SUMMARY

Humans are continuously exposed to a variety of xenobiotics, such as, pharmaceuticals, cosmetics, dietary, occupational or environmental chemicals. Most genotoxic carcinogens are chemically inert, requiring metabolic bioactivation by biotransformation enzymes to form chemically reactive metabolites (CRMs), which may subsequently covalently bind to DNA, RNA and proteins. A diversity of metabolic pathways has been recognized in the bioactivation of carcinogens, with a key role of Cytochrome P540 (CYP) enzymes. In this context, the CYP1A family is of special interest in the bioactivation of specific classes of chemical carcinogens and in the development of cancer.

Ever since the introduction of the revolutionary *S. typhimurium* mutagenicity assay, the "Ames test" over four decades ago, continuous efforts have been made on the improvement and application of additional bacterial-based genotoxicity assays. However, the majority of the available bacterial cell systems used for genotoxicity assessment still lack xenobiotic biotransformation, since they do not contain appropriate metabolic competences.

The *primary aim of the research* described in this thesis was to develop new bacterial genotoxicity test systems, being: i) competent in terms of human CYP-mediated biotransformation and ii) suitable for HTS applications. The bacterial test systems should be suitable for genotoxicity and mechanistic studies, and for co-expression of different CYP enzymes and related polymorphic variants. The *secondary aim of the research* was to investigate the relevance of different CYP alleles of non-synonymous polymorphic forms. For these purposes, their activity towards a diverse group of genotoxic and non-genotoxic substrates should be studied, including the role of the accessory redox partner

Cytochrome b_5 (b_5). Human CYP1A2 and several polymorphic CYP1A2 variants were chosen as model enzymes for proof of concept.

Part I. Introduction

In **Chapter 1**, the pathways of xenobiotic biotransformation and the role of CRMs in genotoxicity and adverse drug reactions (ADRs) are discussed. An overview of the biotransformation enzymes involved in the bioactivation/bioinactivation of foreign compounds and the association between CRMs and toxicity in general, and genotoxicity in particular, is presented. Inter-individual differences in drug metabolism resulting from genetic polymorphisms of drug metabolizing enzymes, and their implications for ADRs and susceptibility to cancer is highlighted. The importance of the CYP superfamily, including the electron donor accessory enzymes b_5 and NADPH cytochrome P450 oxidoreductase (CYPOR), is explained with a special focus on CYP1A2. Chemical carcinogenesis and mutagenesis, and the role of bioactivation of xenobiotics are introduced as well. In this context, HTS-suitability of *in vitro* assays for genotoxicity detection, with emphasis on bacterial systems, is discussed as well.

Part II. Characterization of Human Cytochrome P450 1A2 Polymorphic Variants

Part II of this thesis deals with a comprehensive characterization of WT plus 8 non-synonymous polymorphic variants of human CYP1A2, namely T83M, S212C, S298R, G299S, I314V, I386F, C406Y and R456H; and the role of the accessory redox partner cytochrome b_5.

In **Chapter 2**, a thorough characterization of the WT CYP1A2 plus 8 non-synonymous polymorphic variants (indicated above) is described for their metabolic capacities. Each of the eight CYP1A2 variants plus WT was co-expressed with human CYPOR in *E. coli* strains, using a bi-plasmid co-expression system. All variants showed

similar levels of apoprotein and holoprotein expression, except for I386F and R456H, which showed only apoprotein. However, both were functionally inactive. The CYPOR/CYP ratios for the different variants were found to be in the range of those observed in human liver microsomes, except for I386F and R456H, due to lack of CYP1A2 holoenzyme (*vide supra*). Activities of the CYP1A2 variants were determined, using 8 different substrates. The resulting data set, consisting of 16 different activity parameters for each variant, was extensively analyzed using multivariate analysis methods. These results were subsequently interpreted using the human CYP1A2 crystal structure. It was concluded that among all analyzed human CYP1A2 variants, G299S is the most deviating enzyme in comparison to WT, whereas variant T83M is only slightly but significantly different from the WT. Furthermore, bioactivation of NNK (mutagenicity) was shown to be the most discriminative activity among the CYP1A2 variants. In addition, R456 could be identified as a critical residue for heme anchoring and stabilization.

In **Chapter 3**, the effect of the b_5 protein on the activities of WT CYP1A2 plus the same 8 non-synonymous polymorphic forms is described. A similar approach, as reported in **Chapter 2** was applied. CYPOR contents and b_5 (holo- and apo-protein) expression levels were determined and comparable among the strains. CYP1A2 variant levels, were comparable amongst variants (except for I386F and R456H) and similar to the ones described in **Chapter 2**, indicating that b_5 co-expression had minor effects on CYP expression, in this model. The CYP1A2 variants were screened using the same methods and substrates as described in **Chapter 2**. The results obtained were merged with the ones reported in **Chapter 2**. The combined data set was then analyzed through multivariate analysis and the structural knowledge derived from the human CYP1A2 crystal structure. This indicated that among all human CYP1A2 variants analyzed,

variants T83M and C406Y are the most divergent enzymes in catalytic properties when compared to WT in b_5's presence. Collectively, data indicate that b_5 likely exerts a compensatory effect on the perturbed functional capacities of the polymorphic variants, leading to a more WT-like behavior. Furthermore, the data seems to implicate CYP1A2 residue G299 is in interaction with CYPOR and/or b_5.

Part III. Development of Bacterial HTS-Systems for Detection of Genotoxic Electrophilic Reactive Metabolites

Part III of this thesis describes the engineering of bacterial systems to efficiently detect DNA-damaging CRMs generated by human CYP1A2.

In **Chapter 4**, the development of genotoxicity test systems, metabolically competent in human CYP1A2 and suitable for HTS purposes is presented. The principle of detection was based on a reporter fusion with the promotor sequence of *sulA* (one of the highest inducible bacterial genes upon DNA damage) with GFPmut3.1, a selected GFP reporter. An additional strain containing a constitutive promoter to correct for possible cytotoxic effects of test compounds was included, using the same GFP reporter. The reporter systems were initially used in four different bacterial backgrounds, namely TA1535 and TA100: two *S. typhimurium* strains of the Ames test, and FP401 and PD301, two *E. coli* strains developed in our laboratory in Lisbon. After optimization of assay conditions, all 4 reporter strains were tested with three direct-acting mutagens (4NQO, MNNG and CHP), to determine the sensitivity in genotoxicity detection. Subsequently, the two most effective bacterial systems, namely the ones based on strains TA1535 and PD301, were adapted to co-express human CYP1A2 and CYPOR, using the bi-plasmid system described previously, along with the GFP reporters. All strains demonstrated equivalent CYP and CYPOR levels and CYPOR/CYP ratios were within the range observed in human liver microsomes. The two systems were tested with three pro-

mutagens, namely, 2AA, IQ and 1AP, known to form DNA-reactive metabolites through CYP1A2-mediated metabolism.

The application of the newly developed bacterial genotoxicity assay (described in Chapter 4) for HTS application is reported in **Chapter 5**. This concerns the study of WT CYP1A2 plus the same 8 polymorphic forms, studied in **Chapters 2** and **3**, and using the *S. typhimurium* TA1535 based HTS system. CYP and CYPOR levels were comparable among all strains and CYPOR/CYP ratios were within the range observed in human liver microsomes, except for the strains containing variants I386F and R456H, with the expression issues described in **Chapter 2** and **3.** Still, the bioactivation of two pro-mutagens by the I386F variant could be detected. The CYP1A2-dependent genotoxicity of the pro-mutagens 2AA, IQ and 1AP could be detected and the results were analyzed through multivariate analysis. Variant T83M demonstrated again to be the most divergent isoform from WT among all variants, while variants G299S and I386F were slightly but significantly different from WT. These results were comparable with those obtained in in **Chapter 2**, in which for each CYP1A2 variant 8 different substrates (namely NNK, IQ, 2AA MR, ER, CEC, phenacetine and clozapine) were used and 16 different activity parameters were measured. This particular outcome indicated the effectiveness of the newly developed bacterial HTS system for genotoxicity testing, not only for investigation of the effect of CYP1A2 polymorphisms on pro-mutagens bioactivation, but also for providing insights in the molecular mechanism of CYP1A2 functioning.

Conclusions and perspectives

We designed and engineered the novel SOS-CYPtest system. This system is using several *E. coli* and *S. typhimurium* tester strains, which are competent in human CYP metabolism and are appropriate for *in vitro* HTS genotoxicity testing of chemicals, which was *the primary aim* of this study. Although only applied for a restricted number of

compounds, the newly developed SOS-CYPtest system seems to have the appropriate characteristics for the detection of DNA damaging CRMs in a HTS manner. HTS characteristics include a short assay time (max 120-180 min), the need of only small amounts of test compound (less than a few micrograms), the adaptability to a microplate reader format and real-time measurement.

The *secondary aim* of this thesis was to investigate specific CYP alleles, focusing on non-synonymous polymorphic forms, and the role of the accessory enzyme b_5. CYP1A2 was chosen as a model enzyme. Due to the huge amount of data produced, and to be able to scrutinize appropriately all results, multivariate analysis methods were applied and appeared to be a powerful and very useful tool regarding data analysis. All-together, it is concluded that 5 of the 8 different CYP1A2 variants analyzed, namely variants T83M, G299S, I386F, C406Y and R456H, were of relevance in terms of altered metabolism. Consistently, R456H was inactive. T83M and C406Y were the most different enzymes when compared to WT CYP1A2, while variant I386F is a very unstable enzyme in which b_5 plays a key role in its stability/activity. The results obtained clearly implicated G299 in CYP1A2 to interact with CYPOR/b_5. Collectively, b_5 seems to compensate for structural deviations of the CYP1A2 variants, most likely through extensive allosteric effects. Not only the proximal zones of the active center (I386F) and the b_5/CYPOR/CYP1A2 interface (G299S) are affected, but also more distal zones such as the substrate entrance/product exit channel (T83M and C406Y).

In xenobiotic risk assessment, the potency to induce carcinogenesis is usually provided by genotoxicity or mutagenicity testing. The major handicap of current *in vitro* HTS-systems is mostly due to inadequate representation and/or failure of human biotransformation. The studies in this thesis demonstrate possibilities to overcome this issue. Mechanistic information was also generated. Application and further engineering

of the current *in vitro* HTS-methods to study genotoxicity can contribute to improved selection of safer drug candidates in pharmaceutical R&D, but also for the identification of pollutants and/or hazardous environmental and occupational agents.