# Species-specific developmental toxicity in rats and rabbits: generation of a reference compound list for development of alternative testing approaches

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## Abstract

For regulatory information requirements, developmental toxicity testing is often conducted in two mammalian species. In order to provide a set of reference compounds that could be used to explore alternative approaches to supersede testing in a second species, a retrospective data analysis was conducted. The aim was to identify compounds for which species sensitivity differences between rats and rabbits are not caused by maternal toxicity or toxicokinetic differences. A total of 330 compounds were analysed and classified according to their species-specific differences. A lack of concordance between rat and rabbit was observed in 24% of the compounds, of which 10% were found to be selective developmental toxicants in one of the species. In contrast to previously published analyses the presented comparison is based entirely on publically data allowing validating and comparing alternative approaches for developmental toxicity testing. Furthermore, this list could be useful to identify mechanisms leading to species differences.

**Keywords:** Embryo-fetal developmental toxicity; Database; Alternative methods; cross-species analysis.

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## 1. Introduction

The assessment of potential developmental toxicity is an integral part of international regulations for the risk assessment of pharmaceuticals, industrial chemicals, food additives, biocides and plant protection products [1–3]. At present, such an assessment is typically conducted based on OECD or ICH (for pharmaceuticals) guidelines. These guidelines are primarily using mammalian models, which are laborious, time-consuming and involve the use of animal test. Since the thalidomide tragedy, developmental toxicity studies are often required to be conducted in two mammalian species, in a rodent and a non-rodent species [3]. Therefore, typically rats and rabbits are used as models for developmental toxicity testing. There have been discussions on the principal requirement for mammalian test, for ethical reasons and also with regards to the reliability of extrapolations to humans [4]. Furthermore, it was debated whether testing in rabbits may be waived in certain circumstances [5–9]. Initial reviews on rabbit embryo-fetal development (EFD) toxicity studies versus EFD studies in rat, however, suggested that the overall predictive capacity increases if developmental toxicity is tested in two mammalian species, i.e. in rat and rabbits [10–14]. Only recently the role of toxicokinetic for species-specific sensitivity differences in developmental toxicity of rats and rabbits has been considered [8, 15, 16]. Comparison of rat and rabbit embryo-fetal developmental toxicity data based on maternal systemic doses (AUC or Cmax) suggested that overall both species are equally sensitive [15]. However, for approximately 20 % of the compounds either the rat or the rabbit was found to be more than 10fold sensitive (the 10fold threshold was based on differences between studies that tested the same compound). Hence, despite the overall concordance, testing in two species may still be relevant to capture potential hazards for consumers and patients. Understanding the reasons for species sensitivity may help to develop alternative testing strategies, based on in vitro tests, testing of non-protected vertebrate embryonic stages and/or computational predictions. For the development of chemicals such as pharmaceuticals, screening

strategies to identify developmental toxicity are typically implemented in an early phase of product development. The potential of in vitro, ex vivo and non-mammalian in vivo assays is already considered in the new revision of the ICH S5 (R2) guideline for regulatory purposes under limited circumstances although these circumstances are yet to be defined [17].

The retrospective analyses of Theunissen et al. [8, 15, 16] was based on a rather large set of compounds (379) and clearly indicated that part of the species differences can be attributed to toxicokinetics differences. While they provided a detailed dataset combining external doses, systemic concentrations and details on the type of effect, it was based on a coded dataset that did not reveal the identity of the compounds showing species differences. However, understanding the mechanistic basis of species differences, development of alternative testing approaches and independent validation of the findings by other groups would benefit from a set of known reference compounds. Therefore, we set out to validate the findings of Theunissen et al. [8, 15, 16] by repeating the analysis based on a larger set of non-confidential compounds with available rat and rabbit developmental toxicity data.

## 1. Material and methods

### 1.1. Data Collection

Data were collected for compounds which have been tested in both rat and rabbit embryo-fetal studies (EFD, segment II teratology studies or OECD 414). Compounds showing no developmental toxic effects in both species were not included in the database.

Potential data were identified from various sources. (1) Previous reviews on developmental toxicity studies [5,6,11,18,19], comparing mainly pesticides, veterinary drugs and industrial chemicals were analysed. The data were then retrieved from the original data sources, i.e. peer-reviewed international documents (Table 1). When no or insufficient data for a specific substance were found in those sources, we searched the open literature to retrieve additional data (PubMed). (2) For pharmaceuticals, a list of all drugs in DrugBank [20] was extracted (August, 2015) and developmental toxicity data were collected using original peer-

reviewed, publicly available reports and material safety data sheets (Table 1). (3) Studies were also compiled from PharmaPendium® database (See supporting information, figure 1 for details on obtaining data from the PharmaPendium database ( trademark of Reed Elsevier Properties SA, used under license)) [21]. Verification of the data was conducted for 48.8 % of the compounds for which the original study was available. Around 20% of the populated data was based on limited information on Material Safety Data Sheets.

The following parameters were entered in the database: range of doses tested as mg/kg b.w. per day, route of administration, developmental lowest effect level (dLOEL), developmental non-observed effect level (dNOEL), maternal lowest effect level (mLOEL), and developmental effects observed in the fetus at the dLOEL. These data were retrieved from the original study if available and all manifestations of developmental toxicity were taken into account (like embryofetal death, altered growth and structural changes or abnormalities). The rationale given by Janer et al. [5] was followed: (i) A final reason for considering both teratogenic and other developmentally toxic effects in combination is that the differences in the type of effects observed might not lead to different regulatory decisions in most regulatory frameworks (exceptions are e.g., pharmaceuticals). (ii) Developmental toxicity responses observed in different studies for the same substance are not always of the same type even when examined in the same species. In addition, toxicokinetic data reflecting maternal plasma concentrations (Cmax and AUC) at the dLOEL were included if available. In case that kinetic data were not available for the dLOEL, we attempted to identify kinetic information from different doses of other studies (females only), and linearly extrapolated the data to the dLOEL (toxicokinetic data was extrapolated from different studies for 9 compounds). If more than one study was available for the same chemical, species and route, the lowest dLOEL was taken. Also, the same route of exposure was compared between rat and rabbits to avoid possible differences based on route-specific pharmacokinetics. Data were considered only if the following quality criteria were fulfilled. (i) Exposure of animals was conducted from implantation to the end of gestation as described

for the OECD TG 414. In some cases of data obtained from Safety Data Sheets the exposure period was not indicated. In this case we assumed that the appropriate exposure period was used if data were labelled as embryo fetal developmental effects, (ii) for the conclusion of a species sensitivity difference it was required that both LOEC and NOEC data were available from the less sensitive species. No other quality criteria have been applied.

## 1.2. Data analysis

Comparison of rat versus rabbit developmental toxic exposures was, if available, based on the maternal systemic doses at the dLOEL (AUC or Cmax) as differences between species are largely related to compound kinetics [15]. For those compounds without toxicokinetic data, the dLOEL was scaled by a correction for allometry on the basis of the body surface area to obtain the human-equivalent dose (HED). The HED in mg/kg for the rat and rabbit data was calculated by multiplying the effect concentrations by 0.16 and 0.32, respectively [22]. In agreement with Theunissen et al. [8], a factor of 10 related to overall study variations was selected as threshold to identify potential species-specific developmental toxicants. A factor of 10 is somewhat arbitrary but useful to describe the degree of sensitivity differences for the following reasons: (i) assuming that on average the species sensitivity difference is weak, a descriptive analysis of data with LOEC indicates that 25 % of the data are characterized by a higher difference between species (Fig. 2). This group of compounds exhibits a difference of more than 10. (ii) The factor of 10 has been used in previous studies and allows a better comparison to previous approaches.

The comparison of rat versus rabbit developmental toxicity requires also consideration of maternal toxicity. For some compounds the maternal and developmental effects occurred at similar levels (dLOEL greater or equal to mLOEL). In these cases developmental effects may be secondary to maternal toxicity, though not demonstrated, and represent a confounding factor in the assessment of interspecies differences in developmental toxicity. Hence, the mLOELs were taken into account for the comparison between rat and rabbit

specific developmental toxicants. A compound was considered as a selective developmental toxicant if it was observed at lower levels than the mLOEL or when no maternal toxicity was observed. If no maternal toxicity was reported for a given EFD study it was assumed that no maternal toxicity had occurred. Figure 1 shows how the comparison of the dLOELs between rat and rabbit was used to distribute the compounds in different classes with respect to their species-specificity. The entire database is available from a supplementary file and contains the collected compounds distributed in the different classes.

Species sensitivity was also analysed in relation to the chemical's mode of action (MoA). Five groups that include a minimum of seven compounds were identified and analysed from the entire database. The MoA was in relation to the main pharmacological mechanism (on-target activity) or to its side-effects (e.g. antibiotics, off-target activity on microflora of rabbits). In order to determine whether rat and rabbit exhibit different relative sensitivity within a class of compounds, the cumulative distribution of the developmental LOELs (based on conversion of the effect dose to HED) were plotted separately for each species and mode of action. The median of the distributions were compared to determine if rat or rabbit display developmental specific toxicity to a group of compounds. The distributions were fitted using R software [23–25] with the "fitdistrplus" and "ggplot2" package. The curve fitting model was selected based on the Akaike Information Criterion (AIC) indicating the best fit.

## 2. Results

From the three different data sources described in material and methods, 120 compounds were identified from previous reviews on developmental toxicity studies [5, 6, 18, 19]. The compounds comprised veterinary drugs [11], pesticides [6], industrial chemicals [5, 18] and some pharmaceuticals [19]. Analysis of the list of compounds obtained through DrugBank and other sources (table 1) resulted in the identification of 216 compounds, mainly pharmaceuticals. Additional 30 pharmaceuticals were identified from the PharmaPendium database. In total, 366 LOELs pairs for rat and rabbit were found representing 363

157 compounds based on their CAS registry number. Most of the compounds entered were
158 drugs (75%), followed by pesticides (19.7%), veterinary drugs (4%) and industrial chemicals
159 (1.6%). The main route of exposure was oral (74%), followed by intravenous (7.6%),
160 subcutaneous (5%) and topical administration (1.3%). Thirty-six compounds for which the
161 route of exposure was not indicated were excluded from the analysis. The same route of
162 exposure was compared between studies to avoid differences in internal concentrations (that
163 may result in different dLOELs) due to different application routes.

164 Figure 2 shows the comparison of the dLOELs between rat and rabbit, based on AUC/Cmax
165 or HED in order to account for potential pharmacokinetic differences and species size-
166 dependent effects. For most of the compounds (n= 250, 76% of compounds with a dLOEL in
167 one or both species), rat and rabbit showed a similar sensitivity with respect to
168 developmental toxicity. For 37 compounds (11%) the rat study appeared more sensitive than
169 the rabbit and in 43 compounds (13%) the rabbit study was more sensitive. Similar results
170 were obtained if maternal toxic compounds were not considered (mLOEL≤dLOEL, see
171 supplementary figure 2). In order to identify potential reasons for the difference between rat
172 and rabbit, the mLOELs were included in the analysis and compounds were distributed into
173 different classes according to the difference between dLOEL and maternal toxicity (Fig. 1).

174 Table 2 shows the distribution of compounds within these different classes and all the data
175 collected within the different classes is provided in a supplementary file. Class 1 represented
176 species-specific developmental toxic compounds, with no developmental effects in one
177 species or compounds for which HED or exposure-based dLOEL between rat and rabbits
178 differ 10x or higher. A lack of concordance between rat and rabbit was observed for a total of
179 33 compounds. The rabbit showed to be more sensitive than the rat for 11 compounds either
180 because the rat study did not show developmental toxicity (N=2) or because the dLOEL was
181 10x higher than the rabbit dLOEL (N=9). For 22 substances the rat was found to be more
182 sensitive than the rabbit. For 5 compounds out of these 22, the rabbit study did not show any

183 developmental effect. Class 2 included compounds that are developmental toxic in one
184 species (at maternally non-toxic doses) but the other species showed maternal toxicity at
185 similar doses. There were 17 compounds for which the rat showed to be more sensitive (no
186 effects on rabbit or dLOEL fold change x10 or more) but maternal effects in the rabbit were
187 observed in the range of developmental effects of the rat. Vice versa there were 11
188 compounds for which the rat study did not establish a dLOEL or was 10fold higher but
189 maternal effects were demonstrated. In class 3, compounds that show developmental effects
190 in only one species with evidence to be caused by maternal toxicity were grouped (group of
191 compounds for which relationships/causalities have been previously established). There
192 were a total of 8 compounds for which the animal study reported that the effects observed
193 have probably resulted indirectly from maternal effects (Class 3 list, supplementary file). In
194 class 4, there were 78 compounds for which both species showed similar sensitivity for
195 developmental toxicity (fold change in dLOEL < 10 based on HED or measured exposure
196 data) with no maternal toxicity. There were 15 compounds for which data indicated that
197 species differences of effect concentrations were related to differences in exposure (AUC,
198 $C_{max}$) probably due to differences in toxicokinetics between rat and rabbit (Table 3, 15
199 compounds). These compounds were included in class 4. Data on internal exposure
200 concentrations were, however, only available for 85 compounds out of the 366 entries (23
201 %). Therefore, toxicokinetic differences may also account for the differences of other
202 compounds distributed in class 1 given the lack of appropriate data.

203 The remaining compounds could not be classified into any of the classes due to the lack of
204 appropriate information. This inconclusive group was grouped as Class 5 comprising 183
205 compounds. For 28 compounds from this class there was not enough data to draw a
206 conclusion either because (1) the highest dose tested did not provoke developmental toxicity
207 and maternal toxicity in one species and this dose was equal or less than 10x the dose
208 inducing developmental toxicity in the other species or (2) no dNOEL was established in the
209 less sensitive species (n=7) (when dLOEL differs 10x or more). Hence, an extension of the

dose range may have indicated a similar dLOEL of both species. Information for each case is detailed in the supplementary file by appropriate comments for each compound. The remaining 155 compounds were classified inconclusive because developmental toxicity coincided with maternal toxicity and no causal evidence was found to conclude secondary developmental toxicity due to maternal toxicity. Providing of further data for these compounds may allow grouping them into one of the other five classes.

### 2.1. Species sensitivity to certain MoAs

Comparison of species sensitivity for certain MoAs (On-target or off-target) may indicate mechanisms leading to species differences. However, only five groups that comprised a 48 compounds (i.e. 15% of the compounds) could be identified (glucocorticoid agonists, COX inhibitors, tyrosin-kinase inhibitors, antibiotics and lanosterol 14 α-demethylase inhibitors). In order to demonstrate potential species differences a plot of cumulative distribution of the HED at LOELs for the different classes of compounds was used which indicates species-differences more pronounced (Fig. 3, for corresponding scatter plots see supporting information, figure 3). The MoA-specific analysis did not indicate species-specific differences for tyrosine kinase inhibitors, COX inhibitors and glucocorticoid agonists but for lanosterol 14 α-demethylase (rat 2.7x more sensitive) and antibiotics (rabbit 3.6x more sensitive). However, there was no statistical difference based on the comparison of median of the distributions (data not shown). It should be noted that the analysis did not include compounds for which no dLOEL was established (3 lanosterol 14 α-demethylase inhibitors in rabbit, 1 antibiotic in the rabbit, 5 antibiotics in the rat and 1 COX-inhibitor and glucocorticoid agonist each in the rat, supporting information figure 3).

## 3. Discussion

In this study the analysis of developmental toxicity data – if compared to previous reviews - has resulted in a comparatively high number of non-confidential compounds (N= 363, mostly

pharmaceuticals) for which rat and rabbit developmental toxicity data were available [5, 11, 18, 19]. The compound overlap with existing studies [15] is approximately 20 % (Theunissen, personal communication; based on 177 out of 379 compounds for which the identity was known to the main author). The interpretation of the established data set is partially difficult due to variations in experimental designs, strain of animal used, methods in analysis of foetuses and interpretation of the data. Hence, for some compounds conclusions with respect to species sensitivity differences could be confounded. It should be mentioned that the database included only a few number of industrial chemicals. Potentially the list could be extended by reviewing individual publications or the IUCLID files provided by ECHA.

Previous large-scale comparative study on rat and rabbit developmental toxicity [8, 15, 16] revealed an overall similar sensitivity of the rat and rabbit (80% of all compounds). However, the studies did not reveal the identity of the compounds and information on MoA was only available for a limited number of compounds (135 out of 379). In contrast, our study was based on non-confidential data and information on the MoA was available for all the compounds allowing the comparison of rat and rabbit developmental toxicity on specific mode of actions. Internal exposure data were only available for 85 out of the 366 compounds. Hence, it cannot be excluded that in some cases differences in the systemic doses have caused the species differences. Therefore, for those compounds without internal exposure data the comparison of rat and rabbit developmental exposures was based on the human equivalent dose. In agreement with Theunissen et al. [15] we found that most of the compounds showed similar sensitivity between rat and rabbit (76%) and around 24% of the compounds revealed an increased sensitivity for rat (37 out of 330) or rabbit (43 out of 330).

### 3.2. Role of maternal toxicity in interspecies comparison

Interpretation of developmental toxicity can be difficult due to the indirect role of maternal toxicity [26]. Disturbances of maternal homeostasis or physiology (due to chemical

exposure) may affect normal development of the embryo. The effect would be secondary to maternal toxicity and not a direct effect of the chemical over the embryo caused by direct interference of the compound with important differentiation processes. A common example found in the literature is indacrinone that produces maternal hypokalemia as the principal cause of the fetal skeletal defects observed in rats [27]. In our study, we identified two classes of substances for which maternal toxicity could have an impact on species differences. First, we identified compounds that show developmental toxicity in one species (at maternally non-toxic doses) but the other species shows maternal toxicity at similar doses. In this case developmental toxicity could be masked by a differential sensitivity for maternal toxicity (Class 2 list supplementary file). Second, we identified a set of compounds in which developmental effects were only observed in one species and were secondary to the maternal toxicity observed (Class 3 list supplementary file, it includes 8 compounds: 4 antibiotics, 2 renin-angiotensin inhibitors, 1 colony stimulating factor and 1 dopamine agonist). These differences could also reflect a species-specific maternal sensitivity related to a certain mode of action. For instance, many compounds in Class 3 are antibiotics (e.g. norfloxacin). It is well known that rabbits are not a suitable species to test developmental toxicity of antibiotics because of their gastrointestinal intolerance due to the dependency on microbial activity for digestion [28]. Increased susceptibility of the rabbit was also reported for the group of renin-angiotensin inhibitors [29] (e.g. telmisartan). The adult rabbit is particularly sensitive to renin-angiotensin inhibitors, showing a greater antihypertensive effect than does the rat. Placental blood flow and oxygen delivery decrease in relation to blood pressure and fetal toxicity may result from this decrease in oxygen delivery [30]. Rabbit-specific developmental toxicity potentially caused indirectly by maternal toxicity was also observed for granulocyte colony-stimulating factors (G-CSF), such as filgrastim. The developmental toxicity of these compounds could be related to an exaggerated pharmacodynamic effect specific for rabbits [31]. Neutrophils increase markedly due to G-CSF administration in rabbit causing ischemia of the placenta vessels and consequently fetal

mortality [32]. Class 3 compounds with only developmental toxicity in the rat include dopamine agonists (pramipexole), of which the effects are thought to be secondary to reduction of prolactin levels caused by the drug. Prolactin is required for implantation and pregnancy maintenance in rats but not in rabbits or humans [33]. It can be concluded that the compounds grouped in class 3 in this study (Supplementary file) are likely to be non-developmental or non-teratogenic toxic compounds. Inclusion of compounds showing developmental toxicity as a consequence of maternal influences into a list of model compounds would be of particular concern for the establishment of alternative prediction models. These models may not indicate developmental toxicity resulting from maternal toxicity.

Compounds for which it could not be concluded whether the developmental toxicity was secondary to maternal toxicity (inconclusive compounds, representing about 47% of rat-rabbit comparisons) may principally represent a source of further species-specific developmental toxic compounds provided that further data would confirm that species differences is not based on internal exposure differences and/or maternal toxicity. Many reviews/discussions have been published regarding the relationship between maternal toxicity and embryo-fetal toxicity with differing conclusions [29, 34-38]. Therefore findings should be handled on a case by case basis to establish a causal relationship. The determination of whether or not the relationship is casual is difficult to make and needs a comprehensive assessment of the data coupled with expert judgement.

### 3.2. Role of kinetics/metabolism in interspecies comparison

The key processes that control embryonic development are regulated by a limited number of signalling pathways that are evolutionary conserved over a broad range of species [39]. This suggests that discordance in developmental toxicity testing could often be due to factors not related to the target availability or affinity. For instance, differential pharmacokinetics could have a strong impact on internal bioavailable amount of a compound reaching the embryonic target. In our analysis we found 6 compounds: fingolimod, voriconazole, cabozantinib,

316 alitretinoin, mirabegron and entecavir (Table 3), in which differences of internal exposure
317 concentrations and toxicokinetics may have been involved in the observed species-specific
318 differences in developmental responses. Also, there were 9 compounds that showed a
319 similar sensitivity between rat and rabbit after to the HED conversion. An example is the
320 group of retinoid compounds, for which differences in kinetics among species might play a
321 major role in the different potency rankings in developmental toxic responses between
322 species [40]. This example shows that the knowledge of kinetic processes (absorption,
323 distribution, metabolism and excretion – ADME –) is critical when interpreting the lack of
324 concordance between species in developmental toxicity studies [15, 16, 41]. The HED
325 approach can at least cover systematic species differences provoked by size differences,
326 hence, reducing the number of false positives. However, some drugs are not amenable to
327 simple allometric scaling, i.e. drugs that are highly protein-bound, that undergo extensive
328 metabolism or active transport, significant biliary transport or drugs whose targets are
329 subject to interspecies differences in expression, affinity and distribution [42]. An example of
330 interspecies difference in target affinity is the pesticide flumioxazin (class 1 rat, table 5), a
331 protoporphyrinogen oxidase (PPO) inhibitor. Studies have been shown that protoporphyrin
332 IX accumulation corresponded to the developmental toxicity displayed by flumioxazin and
333 that the rabbit show no accumulation, suggesting a link between PPO inhibition and
334 developmental effects [43].
335 The maternal metabolism can vary widely between species and could also represent a factor
336 for interspecies discordance. The most important drug-metabolising enzyme family is
337 cytochrome P450 (CYP) and differences in CYP isoforms between species are a major
338 cause for differences in species drug metabolism [44]. For instance, similarities can be found
339 for CYP2E1, which is conserved among species and shows no appreciable differences with
340 respect to expression and catalytic activity. CYP1A is also conserved among species, but
341 some catalytic differences have been observed. In contrast, CYP2C, -2D and -3A show
342 substantial species differences in terms of isoform, expression, organ-specificity and

catalytic activity [44]. These differences are more important if the resulting metabolites have pharmacological or toxicological impact. A clear example found in the literature is the discordance response to ethylene glycol (EG) exposure between rat and rabbit. This is due to differences in rates of hepatic metabolism of EG to the toxic metabolite glycolic acid and limited transfer in the rabbit embryo relative to the rat [28]. In our study, additional details on metabolism that could explain species differences to compounds showing increased developmental toxicity are compiled in supplementary file. Much effort should be devoted to profile metabolic pathways, especially in the rabbit, in order to understand differences among species and improve predictivity for the human situation.

### 3.3. On-target/off-target effect based comparison

Only a subset of compounds (10 %) showed increased sensitivity of the developing fetus in one species not related to maternal toxicity (Class 1, table 4 and table 5) being selective developmental toxicants for the rat or rabbit. Given the higher number of available data a comparison with regard to the relation of on-target or off-target effects, cumulative species sensitivity was conducted for five groups of compounds (Figure 3). The results reflected the known species-specific sensitivity of rabbit to antibiotics. The reason for the higher sensitivity of rats to lanosterol 14 α-demethylase inhibitors is not known and due to the lack of data it is difficult to conclude if there are e.g. potential target sensitivity differences. In contrast, rat and rabbit did not show any differential sensitivity to COX-inhibitors, tyrosine kinase inhibitors or glucocorticoid agonists, even though it is been previously reported that rabbits are particularly sensitive to the teratogenic property of glucocorticoids leading to high incidences of cleft palate [45]. This information on overall species differences for groups of compounds could be useful to predict the relative responses of these species to previously untested compounds in such groups.

## 4. Conclusions

The study confirmed the overall similar sensitivity of developmental toxicity in rat and rabbits based on a non-confidential dataset and that a limited number of compounds exhibit a >10fold difference between rabbits and rats. A set of potential reference compounds that could be used to study the mechanistic basis of species differences or to develop alternative testing approaches that may capture differences between rat and rabbit was provided.

Some factors were identified that might be confounding in the assessment of interspecies differences in developmental toxicity, such as the interference of maternal toxicity and differences in pharmacokinetics. Understanding of these confounding factors would be valuable to understand in more detail what drives interspecies differences.

The list of reference compounds could be used to evaluate the potential of in vitro approaches, the culture of whole embryos of rodents and lower vertebrates (e.g. Xenopus and zebrafish) [46–48] computational or read across approaches [7, 49–51], or a combination of these to predict (human) developmental toxicity. Since human developmental toxicity data are limited to anecdotal, accidental or unintended adverse effects, in vivo animal results are at present considered as the reference for the screening and regulatory development of chemicals for human use. This lack of comparative human data has also driven the multiple test species approach, to capture the uncertainty and potential limited concordance between one of the test species with humans – albeit this may increase the number of false positives. Alternative testing approaches like in the lower vertebrate need to be validated as predictive for the in vivo animal models used later in the regulatory part of the development. Hence, the testing in a second species may not be waived until it is demonstrated that other approaches would reveal the same level of information on potential developmental toxicity. Albeit the number of reference compounds is at present limiting, future revisions of the data analysis would increase the basis. Industry and regulators could greatly support any effort to establish alternative testing strategies if confidential data would at least be made available to the scientific community.

## 5. Acknowledgements

Funding: This work was supported by a grant of the German Ministry of Education and Research to the project ZFminus1 (grant number 031A582).

We thank Prof. Aldert Piersma, Dr. Peter Theunissen and Stéphane Barbellion for critical comments on the manuscript. The authors also gratefully acknowledge the helpful comments offered by the anonymous reviewers.

## 6. Conflict of interest

The authors declare that there are no conflicts of interest. The views expressed in this article are those of the authors and do not necessarily reflect the views or policies of the company.

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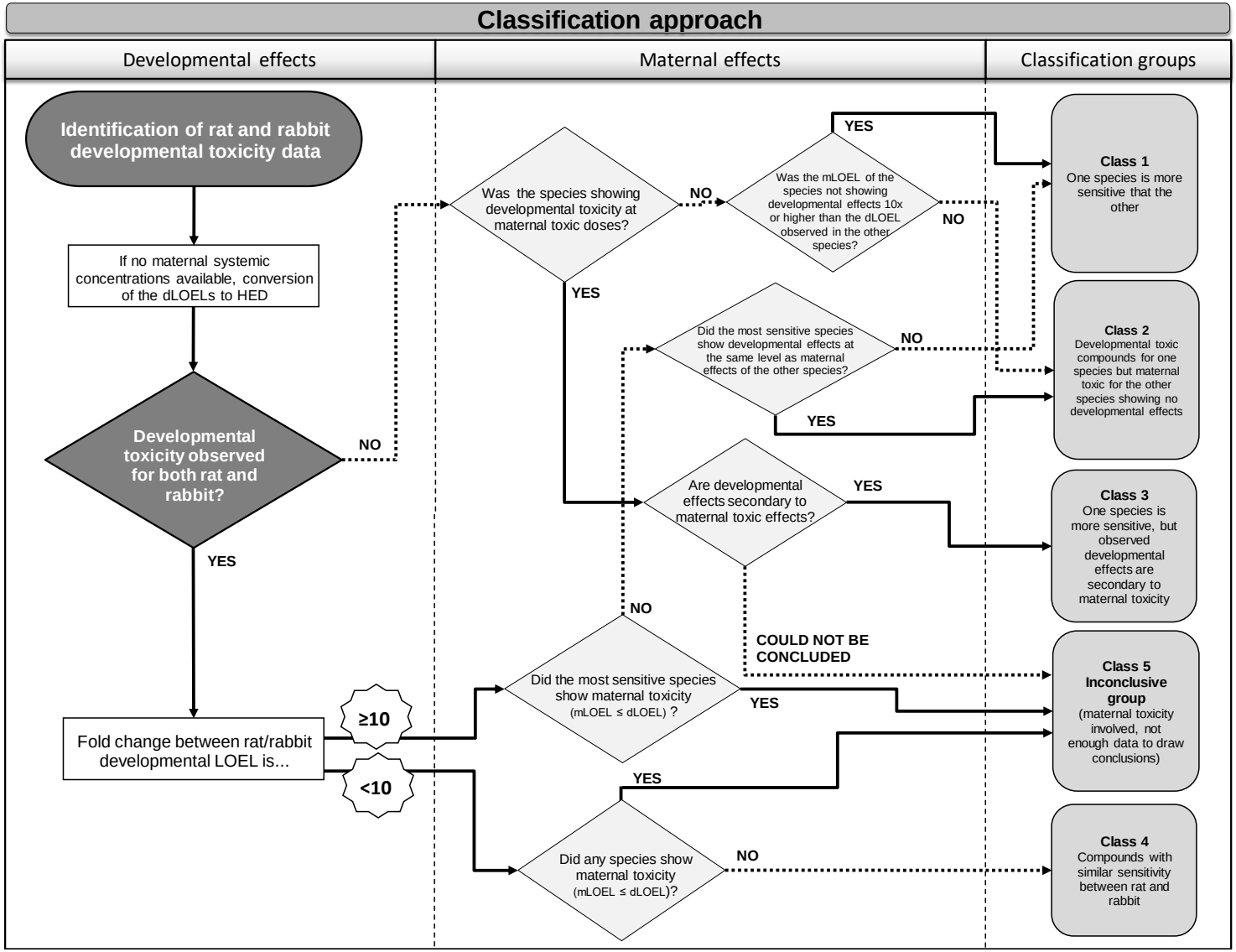
## Figures and tables

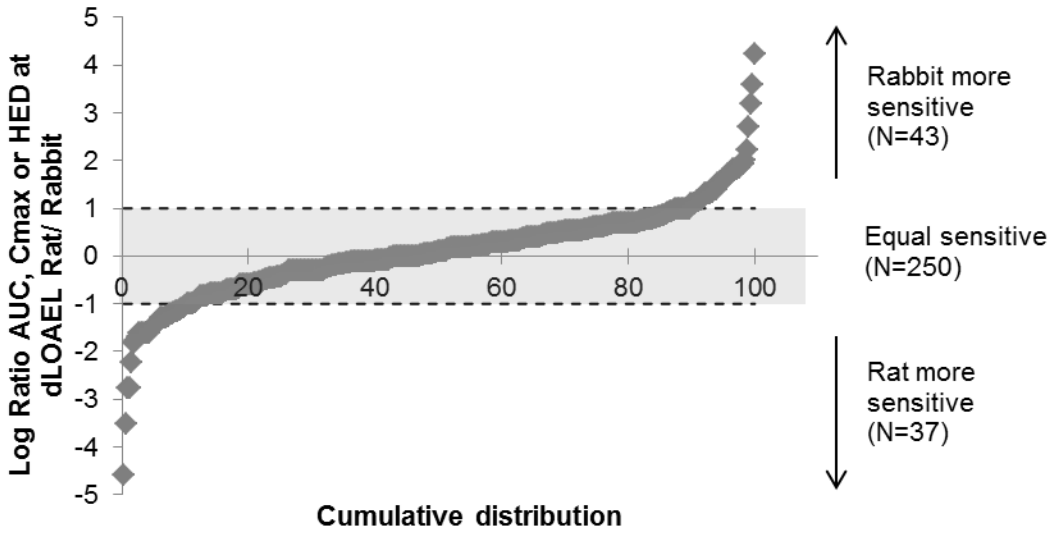
| |
|---|
| **Pesticides and veterinary drugs** |
| Summary Reports of Joint Meeting on Pesticides Residues (JMPR). |
| Concise International Chemical Assessment Documents (CICADs). |
| Joint Expert Committee on Food Additives (JECFA) - Monographs and Evaluations. |
| Screening Information Data Set (SIDS) for High Production Volume Chemicals. |
| European Medicines Agency (EMA) - Maximum residue limit assessment reports for active substances contained in veterinary medicines. |
| **Pharmaceuticals** |
| European Public Assessment Reports (EPAR) from the European Medicine Agency (EMA). |
| FDA drug approval package documents (Drugs@FDA). |
| Safety data sheets on pharmaceutical products available at the respective pharmaceutical company webpage. |
| Summaries of Product Characteristics at the electronic Medicines Compendium (eMC, Datapharm, UK) |

Table 1. Peer-reviewed data sources used




649

650 Fig. 1. Flow chart of the classification approach followed to compare the dLOELs between rat and rabbit. See supporting file, table 1
651 for the detailed description of the process.




652

653 Fig. 2. Range of sensitivity for developmental LOEL in rats and rabbits (n=330). Compounds, for
654 which no dLOEL was reached in the rat or rabbit (n=51 for the rabbit and n=52 for the rat), the
655 maximum concentration was used for rat and rabbit sensitivity comparison. The values were
656 sorted with values>0 indicating a stronger susceptibility of rabbits compared to rat. Differences
657 of the dLOEL (based on the AUC/Cmax or HED conversion) within 10fold between rat and
658 rabbit were considered as similar sensitive.

| | Number of substances | | |
|---|---|---|---|
| Species | Rat | Rabbit | **Total** |
| **Class 1. One species is more sensitive than the other** | | | |
| Fold change in developmental effect level ≥ 10 | 17 | 9 | **26** |
| Failure to detect developmental toxicity in the other species | 5 | 2 | **7** |
| **Class 2. Developmental toxicity in one species but maternal effects observed for the other species** | 17 | 11 | **28** |
| **Class 3. One species is more sensitive, but developmental effects are secondary to maternal toxic effects** | 1 | 7 | **8** |
| **Class 4. Compounds with similar sensitivity between rat and rabbit** | | 63 | |
| Similar sensitivity due to maternal plasma concentrations | | 6 | **78** |
| Similar sensitivity due to HED conversion | | 9 | |
| **Class 5. Inconclusive compounds** | | | |
| Maternal toxicity involved | | 155 | **183** |
| Not enough data to draw conclusions* | | 28 | |

659

660 Table 2. Distribution of the compounds analyzed among the different classes (N=330). * Studies
661 where the higher dose tested without developmental and maternal effects in one species was

10x lower than the dose inducing developmental effects in the other species or studies where dLOEL differs 10x or more but no NOAEL was reached for the less sensitive species. Comparisons were based on HED or maternal plasma concentrations if available.

| Compound | **Cas NR** | Fold change dLOEL external dose | Fold change dLOEL maternal systemic dose or *HED | Reference |
|---|---|---|---|---|
| | | Rabbit/Rat | Rabbit/Rat | |
| Fingolimod hydrochloride | 162359-56-0 | 15.0 | 0.5 | [52] |
| Voriconazole | 137234-62-9 | 10.0 | 4.9 | [53] |
| Cabozantinib (S)-maleate | 1140909-48-3 | 10.0 | 0.6 | [54] |
| | | Rat/Rabbit | Rat/Rabbit | |
| Alitretinoin (9Cis-retinoic acid) | 5300-03-8 | 10.0 | 1.5 | [55] |
| Mirabegron | 223673-61-8 | 10.0 | 1.5 | [56] |
| Entecavir[a] | 142217-69-4 | 20.0 | 2.3 | [57] |
| Triclabendazole | 68786-66-3 | 10 | 5* | [58] |
| Nicardipine Hydrochloride | 199119-58-9 | 10 | 5* | [59] |
| Lamivudine | 54527-84-3 | 10 | 5.0* | [60] |
| Trifloxysulfuron-Sodium | 134678-17-4 | 15 | 7.5* | [61] |
| Beclometasone Dipropionate | 5534-09-8 | 10 | 5* | [45] |
| Fluazinam | 79622-59-6 | 12.5 | 6.25* | [61] |
| Raloxifene Hydrochloride | 82640-04-8 | 10 | 5.00* | [62] |
| Butralin | 33629-47-9 | 11.1 | 5.6* | [61] |
| Naratriptan Hydrochloride | 121679-13-8 | 10 | 5* | [63] |

Table 3. List of compounds for which rat or rabbit showed differences related to absorption and/or kinetics. Apparent specific developmental toxicity (fold change dLOEL external dose between species > 10) but maternal systemic data or HED conversion indicated that species differences are likely related to differences in absorption and/or dose kinetics between rat and rabbit. [a]maternal systemic dose in the rabbit extrapolated.

| Compounds | CasNR | Pharmacological MoA | Rabbit (mg/kg/day) | | | Rat (mg/kg/day) | | | Route of exposure | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | dLOEL | mLOEL | dNOAEL | dLOEL | mLOEL | dNOAEL | | |
| **Pharmaceuticals** | | | | | | | | | | |
| Eletriptan | 143322-58-1 | Selective 5-hydroxytryptamine 1B/1D receptor agonist | 5 | >50 | <5 | 100 | 100 | 30 | po | [64] |
| Finafloxacin | 209342-40-5 | Fluoroquinolone antibiotic[1] | 1 | >9 | <1 | 100 | 500 | 30 | po | [65] |
| Gabapentin | 60142-96-3 | Gabamimetic agent | 60 | 1500 | <60 | 1500 | >1500 | 300 | po | [66] |
| Latanoprost | 130209-82-4 | Selective FP receptor PGF2α agonist | 0.005 | >0.3 | 0.001 | >0.25 | >0.25 | ≥0.25 | iv | [67] |
| Linagliptin | 668270-12-0 | Competitive and reversible DPP-4 enzyme inhibitor | 4 | 150 | <4 | 240 | 240 | 30 | po | [68] |
| Oxaprozin | 21256-18-8 | Selective cyclo-oxygenase inhibitor | 7.5 | N/A | <7.5 | 500 | N/A | 200 | po | [69] |
| Propafenone | 34183-22-7 | Sodium channel blocker | 15 | 150 | <15 | 600 | 600 | 270 | po | [70] |
| Tafluprost | 209860-87-7 | Selective FP receptor PGF2α agonist | 0.00003 | >0.0003 | <0.00003 | 0.01 | >0.030 | 0.003 | iv | [71] |
| **Pesticides** | | | | | | | | | | |
| Isoxaflutole | 141112-29-0 | HPPD inhibitors[2] | 5 | 100 | <5 | 100 | 500 | 10 | po | [61] |
| **Veterinary drugs** | | | | | | | | | | |
| Fenbendazole | 43210-67-9 | Tubulin binding | 63 | >63 | 25 | >2500 | >2500 | ≥2500 | po | [72] |
| Firocoxib* | 189954-96-9 | Selective cyclo-oxygenase inhibitor | 3 | 10 | 1 | 300 | >1000 | 3 | po | [73] |

Table 4. List of compounds for which the rabbit study showed to be more sensitive than the rat study (no effects in rat study were observed or fold change dLOEL was ≥ 10, based on maternal plasma concentrations (for linagliptin and finafloxacin) or HED (rest of compounds). The route of exposure is the same for rat and rabbit: po= oral, sc=subcutaneous, iv= intravenous.

[1]No target in mammals, [2]Target for plants. Abbreviations: FP, fluoroprostaglandin; DPP, dipeptidyl peptidase; HPPD, 4-Hydroxyphenylpyruvate dioxygenase. *Rat and rabbit showed no difference for the NOEL (fold change <10) for this compound, differences for the LOEL may have just been produced by selection of different concentrations for testing.

| Compounds | CasNR | Pharmacological MoA | Rabbit (mg/kg/day) | | | Rat (mg/kg/day) | | | Route of exposure | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | dLOEL | mLOEL | dNOAEL | dLOEL | mLOEL | dNOAEL | | |
| **Drugs** | | | | | | | | | | |
| Artemether | 71963-77-4 | Complexation with heme | 175 | >175 | 105 | 10 | >10 | 3 | po | [74] |
| Bicalutamide | 90357-06-5 | Androgen receptor antagonist | >200 | 200 | ≥200 | 10 | >10 | 1 | po | [75] |
| Cariprazine | 1083076-69-0 | D2 and D3 partial agonist | >5 | 5 | ≥5 | 0.5 | 2.5 | <0.5 | po | [76] |
| Caspofungin Acetate | 179463-17-3 | 1,3-beta-glucan synthase inhibitor[1] | 3 | 6 | 1 | 0.5 | >5 | >0.5 | iv | [77] |
| Clomifene Citrate | 7619-53-6 | Selective estrogen receptor modulator (SERM) | 20 | N/A | 8 | 1 | 40 | <1 | Po | [78] |
| Irinotecan | 100286-90-6 | DNA topoisomerase I inhibitor | 6 | N/A | 0.24 | 1.2 | N/A | 0.06 | Iv | [79,80] |
| Loxapine | 1977-10-2 | Dopamine antagonist and serotonin 5-$HT_2$ blocker | >10 | N/A | N/A | 1 | N/A | N/A | po | [81] |
| Miltefosine | 58066-85-6 | Inhibits cytochrome-c oxidase and protein kinase B | 6 | > 2.4 | 2.4 | 1.2 | >2.4 | 0.6 | po | [82] |
| Nilotinib | 641571-10-0 | Tyrosine kinase inhibitor | 300 | 300 | 100 | 30 | 100 | 10 | po | [83] |
| Olopatadine | 140462-76-6 | Histamine H1 antagonist | 400 | >400 | 100 | 60 | 600 | >60 | po | [84] |
| Ospemifene | 128607-22-7 | Selective estrogen receptor modulator (SERM) | >30 | 10 | ≥30 | 0.1 | 1 | <0.1 | po | [85] |
| Tiotropium bromide | 186691-13-4 | Muscarinic receptor antagonist | 0.05 | >0.05 | 0.011 | 0.007 | >0.139 | 7.50E-04 | inh | [86] |
| **Pesticides** | | | | | | | | | | |
| Atrazine | 1912-24-9 | Photosynthesis II inhibitor[2] | 75 | 75 | 5 | 5 | 25 | <5 | po | [61] |
| Diniconazole | 83657-24-3 | Lanosterol 14 alpha-demethylase (Cyp51) inhibition[1] | >30 | >30 | ≥30 | 1 | 20 | <1 | po | [61] |
| Fluazifop-P-Butyl | 79241-46-6 | ACCase Inhibitor[2] | 50 | 50 | 10 | 5 | 300 | 1 | po | [61] |
| Flumioxazin | 103361-09-7 | Protoporphyrinogen Oxidase (PPG oxidase or Protox) Inhibitor[2] | >3000 | 3000 | ≥3000 | 10 | 30 | 3 | po | [61] |
| Flusilazole | 85509-19-9 | Lanosterol 14 alpha-demethylase (Cyp51) inhibition[1] | 35 | 35 | 12 | 0.4 | 50 | 0.2 | po | [61] |
| Hexaconazole | 79983-71-4 | Lanosterol 14 alpha-demethylase (Cyp51) inhibition[1] | 50 | 100 | 25 | 2.5 | 250 | <2.5 | po | [61] |
| Molinate | 2212-67-1 | Inhibition of lipid synthesis[2] | 200 | 200 | 20 | 35 | 140 | 2.2 | po | [61] |
| Sulfentrazone | 122836-35-5 | Protoporphyrinogen Oxidase (PPG oxidase or Protox) Inhibitor[2] | 250 | 250 | 100 | 25 | 50 | 10 | po | [61] |
| Tralkoxydim | 87820-88-0 | ACCase Inhibitor[2] | 100 | 100 | 20 | 3 | 200 | 1 | po | [61] |
| Triadimenol | 55219-65-3 | Lanosterol 14 alpha-demethylase (Cyp51) inhibition[1] | >125 | 125 | ≥125 | 5 | 15 | <5 | po | [61] |

Table 5. List of compounds classified in class 1 for which the rat study showed to be more sensitive than the rabbit study (no effects in rabbit study were observed or fold change dLOEL ≥ 10, based on maternal plasma concentrations (for cariprazine, nintedanib and ospemifene) or HED (rest of compounds). Route of exposure is the same for rat and rabbit as indicated: po= oral, top= topical. [1]Target for fungi, [2]Target for plants. Abbreviations: ACCase, Acetyl CoA Carboxylase.

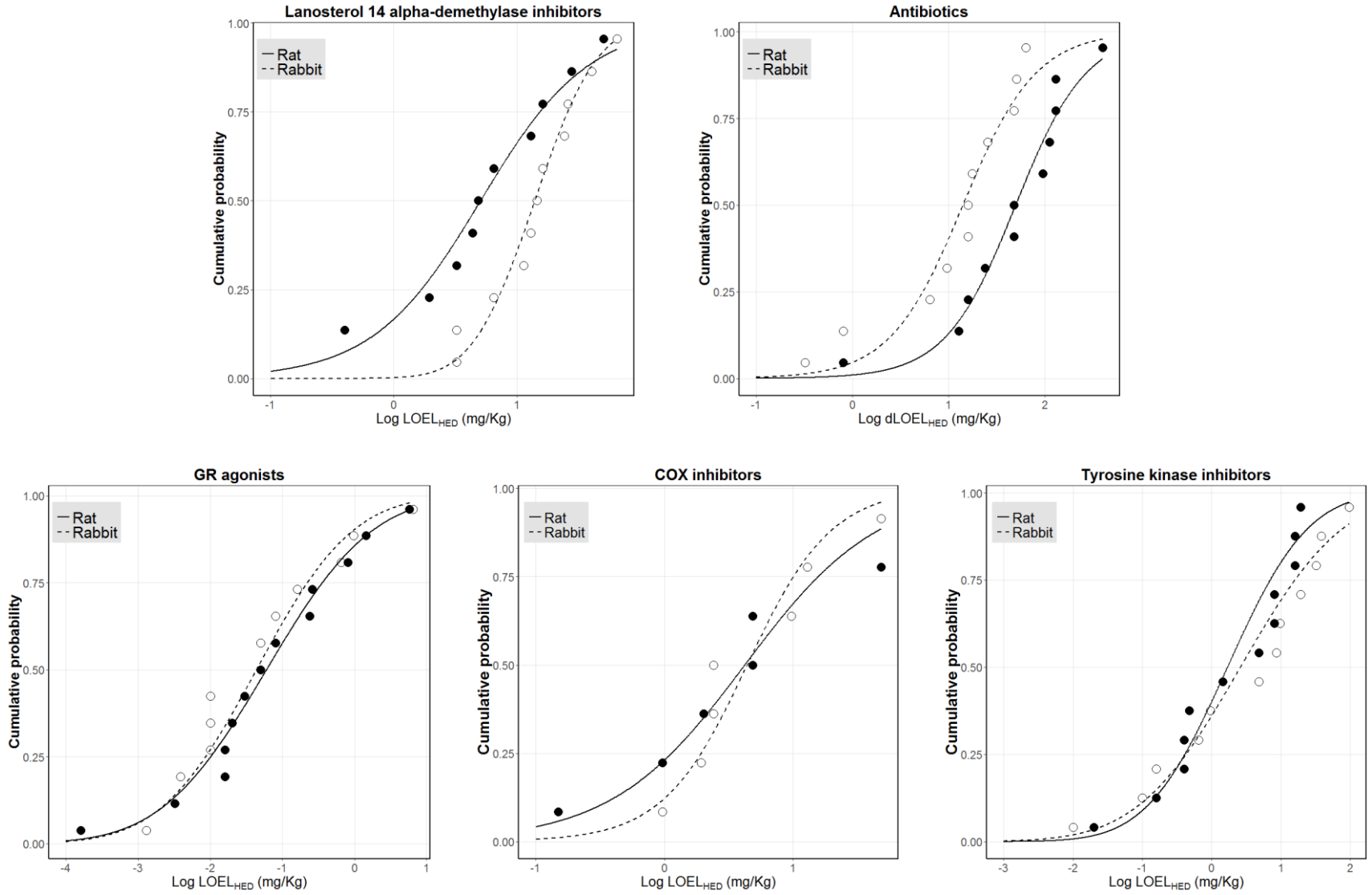



Fig. 3. Relative species sensitivity to specific chemical classes with the same mode of action (on-target activity) or off-target effects (antibiotics and lanosterol 14-alpha demethylase inhibitors). Cumulative distribution for compounds grouped into five different classes. (Data points are percentiles for the rat ● and rabbit ○