Summary

PhD Thesis

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Metabolic activation of pre-teratogens in primary hepatocytes and liver slices of rats for embryotoxicity testing *in vitro*. The murine embryonic stem cell test (EST) and the whole embryo culture (WEC) represent two ECVAM-validated *in vitro* embryotoxicity tests, which are unable to detect proteratogenic compounds due to their insufficient xenobiotic-metabolising enzyme activities. Within the framework of this thesis, the application of primary rat hepatocytes and liver slices to metabolically activate proteratogenic substances was investigated. The integration of metabolic activity into the 3T3 viability assay as a part of the EST was accomplished by a preincubation step of the test compounds in the *in vitro* biotransformation systems followed by the transfer of the obtained supernatant. This thesis is a part of the BMBF-funded project "Development of a biotransformation system for the metabolic activation of two *in vitro* embryotoxicity test systems".

In a first step, the culture conditions of primary rat hepatocytes were optimised for the transfer of supernatants into the 3T3 viability assay. The metabolic activity of the cells was evaluated by the turnover of three alkoxyresorufine derivatives (ethyl-, pentyl- and benzyloxyresorufine) and the model substrate testosterone. Enzyme activity was optimised by incubation of an induction cocktail containing 10 µM β-naphtoflavone, 100 µM phenobarbital and 0.1 µM dexamethasone for 24 hours. The medium of the induction period was replaced by fresh medium, incubated for further 24 hours. The post-induction period was used for substance incubation and transfer of the supernatant into the target culture during method development. Under the conditions described, the EROD-, PROD- and BROD-activities were 96.3 ± 33.4, 1.5 ± 0.8 and 6.3 ± 0.8 pmol/min/10^6 cells after 48 hours. Incubation of a serum buffer mixture (92.5 / 7.5, v/v), required for the transfer into the WEC and hereafter referred to as "WEC medium", during the post-induction period revealed an EROD-, PROD- and BROD-activity of 35.4 ± 2.5, 0.6 ± 0.3 and 2.0 ± 0.4 pmol/min/10^6 cells. The metabolic profile of testosterone (200 µM), which was incubated during the post-induction period, revealed a high conjugation activity as all oxidised metabolites, specifically 6β-, 16α- and 2α-OHT were conjugated with glucuronic acid and/or sulphate. Precision-cut slices from rat liver, used as an alternative *in vitro* biotransformation system, were incubated with the induction cocktail for 6 hours. After a 24 hour post-induction period the EROD- and BROD-activities were 20.4 ± 9.1 and 1.6 ± 1.4 pmol/min/mg protein. PROD-activity was below limit of quantification (0.9 pmol resorufine). There was no difference between the metabolic profile of testosterone in liver slices treated with or without the induction cocktail. The main metabolites were 6β-, 16α- and 2α-OHT and their conjugated forms as well as androstenedione. During method development for integrating the biotransformation system into the 3T3 viability assay, it was shown that 50 % (v/v) of the supernatant from rat hepatocytes and liver slices after a 24 hour culture period did not decrease the viability of the 3T3 cells compared to control, when mixed with fresh medium. 40 % (v/v) of the rat hepatocyte supernatant could be transferred into the differentiation and viability assay in embryonic stem cells (ES-D3). No developmental impairment was observed when 92.5 % (v/v) of WEC medium, incubated in rat hepatocytes for 24 hours was used in the WEC. Within a proof of concept approach cyclophosphamide (CPA), albendazole (ABZ), valpromide (VPD) and 2-acetylaminofluorene (2-AAF) and their defined metabolites acrolein (Acr), albendazole sulfoxide (ABZSO), valproic acid (VPA) and N-hydroxy-2-acetylaminofluorene (NOH-AAF) were used as model compounds. Initially, the half maximal inhibitory concentrations (IC_{50}) of the test substances were evaluated in the 3T3 viability

assay. Defined mixtures of the corresponding proteratogenic and teratogenic compound were used to investigate the turnover required for the detection of a metabolic activation within the viability assay. The IC_{50} values of CPA and Acr were about 1.5 mM and 2 µM. Under the selected conditions, the transfer of CPA supernatants from primary rat hepatocytes revealed a decrease of the IC_{50} value of CPA by a factor of 132 compared to CPA. The embryotoxic potential of CPA was also shown in the differentiation and viability assay in ES-D3 cells, as well as in the WEC after incubation in the induced rat hepatocyte culture. These observations were associated with the analytically detected Phosphoramide Mustard. ABZ was metabolically detoxified *in vitro* as the IC_{50} value of ABZSO was higher than that of ABZ (13 µM and 0.6 µM, respectively). This result was confirmed in all target cultures after the transfer of supernatants from rat hepatocytes. Under the test conditions, no IC_{50} value could be determined, as the concentration of ABZSO was too low to induce cytotoxicity. No IC_{50} value for VPD could be evaluated in the 3T3 viability assay due to limited solubility of the test substance in the culture medium. The IC_{50} value of VPA was about 1.7 mM. There was no evidence for a metabolic activation of VPD by rat hepatocytes as the concentration-response curves of VPD and metabolically activated VPD overlapped. This result was in accordance to the absence of VPA in supernatants of rat hepatocytes as analytically determined. The IC_{50} values of 2-AAF and NOH-AAF were about 77 µM and 10 µM. No decrease of 3T3 viability compared to control was observed after transfer of 2-AAF supernatants, which is in accordance to the absence of the teratogenic metabolite NOH-AAF in the supernatant of rat hepatocytes and liver slices as analytically determined. In conclusion, based on the method developed within the present work, cellular *in vitro* biotransformation systems from rat liver were successfully coupled with the EST and the WEC. However, its use as a standard method is limited by low turnover of substrates, profile of formed metabolites as well as instability of the active metabolites in the culture medium. Within the scope of individual applications of the developed method, analytical examinations of metabolic profiles and stability of formed metabolites are strictly recommended and are assessed as essential for the evaluation of the overall result.