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A rat toxicogenomics study with the calcium sensitizer EMD82571 reveals a pleiotropic cause of teratogenicity

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ABSTRACT

The calcium sensitizer and PDEIII inhibitor EMD82571 caused exencephaly, micrognathia, agnathia and facial cleft in 58% of fetuses. In pursue of mechanisms and to define adverse outcome pathways pregnant Wistar rats were dosed daily with either EMD82571 (50 or 150 mg/kg/day) or retinoic acid (12 mg/kg/day) on gestational days 6–11 and 6–17, respectively. Hypothesis driven and whole genome microarray experiments were performed with whole embryo, maternal liver, embryonic liver and malformed bone at gestational days 12 and 20. This revealed regulation of genes critically involved in osteogenesis, odontogenesis, differentiation and development and extracellular matrix. Importantly, repression of osteocalcin and members of TGF-β/BMP signaling hampered osteo- and odontogenesis. Furthermore, EMD82571 impaired neurulation by inhibiting mid hinge point formation to cause neural tube defects. Taken collectively, a molecular rationale for the observed teratogenicity induced by EMD82571 is presented that links molecular initiating events with AOPs.

1. Introduction

Reprotoxicity testing aims at assessing adverse effects of drugs and chemicals on reproduction and fetal development. Note, the reproductive cycle comprises a multitude of interactions at the molecular, cellular and structural level in a specific chronological sequence. However, mechanisms leading to developmental toxicity as a result of perturbations of maternal and fetal physiology and by acting on the foeto-placental unit are frequently unknown. Owing to its complexity organogenesis is vulnerable to multiple toxic interferences, and the fetal development is dependent on the highly versatile signaling system of calcium (Ca^{2+}) that also functions as a secondary messenger. It is reasonable to assume that any chemical interfering with calcium signaling might cause malformations during embryogenesis or fetal development [1]. Thus, efforts to understand the links between molecular initiation events (MIE) induced by drug and chemicals are critical for the development of an adverse outcome pathway (AOP) and such concept should be based on mechanistic plausibility to enable hypothesis driven research.

The notion that birth defects are a frequent outcome of adverse effects of chemical and drugs on the complex gene regulatory networks and signaling pathways demands the use of enabling technologies in developmental toxicity studies. Surprisingly such methodologies are rarely applied to teratogenicity studies. Toxicogenomics assists in an elucidation of the adverse outcome pathways (AOPs) by searching for genome wide expression changes as related to specific drugs and chemicals with whole genome microarrays being instrumental in deciphering biomarkers and target genes of teratogenic drugs [2]. In conjunction with other molecular endpoints, prediction of drug safety at early stages of drug development is feasible to contribute to an improved understanding of the molecular causes of birth defects.

In the present study a toxicogenomic approach was used to elucidate AOPs associated with craniofacial malformations induced by EMD82571, i.e. a calcium sensitizer developed for the treatment of coronary heart disease. Specifically calcium ions play a pivotal role in the control of the myocardial contractility and a rise in

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contraction force can be elicited by either increasing the transient free Ca^{2+} concentration and/or by augmenting the myofibrillar sensitivity to Ca^{2+}. Traditionally, drugs have been developed which raise the force of contraction by strengthening the myocardial contractility ultimately by modulating intracellular Ca^{2+} stores [3] and this includes different class of drugs such as sympathomimetics, phosphodiesterase III (PDEIII) inhibitors and cardiac glycosides. EMD82571, a pro-drug of EMD 57033, was shown to increase contraction primarily via calcium sensitization. It also has PDEIII inhibitory properties, contributing to its overall cardiovascular profile. The compound acts through "down-stream mechanisms" at the level of the actin-myosin cross-bridges by increasing the contribution of each cross-bridge to the contractile force.

The acute toxicity of EMD82571 in rodents was shown to be low. However, female Wistar rats were considerably more sensitive due to significantly higher bioavailability (60%) of the active drug as compared to males (9%) thus suggesting a gender specific ADME (absorption, distribution, metabolism, and excretion) profile. Repeat dose studies showed a "no observable effect level" (NOEL) of 10 mg/kg in rats and dogs. At higher doses, mild toxicity was observed, with the principle target organ being the liver, which increased in weight and demonstrated centrilobular hypertrophy associated with increase in liver enzyme activities. Histologically, perivasculitis and vacuolation of the centrilobular hepatocytes was observed. The Ames test (+ S9), the rat micronucleus test and the mouse lymphoma cell assay (+S9) were all negative. Mutation frequencies were slightly increased in the absence of S9 activation in the mouse lymphoma cell assay. However, the drug was abandoned in early clinical trials due to its teratogenic effect on some fetuses. Therefore, toxicogenomics was used to elucidate AOPs by linking the molecular initiating events to the observed developmental defects. EMD82571 modifies the actin-myosin based motor complex in cardiomyocytes (MLC1and MLC2) and is known to act on kinases and phosphatases during myofilament response to calcium ions [3]. Hence, the adverse outcome (AO) relates to the organ response upon drug treatment. Next to whole genome gene expression studies the weight of evidence includes bile acid profiling, gross morphology, histopathology and clinical chemistry findings and the strength, consistency and specificity of AOP is considered. Part of this research was briefly summarized in the 'Handbook of Toxicogenomics' [4]. However, the book chapter does not contain the AOP concept and a mechanistic frame work based on biological plausibility. The development of the AOP includes most recent research findings to permit an understanding of the link between the elicited MIE and an identification of intermediated events as part of the AOP.

2. Materials and methods

EMD82571 and its derivatives were prepared by Merck, Darmstadt, Germany and were of >99% purity. Animal studies were carried out either at the Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany or in the Institute of Toxicology at Merck, Darmstadt, Germany.

2.1. Structure of EMD82571

The calcium sensitizer agent EMD53988 (E. Merck KgaA, Darmstadt, Germany) is a thiadiazinone derivative (5-[1-(3,4-dimethoxybenzoyl)1-,2,3,4-tetrahydr0-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one). It increases myocardial contractile force via a dual mechanism of action brought about by the different optical enantiomers: the (−)-enantiomer inhibits phosphodiesterase III (EMD82571), and the (+)-enantiomer increases the Ca^{2+} sensitivity of cardiac contractile proteins (EMD57033). The purity of the drug formulations for animal studies was >99% (Fig. 1).

2.2. Animal studies

Formal approval to carry out animal studies was granted by the animal welfare ethics committee of the State of Lower Saxony, Germany ('Lower Saxony State office for Consumer Production and Food Safety' (LAVES)). The approval ID is Az: 33.9-42502-04-06/1087. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (The National Academy Press, Washington, DC, 2011). Female Wistar rats were obtained from Charles River laboratories (Sulzfeld, Germany). Food and water was given ad libitum to the experimental animals.

2.3. 4-week repeated dose toxicity study

Non pregnant female Wistar rats were dosed daily with either the vehicle (0.25% hydroxyl propyl methylcellulose) or 10, 30, 100, 200 mg/kg/day of EMD82571 for 4 weeks. Each group consisted of 10 animals. At the end of the fourth week of repeated dose study, the animals were euthanized and gross morphological and histopathological changes related to liver were examined. This study was conducted at the Institute of Toxicology, Merck KGaA, Darmstadt, Germany.

2.4. Reprotoxicity study

All-trans retinoic acid (Sigma Chemical, St. Louis, MO) (RA) was chosen as a reference compound due to its well documented teratogenic effects on developing fetuses with similar malformations being reported as observed for EMD82571 [5–7]. Initial experiments with RA were carried out following a dose of 20 mg/kg/day as suggested by Emmanouil and coworkers [8]. However, at this dose all fetuses were resorbed. A repeat of the study resulted in the same findings, i.e. complete resorption of the fetus. Therefore, a third study at a dose of 12 mg/kg/day of RA was performed. Unfortunately, no fetal malformations were observed and the tissues from the third study were used for microarray experiments only.

Pregnant female Wistar rats were dosed daily with either EMD82571 (50 or 150 mg/kg/day, low and high dose of EMD, respectively) or retinoic acid (12 or 20 mg/kg/day) [9] on gestational days 6–11 and 6–17, respectively.

The vehicle was 0.25% aqueous hydroxypropyl methylcellulose. EMD82571 was prepared fresh daily and filled into brown flasks. This formulation was stirred continuously until application. Retinoic acid was dissolved in corn oil. Both the test and reference material were administrated orally in a final volume of 5 ml/kg based on the last weight determination.

On gestational days (GD) 12 and 20, female Wister rats ($n = 5$) per dose, study group and compound were anesthetized by an i.p injection of Ketamine/xylazine and the fetuses were surgically removed ($n = 50$). The maternal liver, whole embryo (day 12), fetal liver (day 20) or fetal cranium and mandibular bone (day 20) were removed and immediately snap frozen in liquid nitrogen to await further analysis.

2.5. Toxicity study with EMD82571 and its derivatives

Safety studies were performed in pregnant Wistar rats with different derivatives of EMD at the Institute of Toxicology, Merck KGaA, Darmstadt, Germany. The rats were dosed daily with either the prodrug EMD82571 (150 mg/kg), the active drug EMD 57033 (100 mg/kg) or the ester cleaved derivative of the prodrug (150 mg/kg/day) on gestational days 6–17.