± values indicating the range of the experimental values.

ATP synthase, BiP and AKR1B10 could be silenced on mRNA-level to the desired threshold knockdown of 75%, and were directly used for further investigations, while Hepcidin- and ALAS-1-targeted siRNAs led to minor knockdowns.

Hepcidin and ALAS-1 that could not be successfully silenced in the first test were re-investigated in HepG2, which was chosen to be the target cell for investigation (see 4.1.2.2.1). Two more siRNAs per gene were tested. For this purpose, HepG2-cells were transfected by electroporation with 300 nM of the corresponding siRNA and mRNA-expression of the target gene was determined after 24 h by qPCR. The mRNA expression of the target-gene in silenced cells was normalised to the corresponding expression in mismatch-transfected cells.

	mRNA-expression [% of mismatch]	
	Hepcidin	ALAS-1
mismatch	100	100
siRNA 3	50	103
siRNA 4	325	65

Table 10: Test of siRNAs targeting Hepcidin and ALAS-1 in HepG2
GAPDH was the housekeeping gene for qPCR. The experiment was executed 2 times, the ± values indicate the range of the experimental values.

One siRNA targeting Hepcidin led to a 50% reduction of mRNA-expression, and one siRNA for ALAS-1 reduced mRNA-expression by 35%, while the other candidate siRNAs were not efficient in silencing or even led to induction of the targeted mRNA.
The three candidate siRNAs with the highest knockdown efficiency were pooled at equivalent proportions. HepG2-cells were transfected by electroporation with the resulting pool at a final siRNA concentration of 300 nM. The mRNA expression of the target-gene in silenced cells was normalised to the corresponding expression in mismatch-transfected cells.

	mRNA-expression [% of mismatch]	
	Hepcidin	ALAS-1
mismatch	100	100
pool of siRNAs 1-3, 1:1:1	24 ± 13	
pool of siRNAs 2-4, 1:1:1		60 ± 21

Table 11: Pooling of siRNAs to increase efficiency
GAPDH was the housekeeping gene for qPCR. The experiment was executed two times, the ± values indicate the range of the experimental values.

For Hepcidin, the pooling led to achievement of the threshold (75% mRNA-reduction). For ALAS-1, pooling of the siRNAs slightly further increased silencing capacity but did not lead to achievement of the threshold.

To identify an efficient siRNA targeting caspase-7 in rat primary hepatocytes, cells were electroporated with 300 nM of four different siRNAs. Caspase-7-mRNA expression was investigated after 24 h by qPCR. The mRNA expression of caspase-7 in silenced cells was normalised to the corresponding expression in mismatch-transfected cells.

	Caspase-7-mRNA [% of control]
mismatch	100
siRNA 1	1
siRNA 2	2
siRNA 3	5
siRNA 4	3

Table 12: siRNA selection on mRNA-level: Caspase-7
The experiment was performed on hepatocytes isolated from one animal.
The housekeeping gene for qPCR was β-actin.

All tested siRNAs were able to reduce caspase-7 mRNA-expression by 98 ± 2%, thereby exceeding the set threshold of 75% knockdown on mRNA-level.

4.1.1.2.2 Selection on mRNA- and protein-expression level

mRNA-knockdown is expected quickly after transfection (24-72h), and protein expression is reduced with delay (48-96 h after transfection) [*Amaxa, 2007*]. To knock down farnesylpyrophosphate synthase (FPPS) in HK-2-cells, single siRNAs were not tested, but four algorithm-selected candidates were directly pooled. Cells were transfected using the formerly established electroporation conditions for HK-2 cells (see table 8) with the siRNA pool at 300 nM. mRNA-expression was investigated 48 h after transfection by qPCR, and protein-expression 72 h after transfection by western blot. The expression of the target-mRNA/protein in silenced cells was normalised to the corresponding expression in mismatch-transfected cells.

	[% of control]	
	FPPS-mRNA expression (48h)	FPPS-protein expression (72h)
mismatch	100	100
siRNA-pool	36 ± 7	30 ± 7

Table 13: Selection of siRNAs on mRNA and protein level
The experiment was performed once in triplicate. The housekeeping gene for qPCR was β-actin.

Under the applied conditions, FPPS mRNA could be decreased by 64 % after 48h, and protein was decreased by 70 % after 72 h.

4.1.1.2.3 Selection on protein-activity level

Determination of protein activity is the most predictive marker for efficient gene silencing and was thus applied for siRNA selection.
Four different siRNAs vs. Caspase-3 were compared in regard to their capability to reduce caspase-3 protein activity. For this purpose, rat primary hepatocytes were electroporated with either 300 nM of mismatch-siRNA or different siRNAs targeting Caspase-3. Cells were kept in culture for 96 h for protein depletion and incubated under serum-free conditions with 50 µM SDZ IMM125, an inducer of caspase-3-activity (see 4.1.4.1), for 4 hours. Caspase-activity in the cytosole of the hepatocytes was determined and compared.

	Caspase-3/7-activity [pmol AMC liberated/min/µg protein at 30°C]	
siRNA	vehicle (0.5% DMSO)	+50 µM SDZ IMM 125
mismatch (GFP)	6 ± 2	270 ± 38
siRNA 1	5 ± 1	109 ± 1
siRNA 2	5 ± 1	49 ± 8
siRNA 3	6 ± 1	216 ± 18
siRNA4	4 ± 1	60 ± 5
pool of siRNAs 2 and 4 (1:1)	4 ± 1	43 ± 16

Table 14: Selection of siRNAs on protein activity level
The experiment was performed in triplicate on hepatocytes isolated from one animal. ± values symbolise the standard deviation of the different wells.

The SDZ IMM125-treatment of the mismatch-transfected hepatocytes led to 45-fold activation of caspase-3/7 compared to non-treated, mismatch transfected cells. Transfection with siRNA 2 led to reduction of this activity signal by 82%, and siRNA 4 reduced activity by

78% compared to mismatch-transfected, SDZ IMM125-treated cells. SiRNAs 1 and 3 were less sufficient, leading to reduction of the activity signal by 40% or 22%, respectively .
To assess possible enhanced silencing capacity by pooling, siRNAs 2 and 4 were pooled 1:1 and rat hepatocytes were electroporated with 300 nM of the resulting pool. Cells were treated with SDZ IMM125 under the same conditions as indicated for the previous experiment.

	Caspase-3/7-activity [pmol AMC liberated/min/µg protein at 30°C]	
siRNA	vehicle (0.5% DMSO)	+50 µM SDZ IMM 125
mismatch (GFP)	6 ± 2	270 ± 38
pool of siRNAs 2 and 4 (1:1)	4 ± 1	43 ± 16

Table 15: Pooling of siRNAs targeting Caspase-3
The experiment was performed in triplicate on hepatocytes isolated from one animal. ± values symbolise the standard deviation of the different wells.

Transfection with the pooled siRNAs led to reduction of the SDZ IMM125-induced caspase-activity by 84% compared to mismatch-transfected, SDZ IMM125-treated cells, which was not superior to application of the single siRNA2 in regard to silencing capacity.

4.1.1.2.4 Selection for *in vivo*-application

For *in vivo*-application of siRNAs, the *in vitro* performance of the candidate siRNAs was checked before application *in vivo*.
For this purpose, 70 siRNAs were designed with support of a computer-based algorithm. The siRNAs were tested in a fluorescent reporter-assay. A mouse CYP2E1-expressing plasmid was amplified in E. coli, isolated and inserted into a reporter vector. The vector expressed CFP and YFP, and mouse CYP2E1 was inserted associated with the YFP-coding region. In a fully automated process, COS-1-cells were cotransfected with the resulting vector and a validated siRNA targeting YFP-expression (2 µl/ml Fugene [Roche, Switzerland, #4709691], 1 ng/µl plasmid, 3.5, 5.3, 7.9, 12, 18, 27 and 40 nM siRNA using 8 µl/ml Oligofectamine [Invitrogen, USA, #12252011]). A mismatch-control siRNA was applied at 40 nM. The fluorescence of CFP and YFP was measured after 72 h. CFP fluorescence served as transfection control. YFP-fluorescence was the reporter-fluorescence for silencing-capacity of the siRNAs and normalised to the YFP-readout achieved with the control siRNA.

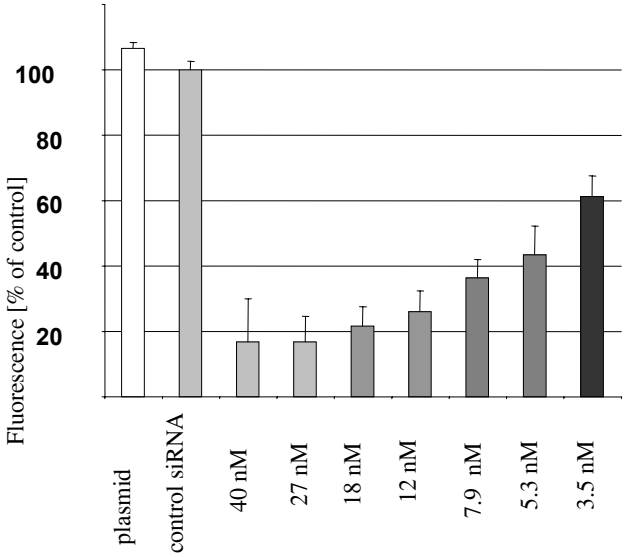


Figure 17: Dose-dependence of reporter-assay

The silencing was dose-dependent. 40 nM of the validated siRNA targeting YFP resulted in 82% reduction of the output signal, and 3.5 nM of the same siRNA achieved reduction of 39%.

The 70 siRNAs designed to target CYP2E1 were cotransfected with the plasmid at the fixed concentration of 15 nM under the conditions mentioned in the previous experiment.

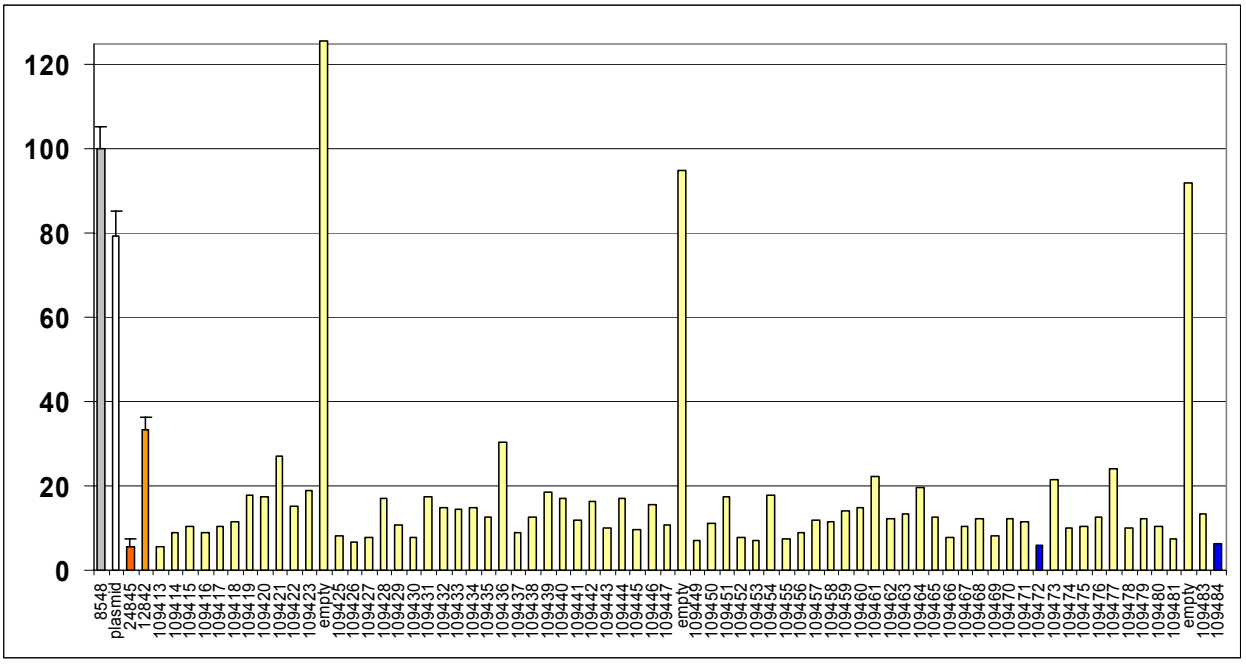


Figure 18: siRNA-screen by reporter-assay

Only 3 siRNAs were not effective in silencing mouse CYP2E1. The two most efficient candidates (indicated in blue) achieved silencing of >95% .

The two candidates identified in the previous experiment were applied for further *in vitro*-testing in primary mouse hepatocytes with protein expression as a predictive marker of gene silencing. Non-modified siRNAs have short *in vivo*-half lives. Thus, the siRNAs were modified for increased RNAse-stability (see 2.4.4.).

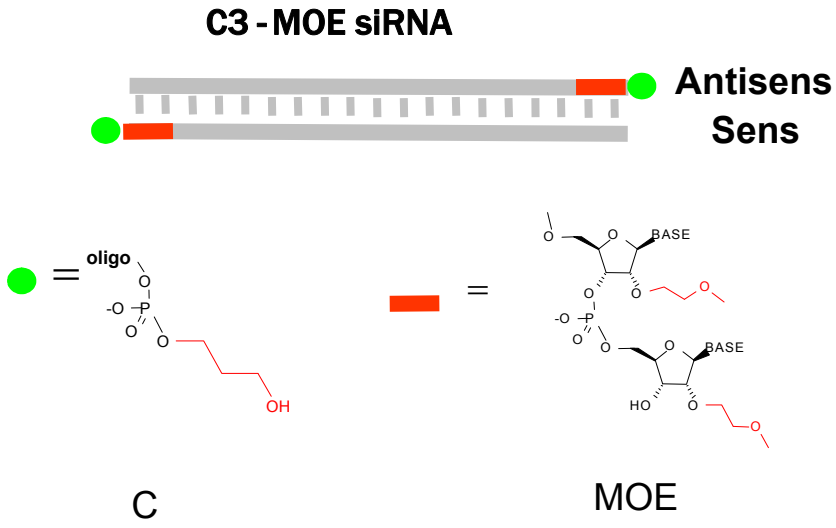
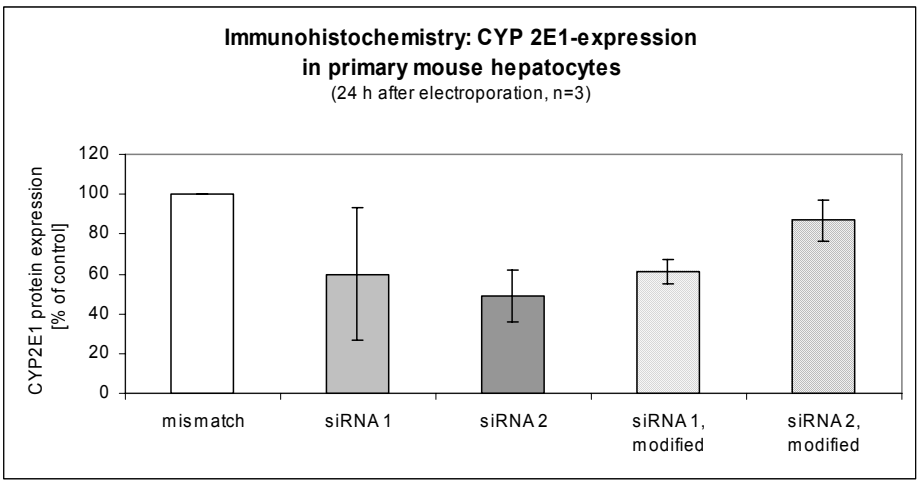
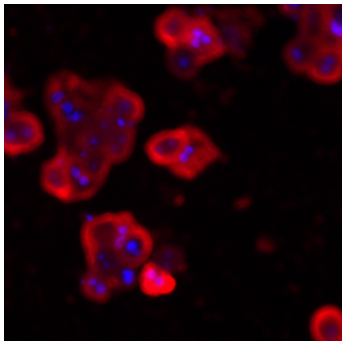


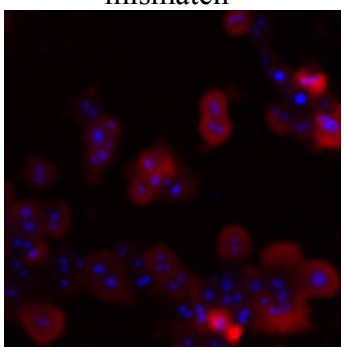
Figure 19: Modification of the siRNAs for *in vivo* application
Both strands were modified at their 3'-end with C3 (i.e. a hydroxypropyl phosphodiester moiety symbolised by a green dot) and by two 2'-moe nucleotides symbolised in red

Modified siRNAs were tested versus their non-modified analogue. For this purpose, primary mouse hepatocytes were electroporated with 300 nM of the native and modified siRNAs and cultured on collagen-gel for 24 h. Cells were stained immunohistochemistrically with an antibody recognising CYP2E1 and DAPI for counterstaining of the nuclei. The antibody-binding was visualised by application of a labelled secondary antibody recognising the primary CYP2E1-antibody. The stained cells were analysed by high-content fluorescence microscopy [Cellomics, USA], and the CYP2E1-originating fluorescence was normalised to the fluorescence obtained from mismatch-transfected hepatocytes.





mismatch



siRNA1

Figure 20: ***In vitro*-evaluation of CYP2E1-siRNAs for *in vivo-use***

DAPI-stained nuclei appear in blue, and CYP2E1-specific staining is displayed in red. All applied siRNAs led to reduction of CYP2E1 protein expression in cell culture. The observed reduction on protein level was ~40% for the unmodified siRNAs and the modified siRNA 1. The modified siRNA 2 had a low silencing capacity of 14 %.

4.1.1.3 Evaluation of interferon-response

Different stimuli like electroporation as transfection method, the siRNAs *per se* or the range of siRNA concentrations used in the experiments could provoke interferon-response in target cells. OAS-1 mRNA was proposed in the literature [*Pebernard et al., 2004*] as a marker for this mechanism and therefore investigated in HepG2.

A long double-stranded RNA-fragment (1kb-fragment of the lacZ-gene) applied as a stress inducer served as a strong positive control. HepG2-cells were electroporated with 10 and 100 nM of long double-stranded RNA-fragments or 300 nM of siRNA targeting ATP synthase β subunit applied in silencing experiments of this thesis. Non-electroporated control cells were used as a control. OAS-1-mRNA expression was investigated after 24 h by qPCR, and normalised to the corresponding expression in cells sham-electroporated without siRNA.

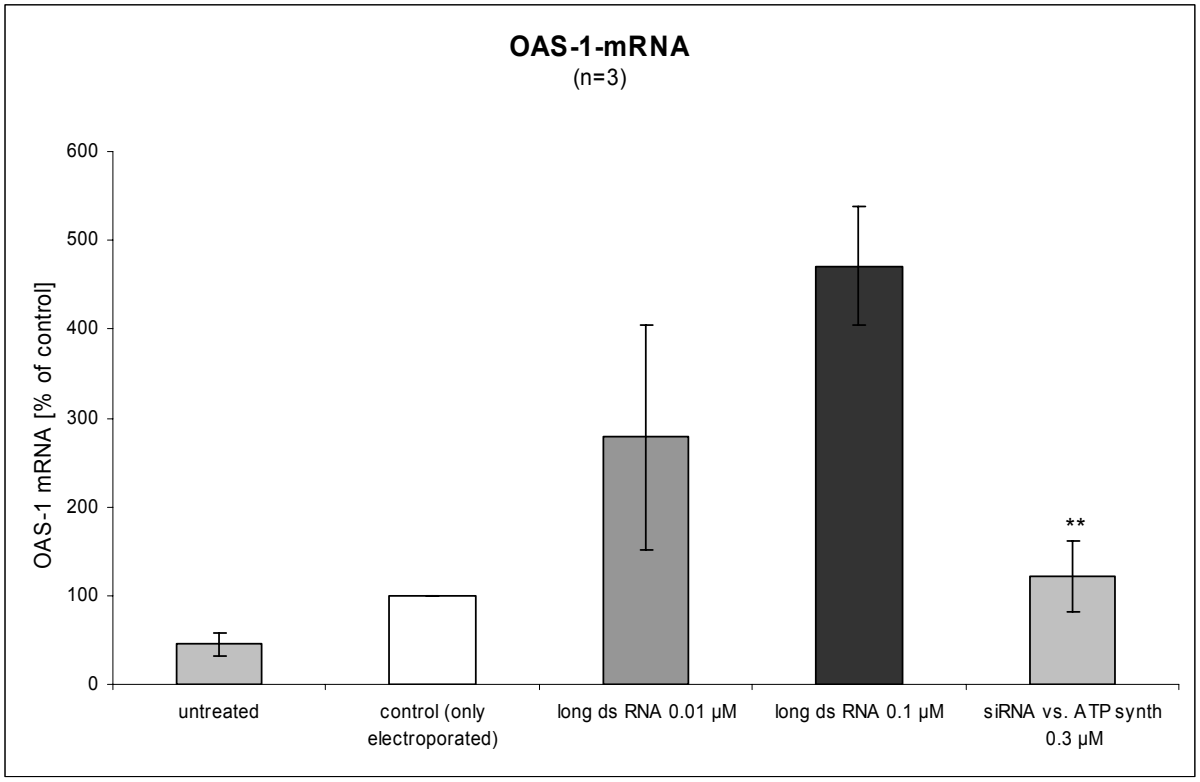


Figure 21: OAS-1-induction upon siRNA-transfection
The housekeeping gene for qPCR was β-actin. Data was acquired in three independent experiments, error bars indicate the standard deviation of the experiments. Statistics was done using One-way ANOVA with SNK post-hoc test (**: $p<0.05$ compared to highest conc. of long ds RNA).

Transfection with long double-stranded RNA led to dose-dependent increase in OAS-1 mRNA-expression. The highest applied concentration of long double-stranded RNA resulted in 4.5-fold induction of OAS-1-mRNA. The applied siRNA targeting ATP synthase did not induce OAS-1 beyond the expression level in sham-transfected cells. Untreated cells without electroporation were marginally lower in OAS-1-expression.

4.1.1.4 Discussion

Electroporation is reviewed in the literature as a universal method for delivery of nucleic acids to hard-to-transfect and primary cells [*Sakai et al., 2005; Liu et al., 2002*].

The suitability of the method for siRNA delivery to target cells could be confirmed in the conducted studies. In a comparison of different chemical and physical transfection methods, electroporation was identified to be more efficient in transfection of human primary hepatocytes with siRNAs compared to the two chemical methods applied and cholesterol-modification of the siRNA as determined by direct assessment of silencing capacity of the siRNA on mRNA-level. The reproducibility of electroporation was superior to chemical transfectants in this investigation, as indicated by lower statistical scatter of the obtained mRNA-readout data. The method demonstrated ability to efficiently deliver plasmids and fluorescence-labelled siRNAs to HepG2-cells. Two cell lines and primary hepatocytes isolated from three different species could be transfected successfully with siRNA as determined by knockdown on mRNA or protein expression or protein activity, demonstrating the universal character of the method. Due to the universal applicability of this method and its strength in transfection of hard-to-transfect and primary cells, the method was selected for transfection of all investigated models. A siRNA with a known silencing potential could be applied for optimisation of electroporation conditions for cells with unknown transfection conditions as demonstrated in HK-2-cells.

According to the literature, standard-concentrations for electroporation of siRNAs are in the range of 100 nM – 1 µM [*Jarvis et al., 2003*]. The application of different concentrations of siRNA within this standard range did not result in elevated gene silencing. The dose of siRNA used for electroporation was in all cases 300 nM. Compared to chemical transfection, the applied dose was high. Electroporation is opening the cellular membrane only for a short period and in this small time window only siRNAs are able to diffuse into the cells. In all experiments cells were diluted in culture medium right after exposure to the electrical square wave-pulses leading to a concentration of 14 nM siRNA during attachment phase. Medium was renewed after attachment of the cells, thereby removing the siRNA from the cell culture. In a typical setup of chemical transfection, the siRNA is present in the cell culture supernatant during the whole period of transfection (typically 24h). During this period, complexed siRNAs are able to fuse with the membrane and deliver siRNA, even enhanced due to gravity force.

Selection of siRNAs could effectively be conducted by direct assessment of knockdown-efficiency on mRNA-/ protein expression level or by means of protein activity.

SiRNAs for specific gene silencing of human ATP synthase β-subunit, AKR1B10, BiP, Hepcidin and rat caspase-7 could be identified by their capacity to reduce target mRNA by >75%. Pooling of siRNAs lead to increased silencing capacity in the case of Hepcidin.

Direct pooling of four siRNAs targeting farnesylpyrophosphate synthase led to efficient knockdown. The silencing capacity of the pool was assessed in one experiment by determination of mRNA- and protein expression at the suspected time points of knockdown at 24 and 72 h after transfection, respectively. Extensive screening of candidate siRNAs could be avoided by this approach, and one experiment provided not only information about mRNA-knockdown, but as well insight on the impact of transfection with the investigated siRNA-pool on protein expression.

Elevated silencing capacity by pooling of siRNAs could not be generalised for all cases, as shown with two siRNAs targeting caspase-3. The pooling of the two potent candidate siRNAs did not lead to elevated silencing capacity. In this case the use of the single effective siRNA was preferred for all further experiments, keeping the system as simple as possible.

A functional siRNA targeting caspase-3 could be identified by means of reduction of specific protein activity. The latter can be suggested to be used as the preferred marker of selection as this is not only demonstrating the ability of a siRNA to interfere with mRNA-expression but also reflecting the capability of the siRNA to reduce the target protein's activity and thereby demonstrating consequences for the cell on functionally relevant levels.

For the application *in vivo* the siRNAs were tested *in vitro* in advance to application to animals. A fluorescence-based reporter assay was shown to reflect siRNA-mediated gene silencing capacity quantitatively and could be applied for pre-selection, narrowing the amount of siRNAs to be tested from 70 to two candidates. The silencing potential of the selected two siRNAs was confirmed on protein level and led to identification of a suitable siRNA.

In vitro, OAS-1 induction as a marker of interferon response could be demonstrated by transfection of HepG2-cells with long double stranded RNA. The observed 5-fold induction of OAS-1 mRNA is low compared to the literature, where up to 1000-fold induction is reported [*Pebernard et al., 2004*]. This is indicating that HepG2 as a permanent cell line could have reduced sensitivity to long ds-RNA-induced interferon response. This is supported by published data [*Invitrogen, 2004*]. The siRNA targeting ATP synthase β subunit applied in the experiments did not provoke OAS-1-induction. By this finding and support from the literature

[*Elbashir et al., 2001*], the applied conditions for siRNA-transfection in this studies can be considered as safe in regard to interferon-response.

In conclusion, electroporation could be demonstrated to be a suitable efficient and reproducible method for siRNA delivery to various cell types. siRNAs for the knockdown of human ATP synthase β-subunit, AKR1B10, BiP, Hepcidin, farnesylpyrophosphate synthase, rat caspase-3 and -7 and mouse CYP2E1 could be identified for application in further experiments by determination of mRNA-/protein expression or protein activity.

4.1.2 Pathway-analysis after silencing ATP synthase

4.1.2.1 Background

The mitochondrial ATP synthase catalyzes regeneration of ADP to ATP using the electromotoric force stored in the electrochemical gradient above the inner mitochondrial membrane. It consists of a membrane-spanning proton channel, the F_O-unit, and a F_1-unit that is able to bind ADP and P_i, regenerating ATP in a conformation-controlled reaction driven by the proton motif force of the gradient.

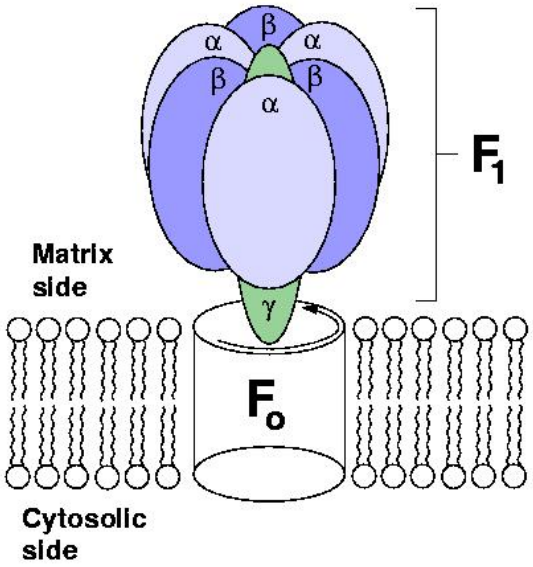


Figure 22: The mitochondrial ATP synthase complex
[http://www.mpibpc.mpg.de/groups/grubmueller/start/projects/atpase/atpase_intro.html]

The β-subunit of the mitochondrial F_1-ATP synthase investigated in this work is part of the ADP binding site and thus critical for the functionality of the complex. Defunctionalisation (or movement-hindering) of one of the three β-subunit completely stops (or slows-down) activity [*Ariga et al., 2002*]. Inhibition of the β-subunit by Aurovertin B, a pyrone produced by *Calcarisporium Arbuscula* [*van Raaij et al., 1996*], leads to ATP depletion but not cytotoxicity in mammalian cells [*Grover et al., 2004; Shchepina et al., 2002*]. A defect of this subunit in a newborn from a consanguineous marriage manifested clinically in severe lactic acidosis, cardio- and hepatomegalie and led to death short after birth [*Houštek et al., 1999*]. In this case the defect was selective with respect to other proteins of the oxidative phosphorylation, leading to mitochondrial hyperpolarisation and a decreased capacity of ATP-production.

The β-subunit of ATP synthase is critical for cell survival in situations, where the mitochondrial membrane potential is compromised. Expression of the pro-apoptotic BAX in yeast leads to mitochondrial hyperpolarisation, growth-arrest and cell death. When the β-subunit of the mitochondrial F1-ATP synthase is knocked out and BAX is expressed cells stay growth-arrested but do not die like wild-type cells [*Gross et al., 2000*].
Down regulation of the β-subunit of ATP synthase has recently been correlated with various types of cancer and their resistance to chemotherapeutics by genomics approaches [*Santamaria et al., 2006; Shin et al., 2005; Cuezva et al., 2002/2004; Isidoro et al., 2004*]. The meaning of this down regulation is not yet elucidated.

The reason for choosing the β-subunit of ATP synthase as a target for mechanistic gene silencing was a top-down, data-driven genomics approach. It was based on a certain mRNA-signature identified *in vivo* in the livers of Diclofenac-treated cynomolgus monkeys and rats. ATP synthase β-subunit was one of the targets regulated upon the treatment, and is supposed to be a critical protein for cellular metabolism.

name	synonyms	Gene accession number	Expression upon Diclofenac-treatment
ATP synthase, mitochondrial F1-complex, beta polypeptide	F1-ATPase, ATP5B, F1-FO-ATPase	NM_001686	↓
ALAS-1 (5-Aminolaevulinate synthase)		NM_000688	↓
AKR1B10 (aldose-keto-reductase 1B10)	ARL-1	NM_020299	↑
Hepcidin (hepatic bactericidal protein)	LEAP=liver expressed antimicrobial peptide	NM_021175	↓
BiP (immunoglobulin binding protein)	GRP78=glucose-regulated protein 78 kDA or HspA5=heat-shock protein A5	NM_005347	↓

Table 16: mRNA expression profile in monkey liver after *in vivo*-Diclofenac-treatment

Diclofenac is a non-selective inhibitor of cyclooxygenases and a non-steroidal, anti-inflammatory drug (NSAID). It is extensively prescribed for the treatment of pain resulting from osteo- and rheumatoid arthritis, ankylosing spondylitis, and acute muscle pain [*Boelsterli, 2003; Tang, 2003*].

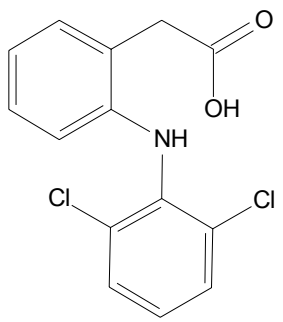


Figure 23: Chemical structure of Diclofenac

Like for other NSAIDs cases of adverse drug-related effects are reported for Diclofenac [*Galati et al., 2002; McCarthy, 1999; Weir et al., 2003; Konstam, 2003; Huang et al., 2004; Auer et al., 2004; Wong et al., 2005; Benvenuti et al., 1992*].
Liver toxicity is a seldom adverse drug reaction (0.0001%-0.0027% of prescriptions), but has a high fatality rate [*Purcell et al., 1991; Rostom et al., 2005 Walker, 1997 Galati et al., 2002 Sgro et al., 2002*]. It is accompanied by opening of the mitochondrial membrane permeability transition pore and depletion of ATP in the hepatocytes [*Berson et al., 2001; Masubuchi et al., 2002; Jaeschke et al., 2002; Lemasters, 1999; Pessayre et al., 1999*].

Investigations in this part of the thesis will focus on the influence of ATP synthase-down regulation on the expression of the other target genes in order to identify eventual interactions. In addition, the functional characteristics of ATP synthase β-subunit down regulation will be investigated.

4.1.2.1.1 Short introduction to the selected genes

ALAS-1

5-Aminolevulinate synthase (ALAS) is the mitochondrial rate-controlling enzyme of heme biosynthesis. ALAS-1 is very sensitive to free heme and can readily be down-regulated by free heme at nanomolar concentrations [*Yamamoto et al., 1988*]. Loosely bound unassigned heme is supposed to have influence on ALAS-1 at transcriptional and post-transcriptional levels [*Kolluri et al., 2005*], especially on mRNA stability [*May et al., 1995*] and mitochondrial import [*Munakata et al., 2004*].

Drugs that typically induce cytochrome P450-expression induce ALAS-1-expression as well via a nuclear xenobiotic-sensing receptor [*Podvinec et al., 2004*]. In patients with acute liver failure, ALAS-1 is suppressed [*Fujii et al., 2004*].

AKR1B10

Aldose-keto-reductase family 1, member B10 (AKR1B10) is a monomeric 37 kDa-protein recently discovered. AKR1B10 is highly expressed in the small intestine and in non-small lung cell carcinomas [*Fukumoto et al., 2005*]. Aldose reductases are efficient in reducing aromatic and aliphatic aldehydes [*Vander Jagdt et al., 1992; Inazu et al., 1994; Vander Jagdt et al., 1995*] suggesting a role in detoxification of harmful metabolites within cells and involvement in multi drug resistance [*Cao et al., 1998; Lee et al., 2001*]. AKR1B10 is able to convert retinals to the corresponding retinols thereby possibly interacting with the retinoic acid signaling and cell differentiation [*Crosas et al., 2003; Penning, 2005*].

BiP

BiP is an immunoglobulin heavy-chain-binding protein of 78 kDa [*Bole et al., 1986*]. It belongs to the heat shock protein70-family of chaperones and is resident in the endoplasmatic reticulum (ER) [*Munro et al., 1987*]. BiP is glucose-, hypoxia- and acidosis-regulated [*Mote et al., 1998; Dong et al., 2005*] and has influence on immunomodulatory pathways [*Corrigall et al., 2001; Matzinger, 2002; Corrigall et al., 2004*]. The function of the protein is to retain misfolded, aberrantly glycosylated and incorrectly disulfide-bonded proteins in the ER [*Kitajewski et al., 1992; Oda et al., 1996*]. ATP has been shown to cause release of BiP from the bound proteins [*Munro et al., 1986*]. BiP is strongly regulated at post-transcriptional level [*Gülow et al., 2002*].

Hepcidin

Hepcidin is a peptide hormone produced in the liver. It shows antimicrobial and antifungal properties and can be detected in urine and plasma [*Ganz, 2003; Kulaksiz et al., 2004*]. Hepcidin is a homeostatic regulator of iron metabolism [*Ganz, 2005*]. Its expression is controlled by oxygen-supply of the organism [*Nicolas et al., 2002*], inflammation [*Nemeth et al., 2004*] and iron load of the organism [*Ganz, 2005*].

4.1.2.2 Results

4.1.2.2.1 Expression of the target genes

HepG2-cells were chosen as the *in vitro*-system to address ATP synthase β-subunit by means of siRNA. In a first control-experiment, the cellular system was tested for the expression of the identified target genes (see table 16). For this purpose, mRNA was extracted from cultured HepG2-cells, and qRT-PCR was performed. A threshold cycle <30 for significant fluorescence was set as the threshold for detectable expression.

	Ct (threshold cycle)
ATP synthase	21.1 ± 0.2
AKR1B10	26.6 ± 0.2
ALAS-1	27.7 ± 0.0
BiP	25.6 ± 0.1
Hepcidin	23.8 ± 0.1

Table 17: Expression-level of the target genes in HepG2.
The experiment was conducted two times.

The fluorescence-signal of the ATP synthase β-subunit-amplicon became significantly detectable after 21 cycles, while detectable signals for AKR1B10, ALAS-1, BiP and Hepcidin were observable ~ 2-6 cycles later, indicating a slightly lower expression as ATP synthase. In summary, all investigated genes were expressed to a detectable extent in HepG2.

The influence of sub-cytotoxic Diclofenac-treatment on mRNA-expression of the target genes in HepG2 was investigated to test the validity of the *in vitro*-system for conclusions on Diclofenac-mediated liver toxicity. HepG2-cells were treated with 50 and 100 µM Diclofenac for 24 h and the mRNA-expression of the target genes was determined by qPCR. The results were normalised to the mRNA-expression of the target gene in vehicle-treated cells.

	mRNA-expression [% of control]				
	ATP synthase	Hepcidin	ALAS1	AKR1B10	BiP
vehicle	100	100	100	100	100
50 µM Diclofenac	125 ± 111	109 ± 112	78 ± 64	70 ± 70	96 ± 59
100 µM Diclofenac	96 ± 77	163 ± 243	82 ± 75	69 ± 68	100 ± 79

Table 18: Influence of Diclofenac-treatment on gene expression in HepG2
The housekeeping gene for qPCR was β-actin. The experiment was executed four times in triplicate. ± values indicate the standard deviation of the experiments.

Treatment of HepG2-cells with non-cytotoxic concentrations of Diclofenac did not lead to significant changes in the expression of ATP synthase β-subunit, AKR1B10, ALAS-1 and BiP.

4.1.2.2.2 Silencing of ATP synthase β-subunit and its functional consequences

The kinetic of the siRNA-mediated silencing of ATP synthase β-subunit was investigated on mRNA and protein level. For this purpose, HepG2-cells were electroporated with 300 nM siRNA targeting ATP synthase β-subunit (see table 9) or mismatch siRNA. The expression of mRNA and protein at different time points were investigated by qPCR or western blot, respectively. MRNA- or protein expression data obtained from ATP synthase-silenced cells was normalised to the corresponding average expression of mismatch-transfected cells.

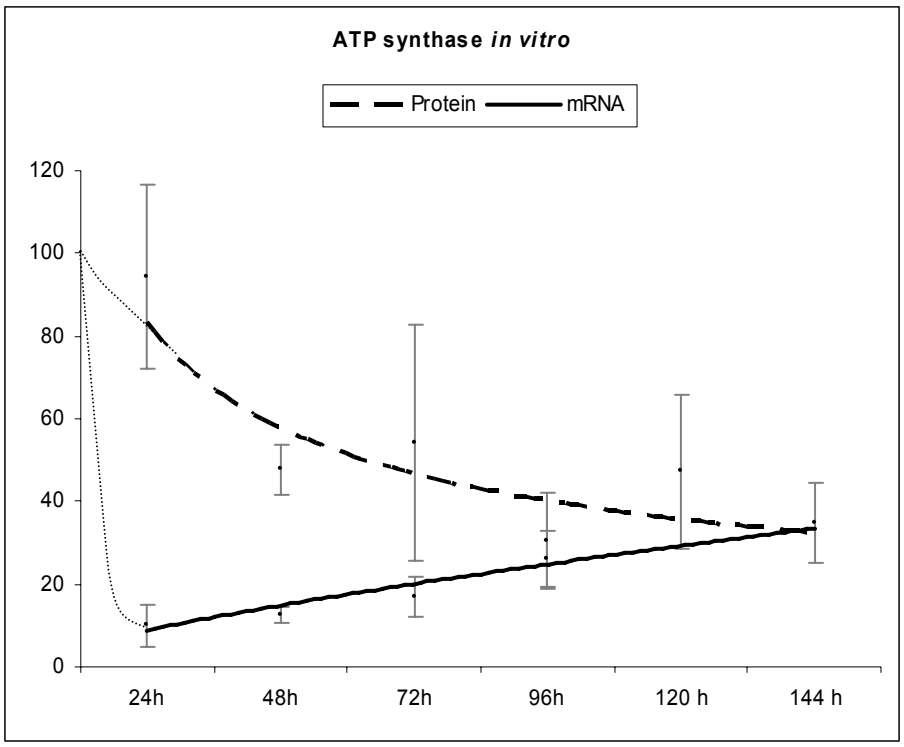


Figure 24: Kinetics of ATP synthase-silencing.
The housekeeping gene for qPCR was GAPDH. The experiment was executed 3 times, error bars symbolise the standard deviation of the different experiments

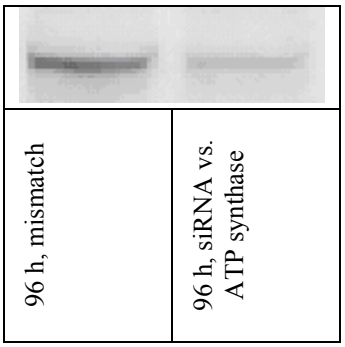


Figure 25: Example western blot of F_1-ATP synthase in HepG2-cells

The selected siRNA decreased ATP synthase β-subunit mRNA expression by 90% (24h after transfection) and the corresponding protein level by 75% (96h after transfection). MRNA-expression slightly recovered starting 72 h after transfection from the initial 90% knockdown to remaining 60% knockdown after 144 h while significant recovery from the transient silencing could not be observed within 6 days on protein level.

The silencing and possible cytotoxicity of BiP, AKR1B10 and Hepcidin was investigated kinetically on mRNA and (for BiP) protein level.
For this purpose, HepG2-cells were transfected with 300 nM either mismatch- or a functional siRNA (in the case of Hepcidin pool) vs. the gene of interest (see tables 9, 11). MRNA- (and for BiP protein-) expression was determined at 24-144 h after transfection by qPCR (or western blot, respectively). LDH-activity in the cell culture supernatant was determined in parallel as a marker of cytotoxicity. All data points were normalised to the average value of the corresponding mismatch-transfected control.

	BiP			AKR 1B10		Hepcidin	
	mRNA [% of control]	Protein [% of control]	LDH-release [% of control]	mRNA [% of control]	LDH-release [% of control]	mRNA [% of control]	LDH-release [% of control]
24 h	21 ± 17	92 ± 3	97 ± 4.4	21 ± 4	87 ± 23	47 ± 29	102 ± 7
48 h	45 ± 9	89 ± 30	98 ± 5.8	38 ± 11	94 ± 20	25 ± 10	103 ± 8
72 h	81 ± 14	82 ± 14	97 ± 7.3	34 ± 7	74 ± 7	60 ± 25	101 ± 5
96 h	68 ± 21	78 ± 10	91 ± 4.7	66 ± 16	81 ± 22	52 ± 15	95 ± 1
120 h	n.d.	n.d.	n.d.	49 ± 12	n.d.	81 ± 77	99 ± 9
144 h	n.d.	n.d.	n.d.	72 ± 11	n.d.	81 ± 44	100 ± 5

Table 19: mRNA-expression and LDH-release after silencing BiP, AKR1B10 or Hepcidin
The experiment was performed thee times in triplicate; ± values show the standard deviation of the average-values from the independent experiments. N.d. = not determined

For BiP, AKR1B10 and Hepcidin, mRNA-expression of the target genes was reduced >75% compared to the respective mismatch-transfected control between 24 and 48 h after transfection. mRNA-expression of BiP and Hepcidin recovered quickly starting 48h after transfection, AKR1B10 mRNA recovered more slowly still showing silencing capacity of 28 ± 11% after 120 h. BiP protein was not significantly reduced by means of siRNA-mediated silencing. None of the silencings led to significant LDH-release.

The influence of the gene silencing of ATP synthase β-subunit on the mRNA-expression of the other target genes was investigated. HepG2-cells were transfected with either 300 nM mismatch- or functional siRNA vs. ATP synthase β-subunit. MRNA-expression of BiP, ALAS-1, AKR1B10 and Hepcidin was investigated by qPCR 96 h after transfection. Data points were normalised to the average value for corresponding mismatch-transfected cells.

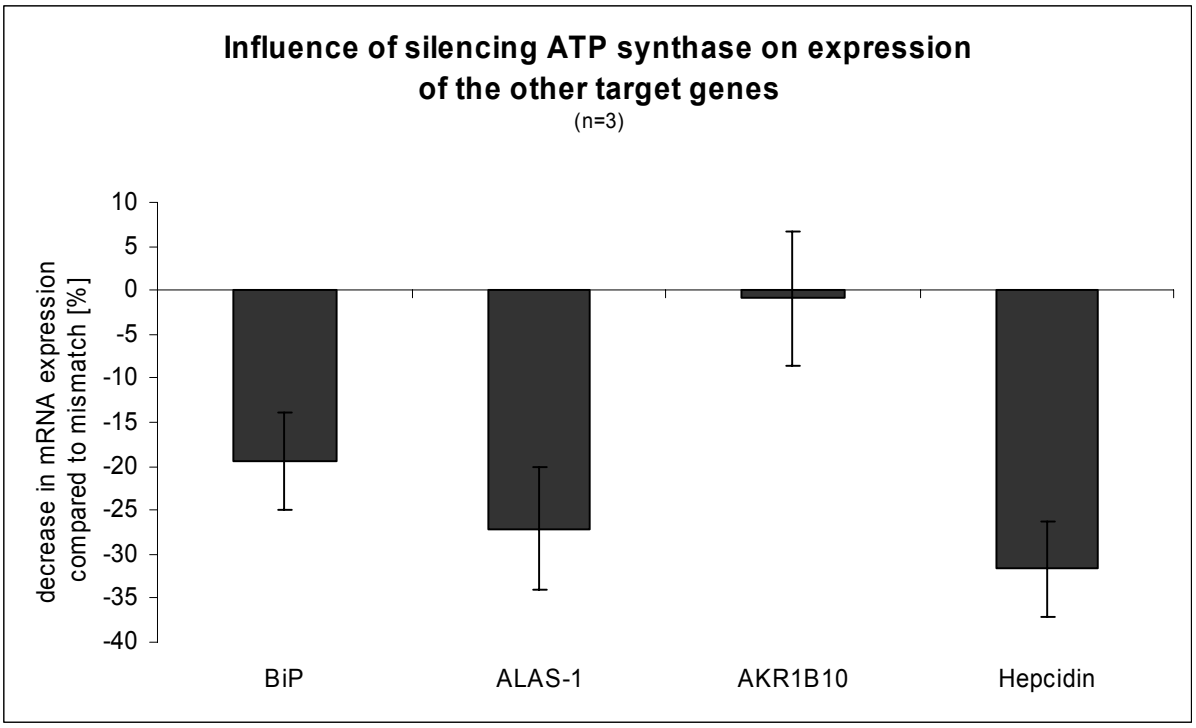


Figure 26: Influence of silencing ATP synthase on expression of the other target genes
The housekeeping gene for qPCR was GAPDH. The experiment was executed three times in triplicate. Error bars symbolise the standard deviation of the different experiments.

MRNA-expression of BiP, ALAS-1 and Hepcidin was reduced by 20-30% 96 h after transfection with siRNA targeting ATP synthase β subunit. AKR1B10 mRNA-expression was not affected significantly under these conditions

The influence of ATP synthase β-subunit silencing on functional cellular consequences was investigated. Intracellular ATP, lactate and glucose consumption as indicators of cellular energy metabolism and LDH-release as a marker of cytotoxicity were assessed. For this purpose HepG2- cells were either treated for 24 h with 0.5, 5 or 50 nM Oligomycin B or electroporated with either 300 nM mismatch- or functional siRNA vs. ATP synthase β-subunit and cultured for 96 h before endpoint-determination. Rhodamin 123-uptake was investigated at the end of incubation, and intracellular ATP was determined from replicate wells. Lactate, glucose concentration and LDH-activity were quantified enzymatically in the cell culture supernatant. All data points were normalised to the average value of the corresponding mismatch-transfected (or vehicle-treated) control.

		ATP [% of control]	$\Delta\psi_m$ [% of control]	Lactate [% of control]	Glucose consumption [% of control]	LDH [% of control]
Oligomycin B [nM]	0	100	100	100	100	100
	0.5	153 ± 34	89 ± 16	97 ± 3	93 ± 8	107 ± 16
	5	74 ± 21	48 ± 13 **	131 ± 12 *	117 ± 13	105 ± 22
	50	57 ± 10	35 ± 13 ***	130 ± 9 *	118 ± 11	125 ± 17
silencing (96 h)	mismatch	100	100	100	100	100
	ATP synthase	124 ± 24	91 ± 13	108 ± 5	103 ± 2	113 ± 14

Table 20: Comparison of Oligomycin B-treatment and ATP synthase-silencing
The experiment was performed three times in triplicate, the standard deviation of the independent experiments is given ±. Mean of vehicle-treated cells in absolute values: ATP=3.16 nmol/ml (cells in 300 µl), ΔΨm=637 F.U. (cells in 500 µl), LDH=24 I.U. /L. Asterisks mark significant differences to the control; data was analysed by one-way-ANOVA with SNK-post-hoc-test (*p<0.05, ** p<0.01, *** p<0.001).

Silencing of ATP synthase β-subunit did not lead to significant changes in intracellular ATP, mitochondrial membrane potential, lactate production, glucose consumption or LDH-release compared to the corresponding mismatch-transfected control. Incubation with Oligomycin B led to dose-dependent ATP depletion (maximal 43% depletion), significant loss of mitochondrial membrane potential (maximal 65% reduction), increased glucose consumption (maximal 20%) and significantly increased lactate production (~ 30%) at concentrations ≥ 5 nM. LDH-release was not affected by Oligomycin B-treatment.

Functional consequences of the siRNA-mediated silencing of ATP synthase β-subunit on ATP and cytotoxicity could possible have occurred during the onset of protein depletion at earlier time points after transfection. To investigate this, earlier time points were investigated. HepG2-cells were transfected with either 300 nM mismatch- or functional siRNA vs. ATP synthase β-subunit. ATP and LDH-release were determined at 24, 48, 72 and 96 h after transfection as indicated in the previous experiment.

	intracellular ATP [% of control]		LDH-release to culture supernatant [% of control]	
time point after electroporation	mismatch	siRNA vs. ATP synthase	mismatch	siRNA vs. ATP synthase
24 h	100	164 ± 26	100	99 ± 3
48 h	100	65 ± 62	100	98 ± 4
72 h	100	186 ± 75	100	102 ± 3
96 h	100	110 ± 28	100	101 ± 4

Table 21: Intracellular ATP and LDH-release after silencing ATP synthase

The experiment was performed three times in triplicate, and the standard deviation of the experiments is given ±.

During 96 h, ATP synthase β-subunit–silencing did neither lead to significant changes in intracellular ATP nor to significant LDH release compared to the corresponding mismatch-transfected control.

Potential sensitisation of the cells by either compound-treatment or ATP synthase β-subunit–silencing to substance-treatment was investigated.

To prove that sensitising of the cells can in principle be investigated in HepG2 cells on the functional parameters, cells were co-incubated for 24 h with Oligomycin B at 0.5, 5, 50 and 500 nM, with Diclofenac at 100 or 800 µM or a combination of both. Intracellular ATP, mitochondrial membrane potential and LDH release were assessed as described in the previous experiments.

Oligomycin B [nM]	Diclofenac [µM]	Δψm [% of control]	LDH-release [I.U./L]
0	0	100	66 ± 21
0	100	56 ± 4 ***	67 ± 8
0	800	51 ± 14 ***	309 ± 76
0.5	0	91 ± 7	59 ± 17
500	0	35 ± 5 ***	52 ± 12
0.5	100	56 ± 2 ***	71 ± 7
500	100	56 ± 10 ***	56 ± 9
0.5	800	39 ± 6 ***	223 ± 70
500	800	25 ± 5 ***	262 ± 91

Oligomycin B [nM]	Diclofenac [µM]	ATP [nmol/ml]
0	0	8.8 ± 2.5
0	100	7.7 ± 1.6
0	800	5.6 ± 1.3
5	0	7.3 ± 1.8
50	0	5.3 ± 1.5
5	100	4.8 ± 1.2 *
50	100	2.0 ± 0.7 ***
5	800	0.8 ± 0.5 ***
50	800	0.2 ± 0.0 ***

Table 22: Effects of Oligomycin B- and Diclofenac (co)-treatments
The experiments were executed three times in triplicate, and the standard deviation of the average of the experiments is given ±. Mean of vehicle-treated cells in absolute values: ΔΨm=2061 F.U. (cells in 500 µl). Asterisks mark significant differences to the control (* $p<0.05$, *** $p<0.001$).

Diclofenac-treatment led to statistically not significant but notable and dose-dependent ATP depletion (maximum 64% depletion) and significant loss of mitochondrial membrane potential (maximum 49% decrease) at both concentrations applied. At the high dose 4.5-fold increase in LDH-release compared to the corresponding vehicle control was observed. Oligomycin B-treatment led to ATP depletion comparable to Diclofenac-treatment (maximal 60% depletion), and at the high dose to significant loss of mitochondrial membrane potential (maximal 65% decrease), while LDH-release was not affected at any concentration applied. Co-treatment with Diclofenac and Oligomycin B led to additional effects in regard to the

decrease of the mitochondrial membrane potential, and ATP depletion became statistically significant upon coincubation in a dose-dependent manner. The cytotoxicity of Diclofenac-treatment was marginally reduced by conincubation with Oligomycin B. In summary, HepG2-cells were a valid for investigations on the chosen parameters and to reflect sensitisation of the cells to substance-treatments.

ATP synthase-silenced cells were treated with Oligomycin B or Diclofenac at the time point of highest protein knock-down to investigate possible sensitisation of the cells to substance treatments due to siRNA-mediated silencing of ATP synthase β-subunit.

For Oligomycin B-treatment, HepG2-cells were transfected with either mismatch- or functional siRNA vs. ATP synthase β-subunit and cultured for 72 h for protein-depletion. After this period, cells were incubated with Oligomycin B at 0.5 and 5000 nM for 24 h. ATP, mitochondrial membrane potential and LDH-release were determined as described in the previous experiments.

	ATP [% of control]		▫▫m [% of control]		LDH [% of control]	
Oligomycin B [nM]	mismatch-transfected	siRNA vs. ATP synthase	mismatch-transfected	siRNA vs. ATP synthase	mismatch-transfected	siRNA vs. ATP synthase
0	100	100±10	100	99±25	100	99 ± 25
0.5	98±16	116±21	101±10	95±15	107 ± 16	113 ± 14
5000	73±7 *	82±35	42±10 ***	42±11 ***	104 ± 24	109 ± 10

Table 23: Oligomycin B-treatment of ATP synthase-silenced cells
The experiment was performed three times in triplicate, and the standard deviation of the three independent experiments is given ±. Asterisks mark significant differences to the control (* $p<0.05$, *** $p<0.001$).

Oligomycin B-treatment led to significant ATP depletion (maximum 27% depletion) and decrease of mitochondrial membrane potential at the high dose (maximum 58% decrease) while LDH-release remained unaffected. Additional silencing of the ATP synthase β-subunit had no significant influence on any of the investigated parameters.

For Diclofenac-treatment, HepG2-cells were transfected with either mismatch- or a functional siRNA vs. ATP synthase β-subunit and cultured for 96 h for protein-depletion. After this period, cells were incubated with Diclofenac at 100, 200 and 800 µM in HBSS with 2 g / l glucose for 2h and intracellular ATP was determined, or for 48 h under serum-free conditions for determination of LDH-release. Incubation medium for LDH was renewed after 24 hrs, and LDH in the supernatant was added to the value after 48 hrs. All values were expressed relatively to the corresponding mismatch-transfected and vehicle-treated control.

	ATP [% of control]		LDH release [% of control]	
Diclofenac [μM]	mismatch	siRNA vs. ATP synthase	mismatch	siRNA vs. ATP synthase
0	100	98 ± 14	100	99 ± 3
100	112 ± 22	94 ± 25	147 ± 1	122 ± 4
200	81 ± 25	77 ± 33	151 ± 17	130 ± 1
800	15 ± 10	12 ± 2	295 ± 116	307± 140

Table 24: Diclofenac-treatment of ATP synthase-silenced cells
The experiments were performed two times in triplicate, ± values indicate the range between the averages of the experiments.

Diclofenac-treatment resulted in dose-dependent ATP-depletion and LDH-release, reaching 75% ATP depletion and 3-fold increase in LDH-release at the highest dose. Additional silencing of the ATP synthase β-subunit had not significant influence on any of the investigated parameters.

4.1.2.3 Discussion

The basis for the investigations was an observed alteration of ATP synthase β subunit-, BiP-, AKR1B10-, ALAS-1- and Hepcidin-mRNAs upon Diclofenac-treatment *in vivo*. The identified target genes were expressed on mRNA-level in the model system, but not affected by Diclofenac-treatment. Thus, the selected cellular system HepG2 can be considered to be not valid for investigations on Diclofenac-induced liver toxicity.

HepG2 cells differ not only by means of sensitivity, but in a lot of functional responses from primary cells [*Bort et al., 1999; Gómez-Lechón et al., 2001/2003; Posonda et al., 1995; Tang, 2003; Wang et al., 2002*]. These differences are supposed to be even bigger comparing *in vivo* conditions and *in vitro*-approaches which could explain the absence of mRNA-regulation in HepG2.

Nevertheless, the system was considered to be capable of serving as a model to investigate the interactions of ATP synthase β subunit down regulation with the expression of the other target genes and the functional effects on cellular functions.

The siRNA-mediated gene silencing of ATP synthase β subunit could be demonstrated on mRNA and protein expression level. The kinetics of the silencing were dominated by quick degradation of the target mRNA while protein depletion followed slowly and continuously over the first 96 h after transfection. The maximal achieved protein depletion was 75% after 96 h indicating incomplete gene silencing.

The observed kinetic and reduction in gene expression is significantly different to chemical inhibition, which is in general quick and quantitative [*Fort et al., 2001*]. Thus, comparison of chemical inhibition and siRNA-mediated gene silencing is critical.

The mRNA expression slowly recovered starting ~72 h after siRNA-mediated gene silencing of ATP synthase β subunit. Recovery can be due to cell division as daughter cells do not have the active siRNA in the cytoplasm, or due to siRNA-degradation in the culture [*Bartlett et al., 2006*].

Maximal protein depletion was observed 96h after transfection. This was chosen to be the time point for all further investigations as protein depletion was at maximum and mRNA-recovery could not yet translate into elevation of protein expression.

SiRNA-mediated down regulation of ATP synthase β-subunit resulted in down regulation of BiP, ALAS-1 and Hepcidin mRNAs.

This coregulation of the four genes suggests a link between the expressions of the four target genes with a key-role of ATP synthase β-subunit-down regulation. Whether this coregulation

has consequences for cellular function or impact on the reported pathologic alterations correlated with ATP synthase β-subunit down regulation [*Santamaria et al., 2006; Shin et al., 2005; Cuezva et al., 2002/2004; Isidoro et al., 2004*] remains unelucidated and requires further investigation. By means of the siRNA-method, one single mRNA-reduction observed *in vivo* by genomics among other regulations could be mimicked *in vitro* in an isolated way and demonstrated impact on other observed mRNA-regulations. This is demonstrating the usefulness of the siRNA-method in investigation of the consequences of a single mRNA-down regulation observed by genomics.

The siRNA-mediated silencing of BiP, AKR1B10 and Hepcidin could be demonstrated on mRNA-level. None of the knockdowns resulted in cytotoxicity. While AKR1B10-knockdown demonstrated similar kinetics as observed upon silencing of ATP synthase β subunit, the kinetics of Hepcidin and BiP were different from this situation. MRNA expressions recovered more quickly, and in the case of BiP no translation of the mRNA-silencing effect to the protein level could be observed. The quick recovery of mRNA-levels could be due to different stabilities of the individual siRNAs, or rapid expression-regulation. BiP and Hepcidin are stress-sensitive proteins [*Mote et al., 1998; Dong et al., 2005; Nemeth et al., 2004; Ganz, 2005*] with a short half-life and extensive regulation on mRNA- and protein level. Thus, they could be delicate candidates for siRNA, as the siRNA-mediated mRNA knockdown could be masked by excessive induction of the target before the siRNA could unfold its effectiveness.

To validate reaction of HepG2 cells to compromittation of mitochondrial ATP synthase, the silencing was compared to treatments with Oligomycin B. Although Oligomycin B is not targeting the identified β subunit of the ATP synthase complex like the siRNA, it was applied for comparison to the siRNA-mediated silencing of the β-polypeptide, as the functionality of the whole ATP synthase complex is compromised by Oligomycin B. Oligomycin B is targeting the F_O-subunit and actually determined its name (O=Oligomycin-sensitive). Oligomycin B-treatment can only be compared with restrictions to the specific siRNA-mediated silencing of the ATP synthase β-subunit. The only two specific inhibitors for the investigated β-subunit are the naturally occurring inhibitory protein and Aurovertin [*van Raaij et al., 1996*]. Both inhibitors could not be applied for comparison, as the protein is not crossing the cellular membrane, and Aurovertin was not available during the course of this dissertation.

Oligomycin B-treatment of HepG2-cells led to reduction of intracellular ATP and the mitochondrial membrane potential in HepG2-cells. Oligomycin B treated cells showed

significantly enhanced lactate-production and marginally enhanced glucose-consumption, suggesting an increase in glycolytic activity compared to oxidative phosphorylation as the source of ATP.

Thus, HepG2-cells are in general reacting to inhibition of mitochondrial ATP synthase and were thus valid for the investigations on functional cellular consequences after specific gene silencing of the ATP synthase β-subunit. The effect of Oligomycin B-treatment on the markers of glycolysis, lactate production and glucose consumption, was not distinctive, indicating that HepG2 as a hepatoma cell line is not completely dependent on oxidative phosphorylation and gaining a lot of its energy via the glycolytic way under normal conditions as proposed in the literature [*Dang et al., 1999; Chevrollier et al., 2005*].

The inhibition of ATP synthase β-subunit by its specific inhibitor Aurovertin is not leading to cytotoxicity, as demonstrated in the literature [*Santamaria et al., 2006; Shchepina et al., 2002*]. This is supported by the experimental results, as specific gene silencing of ATP synthase β-subunit did not result in cytotoxicity. Compromised function of the ATP synthase β-subunit was reported to be critical *in vivo* [*Houštek et al., 1999*], leading to lethality of a newborn with specific defect. *In vitro* on isolated cells, reduced functionality of the β-subunit does not seem to be critical for cell survival, as shown by specific inhibition [*Santamaria et al., 2006; Shchepina et al., 2002*] or the specific protein-depletion achieved in the present study. Even pro-survival effects of β-subunit knockout have been reported in yeast [*Gross et al., 2000*].

Specific inhibition of the ATP synthase β-polypeptide by aurovertin leads to ATP depletion according to the literature [*Grover et al., 2004*]. This effect could not be observed by specific gene silencing of ATP synthase β-subunit, which had no impact on either ATP as the product of the silenced ATP synthase β-subunit or the other investigated parameters of cellular metabolism.

The absence of ATP depletion upon ATP synthase β-subunit-silencing is most likely due to incomplete gene silencing, as observable on protein level. Compensatory mechanisms of the cell due to the slow depletion of the protein would be possible, but compensation would shift the metabolic situation of the cell to glycolytic gain of energy, thereby affecting the investigated markers of glycolysis, lactate production and glucose consumption.

Oligomycin B- and Diclofenac treatment resulted in ATP depletion and loss of mitochondrial membrane potential. Additionally, Diclofenac-treatment triggered cytotoxicity.

Oligomycin B had a clear potential to sensitise HepG2-cells to Diclofenac-treatment, as shown by means of mitochondrial membrane potential and cellular ATP.

Specific siRNA-mediated gene silencing of ATP synthase β-subunit did not result in sensitisation of HepG2-cells to Oligomycin B- or Diclofenac-treatment. The absence of a sensitising potential indicates insufficient gene silencing to observe functional consequences.

In summary, the siRNA method could be used to demonstrate a link between specific silencing of ATP synthase β-subunit-expression and down regulation of BiP, ALAS-1 and Hepcidin on mRNA-expression level. The silencing of ATP synthase β-subunit had no functional consequence on the investigated parameters of metabolism. This is most likely due to incomplete gene silencing, as observable on protein level. The kinetic of siRNA mediated gene silencing is different to chemical inhibition due to slow depletion of protein after transfection.

4.1.3 Evaluation of farnesylpyrophosphate synthase as a target of kidney toxicity

4.1.3.1 Background

Farnesylpyrophosphate synthase (FPPS) in is a ~30 kDa protein converting geranyl pyrophosphate and isopentenyl pyrophosphate to farnesylpyrophosphate. The substrates for the enzymatic reaction come from the mevalonate-pathway, and the product feeds cholesterol biosynthesis and prenylation of small proteins (mainly small GTPases of the Rho/Rac/Rap/Rab-families). The prenylation of GTPases is discussed to be crucial for cell survival [*Reszka et al., 2004*] and the cellular framework [*Lane et al., 2006*].

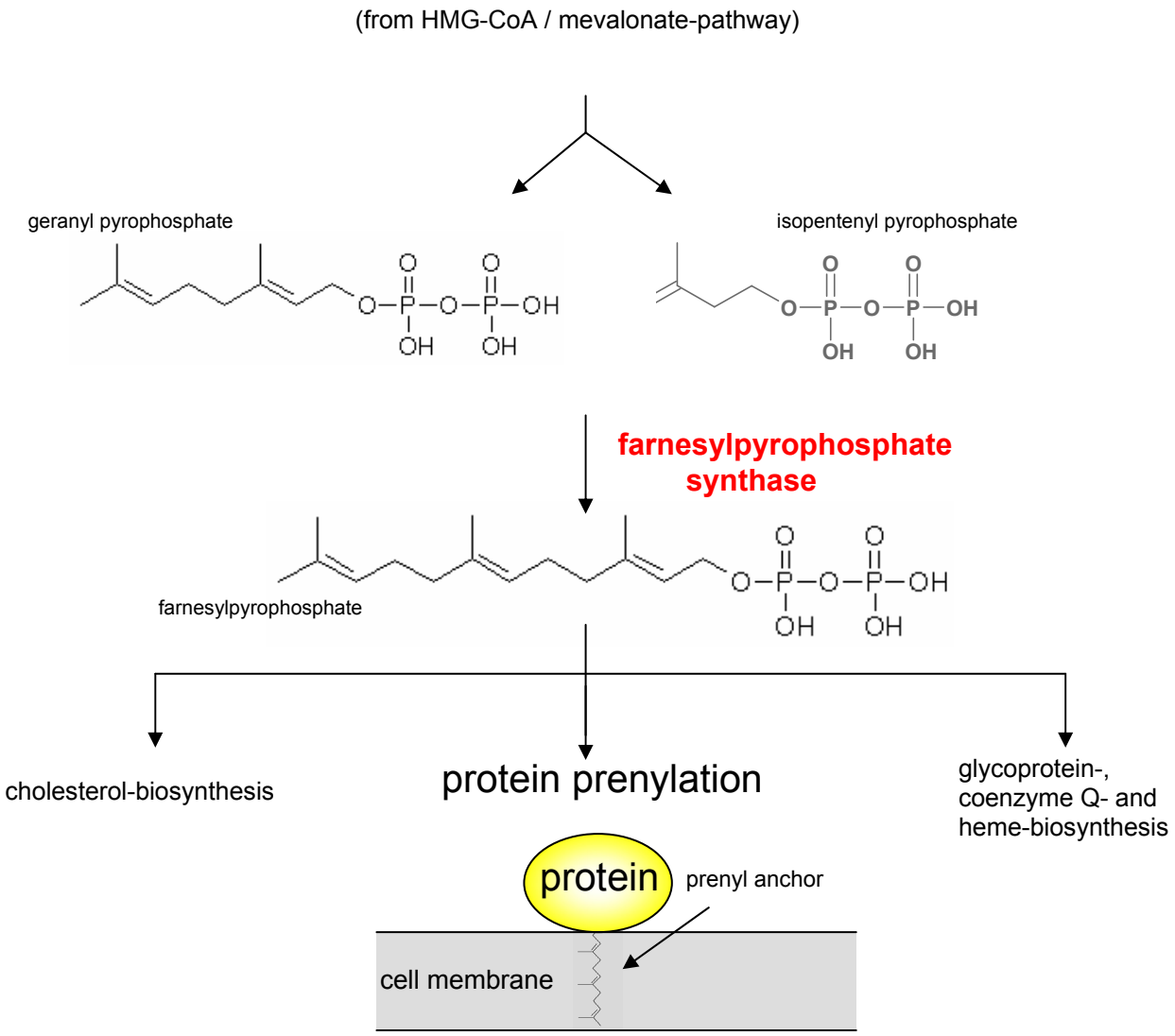


Figure 27: Enzymatic reaction of FPPS

Zoledronic Acid is a nitrogen-containing bisphosphonate, which inhibits FPPS-activity. Bisphosphonates (P-C-P) are analogues of pyrophosphate (P-O-P), the central oxygen being substituted by carbon. As the P-C-P backbone is not a substrate for any known enzyme, the compounds are not metabolised, and the parent compound is renally excreted [*Reszka et al., 2004*].

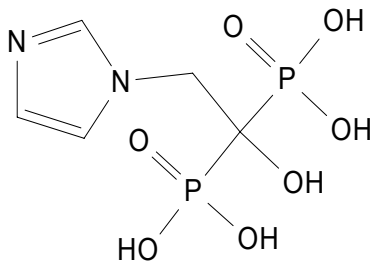


Figure 28: Chemical structure of Zoledronic Acid

Bisphosphonates are used for the treatment of osteoporosis, Paget`s disease and tumor-induced hypercalcemia. Pharmacokinetic is dominated by the high hydrophilicity of the bisphosphonates. Resorption from the gut is very low (~2%), and the small amount reaching the circulation is either quickly bound to the hydroxyapatite component of the bone where it accumulates (~50%), or renally excreted (~50%), with a half-life of ~1h [*Reszka et al., 2004*]. The osteoclast is the target cell of the substance class.

Cases of acute renal failure have been reported, especially when high doses were administered intravenously [*Bounameaux et al., 1983; O`Sullivan et al., 1994*]. This toxic effect on the kidney could be localised in the proximal convoluted tubes [*Pfister et al., 2005*], and does evidently not – as previously considered [*Zojer et al., 1999; Fleisch et al., 2000*]- originate from precipitation of the parent compound in the proximal tubuli.

A role of involvement of the Rho / Rho-kinase-pathway in the renal toxicity of bisphosphonates was proposed. Rho regulates the actin cytoskeleton, the assembly of stress fiber filament and is involved in cell cycle progression. The Rho/Rho kinase-pathway is activated in various clinical pictures of renal diseases [*Wakino et al., 2005*].

The investigations in this part of the thesis will focus on the role of FPPS in Zoledronic Acid-induced cytotoxicity in HK-2-cells originating from the proximal tubulus of the human kidney. Furthermore, the impact of specific gene silencing of FPPS on the prenylation of GTPases will be investigated and compared to the effect of Zoledronic Acid-treatment.

4.1.3.2 Results

To evaluate the effect of Zoledronic Acid-treatment on HK-2-cells, different markers of cytotoxicity were investigated. LDH was applied as a marker of cellular membrane integrity, MTS-reduction as a marker of mitochondrial dehydrogenase-activity and caspase-3-activity as a marker of apoptosis.

HK-2 cells were treated with Zoledronic Acid at 1, 10, 20, 50 and 100 µM for 48 h. LDH-release to the culture supernatant, reductive potential of the cells towards MTS and caspase-3/7-activity were determined at the end of incubation. All experimental data was normalised to the vehicle-treated control.

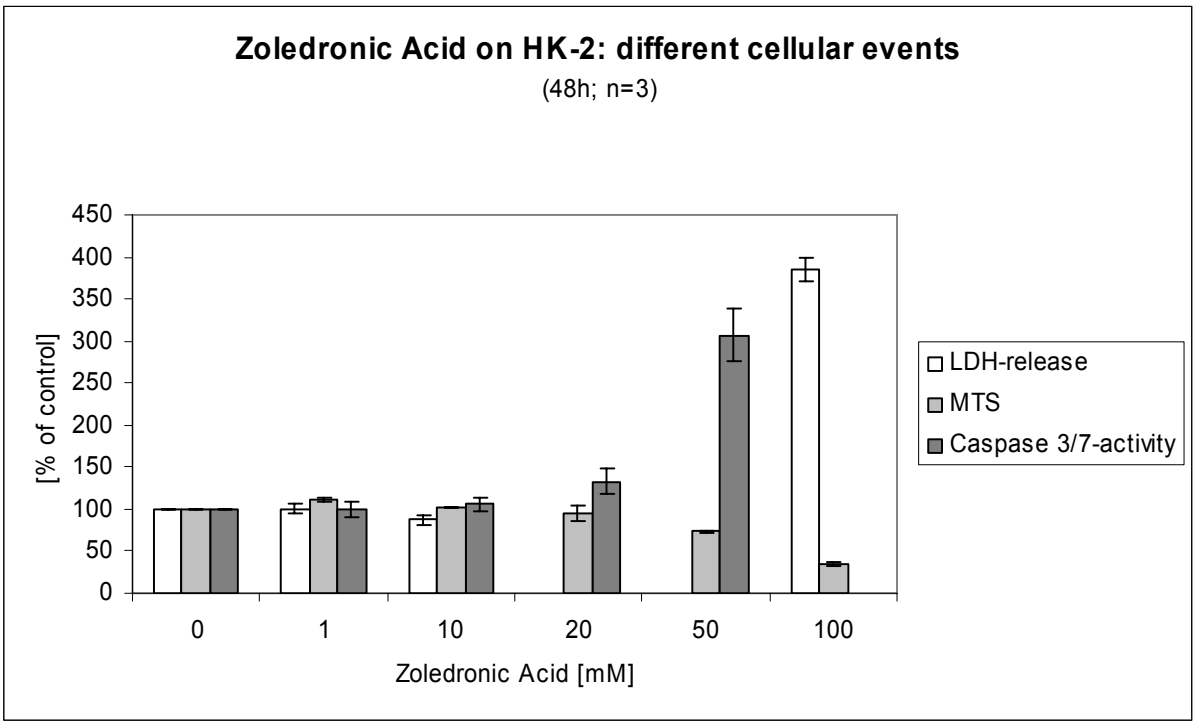
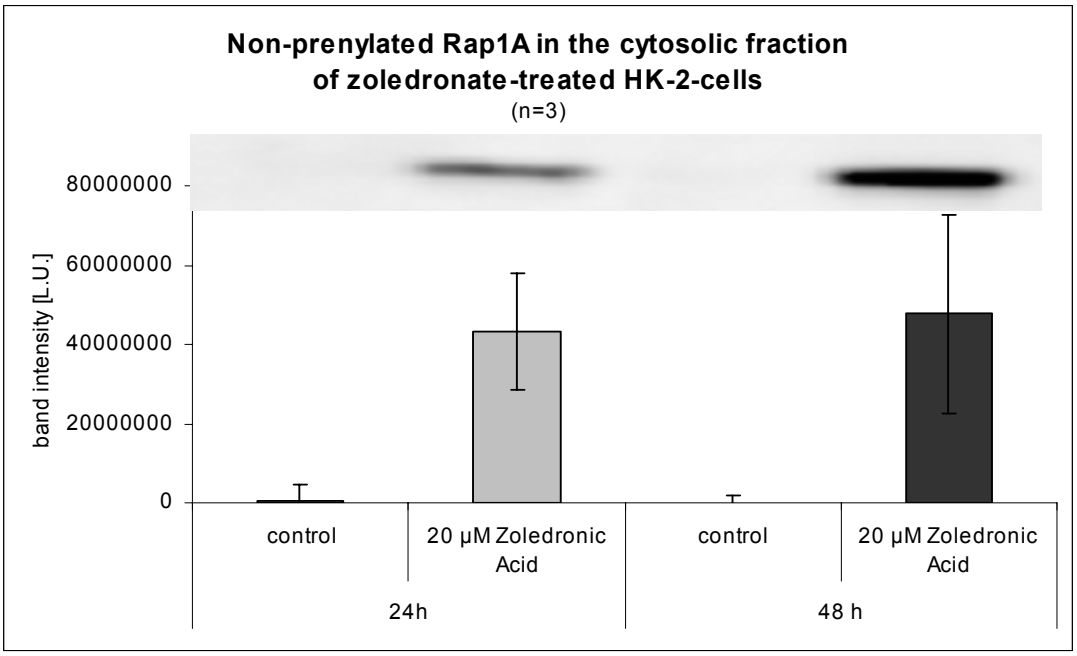


Figure 29: Cellular events in HK-2-cells upon Zoledronic Acid-treatment
The experiments were executed three times in triplicate. ± values indicate the standard deviation of the different experiments.

Incubations with Zoledronic Acid up to 20 µM had no significant impact on the investigated parameters. At 50 µM, MTS-reducing activity of the cells was decreased by 25%, and caspase-activity was increased 3-fold compared to vehicle-treated cells. At the highest dose, LDH-release was increased 4-fold compared to the vehicle-treated control and MTS-reduction was decreased by 80%.

The deprenylation of small GTPases upon Zoledronic Acid-treatment was investigated by assessment of the GTPases RhoA and Rap1A in the membranous- or cytosolic fraction of the cells, respectively. For this purpose HK-2-cells were incubated with 20 µM Zoledronic Acid for 24 and 48 h. The cell lysates were separated into cytosolic and membranous fraction by

high-speed centrifugation, and contents of RhoA and Rap1A were assessed by means Western blot.



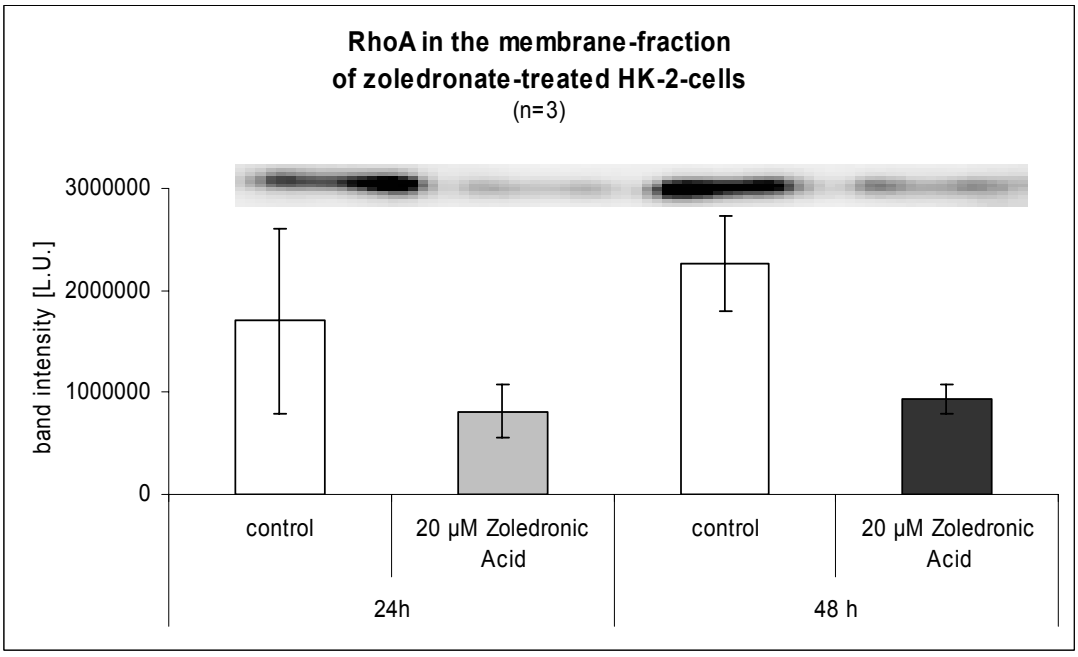


Figure 30: Localisation of small GTPases after Zoledronic Acid-treatment
The experiments were executed three times. Error bars symbolise the standard deviation of the different experiments.

In the cytosolic fraction, no signal for Rap1A was detectable in vehicle-treated control cells. Upon Zoledronic Acid-treatment a strong increase in Rap1A-content became evident after 24 h, which slightly further increased after 48h. In the membrane-fraction RhoA-content was decreased by Zoledronic Acid-treatment to 50% of the vehicle-treated control after 24 h and to 35% of the control after 48h.

Farnesylpyrophosphate synthase was silenced in HK-2-cells. For this purpose cells were electroporated with 300 nM of a pool of four siRNAs targeting FPPS (see table 13). FPPS protein expression in the whole cell lysate was determined 48 and 72 h after transfection by western blot. All experimental data was normalised to the FPPS protein expression of the corresponding mismatch-transfected control.

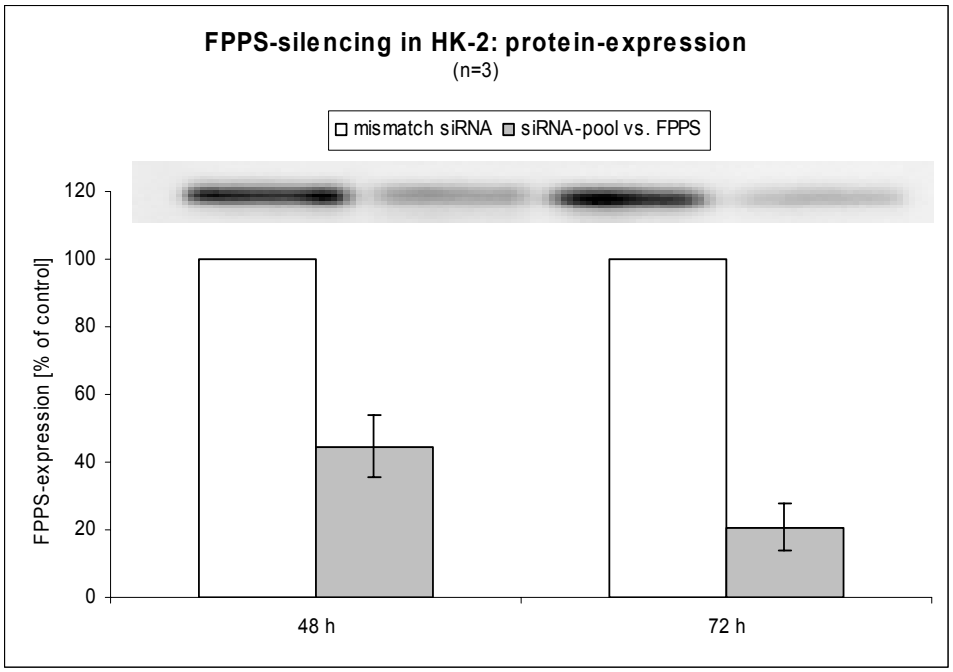


Figure 31: Confirmation of FPPS-silencing on protein expression level.
The experiment was executed three times. Error bars symbolise the standard deviation of the different experiments.

Transfection of the pooled siRNA targeting FPPS led to a 55%-reduction of FPPS-protein expression by after 48 h. Further cultivation resulted in 79% reduction of protein expression 72 h after transfection

The impact of siRNA-mediated gene silencing on the prenylation of small GTPases was investigated. For this purpose HK-2-cells were either transfected with 300 nM siRNA-pool targeting FPPS and cultured for 72 h or treated with 20 µM Zoledronic Acid for 48 h. The protein content of Rap1A in the cytosolic fraction of the cells was determined by western blot.

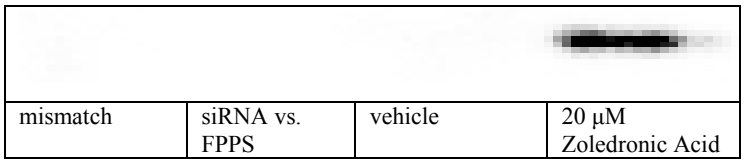


Figure 32: Effect of FPPS-silencing on Rap1A-localisation
The experiment was executed three times; one representative blot is shown.

SiRNA-mediated gene silencing of FPPS had not impact on the prenylation of Rap1A, while 48h- treatment with Zoledronic Acid led to a strong increase of Rap1A in the cytosolic fraction of HK-2-cells.

To investigate possible influence of FPPS-gene silencing or Zoledronic Acid-treatment on cell proliferation, BrdU-incorporation into the cells as a marker of active DNA-synthesis was determined. HK-2-cells were electroporated with 300 nM mismatch-siRNA or 300 nM pooled siRNAs targeting FPPS. The cells were cultured for 48 h and subsequently incubated with 200 µM Zoledronic Acid for additional 48 h. BrdU was added during the last 24 h, and its cellular uptake was determined in by ELISA after incubation. To assess mitochondrial dehydrogenase activity, the same experiment without addition of BrdU was conducted in parallel, and MTS bioreduction was determined.

Zoledronic acid [µM]	siRNA	BrdU-uptake [L.U.]	MTS [L.U.]
0	mismatch	0.81 ± 0.07	0.86 ± 0.08
	siRNA v. FPPS	0.78 ± 0.03	0.75 ± 0.03
200	mismatch	0.44 ± 0.03	0.29 ± 0.06
	siRNA v. FPPS	0.50 ± 0.04	0.17± 0.05

Table 25: Zoledronic Acid-treatment or FPPS silencing: proliferation and cytotoxicity
The BrdU-experiment was performed once in quadruplicate, the MTS experiment was performed 3 times in hexaplicate. ± values symbolise the standard deviation of the replicates or mean values of the experiments, respectively.

The silencing of FPPS alone did not lead to significant reduction of MTS-reducing capacity of the cells or BrdU-uptake. Treatment with Zoledronic Acid resulted in 46 % reduction of BrdU-uptake and 66 % reduction in MTS-reducing capacity. FPPS-silencing had no significant impact on Zoledronic Acid-induced reduction of BrdU-uptake, but demonstrated statistically not significant but notable additional effect to Zoledronic Acid-induced reduction of MTS-reducing capacity of HK-2-cells.

The impact of siRNA-mediated FPPS-silencing on Zoledronic Acid-induced reduction of mitochondrial dehydrogenase-activity was investigated. For this purpose, HK-2-cells were electroporated with either 300 nM mismatch- or active siRNA-pool targeting FPPS. Cells were cultivated for 48 h after transfection and were incubated with either the vehicle or 20, 50 and 200 µM Zoledronic Acid for 48 h. MTS-reducing capacity of the cells as marker of mitochondrial dehydrogenase-activity was determined at the end of incubation. All data

points obtained were normalised to the average value of the corresponding mismatch-transfected and vehicle-treated control.

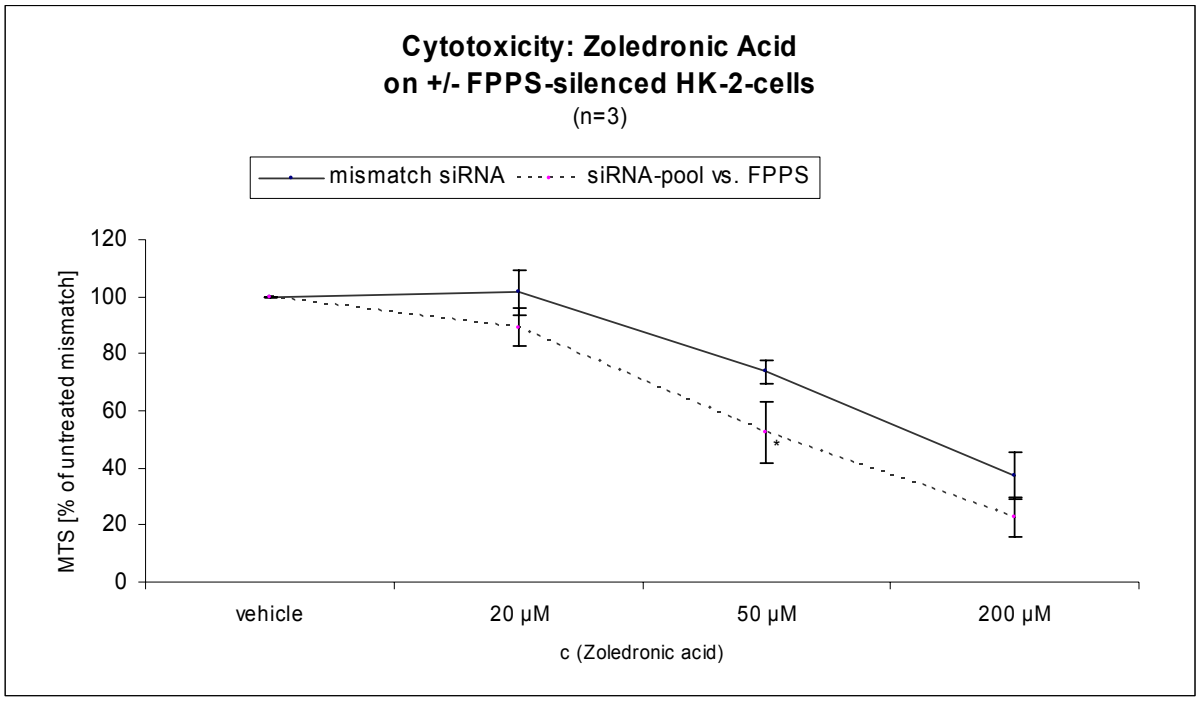


Figure 33: Cytotoxicity of Zoledronic Acid-treatment in FPPS-silenced HK-2-cells
The experiment was executed three times in hexaplicate. Error bars symbolise the standard deviation of the different experiments. Statistics were analysed by ANOVA ($p>0.05$ *).

Zoledronic Acid treatment led to dose-dependent decrease of mitochondrial dehydrogenase-activity in mismatch-transfected cells, reaching a reduction of 63% at the highest dose. In FPPS-silenced HK-2-cells, the Zoledronic Acid-induced decrease of mitochondrial dehydrogenase-activity is intensified by average 15 %, reaching statistical significance at 50 µM.

4.1.3.3 Discussion

Nephrotoxicity of Zoledronic Acid has been describes in the literature [*Bounameaux et al., 1983; O`Sullivan et al., 1994; Pfister et al., 2005*]. This finding could be supported *in vitro* with the acquired experimental data in the human proximal tubular cell line HK-2. Treatment of HK-2-cells with Zoledronic acid led to cytotoxicity, as determined by mitochondrial dehydrogenase activity, cellular membrane integrity (LDH-release) and caspase-3/7-activation. The threshold for cytotoxicity was 20 µM.

Upon Zoledronic Acid-incubations at the threshold-dose of cytotoxicity, Rap1A protein was observable exclusively in the cytosolic fraction of treated cells, and the amount RhoA protein decreased in the membrane-fraction of the cell-lysates. This is indicating that the GTPases lost their attachment to the cell membrane and were liberated into the cytosole. The liberation can be accounted to extensive deprenylation triggered by the bisphosphonate-treatment, as proposed in the literature [*Luckmann et al, 1998*].

Farnesylpyrophosphate synthase-silencing could be demonstrated on protein level. The silencing was incomplete, as shown by a remaining protein expression of ~20% 72 h after silencing. In contrast to treatments with Zoledronic Acid as a chemical inhibitor, silencing of FPPS did not lead to delocalisation of small GTPases, and did not result in cytotoxicity *per se*. The absence of cytotoxicity and GTPase-delocalisation upon specific siRNA-mediated gene silencing of FPPS could be accounted to either remaining FPPS protein activity being sufficient to maintain normal cellular function, compensatory mechanisms of the cell due to the slow siRNA-mediated depletion of FPPS protein compared to quick inhibition of protein activity by Zoledronic Acid, or additional unknown mechanisms by which Zoledronic Acid mediates cytotoxicity and GTPase-delocalisation. The isoprenoid-regulation of the cell is complex, and the cell could possibly replace the farnesylpyrophosphate by another isoprenoid for protein membrane anchoring, or gain farnesylpyrophosphate by alternative enzymatic reactions.

The GTPases –mainly Rho- are supposed to be critical for integrity of the cytoskeleton [*Lane et al., 2006*]. It could be considered that FPPS-silencing could lead to reduced proliferation of the cells due to effects on the GTPases. Reduced proliferation was investigated by BrdU-incorporation during DNA-synthesis. The observed reduced BrdU-uptake under Zoledronic Acid-treatment is most likely due to reduced viability of the cells, which was shown in parallel by reduced dehydrogenase-activity.

The dose-cytotoxicity-relationship of Zoledronic Acid on HK-2-cells could significantly be shifted by means of specific siRNA-mediated silencing of FPPS, indicating sensitisation of the cell. In FPPS-silenced cells, cytotoxicity of Zoledronic Acid became evident at lower doses. The shift of the cytotoxicity-curve indicates at least partial involvement of FPPS-inhibition in the Zoledronic Acid-mediated kidney toxicity. The effect is significant, but not pronounced, leading to the conclusion that either other Zoledronic Acid-induced mechanisms contribute to the renal toxicity or the achieved protein depletion was insufficient to result in extensive cytotoxic actions. Compensatory mechanisms of the cell through the slow course of protein depletion can have diluted the amplitude of the effect, as the kinetics of the knock-down are different to the binding of a chemical inhibitor.

In summary, specific reduction of FPPS protein expression by means of siRNA sensitised the cells to Zoledronic Acid-treatment, demonstrating a participation of FPPS-inhibition in Zoledronic Acid-mediated kidney toxicity. Silencing of FPPS had no impact on the localisation of the GTPase Rap1A, which lost its membranous attachment under Zoledronic-Acid treatment. This was indicating either insufficient down regulation of the protein, compensatory mechanisms due to the slow down regulation of the protein, or involvement of other yet unknown mechanisms of Zoledronic Acid in regard to GTPase-deprenylation.

4.1.4 Comparison of specific caspase-3-silencing to chemical inhibition

4.1.4.1 Background

Caspase-activation is a hallmark of caspase-dependent apoptosis. Upon activation caspases specifically cleave cellular key structural components of the cytoskeleton, the nucleus and proteins involved in signally pathways [*Earnshaw et al., 1999*] and contribute thereby actively to programmed cell death.

SDZ IMM 125-treatment of cultured rat hepatocytes results in apoptotic cell death, as the presence of apoptotic parameters including phosphatidylserine-flip flop to the outer membrane, cytochrome c-release, activation of caspase-3/-7 and nuclear fragmentation was demonstrated [*Grub et al., 2000a/2000b*]. Effects on calcium-homoeostasis and oxidative stress could be shown in parallel [*Grub et al., 2000a/2002*].

SDZ IMM125 is a derivative of the immunosuppressive drug cyclosporine A [*Hiestand et al., 1992*]. Due to its hydroxyethyl-moiety its lipophilicity is lower than that of the parent compound.

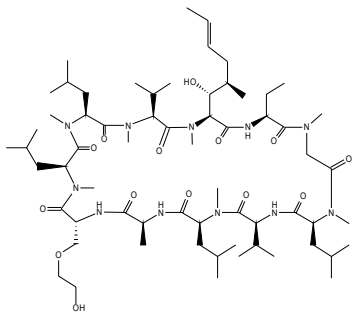


Figure 34: Chemical structure of SDZ IMM 125

Although better tolerated than cyclosporine A in rodent experiments [*Donatsch et al., 1992; Hiestand et al., 1992*] clinical studies with healthy volunteers revealed similar adverse reactions like to cyclosporine A, mainly transient impairment of liver functions, manifesting in elevated aminotransferase-levels in the blood and hyperbilirubinemia [*Witkamp et al., 1995*].

Caspase-3 and -7-activity cannot be distinguished by commercially available assays, as those use the Ac-DEVD-motif for inhibition and as substrate, which is a fine substrate for either of these two caspases [*Sung et al., 2005*]. To distinguish, the special benefit of siRNA-techniques (high specificity) will be used in this work. Furthermore, the effect of specific

siRNA-mediated caspase-3-silencing will be compared to the effect of a chemical caspase-inhibitor.

4.1.4.2 Results

The conditions for caspase-activation by SDZ IMM125 were established and fixed to substance treatment of 96 h-old hepatocyte cultures on collagen gel at a concentration of 50 µM SDZ IMM125 (see 3.2.3) under serum-free conditions for all investigations.

The activities of caspase-2, -3/-7, -8 and -9 were determined upon SDZ IMM125-treatment to identify the time point for investigations on caspase-activity. For this purpose, rat hepatocytes were treated with SDZ IMM125 and investigated at 30, 60, 90, 120, 150, 180, 210 and 240 min after start of treatment for the respective caspase-activities by assessment of reactivity of the cell lysates towards caspase-specific substrates.

	pmol label liberated from the substrate / min / µg protein at 30°C							
	Caspase-2		Caspase-3/7		Caspase-8		Caspase-9	
time [min]	vehicle (x 10^{-4})	+ SDZ IMM125 (x 10^{-4})	vehicle	+ SDZ IMM125	vehicle	+ SDZ IMM125	vehicle	+ SDZ IMM125
30	4.4 ± 1.3	4.3 ± 0.9	143 ± 3	145 ± 7	4.6 ± 3.8	6.9 ± 0.3	9 ± 3	8 ± 2
60	4.7 ± 0.5	4.6 ± 0.8	149 ± 6	131 ± 5	6.9 ± 0.4	6.8 ± 0.4	15 ± 1	11 ± 1
90	5.8 ± 0.5	5.4 ± 1.4	146 ± 2	166 ± 1**	6.8 ± 0.4	8.8 ± 0.2	10 ± 3	10 ± 3
120	2.7 ± 0.8	6.8 ± 0.7	131 ± 5	212 ± 18**	7.3 ± 3.8	9.4 ± 0.3**	9 ± 3	16 ± 9*
150	6.2 ± 1.8	9.0 ± 1.9	133 ± 6	221 ± 20**	6.8 ± 0.1	13.4 ± 1.5***	10 ± 3	22 ± 6**
180	3.0 ± 3.6	8.8 ± 2.2 **	125 ± 2	265 ± 6***	7.5 ± 0.1	14.2 ± 0.1***	10 ± 2	22 ± 3**
210	3.6 ± 1.6	10.4 ± 1.6 ***	137 ± 7	305±14***	6.9 ± 0.4	15.7 ± 1.0***	12 ± 1	29 ± 3***
240	6.6 ± 0.6	13.7 ± 1.6***	180 ± 11	377±68**	7.7 ± 0.6	19.1 ± 2.5***	16 ± 2	27 ± 1**

Table 26: Multicaspase-activation in rat hepatocytes by SDZ IMM125
The experiment was performed in triplicate from one animal. ± values symbolise the standard deviation of the different wells. Statistic was performed using one-way ANOVA with SNK-post-hoc-test, standard deviation from the replicate wells is given ± (* $p<0.05$,**$p<0.01$, *** $p<0.001$)

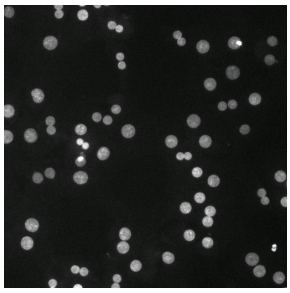
Time-dependent multi-caspase activity was observed upon SDZ IMM125-treatment. After start of treatment, statistical significant activity was reached by Caspase-3/-7 at 90 min , by caspase-8 and -9 at 120 min and by caspase-2 at 180 min The activity for all caspases was 2-fold of the respective vehicle-treated control after 240 min Caspase-3/-7 was the most active of the caspases, as the absolute turnover of substrate-molecules was 14-fold higher than that of caspase-9 at 240 min after start of treatment.

A defined time (4h) was chosen for activation in all further investigations, leading to caspase-activation and starting cytotoxicity (see tables 4,5 and figures 9, 10).

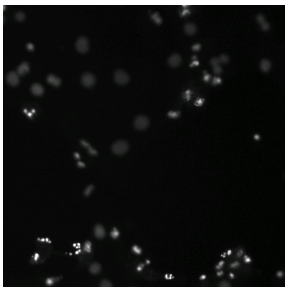
Specific Caspase-3-activation was investigated by immunohistochemistry followed by multiparameter high-content analysis on a Cellomics Arrayscan.

For this purpose, rat hepatocytes were incubated with SDZ IMM125 under the previously defined conditions, stained with the DNA-dye Hoechst 33342 and a Texas Red-labelled anti-cleaved-caspase3-antibody. The antibody recognised only the active form of caspase-3. Intensity of the label-fluorescence can therefore be correlated to caspase-3-activity.

Cells were counted by the number stained nuclei. The cell count / field could be used as a marker of cytotoxicity, as the fields were all of the same defined size. The diameter of the nuclei was recorded in parallel and can be used as a marker of chromatin condensation.



vehicle



+ SDZ IMM125

	cell count/field	nuclear size [pixels]
vehicle	60 ± 15	19.9 ± 0.2
50 µM SDZ IMM 125	47 ± 13	13.2 ± 0.6

Figure 35: Nuclear condensation in rat hepatocytes upon SDZ IMM125-treatment
The experiment was performed from 2 hepatocyte-preparations in hexaplicate ± values symbolise the range of the mean values of the experiments.

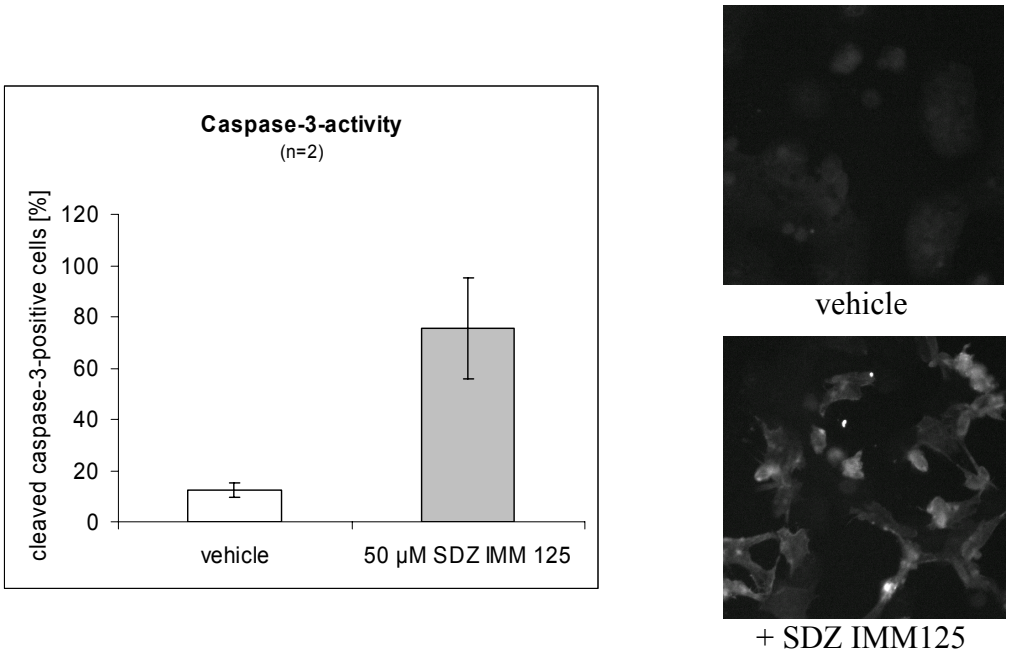


Figure 36: Confirmation of SDZ IMM125-induced caspase-3-activity in rat hepatocytes
The experiment was performed on hepatocytes from two animals in hexaplicate each. ± values symbolise the range of the mean values of the experiments. 70 F.U. was used as the threshold for positive cells

SDZ IMM125-treatment led to reduction of the number of attached cells by 22% and reduced the nuclear diameter by 20%. Specific caspase-3-activity was evident in 70% of the investigated cells.

The effect of siRNA-mediated specific caspase-7-silencing on the indistinguishable caspase-3/-7-activity was investigated. To evaluate the role caspase-7 in SDZ IMM125-induced Caspase-3/-7-signal, caspase-7 was silenced by means of siRNA.
Hepatocytes were transfected with each 300 nM mismatch- or one of the four independent siRNAs targeting caspase-7 (see table 12). After 96h of culture, caspase-activity was triggered by SDZ IMM125-treatment under the established conditions.

	CASP-3/7-activity upon SDZ IMM125-treatment
mismatch	455 ± 16
siRNA 1	372 ± 84
siRNA 2	397 ± 69
siRNA 3	500 ± 71
siRNA 4	431 ± 12

Table 27: Influence of caspase-7-silencing on SDZ IMM125-induced caspase-activity
The experiment was performed in triplicate by using hepatocytes from 1 animal. ± values symbolise the standard deviation of replicates.

None of the four independent siRNAs targeting caspase-7 had significant impact on SDZ IMM125-mediated caspase-3/-7 activity.

To investigate the participation of caspase-3 in SDZ-triggered caspase-3/-7 activity, caspase-3 was specifically silenced by means of siRNA.

Rat hepatocytes were electroporated with 300 nM mismatch- or active siRNA targeting caspase-3 (see table 14). The cells were cultivated for 96 h, and caspase-activity was induced with SDZ IMM125 under the established conditions. One group of the induced mismatch-transfected cells was conicubated with 100 µM of the caspase-inhibitor Ac-DEVD-CHO. At the end of incubation, caspase-3/-7 activity was determined.

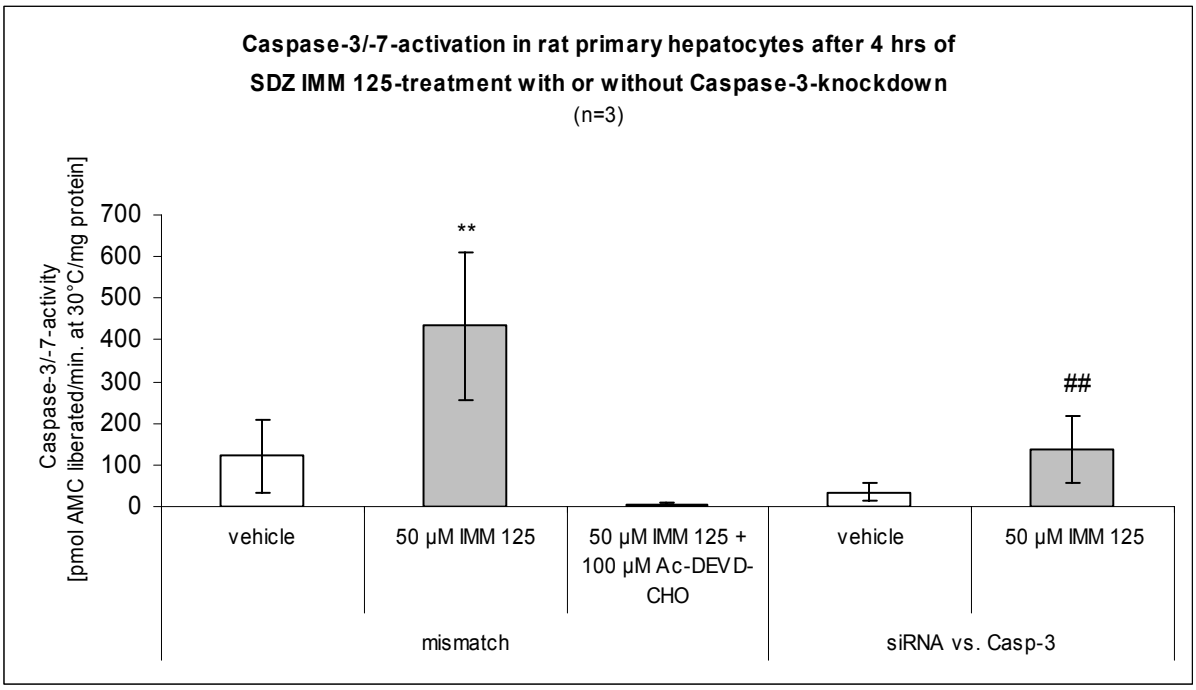


Figure 37: Influence of caspase-3-silencing on SDZ IMM125-induced caspase-activity
The experiment was performed on hepatocytes from 3 animals in triplicate each. Error bars symbolise the standard deviation of the different experiments, statistic was done by one-way-ANOVA with SNK-post-hoc-test ($p<0.01$ =** [to vehicle], $p<0.01$=## [to 50 µM SDZ IMM125])

SDZ IMM125-induction led to 3-fold induction of caspase-3/-7 activity in mismatch-transfected cells. Cotreatment with the caspase-inhibitor Ac-DEVD-CHO resulted in complete inhibition of caspase-activity beyond the background-activity of the culture. Specific siRNA-mediated gene silencing of caspase-3 without SDZ IMM125-induction led to reduction of the background caspase activity compared to the mismatch-transfected cells. The activation of caspase-3/7 by SDZ IMM125 in caspase-3-silenced cells was reduced by 71% compared to mismatch-transfected cells.

To determine the strength of the specific siRNA-mediated caspase-3-silencing, the effect in reducing SDZ IMM125-induced caspase-activity was compared to the effect of different concentrations of caspase-inhibitor.

For this purpose, rat hepatocytes were electroporated with 300 nM mismatch- or active siRNA targeting caspase-3. The cells were cultivated for 96 h and caspase-activity induced with SDZ IMM125 under the established conditions. Mismatch-transfected cells were incubated with SDZ IMM125 alone, or coincubated with 1, 5, and 75 µM caspase-inhibitor Ac-DEVD-CHO. Hepatocytes transfected with active siRNA targeting caspase-3 were incubated with SDZ IMM125 alone.

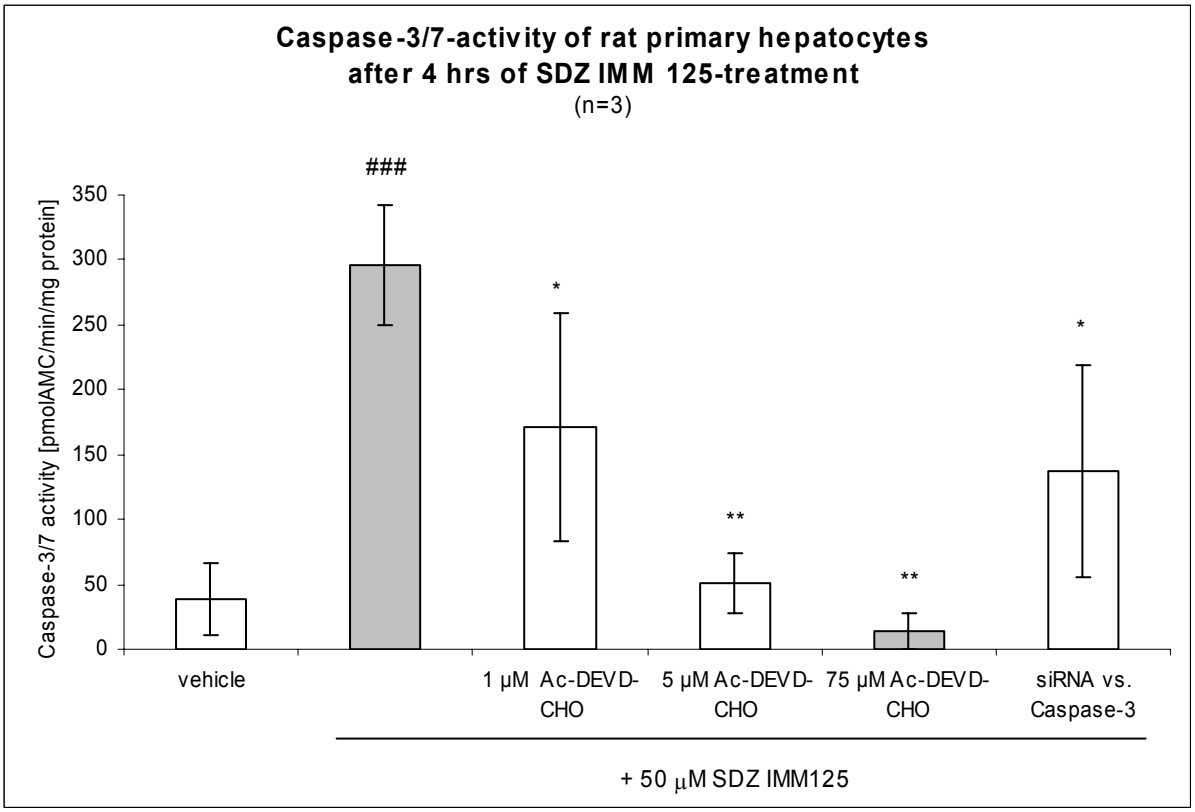
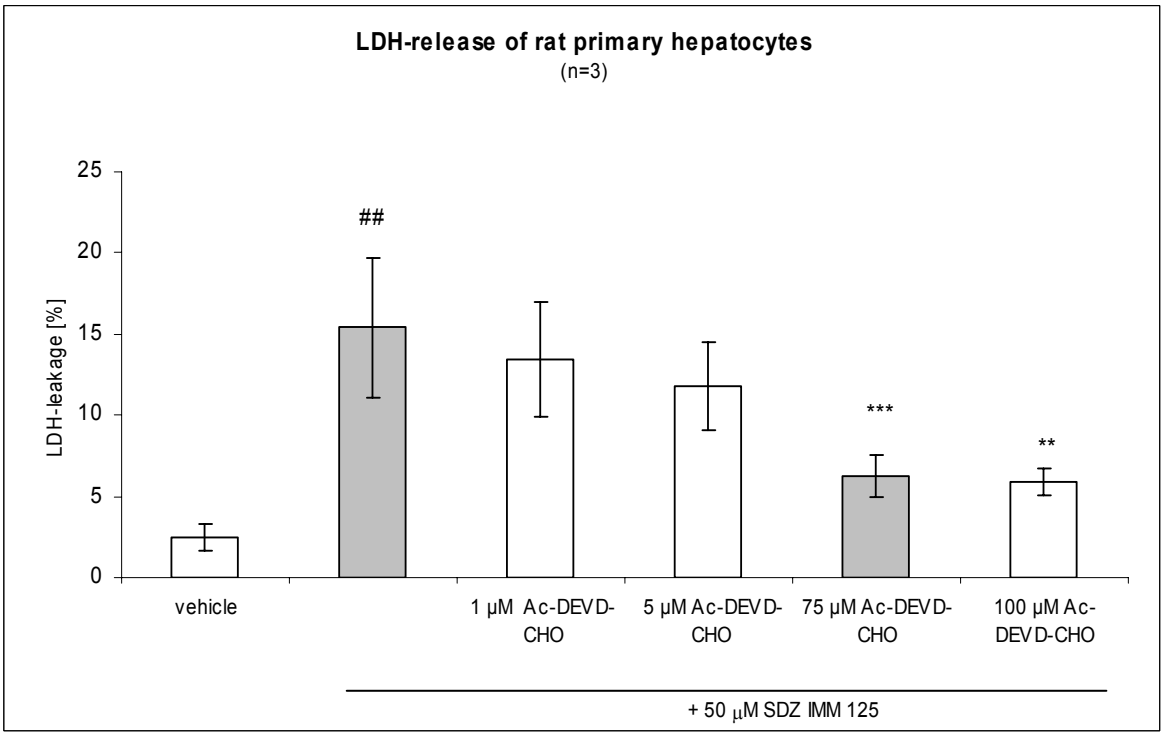


Figure 38: Comparison of caspase-inhibitor and caspase-3-silencing: caspase-activity
The experiment was executed in hepatocytes from three animals in triplicate each. Error bars symbolise the standard deviation of the different experiments. Statistic was analysed by one-way-ANOVA with SNK-post-hoc-test (*: significance vs. SDZ IMM125-treated, $p<0.05$= *, $p<0.01$ =**; #: significance vs. vehicle-treated, $p<0.001$=# # #);

SDZ IMM125-treatment induced caspase-3/-7 activity 6-fold in mismatch-transfected cells compared to the respective control. Coincubation with the caspase-inhibitor Ac-DEVD-CHO led to dose-dependent suppression of caspase-activity. At the dose of 5 µM caspase-inhibitor, the induction mediated by SDZ IMM125 was completely abolished. Incubation with 75 µM caspase-inhibitor decreased caspase-activity below the general background of the cell culture. Specific silencing of caspase-3 by siRNA led to 50% inhibition of the SDZ IMM125-induced caspase-activity compared to induced mismatch-transfected hepatocytes. This inhibitory strength of the specific siRNA-mediated silencing of caspaspe-3 is comparable to the effect of 1 µM chemical caspase-inhibitor Ac-DEVD-CHO.

Effects of specific caspase-3-silencing and chemical caspase-inhibition on the cytotoxicity mediated by SDZ IMM125-treatment of rat hepatocytes were investigated. For this purpose, rat hepatocytes were electroporated with 300 nM mismatch-siRNA. The cells were cultivated for 96 h, and cytotoxicity was induced with SDZ IMM125 under the established conditions. Mismatch-transfected cells were incubated with SDZ IMM125 alone, or coincubated with 1, 5, 75 and 100 µM caspase-inhibitor Ac-DEVD-CHO. For comparison in parallel, rat hepatocytes were electroporated with 300 nM mismatch- or active siRNA targeting caspase-3. The cells were cultivated for 96 h and cytotoxicity was induced with SDZ IMM125 under the established conditions. At the end of incubations, LDH was determined in the culture supernatant and the cell lysate, and the leakage in percent calculated.



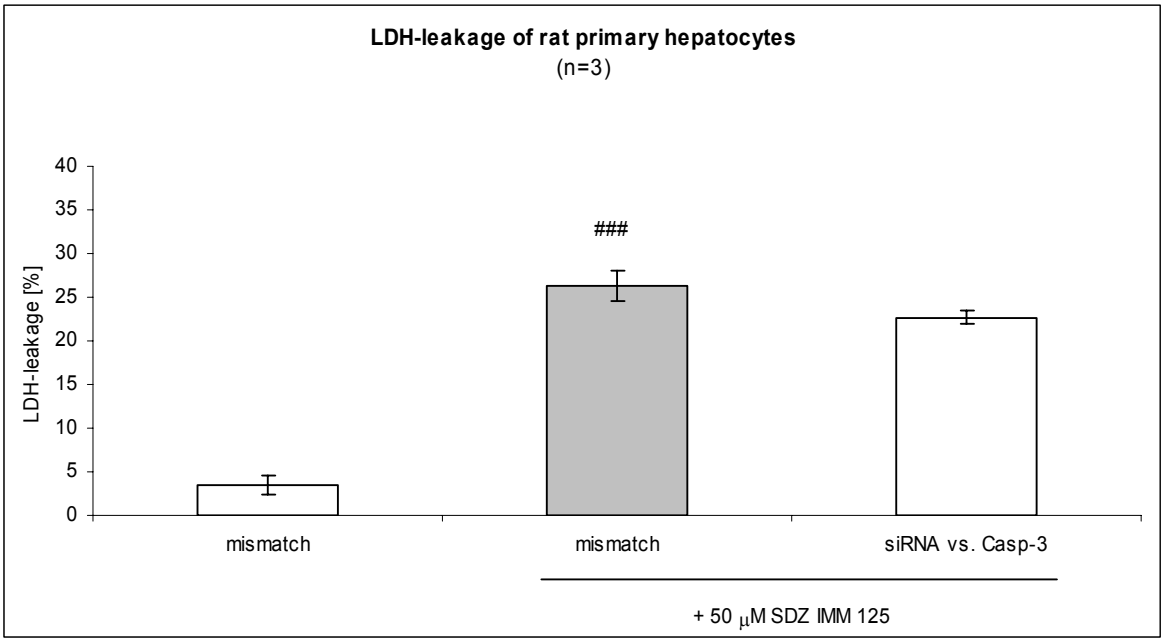


Figure 39: Comparison of caspase-inhibitor and caspase-3-silencing: cytotoxicity
Error bars symbolise the standard deviation of the different experiments, statistic was done by one-way-ANOVA with SNK-post-hoc-test (significance vs. SDZ IMM125-treated, $p<0.05$= *, $p<0.01$ =**; significance vs. vehicle-treated, $p<0.001$=# # #);

Treatment of mismatch-transfected rat hepatocytes with SDZ IMM125 resulted in 5-fold increased LDH leakage compared to vehicle-treated cells. Coincubation with the caspase-inhibitor Ac-DEVD-CHO resulted in decreased cytotoxicity, reaching statistical significance at the two high doses of 75 and 100 µM. The highest does of the inhibitor reduced the SDZ IMM125-induced LDH-leakage by 50%. Silencing of caspase-3 by means of siRNA had no

statistically significant impact on SDZ IMM125-induced cytotoxicity compared to mismatch-transfected cells.

4.1.4.3 Discussion

Induction of apoptosis and secondary necrosis upon SDZ IMM125-treatment of rat hepatocytes is described in the literature [*Grub et al., 2000a/2000b*]. These findings were in congruence with the obtained experimental data of the present study. Specific caspase-3-activity, diminished nuclear diameters and reduced cell count were observed upon SDZ IMM125-treatment, indicating apoptosis (caspase-activation and nuclear condensation) and arising cytotoxicity (cellular detachment) at the treatment conditions applied. Multi-caspase-activation became observable at early time points of compound treatment. The activity of the single caspases found in the cellular protein after SDZ IMM 125-treatment was different, Caspase-3/-7 being the most active signal observed.

The siRNA-mediated specific knockdown of caspase-7 had no influence on the observed indistinguishable caspase-3/-7-activity induced by SDZ IMM 125-treatment. Thus, a contribution of caspase-7 to the observed signal can be excluded.

Specific siRNA-mediated silencing of caspase-3 resulted in reduction of the observed caspase-3/-7 signal, indicating involvement of specific caspase-3-activity. The silencing did not lead to complete abolishment of the observed caspase-activity, indicating incomplete gene silencing on protein level.

The effect of specific silencing of caspase-3 on SDZ IMM125-induced caspase-activity was comparable to incubations with 1µM chemical caspase-inhibitor Ac-DEVD-CHO.

Cytoprotective effects of the chemical caspase-inhibitor towards SDZ-IMM125-induced cytotoxicity became significant at concentrations >75 µM, while siRNA-mediated silencing of caspase-3 did not demonstrate cytoprotective effects. This was most likely due to the incomplete gene silencing comparable to a low dose of the caspase-inhibitor, which is not able to trigger beneficial cytoprotective effects. Non-specific inhibition of other caspases by Ac-DEVD-CHO contribution to the observed cytoprotective effect can also not be excluded.

In summary, the observed SDZ IMM125-induced caspase-3/-7 activity was originating from specific caspase-3-activation. The effect of specific gene silencing of caspase-3 was comparable to the effect of a low dose of the chemical caspase-inhibitor Ac-DEVD-CHO, indicating incomplete gene silencing. Cytoprotective effects of the chemical caspase-inhibitor could not be observed upon specific caspase-3-silencing due to insufficient gene silencing.

4.2 *In vivo*-Investigations

4.2.1 Silencing of Cytochrome P450 2E1

4.2.1.1 Background

The generation of classical knock-out animals is challenging, time-consuming and expansive [*Lee et al., 2006*]. SiRNA-techniques have the potential to circumvent this process in certain cases and to produce transient knock-down animals. The restrictions of the siRNA-method are the remaining enzyme activity (knock-DOWN, not knock-OUT) and adverse reactions to the state-of-the-art-techniques used, which are discussed in the following chapters.

Identification of a route of administration with no interference to the oral administration of test substances and no adverse side-effects would increase the usefulness of siRNA techniques for toxicology. Vector-based delivery techniques to the target cell were not addressed in this thesis work, as these applications exclude proper dosing control, and thus have the hazard of interferon-response and overload of the RNAi-machinery [*Grimm et al., 2006; Sledz, 2003*]; therefore, synthetic siRNAs were applied to the animals in the conducted studies.

In toxicological studies the peroral route is the preferred mode of substance administration. This is due to the facts that on the one hand many pharmacologically active substances are orally administered and thus undergo an extensive gastrointestinal passage before entering the systemic circulation, and on the other hand the minimal-invasive application in animal testing, that is easy-to-apply and robust. For siRNA-delivery to liver cells, intravenous injection of siRNA was identified to be the most efficient way of application in animal studies. Improved results can be obtained by daily repeated dosing [*Soutschek et al., 2004; Song et al., 2003; Mahadevan et al., 2006*]. The best uptake into liver was achieved by high-pressure injection to the tail vein, leading to hydroporation of the hepatocytes. This method was shown to widen the fenestrae of the hepatocytes, leading to extensive uptake of naked DNA or siRNA [*Lewis et al., 2005; Liu et al., 1999; Song et al., 2003; Zhang et al., 2004*]. The observed adverse reactions to this treatment were apnoe, cardiac arrhythmia and a reversible rise in liver transaminases [*Inoue et al., 2004; Zhang et al., 1999; Zhang et al., 2004*]. Alternatives would be of great value, considering the acute liver damage short after high-pressure tail vain injections.

Intraperitoneal application of siRNAs is currently used to target tumors in the peritoneal cavity. Escape from this site to the systemic circulation is reported [*Urban-Klein et al., 2005*],

and this bypass was chosen to be addressed. In parallel to this study, a study with a similar system was conducted at the Oregon State University. In this case, antisense-oligonucleotides against CYP3A2 were administered daily i.p. for 8 days, and Tamoxifen was dosed perorally starting the second day of treatment. The antisense-treatment abolished induction of the CYP3A2 by Tamoxifen, and a significant reduction in tamoxifen-DNA-adducts was observed [*Mahedevan et al., 2006*].

4.2.1.1.1 Cytochrome P450 2E1 as a target for toxicological investigations

All organisms are equipped with plenty of metabolizing enzymes that are capable of executing various functionalisation reactions. These reactions lead in general to higher lipophilicity and undesirable substances can be excreted after a conjugation-step.

The cytochrome P450-family (CYP) assumes a major part of this. The family consists of a big collective of mixed-functional monooxygenases that are anchored in the membrane of the endoplasmatic reticulum. They are generally spoken hemoproteins with a molecular weight of 45-55 kDa, all evolving from a common ancestor that developed 3 – 3.5 million years ago. [*Forth et al., 2001*].

The single CYP-enzymes are not constitutively expressed in constant equal amounts, but are expressed in various, changeable proportions. They are sub classified by means of sequence-homology and uniformly named using the nomenclature proposed in 1996 [*Nebert et al., 1996*].

Cytochrome P-450 2E1 (CYP2E1) is responsible for the metabolism of small neutral hydrophilic molecules. Its average fraction among the CYP-family in man is 3-6%, depending on nutrition, age, gender, polymorphism and health [*Forth et al., 2001*].

The mechanisms of CYP 2E1-induction and inhibition are not fully elucidated. The regulation occurs at transcriptional [*Choi et al., 2002*], post-transcriptional [*Woodcroft et al., 1999; Moncion et al., 2002*] and protein-stability levels [*Choi et al.,2002*; *Wu et al., 2002; Kraner et al., 1992*].

CYP2E1 is involved in the bioactivation and metabolism of ethanol, acrylamide and volatile anesthetics and is thus a target for toxicological investigations. The mechanism of Acetaminophen-induced liver toxicity is well-investigated and briefly described here to demonstrate the relevance of CYP2E1 for toxicology.

Acetaminophen (Paracetamol) is a commonly-used analgesic for the treatment of minor pain with a wide number of users. In high doses, acetaminophen is liver toxic, and has made a sad career as a suicide-poison due to the low price and its easy availability. The mechanism of acetaminophen-induced liver toxicity is well-known [*Nelson, 1990*] and triggered by bioactivation of the compound by CYP2E1 (and a lesser extent CYP1A2 [*Ghanayem et al., 2000; Patten et al., 1993*]) to a thiol-reactive benzoquinone imine. The reactive metabolite is under usual conditions inactivated by extensive binding to glutathione, but in overdoses, the glutathione-pool of the hepatocytes is completely depleted, and excess active metabolite starts to attack hepatic sulfur-containing protein, leading to massive loss of liver cell function and fulminant centrilobular liver necrosis.

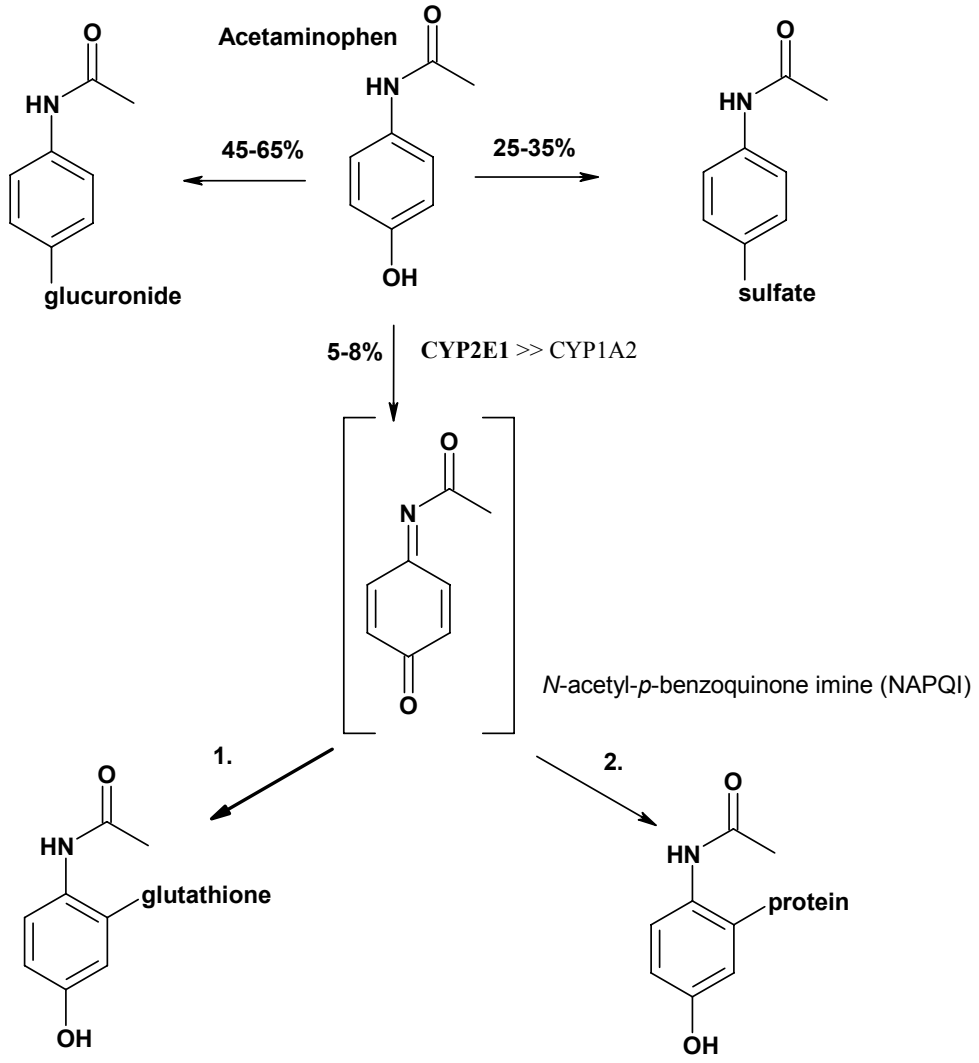


Figure 40: Mechanism of acetaminophen-induced liver toxicity

In animal studies, pre-treatment of mice with chemical inhibitors of CYP2E1 led to decreased toxicity of acetaminophen [*Yim et al., 2005; Kim et al, 1997; Sumioka et al., 1998*], while

pretreatment with CYP2E1-inducers led to increased toxicity [*Lu et al., 2005; McCuskey et al, 2004*]. More specific, CYP2E1-knockout mice showed a higher resistance to acetaminophen than the wild-type [*Lee et al., 1996*], and this resistance could be even improved by establishing a CYP2E1/CYP1A2-double knock-out mouse [*Zaher et al., 1998*].

The purpose of the conducted study was to establish a method for siRNA-delivery to liver cells *in vivo* and to evaluate intraperitoneal injection of non-complexed siRNA as a minimal-invasive method of application to delivery siRNAs to the liver as a target organ of toxicology. Furthermore, the pathologic impact of hydrodynamic injection on the liver will be assessed.

4.2.1.2 Results

A kinetic study (with a modified siRNA targeting CYP2E1 (siRNA1, see figures 19, 20) comparing intraperitoneal and hydrodynamic injection for siRNA-delivery to hepatocytes was performed *in vivo* in male CD-1-mice. The mouse was chosen as the model animal due to the applicability of hydrodynamic injection [*Song et al., 2003; Bartlett et al., 2006; DeSouza et al., 2006; Llacuna et al., 2006*]. The applied dose of siRNA for intraperitoneal injection was chosen to be 50 mg/kg BW, representing a very high dose applied in the literature for silencing a hepatic protein [*Soutschek et al., 2005*]. The dose for hydrodynamic injection was chosen to be 2.5 mg/kg BW. The doses of siRNA applied in the literature for hydrodynamic injection are in the range of 0.6-2.5 mg/kg [*Bartlett et al., 2006; Song et al., 2003; De Souza et al., 2006; Llacuna et al., 2006*].

Single hydrodynamic injection (100 ml/kg, 5-6 sec.) of siRNA into the tail vein was compared to daily intraperitoneal dosing with a high dose of siRNA.

The total number of animals was 36, one treatment group consisted of 6 animals. Each treatment group was split in a subgroup of 3 animals receiving mismatch- and a subgroup of 3 animals receiving functional siRNA targeting CYP2E1.

18 animals received single hydrodynamic injection of siRNA into the tail vein. Animals were sacrificed at 12, 24, and 48 h after injection. 6 animals received one intraperitoneal injection of siRNA and were sacrificed after 24 h, 6 animals received two daily intraperitoneal injections of siRNA and were sacrificed after 48 h and 6 animals received three daily intraperitoneal injections of siRNA and were sacrificed after 72 h (the graphic study schedule can be found in the Material and Methods-section, see figure 11).

RNA was extracted from liver tissue, and CYP2E1 mRNA-expression was determined by qPCR. Microsomes were isolated from liver tissue, and CYP2E1 protein-expression was quantified by western blotting with human recombinant CYP2E1 as a standard.

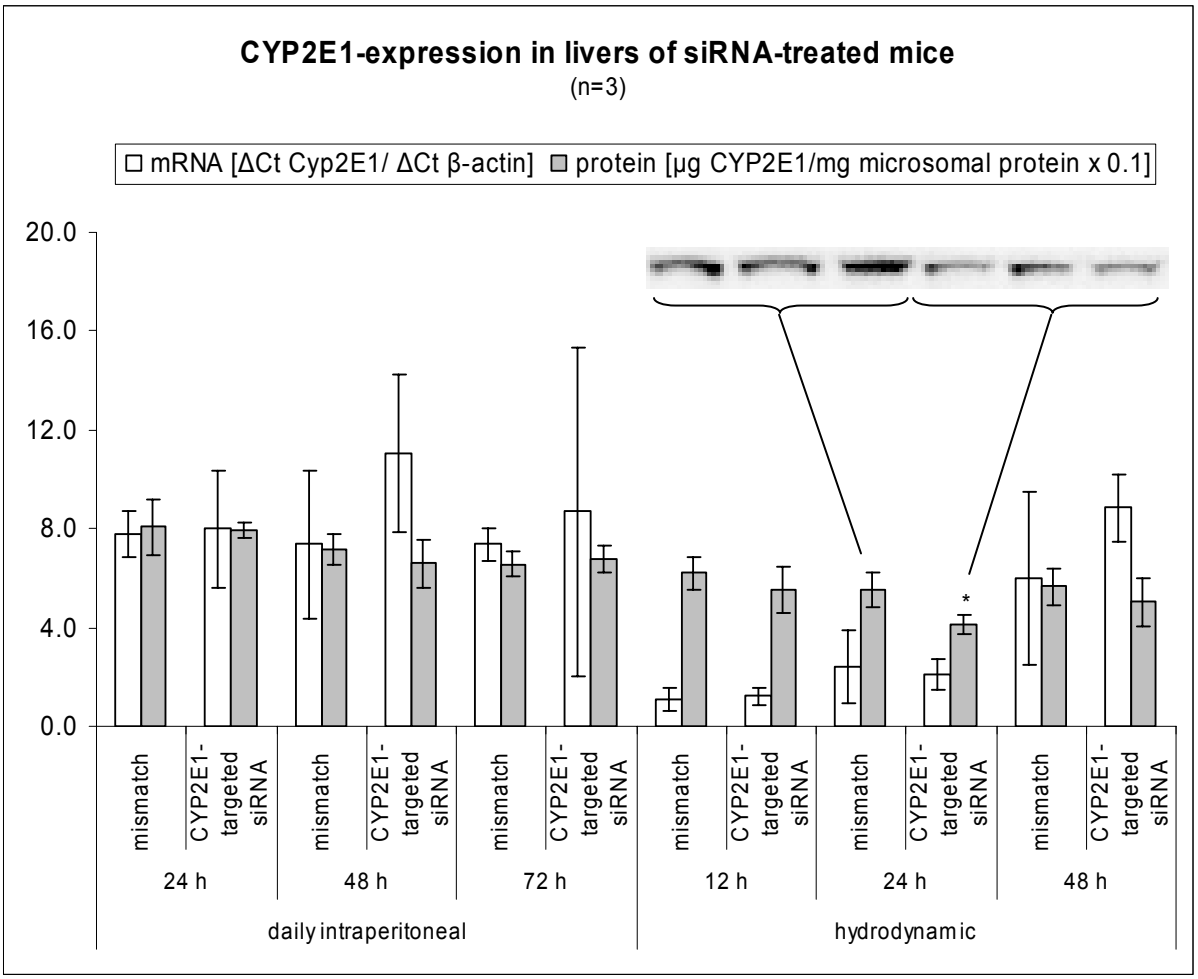
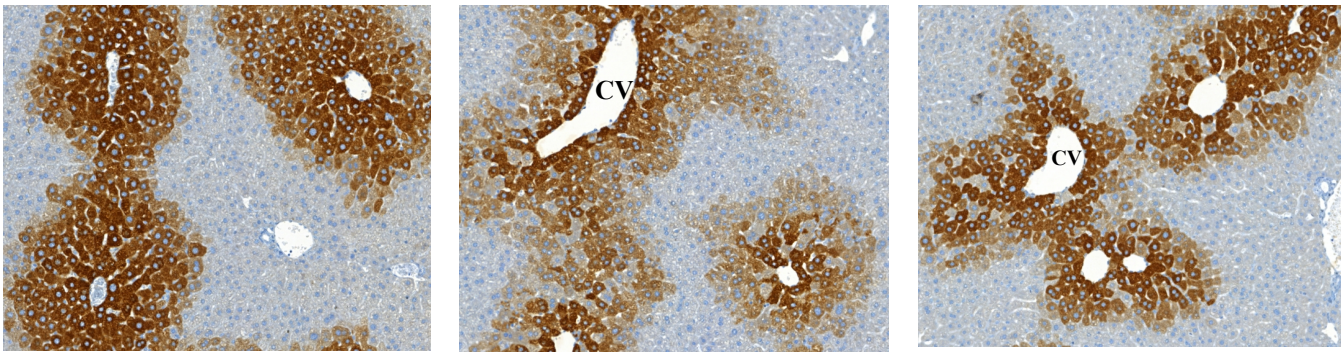


Figure 41: CYP2E1-expression in livers of siRNA-treated mice
The housekeeping gene for qPCR was β-actin, and Calnexin was applied as loading control for western blotting. Statistical analysis by ANOVA with SNK-post-hoc test ($p<0.05$ = *, compared to respective mismatch-treated animals)

Neither single nor repeated intraperitoneal injection of siRNA did not lead to significant alterations in CYP2E1 mRNA- or protein expression. Hydrodynamic injection caused reductions in CYP2E1 mRNA-expression, independent on the use of mismatch- or functional siRNA. The reduction on mRNA-level was most significant 12 h after injection (86% reduction compared to the average of intraperitoneally treated animals), less pronounced 24 h after injection (73% reduction) and only marginally evident after 48 h (12% reduction). No significant difference in CYP2E1 mRNA-expression between mismatch- and siRNA targeting CYP2E1-treated animals was observed. In the group receiving functional siRNA, CYP2E1 protein was significantly reduced 24 h after hydrodynamic injection. 12 and 48 h after injection, reduction of CYP2E1 protein is marginally observable.

To further investigate the observed CYP2E1 protein reduction, immunohistochemistry was performed on liver slices. Formalin-fixed livers from the study animals were sliced and CYP2E1 was visualised by antibody incubation. The slices were counterstained with hematoxylin to visualise non-CYP2E1-expressing cells and nuclei.



mismatch siRNA vs. CYP2E1, 24 h siRNA vs. CYP2E1, 48h

Figure 42: IHC on CYP2E1 in liver slices from hydrodynamically injected mice

CYP2E1 was highly expressed around the central veins (CV), and its expression faded towards the midzonal region. Single-cell protein depletion of CYP2E1 appeared as a mosaic-like pattern of non-stained cells within the hydrodynamically injected group. Single-cell knockdown of CYP2E1 became evident on protein-level 24 and 48h after injection, while livers of mismatch-injected animals did not show this phenotype. The single-cell knockdown was significant, as 3 pathologists were able to identify the slices from all CYP2E1-knockdown animals randomised and blindly out of the collective of all slices obtained from the study. Not the first-contact layer of hepatocytes around the central vein was affected, but randomly distributed cells 2-4 cell layers away from the central vein. The observed patter was not visible in animals who received intraperitoneal injection of siRNA.

Interferon-response was investigated by determination of Interferon-β in the serum of the treated mice, and PCR on interferon-regulated genes in the target-tissues [*Sledz et al., 2003, Patzwahl et al., 2001*].

RNA extracted from liver tissue was used for investigation of PKR and IFIT mRNA expression. Interferon-β concentration in the serum of the animals was determined by ELISA.

				mRNA-expression [□ Ct target mRNA/ □ Ct □-actin]	
			serum	liver	
injection	endpoint	siRNA	Interferon-□ [pg/ml]	PKR (x 10^{-2})	IFIT (x 10^{-3})
daily intraperitoneal	24 h	mismatch-siRNA	(all values below detection limit)	1.6 ± 0.4	1.3 ± 0.4
		CYP2E1-targeted siRNA		2.9 ± 0.5	2.2 ± 0.6
	48 h	mismatch-siRNA		2.1 ± 0.2	1.7 ± 0.5
		CYP2E1-targeted siRNA		3.9 ± 2.2	2.3 ± 0.9
	72 h	mismatch-siRNA		2.6 ± 1.8	1.3 ± 0.8
		CYP2E1-targeted siRNA		3.1 ± 1.7	1.5 ± 0.7
hydrodynamic	12 h	mismatch-siRNA		2.8 ± 1.8	1.4 ± 1.1
		CYP2E1-targeted siRNA		4.2 ± 1.1	4.7 ± 3.6
	24 h	mismatch-siRNA		2.0 ± 0.8	2.0 ± 1.0
		CYP2E1-targeted siRNA		1.9 ± 0.5	1.3 ± 0.5
	48 h	mismatch-siRNA		3.1 ± 1.3	1.6 ± 0.5
		CYP2E1-targeted siRNA		2.9 ± 0.2	2.5 ± 0.1

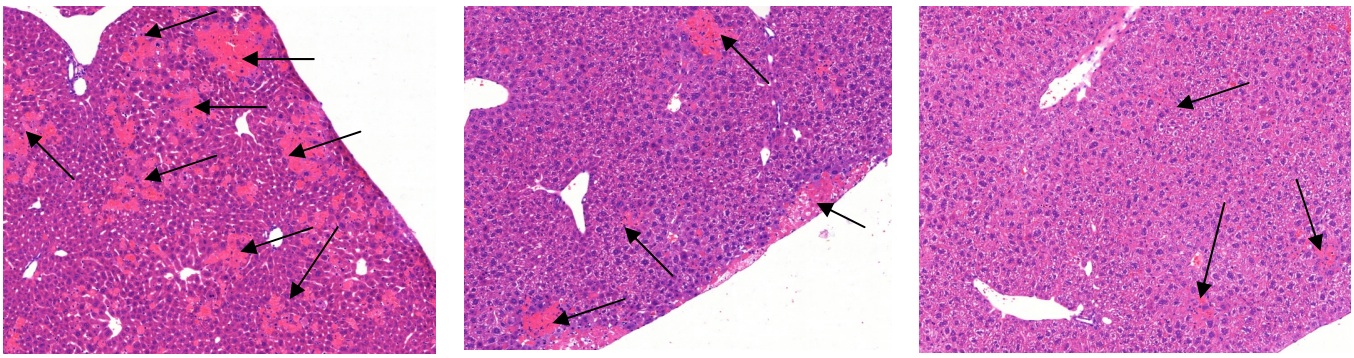
Table 28: Control-parameters for interferon-response
The housekeeping gene for qPCR was β-actin

PKR and IFIT were expressed in the investigated tissues. None of the treatment conditions had significant impact on their mRNA-expression. Interferon-β in the serum was below 16 pg/ml (detection limit of the ELISA).

The impact of the hydrodynamic injection on the liver was investigated by means of symptomatic and by means of macroscopic-/microscopic organotypic observations and circulating liver transaminases AST and ALT in the serum.

From the point of symptomatic, young mice (5-6 weeks of age, <30g) did not react severely within the first 30 min after hydrodynamic injection. Between 30 min and 4 h after injection, the symptomatic was severely increased, as the animals were cold, apatic, slow in movements and displayed in some cases freezing, indicating pain. After this period, all animals returned to a state with no observable symptomatic.

Pathologically, the effect of the hydrodynamic injection became evident macroscopically and microscopically. At dissection, small and located intrahepatic haematoms were observable, especially in the right-lateral liver lobe and the papillaric appendices. Microscopic evaluation of the cellular damage was executed on eosin-hematoxylin-stained liver slices.



liver 12 h after hydrodynamic injection | liver 24 h after hydrodynamic injection | liver 48 h after hydrodynamic injection

Figure 43: Eosin-hematoxylin-stained liver slices from hydrodynamically injected animals

Multifocal to coalescent acute midzonal hepatocellular necrosis with hemorrhage was present in all animals receiving hydrodynamic injection. The hepatocellular damage was markedly decreased 24 h after injection, and further decreased after 48h.

The liver damage detected on the liver slices by eye was semiquantified by grouping into pathology scores. The amount of circulating liver transaminases AST and ALT was determined enzymatically in the serum of the treated mice.

injection	endpoint	siRNA	pathology scores [0 no finding– 5 severe damage]	AST [I.U./L]	ALT [I.U./L]
daily intraperitoneal	24 h	mismatch-siRNA	0 ± 0	85 ± 37	51 ± 33
		CYP2E1-targeted siRNA	0 ± 0	102 ± 28	78 ± 50
	48 h	mismatch-siRNA	0 ± 0	91 ± 20	60 ± 22
		CYP2E1-targeted siRNA	0 ± 0	179 ± 85	141 ±103
	72 h	mismatch-siRNA	0 ± 0	62 ± 3	37 ± 7
		CYP2E1-targeted siRNA	0 ± 0	124 ± 41	56 ± 6
hydrodynamic	12 h	mismatch-siRNA	3 ± 1	3053 ± 769	3564 ±252
		CYP2E1-targeted siRNA	4 ± 1	4217± 250	5539 ± 706
	24 h	mismatch-siRNA	3 ± 1	624 ± 231	945 ± 237
		CYP2E1-targeted siRNA	2 ± 0	407± 96	725 ± 130
	48 h	mismatch-siRNA	1 ± 1	247± 123	213 ± 162
		CYP2E1-targeted siRNA	1 ± 0	124 ± 42	92 ± 12

Table 29: Pathology findings in the livers of siRNA-treated animals
Microscopic damage was expressed in pathology scores 0-5. Circulating transaminases were enzymatically determined in the serum of treated animals.

Intraperitoneal injection of siRNA had no impact on the investigated parameters of liver damage.

The pathological evaluation of the liver damage indicated severe damage 12 h after hydrodynamic injection (pathology score 3.5: medium to heavy damage) and reduced damage after 24h (pathology score 2.5: medium damage) and 48 h (pathology score: 1 light damage). The amount of circulating transaminases in the serum of intraperitoneally injected mice was comparable to non-treated mice (compared to historical data acquired in house). The circulating liver transaminases in the serum were increased by hydrodynamic injection. The increase was most pronounced 12 h after injection (AST 34-fold, ALT 65-fold increased compared to intraperitoneally injected animals), reduced 24 h after injection (AST 5-fold, ALT 12-fold increased compared to intraperitoneally injected animals) and only marginally present 48 h after injection (AST and ALT 2-fold increased compared to intraperitoneally injected animals).

To further evaluate the intraperitoneal administration, a modified study was conducted with 10 animals who received a one-week daily treatment with siRNA. 5 animals received mismatch- and 5 animals functional siRNA targeting CYP2E1 by daily intraperitoneal injection at 50 mg/kg BW for 7 days.
Additionally to the parameters investigated in the first study, CYP2E1 protein activity was investigated by the activity of the liver microsomal fraction towards hydroxylation of Chlorzoxazone at the 6-position *ex vivo*. Interferon-β-detection as a marker of interferon-response was exchanged to interferon-γ-detection in the serum.

		mismatch-siRNA	CYP2E1-targeted siRNA
liver	CYP 2E1 mRNA-expression [Δ Ct target mRNA/ Δ Ct β-actin]	6.4 ± 2.0	6.8 ± 0.4
	CYP2E1 protein expression [µg/mg microsomal protein]	78 ± 9	79 ± 17
	CYP2E1 protein activity [nmol 6-OH-CZX/min/mg microsomal protein]	1.48 ± 0.20	2.08 ± 0.21
	PKR mRNA-expression (x 10^{-3}) [Δ Ct target mRNA/ Δ Ct β-actin]	7.6 ± 1.8	7.2 ± 0.7
	IFIT mRNA-expression (x 10^{-3}) [Δ Ct target mRNA/ Δ Ct β-actin]	1.0 ± 0.4	0.6 ± 0.2
serum	AST [I.U./L]	123 ± 55	103 ± 25
	ALT [I.U./L]	44 ± 10	45 ± 16
	Interferon-γ [pg/ml]	0.56 ± 0.19	0.21 ± 0.13

Table 30: One-week-intraperitoneal treatment with siRNA

None of the investigated parameters was significantly altered in animals treated with active siRNA targeting CYP2E1 compared to the respective mismatch-treated animals.

4.2.1.3 Discussion

Successful gene silencing in the liver of mice by means of hydrodynamic injection of siRNA has been demonstrated in the literature [*Song et al., 2003; Bartlett et al., 2006; DeSouza et al., 2006; Llacuna et al., 2006*]. The acquired experimental data is in congruence to this finding. In mice hydrodynamically injected with chemically stabilised siRNA targeting CYP2E1, significant knockdown of hepatic CYP2E1 protein expression could be observed by means of western blot and immunohistochemistry compared to mismatch-injected animals. Immunohistochemistry revealed single-cell knockdown of CYP2E1 protein expression in hepatocytes situated in the second to fourth cell layer around the central veins. The hepatocytes directly being exposed to daily alterations of extracellular hydrodynamic pressure in the first layer around the blood vessels could be considered to be more pressure-resistant, and thus being not transfected by the injection.

Hydrodynamic injection resulted in heavy, but markedly recoverable hepatocellular damage, as revealed by means of symptomatic, macroscopic and microscopic histopathologic findings, and strongly elevated circulating liver transaminases. These findings are in congruence with the literature [*Liu et al., 1999*]. The papillaric appendices and the triangularic liver lobes were most strongly affected, which can be accounted to their localisation close to the hepatic centrum and thus direct exposure to the hydrodynamic pressure applied. The hydrodynamic injection is not a suitable method for standard application in toxicological studies as the method *per se* is resulting in vigorous liver toxicity.

Hydrodynamic injection of siRNA resulted in decreased CYP2E1 mRNA-expression at early time points after injection, independently of the siRNA (mismatch or active) applied. The observed decrease is most likely due to leakage of the mRNA out of the hepatocytes compromised by the hydrodynamic pressure, as the effect is time-dependently diminished at later time points after injection, correlating with the recovery of the liver from the damage induced by hydrodynamic injection.

Daily repeated intraperitoneal application of non-complexed chemically stabilised siRNA targeting CYP2E1 at a high dose for up to 7 days did not result in reduced CYP2E1 mRNA-expression, protein expression or protein activity levels compared to mismatch-injected animals. Further prolongation of the treatment was not considered to improve gene silencing, as a reported antisense-study with a similar application schedule led to successful knockdown of a cytochrome P450-family member [*Mahedevan et al., 2006*]. Elevation of the applied dose of siRNA could be considered, but the applied dose was chosen high compared to the

commonly applied doses of 1-2 mg/kg BW [*Sorensen et al., 2003; Ma et al., 2005; Urban-Klein et al., 2005*]. Although uptake of siRNAs into the systemic circulation from the intraperitoneal space is reported [*Urban-Klein et al., 2005*], siRNAs are charged polycations, and thus it can be concluded that they do not cross the cell membrane barrier without complexation or hydrodynamic force.

IFIT- and PKR mRNA-expression as well as elevated interferon-β/-γ levels in the serum can be used as markers of interferon response [*Patzwahl et al., 2001; Bridge et al., 2003; Ma et al., 2005*]. SiRNA sequence-specific interferon response was not evident, as demonstrated by unaffected mRNA expressions of IFIT and PKR, and absence of Interferon-β/-γ elevation in the serum compared to mismatch-treated animals. In general, induction of interferon response can be excluded for all applied conditions, as in cases of interferon response, interferons are reported to be present in the low micromolar range in serum [*Ma et al., 2005*], while in this study only picomolar levels or less were detected.

In summary, silencing of CYP2E1 *in vivo* was possible by means of hydrodynamic intravenous injection of chemically modified siRNA. The hydrodynamic injection is not a suitable method for standard application in toxicological studies as it led to recoverable, but initially severe liver damage. Intraperitoneal delivery of non-complexed siRNAs to liver cells was not possible.

5 Summary and Conclusions (Abstract)

The RNAi method plays a great role in target validation in drug discovery. However its usage for toxicology has not been systematically investigated. The aim of this work was to evaluate the RNAi-method for toxicological mechanistic studies and to demonstrate the impact of gene-silencing on biochemical cellular endpoints.

SiRNAs were selected by a computer-supported algorithm. Efficient and reproducible delivery of siRNAs to the target cells *in vitro* was achieved by electroporation. The molecular knockdown of the target was monitored by mRNA- and protein expression or protein activity between 24 and 144 hours after treatment. SiRNAs were tested *in vitro* before application *in vivo*. Intraperitoneal administration of siRNA was evaluated versus hydrodynamic injection into the tail vein as a method for *in vivo* targeting of the liver.

The following enzymes were targeted by RNAi in cell culture: ATP synthase in HepG2, farnesylpyrophosphate-synthase (FPPS) in human kidney (HK-2) cells and caspase-3 in rat primary hepatocytes. In all experiments, RNAi decreased mRNA levels and protein expression or enzymatic activities, which demonstrates the successful gene silencing. The silencing of the mitochondrial F_1-ATP synthase β-subunit had no significant influence on the survival and energy metabolism of HepG2-cells. Although Oligomycin B-treatment resulted in ATP depletion and loss of mitochondrial membrane potential, the silencing did not sensitise the cells towards Oligomycin B- or Diclofenac-induced alterations of mitochondrial functions and cytotoxicity. Silencing of ATP synthase in HepG2 cells resulted in a similar transcriptional signature as Diclofenac treatments *in vivo,* suggesting a potential link between ATP synthase and Hepcidin, BiP and ALAS-1 by co-regulation. The FPPS silencing resulted in tendentially increased cytotoxicity of Zoledronic Acid, but had no influence on the prenylation status of small GTPases. The caspase-3/-7 inhibitor Ac-DEVD-CHO inhibited SDZ-IMM 125-mediated apoptosis. Specific gene silencing of caspase-3 resulted in reduction of SDZ IMM125-induced caspase-activation, while silencing of caspase-7 had no influence in this regard. The threshold effect of gene silencing was assessed by comparing caspase-3 silencing with the chemical caspase-inhibitor Ac-DEVD-CHO on caspase-3 activities and potential cytoprotective effects. The effect of caspase-3 silencing was equivalent to the activity of 1 μM inhibitor. The inhibitor-mediated protection on SDZ-IMM125-induced cytotoxicity was exclusively achieved at higher inhibitor concentrations, indicating that the achieved silencing effect was not sufficient for cytoprotection.

In general, siRNAs have higher specificity compared to enzyme inhibitors. Chemical inhibitors are less specific and can influence enzymatic activities quantitative, in many cases irreversible and fast, thus interfering directly with the targeted pathways. SiRNAs differ in this respect, since decreases in protein expression are incomplete, only transient and slow over a period, during which cells can adapt by compensatory mechanisms and by which primary effects can be masked.

Gene silencing in the liver of CD-1-mice by means of hydrodynamic injection of non-complexed chemically stabilised siRNA was possible and decreased CYP2E1 protein expression significantly. Single or repeated high-dose intraperitoneal injection of siRNAs did not lead to significant effects on either mRNA- or protein level. Further research on stability and efficient delivery of siRNAs to the target cell is inevitable before *in vivo* gene silencing by means of siRNAs can be applied in mechanistic toxicology.

In conclusion, *in vitro* siRNA application is a universal and suitable specific method, which can be used in many mechanistic toxicological studies as a tool for pathway analysis and target validation. The level of inhibition achieved by application of chemical inhibitors cannot be attained by means of siRNA-mediated gene silencing.

In vivo gene silencing can be achieved, but the invasive hydrodynamic method of siRNA application is not suitable for animal toxicity testing. The delivery of siRNA to specific target organs needs significant improvement.

5.1 Zusammenfassung und Schlussfolgerungen (deutsche Übersetzung)

Die RNAi–Methode spielt eine grosse Rolle in der Wirkstoffentwicklung bei der Validierung eines pharmakologischen Ziels. Die Anwendbarkeit in der Toxikologie wurde noch nicht systematisch untersucht. Das Ziel dieser Arbeit ist die Evaluierung der RNAi-Methode für mechanistisch-toxikologische Studien und den Einfluss von posttranskriptioneller Genunterdrückung auf biochemisch-zelluläre Endpunkte zu zeigen.

Die siRNAs wurden mit Hilfe eines computerunterstützten Algorithmus ausgewählt. Effiziente und reproduzierbare Einschleusung der siRNA *in vitro* wurde durch Elektroporation erreicht. Die molekulare Reduktion der Expression des Zielgens wurde auf mRNA- und Proteinexpressionslevel oder auf Proteinaktivitätsebene zwischen 24 und 144 Stunden nach Behandlung überwacht. Die siRNAs wurden *in vitro* getestet bevor sie *in vivo* angewandt wurden. Als Methode zum Erreichen der Leber *in vivo* wurde die intraperitoneale Gabe von siRNAs gegenüber hydrodynamischer Injektion in die Schwanzvene evaluiert.

Auf folgenden Enzyme wurde mit RNAi in der Zellkultur abgezielt: ATP-Synthase in HepG2, Farnesylpyrophosphat-Synthase (FPPS) in humanen Nierenzellen (HK-2) und Caspase-3 in Primärhepatozyten der Ratte. In allen Experimenten war RNAi in der Lage, das mRNA- und Proteinexpressions- oder Proteinaktivitäts-Niveau zu reduzieren, wodurch die erfolgreiche Genunterdrückung gezeigt werden konnte. Die Unterdrückung der mitochondrialen ATP-Synthase β-Untereinheit hatte keinen signifikanten Einfluss auf die Überlebensrate und den Energiestoffwechsel von HepG2-Zellen. Obwohl Oligomycin B-Behandlung zu ATP-Depletion und Verlust des mitochondiralen Membranpotentials führte, war keine Sensitivierung der Zellen gegenüber Oligomycin B- oder Diclofenac-induzierten Veränderungen des mitochondrialen Membranpotentials oder Zytotoxizität zu beobachten. Die Genunterdrückung der ATP-Synthase in HepG2-Zellen führte zu einer ähnlichen transkriptionellen Signatur wie Diclofenac-Behandlung *in vivo*, so dass eine mögliche Verbindung zwischen ATP-Synthase und Hepcidin, BiP und ALAS-1 durch Koregulation nahegelegt wird. Die Genunterdrückung von FPPS führte zu tendenziell erhöhter Zytotoxizität von Zoledronsäure, hatte aber keinen Einfluss auf den Prenylierungsstatus der kleinen GTPasen. Der Caspase-3/7-Inhibitor Ac-DEVD-CHO verhinderte SDZ IMM125-vermittelte Apoptose. Spezifische Genunterdrückung von Caspase-3 führte zur Reduktion der SDZ IMM125-induzierten Caspaseaktivität, während die Unterdrückung von Caspase-7 in dieser Hinsicht keinen Einfluss hatte. Die Effektschwelle des Genunterdrückung wurde durch Vergleich zwischen Caspase-3-silencing und Behandlung mit dem chemischen Caspase-

Inhibitor Ac-DEVD-CHO auf Ebene der Caspase-3-Aktivität und der zytoprotektiven Wirksamkeit bestimmt. Der Effekt von Caspase-3-Unterdrückung war equivalent zur Wirkung von 1 µM Inhibitor. Die inhibitorvermittelte Schutzwirkung im Hinblick auf die Zytotoxizität wurde ausschliesslich bei höheren Inhibitorkonzentrationen erreicht, wodurch gezeigt wurde, dass die erreichte Genunterdrückung für zytoprotektive Wirkungen nicht ausreichend war.

SiRNAs haben verglichen mit Enzyminhibitoren generell eine höhere Spezifität. Chemische Inhibitoren sind weniger spezifisch und können enzymatische Aktivitäten vollständig, in manchen Fällen irreversibel und schnell beeinflussen, so dass sie direkten Einfluss auf die zu untersuchenden Signalwege haben. SiRNAs unterscheiden sich in dieser Hinsicht, da die Abnahme des Proteins nicht vollständig, nur transient und langsam über eine Periode hinweg erfolgt, innerhalb welcher sich die Zellen durch kompensatorische Mechanismen anpassen und Primäreffekte maskiert werden können.

Hydrodynamische Einschleusung von nicht-komplexierter siRNA in die Leber von CD-1-Mäusen war möglich und reduzierte die CYP2E1-Proteinexpression signifikant. Ein- oder mehrfache hochdosierte intraperitoneale Gabe von siRNA führte weder auf mRNA- noch auf Proteinebene zu signifikanten Effekten. Weitere Untersuchungen im Hinblick auf Stabilität und effiziente Einschleusung von siRNAs ist unvermeidlich, bevor siRNAs *in vivo* in der mechanistischen Toxikologie angewandt werden können.

Zusammenfassend kann ausgesagt werden, dass die Anwendung von siRNAs *in vitro* eine universelle und spezifische Methode darstellt, welche in vielen mechanistisch-toxikologischen Studien als Werkzeug zur Signalweganalyse und zur Validierung von Zielproteinen eingesetzt werden kann. Die Stärke der enzymatischen Inhibition, die mit Hilfe eines chemischen Inhibitors erreicht werden kann, ist durch siRNA-vermittelte Genunterdrückung nicht zu erreichen.

Genunterdrückung *in vivo* kann erreicht werden, doch die invasive hydrodynamische Methode ist nicht geeignet für Toxizitätsprüfungen im Tier. Die Einschleusung von siRNA in spezifische Zielorgane benötigt signifikante Verbesserung.

6 Appendix

6.1 Literature references

Abdulla D, Goralski KB, Renton KW: The regulation of cytochrome P450 2E1 during LPS-induced inflammation in the rat. Toxicol Appl Pharmacol 216 (1):1-10 (2006)

Amador E, Dorfman LE, Wacker WEC: Serum Lactic Dehydrogenase Activity: An Analytical Assessmentof Current Assays. Clin Chem. 9:391-99 (1963)

Amaxa GmbH: Determination of Optimal Analysis Time Points. http://www.amaxa.com/sirnakinetics.html (2007)

Ariga T, Masaike T, Noji H, Yoshida M: Stepping rotation of F(1)-ATPase with one, two, or three altered catalytic sites that bind ATP only slowly. J Biol Chem. 277(28):24870-4. (2002)

Auer M, Brezinka C, Eller P, Luze K, Schweigmann U, Schwarzler P: Prenatal diagnosis of intrauterine premature closure of the ductus arteriosus following maternal diclofenac application. Ultrasound Obstet Gynecol. 23(5):513-6 (2004)

Bartlett DW, Davis ME: Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging.Nucleic Acids Res. 12;34(1):322-33 (2006)

Benvenuti LA, Aiello VD, Cury AJ, Ebaid M: Post-ischemic rupture of the anterior papillary muscle of the right ventricle associated with persistent pulmonary hypertension of the newborn: a case report.Am J Cardiovasc Pathol. 4(1):79-84 (1992)

Bernstein E, Caudy AA, Hammond SM, Hannon GJ: Role for a bidentate ribonuclease in the initiation step of RNA interference.Nature 409, 363-366 (2001)

Berry MN, Fried DS: High-yield preparation of isolated rat liver parenchymal hepatocytes: a biochemical and fine structural study. J Cell Biol. 43:506-520 (1969)

Berson A, Descatoire V, Sutton A, Fau D, Maulny B, Vadrot N, Feldmann G, Berthon B, Tordjmann T, Pessayre D: Toxicity of alpidem, a peripheral benzodiazepine receptor ligand, but not zolpidem, in rat hepatocytes: role of mitochondrial permeability transition and metabolic activation.J Pharmacol Exp Ther. 299(2):793-800 (2001)

Bertrand JR, Pottier M, Vekris A, opolon P, Maksimenko A, Malvy C: Comparison of antisnese oligonicleotides and siRNAs in cell culture and in vivo.Biochem. Biophys. Res. Commun. 296, 1000-1004 (2002)

Birnboim HC, Doly J: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7(6):1513-23 (1979)

Boelsterli UA: Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. Toxicol Appl Pharmacol. 192(3):307-22 (2003a)

Boelsterli UA: In: Mechanistic Toxicology. Taylor & Francis London New York (2003)

Bole DG, Hendershot LM, Kearney JF: Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J Cell Biol. 102(5):1558-66 (1986)

Bort R, Ponsoda X, Jover R, Gomez-Lechon MJ, Castell JV: Diclofenac toxicity to hepatocytes: a role for drug metabolism in cell toxicity. J Pharmacol Exp Ther. 288(1):65-72 (1999)

Bounameaux HM, Schifferli J, Montani JP, Jung A, Chatelanat F: Renal failure associated with intravenous diphosphonates. Lancet. 26;1(8322):471 (1983)

Boussif O, Zanta MA, Behr JP: Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold. Gene Ther. 3(12):1074-80 (1996)

Braasch DA, Liu Y, Corey DR: Antisense inhibition of gene expression in cells by oligonucleotides incorporating locked nucleic acids: effect of mRNA target sequence and chimera design. Nucl Ac Res. 30(23):5160-67 (2002)

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248-54 (1976)

Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R: Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet. 34(3):263-4 (2003)

Brunner S, Furtbauer E, Sauer T, Kursa M, Wagner E: Overcoming the nuclear barrier: cell cycle independent nonviral gene transfer with linear polyethylenimine or electroporation. Mol Ther. 2002 Jan;5(1):80-6

Cao D, Fan ST, Chung SS: Identification and characterization of a novel human aldose reductase-like gene. J Biol Chem. 273(19):11429-35 (1998)

Caudy AA, Myers M, Hannon GJ, Hammond SM: Fragile X-related protein and VIG associate with the RNA interference machinery. Genes Dev. 16(19):2491-6 (2002)

Caudy AA, Ketting RF, Hammond SM, Denli AM, Bathoorn AM, Tops BB, Silva JM, Myers MM, Hannon GJ, Plasterk RH: A micrococcal nuclease homologue in RNAi effector complexes. Nature 425(6956):411-4 (2003)

Center for Disease Control, A Proposed Method for Determining Glucose Using Hexokinase and Glucose 6-Phosphatase Dehydrogenase, Public Health Service (1976)

Chevrollier A, Loiseau D, Gautier F, Malthiery Y, Stepien G: ANT2 expression under hypoxic conditions produces opposite cell-cycle behavior in 143B and HepG2 cancer cells. Mol Carcinog. 42(1):1-8 (2005)

Chi JT, Chang HY, Wang NN, Chang DS, Dunphy N, Brown PO: Genomewide view of gene silencing by small interfering RNAs. Proc Natl Acad Sci U S A 100(11):6343-6 (2003)

Choi D, Leininger-Muller B, Kim YC, Leroy P, Siest G, Wellman M: Differential role of CYP2E1 binders and isoniazid on CYP2E1 protein modification in NADPH-dependent microsomal oxidative reactions: free radical scavenging ability of isoniazid. Free Radic Res. 36(8):893-903 (2002)

Chollet P, Favrot MC, Hurbin A, Coll JL: Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. J Gene Med. 4(1):84-91 (2002)

Coons AH, Creech HJ, Jones RN, Berliner E. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J Immunol. 45: 159-170 (1942)
Corrigall VM, Bodman-Smith MD, Fife MS, Canas B, Myers LK, Wooley P, Soh C, Staines NA, Pappin DJ, Berlo SE, van Eden W, van Der Zee R, Lanchbury JS, Panayi GS: The human endoplasmic reticulum molecular chaperone BiP is an autoantigen for rheumatoid arthritis and prevents the induction of experimental arthritis. J Immunol. 166(3):1492-8 (2001)

Corrigall VM, Bodman-Smith MD, Brunst M, Cornell H, Panayi GS: Inhibition of antigen-presenting cell function and stimulation of human peripheral blood mononuclear cells to express an antiinflammatory cytokine profile by the stress protein BiP: relevance to the treatment of inflammatory arthritis. Arthritis Rheum. 50(4):1164-71 (2004)

Crouch SP, Kozlowski R, Slater KJ, Fletcher J: The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Methods. 160(1):81-8 (1993)

Crosas B, Hyndman DJ, Gallego O, Martras S, Pares X, Flynn TG, Farres J: Human aldose reductase and human small intestine aldose reductase are efficient retinal reductases: consequences for retinoid metabolism. Biochem J. 373(Pt 3):973-9 (2003)

Cuezva JM, Krajewska M, de Heredia ML, Krajewski S, Santamaria G, Kim H, Zapata JM, Marusawa H, Chamorro M, Reed JC: The bioenergetic signature of cancer: a marker of tumor progression. Cancer Res. 62(22):6674-81 (2002)

Czauderna F, Fechtner M, Dames S, Aygün H, Klippel A, Pronk GJ, Giese K, Kaufmann J: Structural variations and stabilising modifcations of synthetic siRNAs in mammalian cells. Nucleic Acids Research. 31(11): 2705-2716 (2003)

Dang CV, Semenza GL: Oncogenic alterations of metabolism. Trends Biochem Sci.24(2):68-72 (1999)

Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ: Processing of primary microRNAs by the Microprocessor complex. Nature. 432(7014):231-5 (2004)

De Souza AT, Dai X, Spencer AG, Reppen T, Menzie A, Roesch PL, He Y, Caguyong MJ, Bloomer S, Herweijer H, Wolff JA, Hagstrom JE, Lewis DL, Linsley PS, Ulrich RG: Transcriptional and phenotypic comparisons of Ppara knockout and siRNA knockdown mice. Nucleic Acids Res. 34(16):4486-94 (2006)

DeWitt DL: Cox-2-selective inhibitors: the new super aspirins. Mol Pharmacol. 55(4):625-31 (1999)

Dillin A: The specifics of small interfering RNA specificity. Proc Natl Acad Sci USA. 100(11):6289-91 (2003)

Donatsch P, Mason J, Richardson BP, Ryffel B: Toxicologic evaluation of the new cyclosporin derivative, SDZ IMM 125, in a comparative, subchronic toxicity study in rats. Transplant Proc. 24(4 Suppl 2):39-42 (1992)

Dong D, Ko B,Baumeister P, Swenson S, Costa F, Markland F, Stiles C, Patterson JB, Bates SE, Lee AS: Vascular Targeting and Antiangiogenesis Agents Induce Drug Resistance Effector GRP78 within the Tumor Microenvironment. Cancer Research 65, 5785-5791 (2005)

Dufour DR: Osmometry-The Rational Basis for Use of an Underappreciated Diagnostic tool. Industry Workshop at the Am Ass Clin Chem-meeting, New York (1993)

Dykxhoorn DM, Novina CD, Sharp PA: Killing the messenger: short RNAs that silence gene expression. Nature Reviews Molecular Cell Biology 4, 457-467 (2003)

Earnshaw WC, Martins LM, Kaufmann SC: Mammalian Caspases: Structure, Activation, Substrates, and Function during Apoptosis. Annu Rev Biochem. 68:383-424 (1999)

Elbashir SM, Lendeckel W, Tuschl T: RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 15(2):188-200 (2001)

Elmén J, Thonberg H, Ljungberg K, Frieden M, Westergaard M, Xu Y, Wahren B, Liang Z, Ørum H, Koch T, Wahlestedt C: Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. Nucl Ac Res. 33(1): 439-47 (2005)

Engvall, E. and Perlamnn, P: Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8:871-874 (1971)

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: Potent and specificgenetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806-811 (1998)

Fleisch H: Bisphosphonates: mechanisms of action. Endocr Rev. 19(1):80-100 (1998)

Forth W, Henschler D, Rummel W, Förstermann U, Starke K: Allgemeine und spezielle Pharmakologie und Toxikologie. Urban und Fischer Verlag München, Jena, 8. Auflage (2001)

Frantz S: Studies reveal potential pitfalls of RNAi. Nature Rev. Drug Disc., vol.2, p. 763 (2003)

Freshney, R: Culture of Animal Cells: A Manual of Basic Technique, p.117, Alan R. Liss, Inc., New York. (1987)

Frye RF, Stiff DD: Determination of chlorzoxazone and 6-hydroxychlorzoxazone in human plasma and urine by high-performance liquid chromatography. J Chromatogr B, 686 291-296 (1996)

Fujii H, Takahashi T, Matsumi M, Kaku R, Shimizu H, Yokoyama M, Ohmori E, Yagi T, Sadamori H, Tanaka N, Akagi R, Morita K: Increased heme oxygenase-1 and decreased delta-

aminolevulinate synthase expression in the liver of patients with acute liver failure. Int J Mol Med. 14(6):1001-5 (2004)

Galati G, Tafazoli S, Sabzevari O, Chan TS, O'Brien PJ: Idiosyncratic NSAID drug induced oxidative stress. Chem Biol Interact. 142(1-2):25-41 (2002)

Ganz T: Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood. 102(3):783-8 (2003)

Ganz T: Hepcidin--a regulator of intestinal iron absorption and iron recycling by macrophages. Best Pract Res Clin Haematol. 18(2):171-82 (2005)

Ghanayem BI, Wang H, Sumner S: Using cytochrome P-450 gene knock-out mice to study chemical metabolism, toxicity, and carcinogenicity. Toxicol Pathol. 28(6):839-50 (2000)

Gómez-Lechón MJ, Ponsoda X, O'Connor E, Donato T, Castell JV, Jover R: Diclofenac induces apoptosis in hepatocytes by alteration of mitochondrial function and generation of ROS. Biochem Pharmacol. 66(11):2155-67 (2003)

Gómez-Lechón MJ, Ponsoda X, Bort R, Castell JV: The use of cultured hepatocytes to investigate the metabolism of drugs and mechanisms of drug hepatotoxicity. Altern Lab Anim. 29(3):225-31 (2001)

Grillo MP, Hua F, Knutson CG, Ware JA, Li C: Mechanistic studies on the bioactivation of diclofenac: identification of diclofenac-S-acyl-glutathione in vitro in incubations with rat and human hepatocytes. Chem Res Toxicol. 16(11):1410-7 (2003)

Grimm D, Streez KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA: Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441 (7092):537-41 (2006)

Gross A, Pilcher K, Blachly-Dyson E, Basso E, Jockel J, Bassik MC, Korsmeyer SJ, Forte M: Biochemical and genetic analysis of the mitochondrial response of yeast to BAX and BCL-X(L). Mol Cell Biol. 20(9):3125-36 (2000)

Grover GJ, Atwal KS, Sleph PG, Wang FL, Monshizadegan H, Monticello T, Green DW: Excessive ATP hydrolysis in ischemic myocardium by mitochondrial F1Fo-ATPase: effects of selective pharmacological inhibition of mitochondrial ATPase hydrolase activity. Am J Physiol Heart Circ Physiol. 287: H1747-H1755 (2004)

Grub S, Persohn E, Trommer WE, Wolf A: Mechanisms of cyclosporine A-induced apoptosis in rat hepatocyte primary cultures. Toxicol Appl Pharmacol. 163(3):209-20 (2000a)

Grub S, Persohn E, Trommer WE, Wolf A: Induction of apoptosis by the O-hydroxyethyl-D(Ser)(8)-cyclosporine A derivative SDZ IMM 125 in rat hepatocytes. J Pharmacol Exp Ther. 293(1):24-32 (2000b)

Grub S, Trommer WE, Wolf A: Role of antioxidants in the O-hydroxyethyl-D-(Ser)8-cyclosporine A (SDZ IMM125)-induced apoptosis in rat hepatocytes. Biochem Pharmacol. 64(12):1725-36 (2002)

Gülow K, Bienert D, Haas IG: BiP is feed-back regulated by control of protein translation efficiency. J Cell Sci. 115(Pt 11):2443-52 (2002)

Hannon GJ: RNA interference. Nature 418, 244-251 (2002)

Hiestand PC, Graber M, Hurtenbach U, Herrmann P, Cammisuli S, Richardson BP, Eberle MK, Borel JF: The new cyclosporine derivative, SDZ IMM 125: in vitro and in vivo pharmacologic effects. Transplant Proc. 24(4 Suppl 2):31-8 (1992)

Holen T, Amarzguioui M, Wiiger MT, Babaie E, Prydz H: Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. Nucleic Acids Res. 30(8):1757-66 (2002)

Houstek J, Klement P, Floryk D, Antonicka H, Hermanska J, Kalous M, Hansikova H, Hout'kova H, Chowdhury SK, Rosipal T, Kmoch S, Stratilova L, Zeman J: A novel deficiency of mitochondrial ATPase of nuclear origin. Hum Mol Genet. 8(11):1967-74 (1999)

Huang JB, Yang WC, Yang AH, Lee PC, Lin CC: Arterial thrombosis due to minimal change glomerulopathy secondary to nonsteroidal anti-inflammatory drugs. Am J Med Sci. 327(6):358-61 (2004)

Huesken D, Asselbergs F, Kinzel B, Natt F, Weiler J, Martin P, Haener R, Hall J: mRNA fusion constructs serve in a general cell-based assay to profile oligonucleotide activity. Nucl Ac Res. 31 (17): e102 (2003)

Huesken D, Lange J, Mickanin C, Weiler J, Asselbergs F, Warner J, Meloon B, Engel S, Rosenberg A, Cohen D, Labow M, Reinhardt M, Natt F, Hall J: Design of a genome-wide siRNA library using an artificial neural network. Nature Biotechnology 23: 995 - 1001 (2005)

Hutvagner G, Zamore PD: A microRNA in a multiple-turnover RNAi enzyme complex. Science 297(5589):2056-60 (2002)

Inazu N, Nagashima Y, Satoh T, Fujii T: Purification and properties of six aldo-keto reductases from rat adrenal gland. J Biochem (Tokyo). 115(5):991-9 (1994)

Inoue S, Hakamata Y, Kaneko M, Kobayashi E: Gene therapy for organ grafts using rapid injection of naked DNA: application to the rat liver. Transplantation. 77(7):997-1003 (2004)

Invitrogen: Instruction Manual for Block-iT RNAi Stress Response Control Kit. Version A, ref. K491000 (2004)

Ishizuka A, Siomi MC, Siomi H: A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev. 16(19):2497-508 (2002)

Isidoro A, Martinez M, Fernandez PL, Ortega AD, Santamaria G, Chamorro M, Reed JC, Cuezva JM.: Alteration of the bioenergetic phenotype of mitochondria is a hallmark of breast, gastric, lung and oesophageal cancer. Biochem J. 378:17-20 (2004)

Ito H, Fukuda Y, Murata K, Kimura A: Transformation of intact yeast cells treated with alkali cations. J Bacteriol. 1983 January; 153(1): 163-168

Jackson PJ, Harris DA: Sites of protein-protein interaction on the mitochondrial F_1-ATPase inhibitor protein. Biochem J. 235, 577-583 (1986)

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS: Expression profiling reveals off-target gene regulation by RNAi. Nat Biotechnol. 21(6):635-7 (2003)

Jackson AL, Burchard J, Schelter J, Chau BN, Cleary M, Lim L, Linsley PS: Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. RNA. 12(7):1179-87 (2006)

Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, Guo J, Johnson JM, Lim L, Karpilow J, Nicols K, Marshall W, Khvorova A, Linsley PS: Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA. 12(7):1197-205 (2006a)

Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ: Mechanisms of hepatotoxicity. Toxicol Sci. 65(2):166-76 (2002)

Jarvis R, King A: Optimizing chemical transfection and electroporation of siRNAs. Ambion TechNotes 10 (5): 12-15 (2003).

Jason TLH, Koropatnick J, Berg RW: Toxicology of antisense therapeutics. Toxciol. Appl. Pharmacol. 201; 66.83 (2004)

Jorgensen R: Altered gene expression in plants due to trans interactions between homologous genes. Trends in Biotechnology 8, 340-344 (1990)

Karmen A, Wroblewski F, LaDue JS: Transaminase activity in human blood. J Clin Inv. 34:126-133 (1955)

Kharasch ED, Thummel KE: Identification of cytochrome P450 2E1 as the predominant enzyme catalyzing human liver microsomal defluorination of sevoflurane, isoflurane, and methoxyflurane. Anesthesiology. 79(4):795-807. (1993)

Kim ND, Kwak MK, Kim SG: Inhibition of cytochrome P450 2E1 expression by 2-(allylthio)pyrazine, a potential chemoprotective agent: hepatoprotective effects. Biochem Pharmacol. 53(3):261-9 (1997)

Kitajewski J, Mason JO, Varmus HE.: Interaction of Wnt-1 proteins with the binding protein BiP. Mol Cell Biol.12(2):784-90 (1992)

Kolluri S, Sadlon TJ, May BK, Bonkovsky HL: Heme repression of housekeeping 5-aminolevulinate synthase gene in the LMH hepatoma cell line. Biochem J. Jul 21; [Epub ahead of print] (2005)

Konstam MA: Matters of the heart: assessing the cardiovascular safety of new drugs. Am Heart J. 146(4):561-2 (2003)

Kraner JC, Lasker JM, Corcoran GB, Ray SD, Raucy JL: Induction of P4502E1 by acetone in isolated rabbit hepatocytes. Role of increased protein and mRNA synthesis. Biochem Pharmacol. 45(7):1483-92 (1993)

Kulaksiz H, Gehrke SG, Janetzko A, Rost D, Bruckner T, Kallinowski B, Stremmel W: Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. Gut. 53(5):735-43 (2004)

Lane KT, Beese LS: Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. J Lip Res. 47(4):681-99 (2006)

Lee SH, Sinko PJ: siRNA--getting the message out. Eur J Pharm Sci. 27(5):401-10 (2006)

Lee SS, Buters JT, Pineau T, Fernandez-Salguero P, Gonzalez FJ: Role of CYP2E1 in the hepatotoxicity of acetaminophen. J Biol Chem.271(20):12063-7 (1996)

Lee KW, Ko BC, Jiang Z, Cao D, Chung SS: Overexpression of aldose reductase in liver cancers may contribute to drug resistance. Anticancer Drugs. 12(2):129-32 (2001)
Lehner B, Fraser AG, Sanderson CM: Technique review: how to use RNA interference. Brief Funct Genomic Proteomic. 3(1):68-83 (2004)

Lemasters JJ: V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. Am J Physiol. 276(1 Pt 1):G1-6 (1999)

Lewis DL, Wolff JA: Delivery of siRNA and siRNA expression constructs to adult mammals by hydrodynamic intravascular injection. Methods Enzymol. 392:336-50 (2005)

Liu F, Song Y, Liu D: Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 6(7):1258-66 (1999)

Liu F, Huang L: Electric gene transfer to the liver following systemic administration of plasmid DNA. Gene Ther. Aug;9(16):1116-9 (2002)

Livak KY, Flood SJ, Marmaro J, Giusti W, Deetz K: Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 4(6):357-62 (1995)

Llacuna L, Mari M, Garcia-Ruiz C, Fernandez-Checa JC, Morales A:
Critical role of acidic sphingomyelinase in murine hepatic ischemia-reperfusion injury. Hepatology. 44(3):561-72 (2006)

Lu Y, Cederbaum AI: Cisplatin-induced hepatotoxicity is enhanced by elevated expression of cytochrome P450 2E1. Toxicol Sci. 89(2):515-23 (2006)

Luckmann SP, Hughes DE, Coxon FP, Graham R, Russel D, Rogers MJ: Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. J Bone Miner Res. 13(4):581-9 (1998)

Ma Z, Li J, He F, Wilson A, Pitt B, Li S: Cationic lipids enhance siRNA-mediated interferon response in mice. Biochem Biophys Res Commun. 2005 May 13;330(3):755-9

Mahedevan B, Arora V, Schild LJ, Keshava C, Cate ML, Iversen PL, Poirier MC, Weston A, Pereira C, Baird WM: Reduction in Tamoxifen-Induced CYP3A2 Expression and DNA Adducts Using Antisense Technology. Mol. Carcinog. 45:118-125 (2006)

Malich G, Markovic B, Winder C: The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals unsing human cell lines. Toxicology. 124(3):179-92 (1997)

Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T: Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell. 110(5):563-74 (2002)

Masubuchi Y, Ose A, Horie T: Diclofenac-induced inactivation of CYP3A4 and its stimulation by quinidine. Drug Metab Dispos. 30(10):1143-8 (2002)

Matzinger P: The danger model: a renewed sense of self. Science. 296(5566):301-5 (2002)

May BK, Dogra SC, Sadlon TJ, Bhasker CR, Cox TC, Bottomley SS: Molecular regulation of heme biosynthesis in higher vertebrates. Prog Nucleic Acid Res Mol Biol. 51:1-51 (1995)

McCarthy DM: Comparative toxicity of nonsteroidal anti-inflammatory drugs. Am J Med. 107(6A):37S-46S (1999)

McCuskey RS, Bethea NW, Wong J, McCuskey MK, Abril ER, Wang X, Ito Y, DeLeve LD: Ethanol binging exacerbates sinusoidal endothelial and parenchymal injury elicited by acetaminophen. J Hepatol. 42(3):371-7 (2005)

McManus MT, Petersen CP, Haines BB, Chen J, Sharp PA: Gene silencing using micro-RNA designed hairpins. RNA 8, p. 842-850 (2002)

McNamara JO 2nd, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA, Giangrande PH: Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol. 24(8):1005-15 (2006)

Mesulam MM: Tetramethyl benzidine for horseradish peroxidase neurohistochemistry: a non-carcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents. J Histochem Cytochem. 26(2): 106-117 (1978)

Mitoma C, Posner HS, Reitz HC, Udenfriend S: Enzymatic hydroxylation of aromatic compounds. Arch Biochem Biophys. 61(2):431-41 (1956)

Miyachi H, Masukawa A, Ohshima T, Hirose T, Impraim C, Ando Y: Automated specific capture of hepatitis C virus RNA with probes and paramagnetic particle separation. J Clin Microbiol. 38(1):18-21 (2000)

Moncion A, Truong NT, Garrone A, Beaune P, Barouki R, De Waziers I: Identification of a 16-nucleotide sequence that mediates post-transcriptional regulation of rat CYP2E1 by insulin. J Biol Chem.277(48):45904-10 (2002)

Mote PL, Tillman JB, Spindler SR.: Glucose regulation of GRP78 gene expression. Mech Ageing Dev. 104(2):149-58 (1998)

Munakata H, Sun JY, Yoshida K, Nakatani T, Honda E, Hayakawa S, Furuyama K, Hayashi N: Role of the heme regulatory motif in the heme-mediated inhibition of mitochondrial import of 5-aminolevulinate synthase. J Biochem (Tokyo). 136(2):233-8 (2004)

Munro S, Pelham HR: A C-terminal signal prevents secretion of luminal ER proteins. Cell. 48(5):899-907 (1987)

Munro S, Pelham HR: An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell. 46(2):291-300 (1986)

Napoli C, Lemieux C, Jorgensen R: Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell. 2(4):279-289 (1990)

Nebert DW, Nelson DR: P450 gene nomenclature based on evolution. Methods Enzymol 206:3-11 (1996)

Nelson SD: Molecular mechanisms of the hepatotoxicity caused by acetaminophen. Semin Liver Dis. 10(4):267-78 (1990)

Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T: IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest. 113(9):1271-6 (2004)

Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH: Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J. 1(7):841-5 (1982)

Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S: The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest. 110(7):1037-44 (2002)

Nykanen A, Haley B, Zamore PD: ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell. 107(3):309-21 (2001)

Oda K, Wada I, Takami N, Fujiwara T, Misumi Y, Ikehara Y: Bip/GRP78 but not calnexin associates with a precursor of glycosylphosphatidylinositol-anchored protein. Biochem J. 316 (Pt 2):623-30 (1996)

O'Sullivan TL, Akbari A, Cadnapaphornchai P: Acute renal failure associated with the administration of parenteral etidronate. Ren Fail. 16(6):767-73 (1994)
Ovcharenko D: Efficient Delivery of siRNAs to Human Primary Cells: Electroporation vs. Chemical Transfection. Ambion TechNotes 10(5):1516 (2003)

Patten CJ, Thomas PE, Guy RL, Lee M, Gonzalez FJ, Guengerich FP, Yang CS: Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. Chem Res Toxicol. 1993 Jul-Aug;6(4):511-8 (1993)

Patzwahl R, Meier V, Ramadori G, Mihm S: Enhanced expression of interferon-regulated genes in the liver of patients with chronic hepatitis C virus infection: detection by suppression-subtractive hybridization. J Virol. 75(3):1332-8 (2001)

Pebernard S, Iggo RD: Determinants of interferon-stimulated gene induction by RNAi vectors. Differentiation. 72(2-3):103-11 (2004)

Penning TM: AKR1B10: a new diagnostic marker of non-small cell lung carcinoma in smokers. Clin Cancer Res. 11(5):1687-90 (2005)

Pessayre D, Haouzi D, Fau D, Robin MA, Mansouri A, Berson A: Withdrawal of life support, altruistic suicide, fratricidal killing and euthanasia by lymphocytes: different forms of drug-induced hepatic apoptosis. J Hepatol. 31(4):760-70 (1999)

Podvinec M, Handschin C, Looser R, Meyer UA: Identification of the xenosensors regulating human 5-aminolevulinate synthase. Proc Natl Acad Sci U S A. 101(24):9127-32 (2004)

Porstmann T, Ternynck T, Avrameas S: Quantitation of 5-bromo-2-deoxyuridine incorporation into DNA: an enzyme immunoassay for the assessment of the lymphoid cell proliferative response. J Immunol Methods.82(1):169-79 (1985)

Prud'homme GJ, Glinka Y, Khan AS, Draghia-Akli R: Electroporation-Enhanced Nonviral Gene Transfer for the Prevention or Treatment of Immunological, Endocrine and Neoplastic Diseases. Curr Gen Ther. 6, 243-273 (2006)

Purcell P, Henry D, Melville G: Diclofenac hepatitis. Gut 32(11):1381-5 (1991)
Raleigh EA, Elbing K, Brent R: Selected Topics from Classical Bacterial Genetics. Current Protocols in Molecular Biology, 1.4.1.-1.4.14. (2002)

Rana TM: Illuminating the silence: understanding the structure and function of small RNAs. Nat Rev Mol Cell Biol. 8, 23-36 (2007)

Renart, J. et al.: Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. Proc Natl Acad Sci. USA. 76: 3116-3120 (1979)

Reszka AA, Rodan GA: Nitrogen-containing bisphosphonate mechanism of action. Mini Rev Med Chem.4(7):711-9 (2004)

Robbins MA, Maksumova L, Pocock E, Chantler JK: Nuclear Factor-κB Translocation Mediated Double-Stranded Ribonucleic Acid-Induced NIT-1 β-Cell Apoptosis and Up-Regulated Caspase-12 and Tumor Necrosis Factor-Associated Ligand (TRAIL). Endocrinology. 144(10):4616-4625 (2003)

Roempp: Lexikon der Chemie online. Georg Thieme Verlag Stuttgart http://www.roempp.com/prod/index1.html)

Rostom A, Goldkind L, Laine L: Nonsteroidal anti-inflammatory drugs and hepatic toxicity: a systematic review of randomized controlled trials in arthritis patients. Clin Gastroenterol Hepatol. 3(5):489-98 (2005)

Sakai M, Nishikawa M, Thanaketpaisarn O, Yamashita F, Hashida M: Hepatocyte-targeted gene transfer by combination of vascularly delivered plasmid DNA and in vivo electroporation. Gene Ther. Apr;12(7):607-16 (2005)

Salomon AR, Voehringer DW, Herzenberg LA, Khosla C: Understanding and exploiting the mechanistic basis for selectivity of polyketide inhibitors of F(0)F(1)-ATPase. Proc Natl Acad Sci USA. 19;97(26):14766-71 (2000)

Sandy P, Ventura A, Jacks T: Mammalian RNAi: a practical guide. Biotechniques.;39(2):215-24 (2005)

Santamaria G, Martínez-Diez M, Fabregat I, Cuezva JM: Efficient execution of cell death in non-glycolytic cells requires the generation of ROS controlled by the activity of mitochondrial H^+-ATP synthase. Carcinogenesis 27(5): 925-935 (2006)

Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, Wolfsberg TG, Umayam L, Lee JC, Hughes CM, Shanmugam KS, Bhattacharjee A, Meyerson M, Collins FS: Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. Proc Natl Acad Sci U S A 101(7):1892-7 (2004)

Schägger, H., von Jagow G.: Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166, 368-379 (1987)

Schuetz EG, Schuetz JD, May B, Guzelian PS: Regulation of cytochrome P-450b/e and P-450p gene expression by growth hormone in adult rat hepatocytes cultured on a reconstituted basement membrane. J Biol Chem. 1990 Jan 15;265(2):1188-92 (1990)

Schuetz EG, Wrighton SA, Safe SH, Guzelian PS: Regulation of cytochrome P-450p by phenobarbital and phenobarbital-like inducers in adult rat hepatocytes in primary monolayer culture and in vivo. Biochemistry. 25(5):1124-33 (1986)

Seglen PO: Preparation of isolated rat liver sells. Methods Cell Biol. 113:29-83 (1976)

Seitz WR: Immunoassay labels based on chemiluminescence and bioluminescence. Clin Biochem. 17(2):120-5 (1984)

Semizarov D, Frost L, Sarthy A, Kroeger P, Halbert DN, Fesik SW: Specificity of short interfering RNA determined through gene expression signatures. Proc Natl Acad Sci U S A. 100(11):6347-52 (2003)

Sgro C, Clinard F, Ouazir K, Chanay H, Allard C, Guilleminet C, Lenoir C, Lemoine A, Hillon P: Incidence of drug-induced hepatic injuries: a French population-based study. Hepatology. 36(2):451-5 (2002)

Shchepina LA, Pletjushkina OY, Avetisyan AV, Bakeeva LE, fetisova EK, Izyumov DS, Saprunova VB, Vyssokikh MY, cernyak BV, Skulachev VP: Oligomycin, inhibitor of the F_O part of H^+-ATP-synthase, suppresses the TNF-induced apoptosis. Oncogene 21: 8149-8157 (2002)

Shin YK, Yoo BC, Chang HJ, Jeon E, Hong SH, Jung MS, Lim SJ, Park JG: Down-regulation of mitochondrial F1F0-ATP synthase in human colon cancer cells with induced 5-fluorouracil resistance. Cancer Res. 65(8):3162-70 (2005)
.
Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, Plasterk RH, Fire A: On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 107(4):465-76 (2001)

Skipper, M: Have our dreams been shattered? Nature Rev. Gen. 4, p.671 (2003)

Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR: Activation of the interferon system by short-interfering RNAs. Nat Cell Biol. 5(9):834-9 (2003)

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC: Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85 (1985)

Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, Lieberman J: RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med. 9(3):347-51 (2003)

Sorensen DR, Leirdal M, Sioud M: Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J Mol Biol. 327(4):761-6 (2003)

Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Rohl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliansky V, Limmer S, Manoharan M, Vornlocher HP: Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature. 432(7014):173-8 (2004)

Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD: How cells respond to interferons. Annu Rev Biochem. 67:227-64 (1998)

Stein P, Svoboda P, Anger M, Schultz RM: RNAi: mammalian oocytes do it without RNA-dependent RNA polymerase. RNA 9(2):187-92 (2003)

Sumioka I, Matsura T, Kasuga S, Itakura Y, Yamada K: Mechanisms of protection by S-allylmercaptocysteine against acetaminophen-induced liver injury in mice. Jpn J Pharmacol. 1998 Oct;78(2):199-207 (1998)

Sung YH, Hwang SJ, Lee GM: Influence of down-regulation of caspase-3 by siRNAs on sodium-butyrate-induced apoptotic cell death of Chinese hamster ovary cells producing thrombopoietin. Metab Eng. 2005 Sep 16 (ePub)

Tang W, Stearns RA, Bandiera SM, Zhang Y, Raab C, Braun MP, Dean DC, Pang J, Leung KH, Doss GA, Strauss JR, Kwei GY, Rushmore TH, Chiu SH, Baillie TA: Studies on cytochrome P-450-mediated bioactivation of diclofenac in rats and in human hepatocytes: identification of glutathione conjugated metabolites. Drug Metab Dispos. 27(3):365-72 (1999)

Tang W: The metabolism of diclofenac--enzymology and toxicology perspectives. Curr Drug Metab. 4(4):319-29 (2003)

Terauchi S, Yamamoto T, Yamashita K, Kataoka M, Terada H, Shinohara Y: Molecular basis of morphological changes in mitochondrial membrane accompanying induction of permeability transition, as revealed by immuno-electron microscopy. Mitochondrion. 5(4):248-54 (2005)

Thornberry NA: Interleukin-1β converting enzyme. Meth Enzymol. 244, 615 (1994)

Tyson CA, Green CE: Cytotoxicity measures: Choice and methods. In: Rauckmann EJ, Padilla GM, eds. The isolated hepatocyte, use in Toxicology and Xenobiotic Biotransformation. Academic Press (Orlando), 119-158 (1987)

Urban-Klein B, Werth S, Abuharbeid S, Czubayko F, Aigner A: RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. Gene Ther. 12(5):461-6. (2005)

Vander Jagt DL, Kolb NS, Vander Jagt TJ, Chino J, Martinez FJ, Hunsaker LA, Royer RE: Substrate specificity of human aldose reductase: identification of 4-hydroxynonenal as an endogenous substrate. Biochim Biophys Acta. 1249(2):117-26 (1995)

Vander Jagt DL, Robinson B, Taylor KK, Hunsaker LA: Reduction of trioses by NADPH-dependent aldo-keto reductases. Aldose reductase, methylglyoxal, and diabetic complications. J Biol Chem. 267(7):4364-9 (1992)

van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR: Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell. 2(4):291-9 (1990)

van Ness, J, Hahn WE: Sequence complexity of cDNA transcribed from a diverse mRNA population. Nucleic Acids Res. 8(18):4259-70 (1980)

van Raaij MJ, Abrahams JP, Leslie AGW, Walker JE: The structure of bovine F1-ATPase complexed with the antibiotic inhibitor aurovertin B. Proc Natl Acad Sci USA. 93, 6913-6917 (1996)

Wakino S, Kanada T, Hayashi K: Rho/Rho Kinase as a Potential Target for the Treatment of Renal Disease. Drug News Perspect 18(10): 639-643 (2005)

Wang K, Shindoh H, Inoue T, Horii I: Advantages of in vitro cytotoxicity testing by using primary rat hepatocytes in comparison with established cell lines. J Toxicol Sci. 27(3):229-37 (2002)

Walker, AM: Quantitative studies of the risk of serious hepatic injury in persons using nonsteroidal intiinflammatory drugs. Arthitis Rheum. 40, pp. 201-208 (1997)

Warner TD, Mitchell JA: Cyclooxygenases: new forms, new inhibitors, and lessons from the clinic. FASEB J. 18(7):790-804 (2004)

Weir MR, Sperling RS, Reicin A, Gertz BJ: Selective COX-2 inhibition and cardiovascular effects: a review of the rofecoxib development program. Am Heart J. 146(4):591-604 (2003)

Welder AA, Acosta D: Membrane integrity and function. In: Tyson CA, Frazier JM. Methods in Toxicology. Vol 1. Part B. Academic Press (San Diego), 46-49 (1994)

Wianny F, Zernicka-Goetz M: Specific interference with gene function by double-stranded RNA in early mouse development. Nature Cell Biol. 2, p. 70-75 (2000)

Witkamp L, Zonneveld IM, Jung EG, Schopf RE, Christophers E, Grossman R, Meffert H, Belaich S, Mahrle G, Van Joost T, et al.: Efficacy and tolerability of multiple-dose SDZ IMM 125 in patients with severe psoriasis. Br J Dermatol. 133(1):95-103 (1995)

Williams BR: Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation. Biochem Soc Trans. 25(2):509-13 (1997)

Wong D, Wang M, Cheng Y, FitzGerald GA: Cardiovascular hazard and non-steroidal anti-inflammatory drugs. Curr Opin Pharmacol 5: 1-7 (2005)

Woodcroft KJ, Novak RF: Insulin differentially affects xenobiotic-enhanced, cytochrome P-450 (CYP)2E1, CYP2B, CYP3A, and CYP4A expression in primary cultured rat hepatocytes. J Pharmacol Exp Ther. 289(2):1121-7 (1999)

Wu D, Cederbaum AI: Cyclosporine A protects against arachidonic acid toxicity in rat hepatocytes: role of CYP2E1 and mitochondria. Hepatology. 35(6):1420-30 (2002)
Wu EY, Smith MT, Bellomo G, Di Monte D: Relationships between the mitochondrial transmembrane potential, ATP concentration, and cytotoxicity in isolated rat hepatocytes. Arch Biochem Biophys. 282:358-362 (1990)

Xie FY, Woodle MC, Lu PY: Harnessing in vivo siRNA delivery for drug discovery and therapeutic development. Drug Discov Today. 11(1-2):67-73 (2006)

Yamamoto M, Kure S, Engel JD, Hiraga K: Structure, turnover, and heme-mediated suppression of the level of mRNA encoding rat liver delta-aminolevulinate synthase. J Biol Chem. 263(31):15973-9 (1988)

Yamazaki H, Guo Z, Guengerich FP: Selectivity of cytochrome P4502E1 in chlorzoxazone 6-hydroxylation. Drug Metab Dispos. 1995 Mar;23(3):438-40

Yim HK, Jung YS, Kim SY, Kim YC: Contrasting changes in phase I and phase II metabolism of acetaminophen in male mice pretreated with carbon tetrachloride. Basic Clin Pharmacol Toxicol. 98(2):225-30 (2006)

Yu J, McMahon AP: Reproducible and inducible knockdown of gene expression in mice. Genesis. 44(5):252-61 (2006)

Zaher H, Buters JT, Ward JM, Bruno MK, Lucas AM, Stern ST, Cohen SD, Gonzalez FJ: Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice. Toxicol Appl Pharmacol. 152(1):193-9 (1998)

Zamore PD: Ancient Pathways Programmed by Small RNAs. Science 296, pp. 1265-1269 (2002)

Zhang G, Budker V, Wolff JA: High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther. 10(10):1735-7 (1999)

Zhang G, Gao X, Song YK, Vollmer R, Stolz DB, Gasiorowski JZ, Dean DA, Liu D: Hydroporation as the mechanism of hydrodynamic delivery. Gene Ther. 11(8):675-82 (2004)

Zhang HY, Du Q, Wahlestedt C, Liang Z: RNA Interference with Chemically Modified siRNA. Curr Top Med Chem. 6(9);893-900 (2006)

Zhou D, He QS, Wang C, Zhang J, Wong-Staal F: RNA Interference and Potential Applications. Curr Top Med Chem. 6, 901-911 901 (2006)

Zojer N, Keck AV, Pecherstorfer M: Comparative tolerability of drug therapies for hypercalcaemia of malignancy. Drug Saf. 21(5):389-406 (1999)

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