Serious adverse drug reactions (ADRs) remain a significant problem during drug development and post-marketing stages. Even though extensive research has been performed, the understanding and prediction of diverse types of ADRs is still far from complete. Especially considering the most problematic ADRs, the idiosyncratic drug reactions (IDRs), which are rare and unpredictable and in several cases lethal [1]. Several hypotheses for the mechanisms of underlying IDRs have been proposed, however, not all cases can be fully explained by these mechanisms. The formation of and human exposure to chemically reactive metabolites (CRMs) is generally considered to play an essential role in ADRs and IDRs, in different manners [2-4]. Essential macromolecules such as proteins can be modified or oxidized by CRMs, which triggers the formation of reactive oxygen species (ROS) and toxicity, in some cases leading to immune-mediated toxicity. The internal exposure of CRMs in tissues is determined by formation and protection processes, which are predominantly catalyzed by drug metabolizing enzymes (DMEs). The disbalance between bioactivation and inactivation processes leading to high internal exposure of CRMs is suspected to be a potential risk factor explaining the susceptibility to ADRs. Individuals with high bioactivation combined with lower inactivation capacities of toxic drugs are suspected to be more susceptible to ADRs and IDRs.

Over decades, many studies have been performed to elucidate the risk factors for ADRs and IDRs and to improve the predictability of working models, however, not all risk factors in the bioactivation and inactivation processes are yet understood.

The general aim of the research presented in this thesis was to study the possible role of DMEs and their interplay in determining the toxicological outcome of drugs at the molecular and cellular level, by using the anti-malaria drug Amodiaquine (AQ) and its CRMs as model compounds. To this end, in chapter 2 and chapter 3, we characterized the involvement of phase I (CYPs and NQOs) and phase II (GSTs) isoenzymes in the metabolism of AQ and in the formation and detoxification of related CRMs. These studies aimed at the elucidation of the inter-individual variation of DMEs as a potential risk factor in AQ-induced toxicity. In chapter 4, the AQ-derived quinoneimines (AQ-QI and DEAQ-QI) were employed as model compounds to elucidate cellular mechanisms and to identify both risk and protective factors of CRM-induced toxicities in a cellular system. In chapter 5, the role of human glutathione S-transferase (GST) isoform T2-2, a previously overlooked phase II enzyme, in detoxifying CRMs and environmental carcinogens was characterized.

Chapter 1 introduced the concept of ADRs and IDRs, with a brief description of their characteristics. Several hypotheses and currently known risk factors for ADRs and IDRs, such as immune system involvement, mitochondrial injury, inhibition of bile salt excretion pump (BSEP) and genetic variations were discussed. The formation of CRMs was introduced as a key factor in the onset of ADRs and IDRs. Additionally, biological functions, expression levels, inter-individual variability of three representative DMEs investigated in this thesis, namely CYPs, GSTs, and NQO1, and their potential impact on susceptibility to ADRs and IDRs were summarized. A few human liver cell-derived models,
new cell culturing techniques and their applications in studies on the roles of DMEs in drug toxicity were overviewed as well. Moreover, the role of DMEs and CRMs formation in other proposed mechanisms of ADRs and IDRs were introduced. Besides, DME-catalyzed formation and elimination of CRMs as important molecular initiating events (MIEs) in the adverse outcome pathways (AOPs) concept and the prediction of drug toxicity in the hazard matrix assessment strategy was discussed. Lastly, AQ as a model drug for the study of roles of DMEs and CRMs in IDRs, and currently known mechanisms of AQ-induced idiosyncratic toxicity were summarized.

Chapter 2 and chapter 3 focused on the characterization of phase I and phase II DMEs involved in the formation and elimination of CRMs of the model drug used throughout this thesis, AQ. AQ is widely used in the endemic area of Africa and Asia for more than 50 years for the treatment of malaria. The ADRs of AQ reported are severe idiosyncratic agranulocytosis and hepatotoxicity, with very low incidences of around 0.05%. Oxidative bioactivation to protein-reactive quinonemines (QIs) is considered to play an important role in the onset of AQ-induced toxicity [5, 6]. Previous studies showed that hepatic metabolism is important in the formation of a reactive QI of AQ (AQ-QI), and that GSH is able to spontaneously conjugate to AQ-QI, thus functioning as a protective factor. However, the individual DME-isoenzymes catalyzing these steps were not known at the start of this thesis. Another important feature of AQ is that after oral administration, the major metabolite, N-desethylamodiaquine (DEAQ) is the principle pharmacologically-active form in vivo while AQ plasma concentrations have a 100- to 240-fold lower AUC [7, 8]. In chapter 2 human liver microsomes (HLM) and recombinant CYP isozymes (a panel of 14 isoforms) were used to study the kinetics of bioactivation of AQ and DEAQ and to characterize the CYP isoforms involved in the formation of reactive QIs of AQ and DEAQ, namely AQ-QI and DEAQ-QI. GSH was used to trap reactive QIs formed in incubations of AQ and DEAQ. The results showed that even though AQ and DEAQ are prone to auto-oxidation, also resulting in the formation of the two QIs, the enzyme-catalyzed formation of the QIs remains the major contributor to CRMs exposure in vivo (Figure S4, chapter 2). Bioactivation of AQ and DEAQ to the respective QIs in HLM both exhibited Michaelis–Menten kinetics. For AQ bioactivation, the enzyme kinetical parameters were $K_m$, 11.5 ± 2.0 μmol/L, $V_{max}$, 59.2 ± 3.2 pmol/min/mg and $CL_{int}$, 5.15 μl/min/mg. For DEAQ, parameters were $K_m$, 6.1 ± 1.3 μmol/L, $V_{max}$, 5.5 ± 0.4 pmol/min/mg and $CL_{int}$, 0.90 μl/min/mg. Recombinant CYPs and inhibition studies with HLM showed that CYP3A4, CYP2C8, CYP2C9 and CYP2D6 are major isozymes contributing to the bioactivation of AQ and DEAQ. Collectively, chapter 2 showed that, considering its much higher AUC, DEAQ is likely to be quantitatively more important than AQ with respect to hepatic exposure to CRMs in vivo. Since it is well known that the expression levels and activity of hepatic human CYPs can vary strongly amongst populations [9, 10], Individuals with high levels of CYP3A4, CYP2C8, CYP2C9, and CYP2D6 may be more susceptible to hepatotoxicity upon AQ-therapy.
Chapter 3 focused on the identification and characterization of human DMEs involved in the elimination of CRMs of AQ and DEAQ, in particular GSTs and NQO1. Human GSTs are important detoxifying DMEs protecting against various electrophilic compounds by catalyzing the conjugation of electrophiles to GSH. Besides GSTs, human NQO1 is also known as an important detoxifying DME, by catalyzing the two-electron reduction of quinoneimines. The activities of 15 recombinant human GSTs and NQO1 in the inactivation of the reactive QIs of AQ and DEAQ were determined, using HLM as a bioactivation system. The results showed that GSTP1-1, GSTA4-4, GSTM4-4, GSTM2-2 and GSTA2-2 (activity in decreasing order) were active isoforms in catalyzing GSH conjugation of the QIs of AQ and DEAQ. Additionally, NQO1 showed competing activity to GSTP1-1 in the inactivation of the reactive QIs of AQ and DEAQ. By applying the major hepatic GSTs known from literature, the variability in the GST-catalyzed GSH-conjugation profile in 22 liver donors was simulated in this study. Results showed a large variation in cytosolic GSH-conjugation of the QIs of AQ and DEAQ, especially at a reduced GSH-concentration condition (up to 59-fold difference). Altogether, these results indicate that both human GSTs and NQO1 may play important roles in the detoxification of the reactive QIs from AQ and DEAQ, via conjugation and reduction pathways, respectively. Especially at low GSH-concentrations, e.g. occurring under hepatotoxicity conditions, GSH-conjugation is strongly dependent on catalysis by GSTs. Thus variability in GST expression will have the most significant consequences at reduced GSH concentrations, e.g. under conditions of oxidative stress. Inter-individual variability in expression levels of GSTs and NQO1 will have a significant influence on the inactivation of the QIs of AQ and DEAQ. Combined with the conclusions from chapter 2 and chapter 3, high(er) susceptibility to AQ-induced toxicity might occur in individuals carrying high expression levels of bioactivating CYP isoforms (CYP3A4, CYP2C8, CYP2C9, and CYP2D6) in combination with low expression levels of inactivating GST isoforms (GSTA2-2 and GSTA1-1) and NQO1.

To translate and validate the findings at the molecular level to the cellular level, we applied a transgenic HepG2 cell model to unravel the mechanisms of cytotoxicity of the QIs of AQ and DEAQ, namely AQ-QI and DEAQ-QI, and to assess the protective role of GSTs in chapter 4. HepG2 cells were selected based on certain advantages as described in chapter 1. With a transient transfection approach, a high expression level of GSTP1-1 in HepG2 cells was achieved, as indicated by the CDNB assay. Multiple parameters, including loss of cell viability, caspase 3 activation, GSH homeostasis, activation of adaptive stress response pathway and the cellular QIs disposition, were assessed in mock- and GSTP1-transfected cells. Additionally GSH-depletion, achieved by L-Buthionine-sulfoximine (BSO) pre-treatment, was created to mimic an oxidative stress situation. The results showed that both QIs induced a concentration-dependent loss of cell viability, which was significantly aggravated by GSH-depletion. Activation of caspase 3 is indicative for stimulation of apoptosis. Exposure of the cells to QIs was accompanied by a significant decrease of intracellular GSH and GSSG levels and a moderate increase in GSSG/GSH ratio. Transfection of HepG2 cells with GSTP1 resulted in a 2-fold increase in GSH-conjugates of
both QIs and provided protection against their cytotoxicity. Interestingly, caspase 3 activation by the QIs was completely blocked by GSTP1 transfection, even in GSH-depleted cells. Application of a high-content imaging-based adaptive stress response fluorescent protein reporter assay demonstrated a rapid activation of the adaptive endoplasmic reticulum (ER) stress response pathway in the HepG2 cells by both QIs, which was not alleviated by GSTP1 in transfected cells.

The subject of chapter 5 was the characterization of GSTT2-2 and its variability in human livers. Human GSTT2-2 is one of the overlooked DMEs in this context. Human GSTT2-2 has been isolated from human liver and identified for over three decades, however, a sensitive activity assay and the elucidation of its role in the detoxification of toxic compounds are still missing. In this chapter, we established a sensitive HPLC-based method for the quantification of S-(1-menaphthyl) glutathione (MSG), the product of the GSTT2-2 specific substrate 1-menaphthyl sulfate (MSu). This assay was more sensitive and more accurate when compared to the currently used spectrophotometric assay. By applying this new assay, the specificity of GSTT2-2, amongst 15 recombinant human GST isoforms, was confirmed. The enzyme kinetical properties of GSTT2-2 with recombinant GSTT2-2 and in pooled human liver cytosol (HLC) were also characterized. Moreover, a 65-fold inter-individual variation of GSTT2-2 activity was discovered in 20 individual HLCs. In addition, a selective role of GSTT2-2 in detoxifying the environmental carcinogen, 1-methylpyrene sulfate (MPS), was confirmed by using 15 recombinant GSTs screen and correlation analysis. Combined with the large inter-individual variability of GSTT2-2 activity observed, it is concluded that low expression levels of GSTT2-2 may be a risk factor underlying susceptibility to MPS carcinogenicity.

In conclusion, in this thesis, several in vitro approaches were used for the characterization of the roles of bioactivating and inactivating DMEs in determining the levels of as well as the exposure to CRMs, and its impact on the toxicological outcome of xenobiotics, with a special emphasis on the anti-malaria drug AQ. With these approaches, we successfully identified the balance between the bioactivation and inactivation of reactive QIs of AQ, its potential consequences for AQ-induced idiosyncratic toxicity, and gained insights into the cellular mechanisms underlying reactive QIs-mediated hepatotoxicity. These approaches provided rapid, reproducible, and cost-efficient strategies for the identification of risk factors of drugs causing ADRs or IDRs and for the evaluation of potential risks of new drug candidates and chemicals. The mechanistic understanding of ADRs and IDRs has been evolving over years, however, despite that still a lot of work remains to be done to fully comprehend the molecular and cellular mechanisms of ADRs and IDRs.

Toxicology is a field requiring the integration of knowledge and expertise also from medicinal chemists, biologists, pharmacologists, pathologists, and clinicians. With unraveling a small piece of the big puzzle, the work presented in this thesis has contributed further to the understanding of mechanisms, the rationalization of human susceptibility to
ADRs and IDRs, and the identification and assessment of risks and hazards of drug candidates and chemicals.