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Retrospective Mining of Toxicology Data to Discover Multispecies and Chemical Class Effects: Anemia as a Case Study

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Abstract

Predictive toxicity models rely on large amounts of accurate *in vivo* data. Here, we analyze the quality of *in vivo* data from the U.S. EPA Toxicity Reference Database (ToxRefDB), using chemical-induced anemia as an example. Considerations include variation in experimental conditions, changes in terminology over time, distinguishing negative from missing results, observer and diagnostic bias, and data transcription errors. Within ToxRefDB, we use hematological data on 658 chemicals tested in one or more of 1738 studies (subchronic rat or chronic rat, mouse, or dog). Anemia was reported most frequently in the rat subchronic studies, followed by chronic studies in dog, rat, and then mouse. Concordance between studies for a positive finding of anemia (same chemical, different laboratories) ranged from 90% (rat subchronic predicting rat chronic) to 40% (mouse chronic predicting rat chronic). Concordance increased with manual curation by 20% on average. We identified 49 chemicals that showed an anemia phenotype in at least two species. These included 14 aniline moiety-containing compounds that were further analyzed for their potential to be metabolically transformed into substituted anilines, which are known anemia-causing chemicals. This analysis should help inform future use of *in vivo* databases for model development.

Keywords

in vivo; pathology; database; anemia; uncertainty; bioinformatics; aniline

Introduction

The field of predictive toxicology aims to integrate different data streams, from *in vitro* effects to information on chemical structure and physicochemical properties, in order to make accurate predictions of adverse health outcomes associated with chemical exposure. To develop, evaluate, and apply better predictive methods, rich and reliable sets of *in vivo* toxicity data are needed. Significant effort has gone into developing these reference data sets

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through projects such as HESS (Hazard Evaluation Support System) (Sakuratani, Zhang et al. 2013), RepDose (Bitsch, Jacobi et al. 2006), NTP CEBS (National Toxicology Program Chemical Effects in Biological Systems) (NTP 2011), COSMOS (COSMOS 2016), REPROTECT (Hareng, Pellizzer et al. 2005) and the U.S. EPA Toxicity Reference Database (ToxRefDB) (U.S. EPA 2008, Knudsen, Martin et al. 2009, Martin, Judson et al. 2009, Martin, Mendez et al. 2009). Used uncritically, however, these databases can be problematic as sources of *in vivo* anchors for predictive modeling. Here we describe some of these issues, using ToxRefDB as a referent data source, and demonstrate approaches to data curation by examining chemically-induced anemia as a case study.

Toxicological pathology databases vary widely in design and data structure, and it is important to understand potential strengths and weaknesses of an extracted data set prior to analysis. Some key considerations include the following:

(1) Experimental variability.

Even when animal studies follow well-established guidelines (e.g., those developed by the U.S. EPA or the Organisation of Economic Co-operation and Development (OECD)), there are inevitable sources of variation at the experimental level. Some of these factors are known and accepted (e.g., variability among species and strains, or within individuals of the same species or strain, age gender), while others are suspected but whose consequence is not well known (e.g., exact details of diet, sample collection, and animal handling protocols) (Claassen 1994).

(2) Statistical issues.

Studies (one chemical in one protocol) are rarely repeated due to cost and animal welfare concerns, thus experiment-to-experiment variability is rarely characterized. Often, the number of animals used in each dose group is insufficient to observe rare events (statistical power) (Bailey, Thew et al. 2014), and relatively few doses and time points are used, meaning that effects that are only manifested in particular regions of time-dose space may be missed (Martin and Li, in preparation). Conversely, larger studies with many potential endpoints may increase type I errors (false positive rate) resulting from multiple tests (Fisher and van Belle 1993).

(3) Reporting bias.

It can be difficult to distinguish a negative result from an endpoint that was not evaluated. In guideline studies, specified effects should be examined, and any treatment-related effects recorded. However, in some cases there may be insufficient documentation of negative findings. In other cases, observed effects not specified by the guidelines may be sporadically recorded when seen. Recording bias may also occur when a higher-grade effect (e.g., carcinoma) is seen and an associated less severe or benign effect (e.g., hyperplasia) is ignored, or not recorded, or recorded in a limited way

(4) Observer bias.

Pathology is a largely interpretive science that is subject to inter-observer variability (Marchevsky and Wick 2011). These differences can relate to variation in diagnostic

thresholds or experience across study pathologists and use of peer review procedures, all of which can affect lesion incidence. Similarly, there may be differences in sampling intensity or the number of sections examined for particular tissue sites when not explicitly mandated by study guidelines (Marchevsky and Wick 2011).

(5) Diagnostic drift.

Chemical or pharmaceutical databases typically include studies that have been performed in many different laboratories over a period of many years. This can lead to variability in the methodologies used (e.g., for clinical pathology readouts) and terminology used to describe pathologic effects (Haschek, Wallig et al. 2010). While this can be managed to some extent by the use of controlled vocabulary or defined ontologies onto which the original effect descriptions can be mapped, there are cases where diagnoses shift over time and one original term is split into two, so that the exact effects seen in earlier studies are obscured (Haschek, Wallig et al. 2010).

(6) Level of review.

Treatment-related effects present in a database may not ultimately represent the final calls determined after regulatory or committee review (Dorato and Engelhardt 2005). For example, some outcomes, while statistically different from the concurrent control group (and thus captured by the database), may simply be due to age-related variation or some other experimental factor and not included in the final assessment. In other cases an effect may be considered treatment-related but not adverse.

(7) Data assimilation and analysis.

In the process of transcribing data from notebooks or spreadsheets to initial study reports to published study summaries to a multi-chemical database (including enforcing controlled vocabularies), there will inevitably be transcription errors and missed data. Further discrepancies in chemical names, identifier codes, or other study records, even if minor, can lead to mistakes during data extraction, collation, and analysis.

Users of databases of *in vivo* effects must recognize that the information is confounded by these numerous sources of variation and potential error. While these issues may not necessarily affect the utility of these studies for their original purpose of evaluating a single compound, they do create issues when trying to compare results across chemicals at the level of individual effects. Several related challenges, including interpretation of the biological significance of responses at the level of the study, were also highlighted in a recent case study of reproductive toxicity using ToxRefDB (Plunkett, Kaplan et al. 2015).

In this study we critically examine some of these issues using data from ToxRefDB, which is largely composed of guideline studies for pesticidal active ingredients. Our case study focuses on chemically-induced anemia. Anemia (or decreased red cell mass) is defined by a decrease in the ability of blood to carry oxygen and typically characterized by a lower number of red blood cells (RBC), hemoglobin levels (HGB), and/or hematocrit (HCT) (Kaushansky, Lichtman et al. 2010). These and other hematological parameters are measured using largely automated procedures as part of standard protocols for subchronic

and chronic animal studies. The basic hematology protocols and terminology have been largely stable for many decades (Coller 2015), and the use of quantitative data avoids many of the interpretive issues that may affect analyses of subjective endpoints, such as histopathology data. Using ToxRefDB, we explore how these issues affect the ability to draw conclusions about the toxicity of chemicals, using anemia as a case study. In particular, we explore the issue of cross-species hematological effects. This information may provide an important piece of evidence in deciding on the adversity or biological importance of study findings. For clinical pathology measures, it is often difficult to determine whether a change is treatment-related and, if so, whether it represents an adverse biological effect relevant to human health. The presence of anemia in more than one species could thus add weight to selection of this endpoint in the final integrative human health assessment. We stress that this work is not intended to imply that anemia due to exposure to environmental chemicals is necessarily of significant human clinical concern, only that it provides a convenient case study for understanding the strengths and limitations of mining a large *in vivo* database.

Methods

Database:

We used data from the U.S. EPA ToxRefDB, extracted August 2014. ToxRefDB compiles treatment-related effects at the dose-group level for guideline or guideline-like studies. The large majority of studies analyzed here were submitted by companies registering pesticidal active ingredients. In this case, the registrant provides the U.S. EPA with the original study document, which is treated as confidential business information (CBI). The U.S. EPA then reviews the original study and develops a Data Evaluation Record (DER), which summarizes the key findings, assesses data quality, defines critical effects, and often provides Low / No Observed Adverse Effect Level (LOAEL / NOAEL) values (at the study level). In principle, the DERs can be made public, but they may not be publicly available. For each study (one chemical in one protocol), all treatment-related effects in each dose group were recorded in ToxRefDB. Note that the DERs and other study documents were written over 30–40 years by many reviewers and authors with various levels of expertise, all of which impacts treatment-related and critical effect determinations. Additionally, there can be omissions or transcription errors at several steps, from study notebooks to the original study report, from the study report to the DER, and from the DER to the database. ToxRefDB has been extensively documented (U.S. EPA 2008, Martin, Mendez et al. 2009), including descriptions of the data entry and data quality control procedures. A subset of studies used here were from other sources, including from the NTP and open literature. For this analysis, we used data from studies in rat, mouse and dog for chronic (CHR, equivalent or closely related to 870.4100, 870.4200 and 870.4300 protocols) and subchronic studies (SUB, equivalent or closely related to protocol 870.3100). Only studies that met the “guideline acceptable” criteria were used (Martin, Mendez et al. 2009). Other study types occasionally had hematology data (e.g. multigenerational reproductive or developmental studies), but the coverage was so sparse that these were excluded. The number of chemicals covered by the study types are: rat CHR (446), rat SUB (438), mouse CHR (376) and dog CHR (259). The database download used for this study (August 2014 public release) is given in Supplemental File S1.

Definition and Selection of Effects:

ToxRefDB characterizes effects into a hierarchy of [Species] : [Study Type] : [Effect Type] : [Effect Target] : [Effect Description] : [Direction]. Study Type is one of CHR, SUB. Effect types used are In-Life Observations, Organ Weight, Pathology (Clinical), Pathology (Gross), Pathology (Neoplastic) and Pathology (Non-neoplastic). There are a large number of effect targets, including individual organs and results of clinical observation or analytical techniques. Prominent non-organ effect targets are Clinical Signs, Food Consumption, Water Consumption, Body Weight, Clinical Chemistry, Urinalysis, Hematology and Mortality. There are a large number of effect descriptions (7138 across all study types) that offer the most detailed definition of what was observed. Direction is either increase or decrease. An example of an overall effect is “Rat : CHR : Pathology (Clinical) : Hematology Mean Corpuscular Hemoglobin (MCH) : Increase”.

Data preparation:

ToxRefDB contains data from studies that were run in many different labs over approximately 40 years, meaning that there have likely been changes in diagnostic terminology that will need to be reconciled. Additionally, there are multiple steps of data transcription from the initial study report to the public ToxRefDB version, so that some level of errors are present. These range from spelling or coding mistakes (including subtle issues such as capitalization and addition of extra spaces) that can make direct comparison from one study to the next problematic. To address these issues, software was developed to create all composite endpoints for each effect and to clean these by replacing variant terms with standard ones, including correction of spelling mistakes, extra spaces, etc. This process could have been carried out manually, but by implementing with software, the process can be repeated with later database releases, with documentation of changes made by the software. The complete list of endpoints is given in the Supplemental File S2. For the current anemia case study, a subset of the total list of effects was selected and in some cases mapped to common terms that would be more informative for identifying effect groupings or patterns. For instance, there are many terms for specific findings that point to bone marrow hyperplasia. This final mapping is contained in the Supplemental File S3. All composite endpoints were then mapped to a set of anemia “effect classes,” listed in Table 1. These are the effects used in the clinical classification of anemia.

A common cause of anemia in toxicity studies is chronic stress or systemic illness, often described clinically as “anemia of chronic disease” (Weiss and Goodnough 2005, Everds, Snyder et al. 2013, Everds 2015). To account for this type of anemia, we track the doses at which anemia-related effects are seen, and the doses in the same studies where a statistically significant loss in body weight or increase in mortality is seen. For each effect class for each chemical, a score is calculated which is equal to 0 (effect not seen), 1 (effect seen at or above the dose where significant body weight loss is seen), or 2 (effect is seen at doses below where body weight loss is seen). We make the assumption that the standard anemia-related effects were assessed in these guideline-like studies (*i.e.*, no survey or diagnostic bias), but this assumption is not always true and will account for some fraction of discrepancies observed. The doses are the “Lowest Effect Level” or LEL values, which indicate the lowest dose where the effect was determined to be statistically significantly

changed relative to that in the control group, and/or to be treatment-related by the DER author. Note that the LEL for anemia may or not be the ultimate LOAEL for the study.

Review of Primary Reports:

All studies were classified as positive (two or three of RBC, HGB, or HCT significantly decreased in the same dose group); ambiguous (one of RBC, HGB, or HCT significantly decreased in a dose group); or negative (no dose group showed a statistically significant decrease in any of the three anemia markers). We assume that these three variables are correlated, and by requiring at least two to be changed, decrease the rate of false positives. All study reports (DERs and primary literature papers) were manually reviewed for each study for each chemical with at least one study showing anemia (positive class). This review was carried out to check for causes of discrepancies in anemia classification between studies of the same chemical. If the database classification (positive, ambiguous, or negative) was inconsistent with the text of the original report, a note was made. Studies were placed into three classes: (1) the manual review agreed with the database (classification would not change); (2) the manual review found some discrepancy with the database, but the difference would not have changed the call of positive, ambiguous, or negative; (3) the manual review uncovered a reporting error that would have changed the classification (e.g., negative to positive). The workflow first reviewed each DER to ensure that the database was correct or not regarding statistically significant changes in these parameters. We also checked for missing data. In some cases the standard hematology parameters were not measured in a study and therefore were assumed to be negative in the database. Note that the DER authors were not consistent in including statistically significant results (e.g., decreases in RBC, HGB, or HCT) if they had determined that these changes were of no toxicological importance. A second round of review was then run to fill in missing parameters for those chemicals that had been missed in the original reviews to populate ToxRefDB (i.e. missing data was taken from the DERs and entered into the project data files). This review was carried out for all studies of all chemicals with at least one positive study. In addition to filling in effect dose values, notes from the reviewer were transcribed if they pertained to the cause or mechanism of anemia (e.g., hemolysis).

Anemia decision tree:

The anemia decision tree (Figure 2) was developed by one of the authors, a trained pathologist (CW).

Structure groupings:

The structural groupings in Figure 4 were carried out by visual inspection by a trained chemist (RJ).

Reaction schema diagrams:

The reaction schemas describing the metabolic pathways were either taken from the cited literature sources or derived based on the predicted metabolites produced using the rat liver S9 metabolism simulator which is implemented in the OECD Toolbox v3.3 (<https://www.qsartoolbox.org/>). The publicly available OECD Toolbox is designed to assist users in

the development, evaluation, justification, and documentation of chemical categories and to provide support for “read-across” chemical safety assessments within those categories following recent OECD grouping guidance (OECD, 2014). The OECD Toolbox contains a number of chemical profiling schemes for comparing substances on the basis of their mechanistic similarity in relation to various types of toxicity. Some of the profilers consist only of broad categories, whereas others contain endpoint-specific rule-bases that consist of structural alerts as are contained in knowledge expert systems, such as Derek Nexus. In addition, the OECD Toolbox contains a number of degradation and metabolism simulators.

The rat liver S9 metabolism simulator was developed by the Laboratory of Mathematical Chemistry (LMC) at the University As Zlatarov, Bourgas, Bulgaria. This approach represents a set of 509 structurally generalized, hierarchically arranged biotransformation reactions, which are characteristic for the metabolism for *in vitro* experimental systems such as rodent (mostly rat) liver microsomes and S9 fraction. The principal applicability of the simulator is associated with the reproduction as well as the prediction of the metabolic activation reactions and pathways of xenobiotic chemicals, which may elicit *in vitro* genotoxicity effects such as bacterial mutagenicity and chromosomal aberrations. Each transformation in the simulator consists of source and product structural fragments and inhibiting “masks.” A probability of occurrence is ascribed to each principal transformation, which determines its hierarchy in the transformation list. A training set of xenobiotic chemicals of a wide structural diversity, with experimentally observed metabolic reactions and pathways, was built using published data on their metabolism in rodent liver microsomes and S9 fraction, as taken from primary articles, monographs, or websites (Kolanczyk et al., 2012). The organic compounds in the training set belong to different classes of industrial chemicals, including single and fused-ring arenes, phenols, haloalkanes and haloarenes, aromatic and aliphatic amines, nitroarenes, alkanes and cycloalkanes, alkenes, ethers, carboxylic acids and their derivatives, halogenated hydrocarbons, alcohols, epoxides, N-nitrosoamines, and azo chemicals. The molecular transformations set consists partly of 25 – 30 abiotic and a few enzyme-controlled reactions that occur at a very high rate compared to the duration of the tests, and the highest priority is assigned to these reactions. This subset of reactions also includes transformations of highly-reactive functional groups and intermediates, such as tautomerizations and arene epoxide rearrangements to phenols. Overall, the simulator contains 450 – 470 enzymatic phase I transformations and 15 – 20 enzymatic phase II transformations. The reported average sensitivity and predictivity of the simulator are 81.6% and 39.1% respectively (Kolanczyk et al., 2012; Mekenyan et al., 2012; Serafimova et al., 2007). Within the Toolbox platform, a list of metabolites simulated are provided but without any additional information on the relative proportions or rates.

Results

The database included a total of 658 chemicals tested in one or more studies (CHR in rat, mouse, or dog; SUB in rat). There were 1738 total studies, of which 1224 (70%) showed no treatment-related decrease in any of the three anemia parameters, 73 (4%) showed a decrease in a single parameter, 117 (7%) showed a decrease in two parameters, and 324 (19%) showed a decrease in all three parameters. Some basic trends in the data are illustrated in Figure 1. For example, anemia was seen more often in the SUB compared to

CHR rat studies. One potential reason is that the SUB studies (first three rows from the top) often include higher doses, as shown in Figure 1 (bottom). In the CHR studies, anemia was more common in dog studies compared to rat or mouse studies, despite the fact that the upper doses tested in the dog studies tended to be below those in the rat or mouse CHR studies (and the fact that dog studies typically have fewer individuals). Using a proportion test, the fraction of positive studies in dog CHR or rat SUB are statistically significantly greater ($p < 0.005$) than in the mouse CHR or the rat CHR. There is no statistically significant difference in the fraction of positive studies between the mouse CHR and rat CHR, or between the dog CHR and the rat SUB studies. There is not a correlation between the maximum tested dose (MTD) and the likelihood that anemia will be seen in the absence of body weight decrease (a possible confounder). Finally, the majority of chemicals causing anemia do so at doses where body weight loss is seen. The information on this per-chemical classification is given in Supplemental File S4. Because of the many indirect factors that may contribute to anemia in the face of significant body weight loss, including dietary intake, stress, and systemic disease, we focused much of the subsequent analyses on chemicals in which anemia occurred at doses below those at which body weight loss was observed.

Concordance Prior and Subsequent to Manual Review

We next examined the concordance of anemia between one study type and another, prior to the manual review. Here we use concordance as a general term meaning that the anemia call (positive or negative) is the same in two studies for the same chemical tested over approximately the same dose range, regardless of whether the two studies use the same species (mouse, rat, or dog) or time frame (chronic or subchronic). This allowed us to assess the impact of the data quality review. Table 2 shows the concordance rate where the same chemical was run in both studies, and the top tested concentrations in the two studies were within a factor of two. From this analysis, we can see that there is relatively high concordance between certain pairs of study type. The low concordance rates at the bottom of the table reflect the different overall positive rates stratified by study type (Figure 1).

The next step was to carry out a manual review of all studies for each chemical with at least one positive study (two or more parameters decreased out of RBC, HGB, and HCT). A total of 429 study reports (25% of all studies) were manually reviewed across 70 chemicals (11% of all chemicals). Table 3 summarizes the concordance both before and after database cleanup. A total of 6% of studies (24 of 429) did not measure (or report) hematology data. This missing information is notable given that hematology data are standard for these guideline or guideline-like studies. Of the remaining 163 studies that were initially negative, 11 (6%) had a statistically significant decrease in one of the anemia parameters, and 24 (14%) had decreases in two or three of the parameters. In most of these cases, the author of the study summary (DER) made a statement to the effect that any changes in hematology values were of no toxicological significance. The person transcribing from the summary statement of the DER into ToxRefDB may have then failed to investigate further details of the report to determine if there were indeed any statistically significant changes, regardless of their biological significance. Of the 218 studies that were initially positive, 4 (2%) switched to the ambiguous class and 2 (1%) switched to the negative class after manual

review. In most of these cases, there was a change in one or more anemia parameters (RBC, HGB, or HCT) noted in the study report, but it was not statistically significant. Of the 24 ambiguous cases, 6 (25%) moved to the positive class after the review discovered one or two other parameters that were statistically significantly decreased, and 2 (1%) switched to the negative class because the single effect noted in the database was discovered to be not statistically significant. Overall, 97% of positive findings were confirmed. A number of statistically significant effects were missed. In the manual review, we observed that the majority of these were deemed by the study summary authors to be of no biological or toxicological significance. Manual curation resulted in significant improvements in the concordance from one study type to another, as shown in Table 2. This effect was mainly driven by the relatively large number of studies that changed from an initial negative or ambiguous call to a positive call.

Chemicals positive for anemia in multiple species

After this review process, there were 49 chemicals positive for anemia in at least two species. These chemicals are summarized in Table 4, and full details are provided in Supplementary File S5. Anemia-related literature on each chemical is provided, along with a summary of the classifications from the decision tree in Figure 2. The term “MIX” indicates that at least one study was classified as non-regenerative and at least one as regenerative anemia. (Regenerative anemia is characterized by the loss of RBCs due to hemorrhage or hemolysis in which there is a compensatory response by the bone marrow, while non-regenerative anemia is characterized by decreased red cell mass without increased production of red cells in the bone marrow.) Note that there are many chemicals with a finding of regenerative anemia in one study type and non-regenerative anemia in another. Specific examples will be described below. Overall, 14 of the 49 chemicals (29%) have literature data on anemia. Note that this does not count the paper by Leet et al. in which they tested 10 of these chemicals for anemia in zebrafish (Leet, Lindberg et al. 2014); the only one of these chemicals that caused anemia in their screen was butafenacil. Figure 3 shows the distribution of anemia-related endpoints across the studies.

We observed differential species sensitivity, both in overall activity and in potency (Supplementary Files S5 and S6). Our analysis is the first report of cross-species anemia (defined here as positive for at least two of the three anemia metrics) for 70% of these chemicals. However, only 6 of these chemicals show multi-species anemia below the doses where significant body weight loss and systemic toxicity occurs. This group includes metiram zinc, molinate, and 4 aniline-related compounds (novaluron, fenhexamid, propanil and flufenacet), described below.

Within the larger set of 49 chemicals, there are several sets of structurally related compounds including the following: (1) the molting hormone agonists methoxyfenozide and tebufenozide; (2) carbamates methomyl, thiodicarb and metiram-zinc; (3) aryloxyphenoxypropionic herbicides clodinafop-propargyl and fluazifop-butyl; (4) organothiophosphates fosthiazate and tribufos; (5) pyrethroids etofenprox and pyriproxyfen; (6) phenyl-nitro containing compounds benfluralin, lactofen, oryzalin and oxyfluorfen; and (7) aliphatic amino-thio compounds ametryn, maneb, methylene bis(thiocyanate) and thiram.

The observation of anemia in structurally similar chemicals in multiple species supports the idea that this finding is a direct toxicity outcome and not a secondary effect or artifact of specific studies.

Analysis of aniline-containing chemicals

The primary group of interest was a set of 14 chemicals containing an aniline-like moiety. The structures of these chemicals are shown in Figure 4, with anemia classification for group A listed in Table 5. Out of the complete set of 658 chemicals evaluated, 81 contain an aniline moiety, and 48 of these are anemia positive in at least one study (59%). Of the non-aniline containing chemicals, 269/577 caused anemia in at least one study (47%). (See Supplemental Table S4.) The odds-ratio for anemia for chemicals with an aniline moiety is 1.66 (one-sided Fisher's exact test $p=0.022$). For chemicals that were anemia-positive in 2 or more species, 29/81 aniline-containing compounds are positive (36%), while 82/577 non-anilines are positive (14%). The odds ratio of anemia for chemicals with an aniline moiety is 2.69 ($p=0.0003$).

Not only do these constitute a significant fraction of the total set (14/49, 28%), they also contain 4 out of 6 (67%) of the chemicals that cause anemia below doses where body weight decrease is seen. In addition, they contain 5 out of 6 of the chemicals annotated as causing heme oxidation. Figure 5 shows dose-response "lane plots" for 4 chemicals (novaluron, fenhexamid, propanil and flufenacet) showing the relative doses where the anemia-related effects are seen. Since aniline and chloroanilines are known to cause anemia (<http://www.atsdr.cdc.gov/mmg/mmg.asp?id=448&tid=79>), we investigated whether there is evidence for these chemicals having (substituted) anilines as metabolites. All 14 aniline containing chemicals were profiled using the *in vitro* rat liver S9 metabolism simulator within the OECD Toolbox in conjunction with the National Institute of Technology and Evaluation (NITE) repeated dose HESS profiler (Sakuratani, Zhang et al. 2013) to systematically evaluate whether formation of an aniline metabolite was a plausible explanation for the anemia observed. The HESS profiler contains 61 category boundaries which are akin to Structure Activity Relationships (SARs) for a number of repeated dose toxicity effects including anemia. These category boundaries were developed based on repeated dose toxicity test data in the database underpinning HESS. Most of the positive studies for the aniline-containing chemicals from Figure 4 are classified as regenerative, with several being further classified as hemolytic or heme oxidation, which is a common mechanism of action of anilines (Kiese 1966, Bus and Popp 1987, Sabbioni 1992).

Group A consists of 4 structurally similar analogs: carboxin, propanil, fenhexamid, and novaluron, as shown in Figure 4 and described below.

- Carboxin (5234-68-4) is predicted by the ACD Inc. tautomers algorithm (<http://www.acdlabs.com>) to tautomerize from an imidic acid form to its amide major form. As a parent structure, the repeated dose HESS profiler flags carboxin for potential liver effects based on the sulfide moiety. However, carboxin is predicted by the *in vitro* rat liver S9 simulator to hydrolyze to release aniline, which subsequently can be transformed to cause methemoglobinemia. The proposed pathway, as described in the HESS alert, begins with oxidation of aniline by

P450 enzymes to N-hydroxyl aniline (Kiese, M. (1966), Bus, J. S. and J. A. Popp (1987), Sabbioni, G. (1992)). The N-hydroxylaniline reacts with hemoglobin in erythrocytes to produce nitrosoaniline. Nitrosobenzene reduction back to the N-hydroxylamine leads to the formation of methemoglobin. The nitrosobenzene has also been implicated in the selective splenic toxicity exhibited by aniline and structurally related compounds (Bus and Popp 1987). It has been suggested that the nitrosobenzene binds to erythrocyte proteins, and that damaged erythrocytes accumulate in the spleen, leading to local oxidative DNA damage and ultimately the formation of tumors. Erythrocyte overload and the build-up of the aniline, its metabolites and erythrocytic debris in the spleen have been suggested to contribute to the mechanism. Bus and Popp propose that such splenic tumorigenicity is species- and sex-specific and occurs only above a threshold dose. Male rats, as opposed to female rats and male and female mice, have been found to be more susceptible to the development of splenic tumors following administration of aniline, p-chloroaniline and o-toluidine (Bus and Popp 1987). The metabolic route is illustrated in Scheme I.

- Propanil (709-98-8) is predicted using the profilers within the OECD Toolbox to undergo a similar pathway to that of carboxin to result in the formation of 3,4-dichloroaniline and propanoic acid, as illustrated in Scheme II. In the repeated dose HESS profiler within the OECD Toolbox, there are 29 monocyclic anilines, including m-chloroaniline and p-chloroaniline, with findings related to hemolytic anemia. Based on the repeated dose toxicity studies collected, it is noted in the category rationale that hemolytic anemia is frequently cited as the primary reason for setting a study NOAEL. The predictions for propanil are complementary to the experimental findings described later.
- Fenhexamid (126833-17-8) is predicted by ACD Inc. to tautomerise to form its corresponding amide, which can then hydrolyze (per the *in vitro* rat liver S9 simulator predictions) to release an aminophenol as shown in Scheme III. Ortho- and para-aminophenols are thought to cause hemolytic anemia with methemoglobinemia as a result of activation. Ortho- and para-aminophenols can oxidize to quinoneimines in the blood in the presence of oxygen. These quinoneimines oxidize hemoglobin to methemoglobin (Kiese 1966). Example substances that do cause hemolytic anemia in this manner include para-aminophenol (123-30-8) and 2-amino-4-chlorophenol (95-85-2) which are both listed as part of the alert description in the repeated dose HESS profiler.
- Novaluron (116714-46-6) is able to undergo amide hydrolysis to form the corresponding substituted aniline, as shown in Scheme IV per the *in vitro* rat liver S9 simulator. The remaining pathway would then be the same as that described for carboxin (i.e., oxidation to an N-hydroxyl aniline and subsequent formation of a nitrosoaniline).

Group B is comprised of indoxacarb, flufenacet, acetochlor, and linuron.

- The metabolic pathway in rats for indoxacarb (173584-44-6) is shown in part in Scheme V (adapted from (Mueller and Moretto 2005)). The major metabolites

formed are JT333 and 5HO-JW062. Neither of these metabolites was predicted by the *in vitro* rat liver S9 simulator contained within the OECD Toolbox. The predicted metabolites for indoxacarb do not correspond with the experimental findings. If the major metabolite JT333 is considered in terms of how it could undergo further transformations, a hydrolysis transformation can be predicted by the *in vitro* rat liver S9 simulator, which would then result in the formation of a substituted aniline. This substituted aniline could then undergo further transformation to result in hemolytic anemia (Scheme VI).

- Flufenacet (142459-58-3) is flagged by the repeated dose HESS profile to induce adverse effects in the liver based on the presence of a thiocarbamate moiety. The *in vitro* rat liver S9 simulator predicts a number of metabolites that could form from several competing pathways. One of these involves an N-dealkylation of the amine group with subsequent amide hydrolysis to yield 4-fluoroaniline which could then become undergo further transformation to cause hemolytic anemia (Scheme VII).
- According to the *in vitro* rat liver S9 simulator, acetochlor (34256-82-1) can undergo oxidative N-dealkylation, followed by amide hydrolysis to release a substituted aniline which could then undergo subsequent activation to cause hemolytic anemia. This is corroborated by the experimental findings discussed later (Scheme VIII).
- Linuron (330-55-2) is expected to undergo an amide hydrolysis to release 3,4-dichloroaniline, which can cause hemolytic anemia (Scheme IX).

Group C is comprised of desmedipham and chlorpropham.

- Desmedipham (13684-56-5) has the potential to undergo ester hydrolysis followed by decarboxylation to release aniline or 3-hydroxyaniline (Scheme X).
- Chlorpropham (101-21-3) is also predicted to undergo an ester hydrolysis followed by decarboxylation to release 3-chloroaniline. The anemia effects reported are consistent with the formation of this metabolite (Scheme XI).

Group D is comprised of Bifenazate, Diphenylamine, Fenamidone and Triflumizole.

- Bifenazate (149877-41-8) is likely to oxidize to a diazene before releasing a biphenyl. No plausible explanation can be offered to rationalize the anemia observed since none of the metabolites predicted within the OECD Toolbox flag any alerts for anemia (Scheme XII). This substantiates the types of metabolites identified experimentally, including 3-hydroxy-4-methoxybiphenyl, 4-hydroxy-4'-methoxybiphenyl, 4,4'-dihydroxybiphenyl and 4-hydroxybiphenyl (see (Hamilton 2006)).
- Diphenylamine (122-39-4) could form a quinone imine upon aromatic C-hydroxylation to act similarly to other aminophenols already discussed. Rat studies show that metabolites include 4-hydroxy or 4,4-dihydroxydiphenylamine. Aniline was not found to be a metabolite in rat studies (JMPR 2000), in contrast with environmental degradation studies (discussed later). The OECD Toolbox

does contain a microbial metabolism simulator that has been developed by LMC which is the original simulator from the CATABOL expert system (Jaworska, Dimitrov et al. 2002). Microbial degradation products predicted for diphenylamine by this simulator do include aniline.

- Fenamidone (161326-34-7) was profiled by the *in vitro* rat liver S9 simulator to elucidate its predicted metabolic pathway. Aromatic C-hydroxylation or arene epoxide formation tended to be the initial transformations that were postulated to occur, which is in conflict with the proposed metabolic pathway described for fenamidone based on experimental studies (see Scheme XIII adapted from the WHO report on fenamidone (O'Mullane and Tasheva 2013)).
- Triflumizole (68694-11-1) was simulated to hydroxylate on either of its ring structures according to the *in vitro* rat liver S9 simulator but without any ring opening to release an aniline derivative. On the other hand, the metabolic pathway proposed based on experimental findings includes a substituted aniline, specifically 4-chloro-2-(trifluoromethyl)aniline, as one of the minor metabolites (see WHO/JMPR report on triflumizole (Busschers and Buffinton 2013)). Gomyo et al. also reported formation of chloroanilines (Gomyo, Morishima et al. 1991).

Summarizing this section, we examined 14 compounds that cause anemia in two or more species and contain an aniline moiety. The data suggested that these compounds could be causing anemia through a metabolic product that was aniline itself or a substituted aniline, which are both well documented to cause anemia. Table 6 compares the predicted and experimental metabolites where available. All but two of the 14 are either experimentally documented to have a substituted aniline as a metabolic product, or to have a substituted aniline as a predicted metabolite using the *in vitro* rat liver S9 simulator. Only Bifenazate and Fenamidone did not plausibly produce an aniline metabolite. Predictions made of the pathways for indoxacarb, fenamidone, and triflumizole using the simulator were poorly aligned with the experimental metabolic pathways reported.

Discussion

Guideline animal studies have been conducted to support safety assessments for thousands of chemicals, including pharmaceuticals, agrochemicals, and industrial agents. These data are used by regulatory and other public health agencies to identify adverse effects and set various guidance values. For hematological data, determining whether a statistically significant change in one or more RBC parameters qualifies as an adverse effect can be a challenging call during regulatory review (Everds 2015). This is especially true when hematological parameters are not clearly correlated with one another or other toxicity endpoints. The use of large databases such as ToxRefDB to find consistent patterns across chemicals, species, and study types can help provide context for these decisions. In addition, this information can be used for building predictive models of toxicity based on *in vitro* data and *in silico* predictions as inputs. A future goal of these models, once validated against legacy *in vivo* data, is to estimate toxicity of chemicals in the absence of traditional animal tests (Kavlock, Chandler et al. 2012). In this study we evaluated approaches for mining and

curating information from a large *in vivo* toxicology database. Our goal was to assess how different issues with data uncertainty can affect analysis and conclusions. We examined these issues using a case study to identify chemicals and chemical classes with strong evidence for causing anemia.

Predictive models can only be as informative as the source data. One aim of this study was to assess the level of data quality in ToxRefDB, based on our knowledge of certain types of issues inherent to large *in vivo* databases. These include experimental variability in animal studies; biostatistical considerations including false-positive and false-negative errors; detection and observer bias; inconsistency in terminology across time (years to decades); and transcription and data assimilation errors (Claassen 1994). In the present study, we attempted to address several of these issues. We adjusted for the possibility of false-positive effects by classifying chemicals as positive for anemia in the initial screen only if at least two different redundant measures (out of RBC, HGB, and HCT) were decreased and then focused on chemicals showing anemia in at least two species to increase confidence that the effects were treatment-related.

The next issue was distinguishing missing data from negative findings. Because a basic hematology panel is a standard part of subchronic and chronic studies, we initially assumed that this would not be an issue with the current analysis; however, we found that a subset of the studies (6%) did not run or report hematology measurements. We were then able to review the study reports and exclude those studies where relevant data were not collected. For anemia, observer bias was also not thought to be an issue because the basic measurements are automated or well-standardized. However, in reviewing study summaries (DERs), we found multiple cases where a statistically significant change in the original study report was dismissed as being of no biological or statistical significance, and so it was not recorded in enough detail to allow for inclusion in the database. Further studies using dose-response analysis would be needed to determine patterns in the inclusion or exclusion of statistically significant changes in anemia parameters.

We managed diagnostic drift by developing software to map terms onto a common set of vocabulary, including correction of common spelling mistakes. For anemia, this process was relatively straightforward given that terms and methods have been relatively stable over time. However, this effort was laborious even for anemia, and may be more difficult to manage for other broad domains (e.g., liver toxicity). In future studies, terminology mapping/grouping will need to be carefully implemented separately for each toxicology domain in turn, with input from domain experts. Finally, in the case of transcription errors, we carried out a second round of manual review of several hundred reports to quantify the level of error between the DERs and the final database. Overall, it appears that clear/strong findings are accurately captured and recorded (at least for ToxRefDB), but that there is a potential bias to miss weak or equivocal findings.

To demonstrate the utility of the cleaned database, we looked for similarities between chemicals causing anemia across multiple species, in particular to see if there were chemicals where the treatment-related anemia could be relevant to humans. We identified 49 chemicals that appear to cause anemia, based on positive findings in at least two parameters

in at least two species. The majority of these effects had not been reported previously in the literature. However, only six of these chemicals consistently caused anemia at doses below those where significant weight loss or mortality occurred. Of these, four were aniline derivatives (novaluron, fenhexamid, propanil, and flufenacet). Overall, aniline moiety-containing chemicals had an elevated probability of being anemia positive in 2 or more species over non-aniline containing chemicals (odds-ratio=2.69, $p=0.0003$). We hypothesized that the ultimate cause of anemia was a metabolite, based on a combination of information from the literature and predicted metabolic pathways. The other two chemicals are metiram-zinc and molinate. Predictions using the *in vitro* rat liver S9 metabolism simulator within the OECD Toolbox in conjunction with the repeated dose HESS profiler were found to be useful in identifying substituted aniline metabolites for the large majority of the 14 substances and providing a structural basis for the anemia observed. The exception was the metabolic profile of Group D substances (Figure 4), which were poorly predicted in contrast to any experimental information that was available.

Several of these aniline-containing chemicals have published literature linking them to anemia, some from animal studies, and some from human case studies for accidental or intentional poisonings. Eddleston et al. report case studies of self-poisoning with propanil which produce hemolysis, methemoglobinemia and other symptoms (Eddleston, Rajapakshe et al. 2002). Indoxacarb is known to cause methemoglobinemia in humans through a series of accidental poisonings (Park, Kim et al. 2011). Wu and colleagues published a case study of a 68-year old male with methemoglobinemia following ingestion of indoxacarb (Wu, Lin et al. 2010), and Shih et al. report a similar case (Shih and Tsai 2011). Jung et al. review several similar case studies of indoxacarb-caused methemoglobinemia (Jung, Lee et al. 2013). Gadagbui et al. derived reference doses for acetochlor using anemia as one input to the LOAEL/NOAEL determination. However, the underlying data that were used in this publication appear to be a summary of the DERs used in our work (Gadagbui, Maier et al. 2010). Cabagna showed that toads (*Bufo arenarum*) exposed to pesticides in the acetochlor class displayed evidence of normochromic, normocytic anemia (Cabagna 2005). Fujitani et al. studied the effects of chlorpropham in rats including splenotoxicity, anemia and methemoglobinemia (Fujitani, Tada et al. 1997, Fujitani, Tada et al. 2000, Fujitani, Tada et al. 2001, Fujitani, Tada et al. 2004). They found that some of these effects were reversible, but that hemosiderin deposition and fibrosis in the spleen were not. Thomas et al. demonstrated the appearance of anemia in diphenylamine-dosed albino rats (Thomas, Ribelin et al. 1967) and dogs (Thomas, Ribelin et al. 1967). Yoshida et al. also detected anemia in diphenylamine-dosed F344 rats (Yoshida, Shimoji et al. 1989). We found no anemia-related literature for novaluron, fenhexamid, flufenacet, linuron, desmedipham, bifenazate, fenamidone, or triflumizole.

There is also evidence from the literature that a subset of these compounds can be metabolized to anilines either *in vivo* or in the environment. Propanil has been shown to be metabolically converted to chloroanilines (Chow and Murphy 1975). McMillan et al. studied the role of the metabolites of propanil in causing hemolytic anemia (McMillan, Bradshaw et al. 1991, McMillan, Bradshaw et al. 1991). Using rats, they showed that propanil caused hemolytic anemia, likely via action of its metabolite N-hydroxy-3,4-dichloroaniline. Chow and Murphy demonstrated that propanil can cause methemoglobinemia via an active

metabolite in an *in vitro* system (Chow and Murphy 1975). Malerba and coworkers investigated the effects of propanil and dichloro aniline (also a metabolite of linuron) and showed that erythrocyte precursors were sensitive to both propanil and dichloro aniline (Malerba, Castoldi et al. 2002). Pastorelli et al. detected dichloro aniline-hemoglobin adducts in propanil-exposed individuals (Pastorelli, Catenacci et al. 1998). Jefferies et al. demonstrated that anilines are metabolic products of acetochlor *in vivo* (Jefferies, Quistad et al. 1998). Qing et al. showed that acetochlor was degraded by sludge-dwelling bacteria to an aniline compound 2-methyl-6-ethyl aniline (MEA) (Qing, Li et al. 2013). MEA has been reported to be a hydrolysis product of acetochlor metabolites (Barr, Hines et al. 2007). Linuron has been shown to be metabolically converted to chloroanilines (Badawi, Ronhede et al. 2009). There is some evidence that aniline is a metabolite of desmedipham (Weiss and Angerer 2002). Chlorpropham has been shown to be metabolically converted to an aniline (Nakagawa, Nakajima et al. 2004, Nakagawa, Nakajima et al. 2004). Aniline is one environmental degradation product of diphenylamine (Drzyzga 2003). Triflumizole has been shown to be metabolically converted to chloroanilines (Gomyo, Morishima et al. 1991). It is worth noting that a typical requirement of pesticide registrations is the need to work out the metabolic map of the active ingredient. In 2012, Kolanczyk et al. published a report on MetaPath, a software system that was to capture metabolic pathway maps from pesticides and other commercial chemicals (560 chemicals total). However, this database has never been made public (Kolanczyk, Schmieder et al. 2012) and thus was not used in this effort.

The set of 14 aniline containing substances were also profiled within the OECD Toolbox using the Laboratory of Mathematical Chemistry (LMC) *in vitro* rat liver S9 metabolism simulator and the National Institute of Technology and Evaluation (NITE) repeated dose HESS profiler (Sakuratani, Zhang et al. 2013) in order to systematically evaluate the likely explanation for the anemia observed. Where experimental data was available, the predictions whilst qualitative in nature were concordant in many cases.

Currently there is limited guidance on what degree of change in different clinical pathology parameters constitutes a toxicologically important or adverse effect. Working estimates have been provided in some cases for considering adversity (e.g., 10% of number of RBCs, hematocrit or hemoglobin) (WHO Core Assessment Group on Pesticide Residues 2015), but these thresholds are somewhat arbitrary and vary with study type, model species, analytical methods, and other factors. In many cases clinical pathology changes may not be considered adverse in isolation but rather serve as corroborating evidence for associated anatomic pathology or clinical findings. Future analysis of databases such as ToxRefDB can provide important information for establishing these relationships and supporting interpretation of statistically significant, but perhaps not clinically significant, clinical pathology effects. In addition, these types of studies may provide new information on species-specific pathways, given known species differences in susceptibility to hemolytic injury, regenerative bone marrow responses, and iron metabolism.

ToxRefDB has served as a primary resource for retrospective (Piersma, Rorije et al. 2011, Theunissen, Beken et al. 2014) and predictive (Martin, Knudsen et al. 2011, Sipes, Martin et al. 2011, Kleinstreuer, Dix et al. 2013) toxicology analyses. However, the automated and manual data curation and cleanup for a specific endpoint, in this case anemia, identified a

clear need for improved endpoint vocabulary within ToxRefDB as well as improved understanding of the testing status for particular endpoints. To this end, we have begun the process of creating an updated version of ToxRefDB. The improved and expanded version of ToxRefDB will focus on four areas of improvement:

1. Systematic study quality evaluation of all open literature studies (ToxR-Tool);
2. Updated endpoint and effect vocabulary with clear mapping to harmonized test guidelines and normalization of vocabularies and ontologies across other toxicology database resources (e.g., RepDose, eTox, HESS);
3. Guideline adherence and testing status information (e.g., clearly defining whether an endpoint was tested or not within each study); and
4. Curation of quantitative values on all observed effects (e.g., group-level means and standard errors and incidence values)

Once complete, ToxRefDB V2 will require less manual curation of specific endpoints or classes of endpoints (as performed here) to derive a dataset ready for analysis. Additionally, this version will better estimate the underlying uncertainty around quantitative values and variability introduced by differing study conditions and study quality metrics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

(Top) Fraction of tested chemicals showing anemia below doses where body weight decreases are seen (black, bottom), at doses equivalent to where body weight decrease is seen (dark gray, middle), and where anemia is not seen (light gray, top). The numbers indicate the total number of chemicals in the database for each species / study-type combination. (Bottom) Ranges of dose tested for the different study type, broken out by the three activity classes. In the box-and-whiskers plot, the box covers the 2nd and 3rd quartiles

of the distribution around the median, which is indicated by a dark line. The whiskers denote the 95% confidence intervals, and circles are outliers beyond 95%.

Figure 2:
Decision tree for classifying chemical / study pairs.

Figure 3:
Heatmap of the effects as a function of study (x-axis). Orange cells indicate an effect occurring at or above doses where body-weight loss is seen, and brown cell indicate effects seen at doses below where body-weight loss is seen. Effects showing up in fewer than 10 of the studies are not shown. This information is taken from in Supplementary File S5.

Figure 4:

Aniline-containing compounds from Table 5. Groups of compounds in each box have a similar substituent to the aniline nitrogen. The overall classification from Table 5 is given for each chemical. Chemicals with an asterisk (*) are active well below doses where body weight decreases are seen.

Figure 5:

Lane plots for novaluron, fenhexamid, propanil and flufenacet. For each study that was run for a chemical, there is a rectangle ranging from the lowest to the highest doses tested. A grey area indicates the range of doses where statistically significant body weight decreases were seen. A corresponding red area (not seen in these examples) shows doses where statistically significant increases in mortality rates are seen. Doses where RBC (red), HGB (green) and HCT (grey) decrease are indicated by diamonds. Doses where increases in any of the hemolysis markers are seen are indicated by a star. Doses where macrocytosis or microcytosis is seen are indicated by up or down blue arrowheads, respectively. Lane plots for all chemicals are in Supplemental File S6.

Scheme I.

Simulated metabolic pathway of Carboxin (5234-68-4) that leads to aniline formation and subsequent methemoglobinemia

Scheme II.

Simulated metabolic pathway of Propanil (709-98-8) to result in the formation of a substituted aniline

Scheme III.

Proposed formation of an aminophenol from Fenhexamid (126833-17-8)

Scheme IV.

Proposed formation of a substituted aniline from parent substance Novaluron (116714-46-6)

Scheme V.

Known metabolic pathway in rats for Indoxacarb (173584-44-6)

Scheme VI.

Proposed hydrolysis of Indoxacarb (173584-44-6) metabolite JT333

Scheme VII.

Proposed metabolic pathway of Flufenacet (142459-58-3)

Scheme VIII.

Proposed metabolic pathway of Acetochlor (34256-82-1)

Scheme IX.
Proposed metabolic pathway of Linuron (330-55-2)

Scheme X.

Simulated transformation pathway of Desmedipham (13684-56-5)

Scheme XI.

Simulated transformation pathway of Chlorpropham (101-21-3)

Scheme XII.

Simulated transformation pathway of Bifenazate (149877-41-8)

Scheme XIII.

Proposed metabolic pathway of Fenamidone (161326-34-7) based on experimental studies

Table 1:

Classes of endpoints used in the analysis. Note that for changes to be recorded, they must be statistically significant relative to concurrent controls.

Variable	Definition
Anemia count	number of parameters (RBC, HCT or HGB) that are decreased
Erythrocyte count (RBC)	dose at which RBC count first decreases
Hematocrit (HCT)	dose at which HCT first decreases
Hemoglobin (HGB)	dose at which HGB first decreases
LDT	lowest tested dose in the study
HDT	highest tested dose in the study
Body Weight	dose at which body weight decreases are first seen
Mortality	dose at which increased mortality is first seen
Anemia Hemolytic	first dose at which reviewer notes an increase in hemolytic anemia
Anemia Macrocytic	first dose at which reviewer notes an increase in macrocytic anemia
Anemia Microcytic	first dose at which reviewer notes an increase in microcytic anemia
Anemia NOS	first dose at which reviewer notes an increase in anemia (not otherwise specified)
Erythrocyte Anisochromia	dose at which anisochromia change is first seen
Erythrocyte Anisocytosis	dose at which anisocytosis is first seen
Erythrocyte Hypochromia	dose at which hypochromia is first seen
Erythrocyte Macrocytosis	dose at which macrocytosis is first seen
Erythrocyte Microcytosis	dose at which microcytosis is first seen
Reticulocyte	dose at which first increase in reticulocyte is first seen
Mean Corpuscular Volume (MCV)	dose at which change in MCV is first seen
Heinz Bodies	dose at which Heinz bodies are first seen
Howell-Jolly Bodies	dose at which Howell-Jolly bodies are first seen
Iron	dose at which a decrease in serum? iron levels is first seen
Methemoglobin	dose at which methemoglobin is first seen
Sulphhemoglobin	dose at which sulphhemoglobin is first seen
Spherocytes	dose at which spherocytes are first seen
Nucleated red blood cell (nRBC)	dose at which nucleated RBCs are first seen
Betaglobulin	dose at which changes in betaglobulin levels are first seen
Leukocyte (WBC)	dose at which WBC counts first changes (up and down recorded separately)
Lymphocytes	dose at which lymphocyte counts first changes (up and down recorded separately)
Monocytes	dose at which monocytes counts first changes (up and down recorded separately)

Variable	Definition
Neutrophils	dose at which neutrophil counts first changes (up and down recorded separately)
Eosinophils	dose at which eosinophil counts first changes (up and down recorded separately)
Platelets	dose at which platelet counts first changes (up and down recorded separately)
Bilirubin	dose at which bilirubin levels first increase
Lactic acid dehydrogenase (LDH)	dose at which LDH levels first increase
Bone Marrow Cellular Alteration	dose at which bone marrow cellular alteration first increases
Bone Marrow Hyperplasia	dose at which bone marrow hyperplasia is first seen
Bone Marrow Congestion	dose at which bone marrow congestion is first seen
Bone Marrow Hypoplasia	dose at which bone marrow hypoplasia is first seen
Bone Marrow Pigmentation	dose at which increased bone marrow pigmentation is first seen
Hemosiderosis Bone Marrow	dose at which bone marrow hemosiderosis is first seen
Spleen Congestion	dose at which spleen congestion is first seen
Spleen Hyperplasia	dose at which spleen hyperplasia is first seen
Spleen Pigmentation	dose at which spleen pigmentation is first seen
Hemosiderosis Spleen	dose at which spleen hemosiderosis is first seen
Hemosiderosis Kidney	dose at which kidney hemosiderosis is first seen
Creatinine	dose at which creatinine levels first increases
Hemosiderosis Liver	dose at which liver hemosiderosis is first seen
Alanine Aminotransferase (ALT/SGPT)	dose at which ALT/SGPT first increases
Alkaline phosphatase (ALP/ALK)	dose at which ALP/ALK first increases
Aspartate Aminotransferase (AST/SGOT)	dose at which AST/SGOT first increases

Table 2:

Concordance rates for the same chemical tested in two different study types, prior to and after the manual review. The table only includes cases where anemia was seen at doses below those at which significant body weight loss was observed in the first study. Study pairs are only included if the highest dose tested in the two was within a factor of two. A chemical is considered concordant if the second study showed anemia at any dose. We only include study-type pairs with 5 or more studies. Ambiguous studies are not included.

Species / Study Pairs		Before Cleanup			After Cleanup		
Species / study 1	Species / study 2	Concordant	Not Concordant	Fraction Concordant	Concordant	Not Concordant	Fraction Concordant
rat SUB	rat CHR	15	6	0.71	18	2	0.90
rat CHR	dog CHR	4	5	0.44	13	2	0.87
rat CHR	rat SUB	14	4	0.78	18	4	0.82
rat SUB	rat SUB	8	5	0.62	16	4	0.80
rat SUB	dog CHR	6	9	0.40	11	4	0.73
mouse CHR	rat CHR	7	5	0.58	11	4	0.73
mouse CHR	rat SUB	5	7	0.42	13	7	0.65
dog CHR	rat SUB	6	5	0.55	11	6	0.65
dog CHR	rat CHR	6	11	0.35	13	8	0.62
rat CHR	mouse CHR	3	15	0.17	11	11	0.50
mouse CHR	dog CHR	3	5	0.38	6	6	0.50
rat SUB	mouse CHR	9	9	0.50	13	14	0.48
dog CHR	mouse CHR	1	7	0.13	6	8	0.43
mouse CHR	mouse CHR	2	4	0.33	2	3	0.40

Table 3:

Concordance between initial database entry and results of manual review. Each cell contains the number of studies in each class before and after the review. Positive (decrease in two or more of RBC, HGB, and HCT), Negative (0), Ambiguous (1), Unknown (manual review found that hematology values were not measured in the study).

	DB class	After manual review			
		Positive	Negative	Ambiguous	Unknown
Before manual review	Positive	212	2	4	0
	Negative	24	128	11	24
	Ambiguous	6	2	16	0
	Total	242	132	31	24
					429

Table 4:

Summary of anemia classes of 49 chemicals broken out by species and study type. Full details are given in Supplementary File S5. REG=regenerative; nonREG=non-regenerative; NEG=negative for anemia; MIX=both regenerative and non-regenerative studies.

CASRN	Name	Positive Studies	Rat CHR	Rat SUB	Mouse CHR	Dog CHR	Literature
34256-82-1	Acetochlor	5	MIX	NEG	nonREG	REG	(Cabagna 2005, Gadagbui, Maier et al. 2010)
135158-54-2	Acibenzolar-S-methyl	3	REG		REG	REG	No positive reports found
834-12-8	Ametryn	3	nonREG	REG		nonREG	(Santos, Cancian et al. 2015)
68049-83-2	Azafenidin	3	REG	REG		nonREG	No positive reports found
1861-40-1	Benfluralin	2		REG	nonREG		No positive reports found
149877-41-8	Bifenazate	3	nonREG	REG	NEG	REG	No positive reports found
5234-68-4	Carboxin	2	nonREG		NEG	REG	No positive reports found
1897-45-6	Chlorothalonil	2	REG	NEG	REG	NEG	(Leet, Lindberg et al. 2014)
101-21-3	Chlorpropham	3	REG	REG		REG	(Fujitani, Tada et al. 1997, Fujitani, Tada et al. 2000, Fujitani, Tada et al. 2004)
105512-06-9	Clodinafop-propargyl	3	nonREG	nonREG	NEG	nonREG	(Leet, Lindberg et al. 2014)
1702-17-6	Clopyralid	2	nonREG		NEG	nonREG	No positive reports found
91-64-5	Coumarin	3	nonREG	REG	nonREG		No positive reports found
57966-95-7	Cymoxanil	2	NEG	NEG	nonREG	nonREG	No positive reports found
13684-56-5	Desmedipham	6	REG	REG	REG	REG	No positive reports found
115-32-2	Dicofol	2	NEG	nonREG		REG	No positive reports found
75-60-5	Dimethylarsinic acid	3	nonREG	REG		nonREG	(Zhang, Cai et al. 2002, Tchounwou, Patlolla et al. 2003, Cheng, Li et al. 2004)
122-39-4	Diphenylamine	4	REG	REG	REG	REG	(Thomas, Ribelin et al. 1967, Yoshida, Shimoji et al. 1989)
80844-07-1	Etofenprox	5	nonREG	MIX	REG	nonREG	No positive reports found
161326-34-7	Fenamidone	5	nonREG	MIX	NEG	nonREG	No positive reports found
126833-17-8	Fenhexamid	2	REG	NEG	NEG	REG	No positive reports found
69806-50-4	Fluazifop-butyl	3	nonREG	nonREG		REG	No positive reports found
142459-58-3	Flufenacet	4	REG	REG	REG	REG	No positive reports found
98886-44-3	Fosthiazate	3	REG	nonREG		REG	No positive reports found
173584-44-6	Indoxacarb	5	REG	REG	NEG	REG	(Wu, Lin et al. 2010, Park, Kim et al. 2011, Jung, Lee et al. 2013, Leet, Lindberg et al. 2014)
77501-63-4	Lactofen	4	nonREG	REG	nonREG	nonREG	(Leet, Lindberg et al. 2014)
330-55-2	Linuron	2	REG		NEG	REG	No positive reports found
12427-38-2	Maneb	2	NEG		nonREG	nonREG	(Pinkhas, Djaldetti et al. 1963)

CASRN	Name	Positive Studies	Rat CHR	Rat SUB	Mouse CHR	Dog CHR	Literature
16752-77-5	Methomyl	2	nonREG			REG	(Aziz and Zabut 2014)
161050-58-4	Methoxyfenozide	3	REG	nonREG		REG	No positive reports found
1910-42-5	Methyl viologen	2	nonREG		REG		(Clark, Gildersleeve et al. 1988, Bhardwaj and Saxena 2014, Jang, Bae et al. 2014)
6317-18-6	Methylene bis(thiocyanate)	2	NEG	nonREG		REG	(Burka 1993, Braun, Birek et al. 2006)
9006-42-2	Metiram-zinc	2		nonREG		REG	No positive reports found
21087-64-9	Metribuzin	2	NEG		nonREG	REG	(Velisek, Svobodova et al. 2008)
2212-67-1	Molinate	3	nonREG		nonREG	REG	(Kawatsu 1977, Sancho, Ceron et al. 2000)
116714-46-6	Novaluron	4	REG	REG	REG	NEG	No positive reports found
19044-88-3	Oryzalin	3	nonREG	REG	nonREG		No positive reports found
19666-30-9	Oxadiazon	5	REG	REG	nonREG	NEG	(Leet, Lindberg et al. 2014)
42874-03-3	Oxyfluorfen	4		MIX	NEG	nonREG	(Leet, Lindberg et al. 2014)
709-98-8	Propanil	2	REG		NEG	REG	(McMillan, Bradshaw et al. 1991, Pastorelli, Catenacci et al. 1998, Eddleston, Rajapakshe et al. 2002, Malerba, Castoldi et al. 2002)
129630-19-9	Pyraflufen-ethyl	3	nonREG	REG	nonREG		(Leet, Lindberg et al. 2014)
95737-68-1	Pyriproxyfen	3	nonREG	nonREG		nonREG	No positive reports found
123343-16-8	Pyriithiobac-sodium	3	nonREG	REG	NEG	nonREG	No positive reports found
74051-80-2	Sethoxydim	3	REG	NEG	REG	REG	No positive reports found
122836-35-5	Sulfentrazone	4	REG	REG	nonREG	nonREG	(Leet, Lindberg et al. 2014)
112410-23-8	Tebuufenozide	3	REG	REG		REG	No positive reports found
59669-26-0	Thiodicarb	3	REG		REG	nonREG	No positive reports found
137-26-8	Thiram	2	REG		REG		(Maita, Tsuda et al. 1991, Leet, Lindberg et al. 2014)
78-48-8	Tribufos	3	nonREG		nonREG	nonREG	(Leet, Lindberg et al. 2014)
68694-11-1	Triflumizole	3	NEG	REG	NEG	REG	No positive reports found

Table 5:

Summary of classifications for the 4 chemicals in Figure 5, group A.

Chemical	Rat CHR	Rat SUB	Mouse CHR	Dog CHR
Novaluron	regenerative / heme oxidation	(1) regenerative (2) regenerative	regenerative / heme oxidation	negative
Fenhexamid	regenerative	negative	negative	regenerative / heme oxidation
Propanil	regenerative		negative	regenerative / heme oxidation
Flufenacet	regenerative	regenerative / hemolytic	(1) regenerative (2) negative	regenerative

Table 6:

Comparison of predicted and experimental metabolite information

Group	Name	Available experimental metabolism information	Predicted metabolism	Comments
A	Novaluron		Substituted aniline	
	Fenhexamid		aminophenol	
	Propanil	Yes	3,4-dichloroaniline	Reasonable concordance
	Carboxin		Aniline	
B	Indoxacarb	Scheme V	Metabolites are demethylated products of parent	Predicted and experimental pathways discordant
	Flufenacet		4-fluoroaniline	
	Acetochlor	2-methyl-6-ethyl aniline	2-methyl-6-ethyl aniline	Concordant prediction
	Linuron	dichloroaniline	dichloroaniline	Concordant prediction
C	Desmedipham	Aniline	Aniline	Concordant prediction
	Chlorpropham	3-chloroaniline	3-chloroaniline	Concordant prediction
D	Bifenazate	Biphenyl derivatives	Biphenyl derivatives	Reasonable concordance but no aniline derivative postulated
	Diphenylamine	Aniline (e-fate) 4-hydroxy or 4,4-dihydroxydiphenylamine	Aniline (e-fate) 4-hydroxy or 4,4-dihydroxydiphenylamine	Concordant predictions but aniline produced only by environmental degradation
	Fenamidone	Scheme XIII		Predicted and experimental pathways discordant
	Triflumizole	4-chloro-2-(trifluoromethyl)aniline		Predicted and experimental pathways discordant