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Minimizing Experimental Testing on Fish for Legacy Pharmaceuticals

Anja Coors,* A. Ross Brown, Samuel K. Maynard, Alison Nimrod Perkins, Stewart Owen, and Charles R. Tyler

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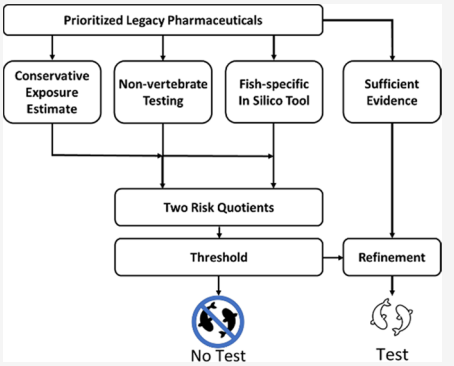
ABSTRACT: There was no regulatory requirement for ecotoxicological testing of human pharmaceuticals authorized before 2006, and many of these have little or no data available to assess their environmental risk. Motivated by animal welfare considerations, we developed a decision tree to minimize in vivo fish testing for such legacy active pharmaceutical ingredients (APIs). The minimum no observed effect concentration ($NOEC_{min}$, the lowest NOEC from chronic *Daphnia* and algal toxicity studies), the theoretical therapeutic water concentration (TWC, calculated using the fish plasma model), and the predicted environmental concentration (PEC) were used to derive API risk quotients ($PEC/NOEC_{min}$ and PEC/TWC). Based on a verification data set of 96 APIs, we show that by setting a threshold value of 0.001 for both risk quotients, the need for in vivo fish testing could potentially be reduced by around 35% without lowering the level of environmental protection. Hence, for most APIs, applying an assessment factor of 1000 (equivalent to the threshold of 0.001) to $NOEC_{min}$ substituted reliably for $NOEC_{fish}$, and TWC acted as an effective safety net for the others. In silico and in vitro data and mammalian toxicity data may further support the final decision on the need for fish testing.



KEYWORDS: *animal welfare, ecotoxicity, environmental risk assessment, vertebrate testing, fish plasma model*

BACKGROUND

Over the last three decades, a wide range of pharmaceuticals have been detected in aquatic environments globally, raising concerns about their potential impact on environmental and human health.[1−4] In 2001, the European Union (EU) implemented a legal requirement for an environmental risk assessment (ERA) within the market authorization process for new medicinal products for human use.[5] Subsequently, the European Medicines Agency (EMA) issued the relevant guideline for this ERA.[6] This action resulted in a substantial increase in the amount of data on the environmental fate and effects of active pharmaceutical ingredients (APIs) for use in regulatory ERAs. However, data available for legacy pharmaceuticals, i.e., medicines that were on the market before the implementation of the ERA guideline, are still scarce. Around 3000 APIs are marketed in Europe[7] with an estimated 11% of these having ERA data available.[8] Various prioritization approaches have been proposed to address this shortfall and to assess the environmental risks of these legacy pharmaceuticals.[8] However, they have usually concluded with a listing of prioritized APIs, rather than detailing a testing strategy to close the data gaps.

Minimizing, and ideally avoiding, the use of intact vertebrates (i.e., the in vivo testing of fish) in the experimental testing of legacy APIs, and in the assessment of chemicals in general, is an important ethical and legal obligation.[9] Any routine animal testing in the absence of a regulatory requirement, as would usually be the case for legacy pharmaceuticals, warrants appropriate justification that takes into account the 3Rs: reduction, refinement, and replacement.[10] This is particularly the case for human-use APIs because many do not appear to pose a significant risk to the environment when assessed according to the current EMA guideline.[3] In this context, we sought to establish an approach that minimizes the use of vertebrates in the testing of legacy APIs, particularly for groups of legacy APIs that have already been highlighted by a prioritization approach. To this end, we have developed a decision tree to ensure that in vivo testing with fish is avoided for those legacy APIs where other data allow for reliable substitution. Particularly, the fish early life stage test (OECD technical guideline 210) as one of the three aquatic toxicity endpoints required for an ERA in the European regulatory context[6] shall be substituted by nonanimal methods in our proposed approach.

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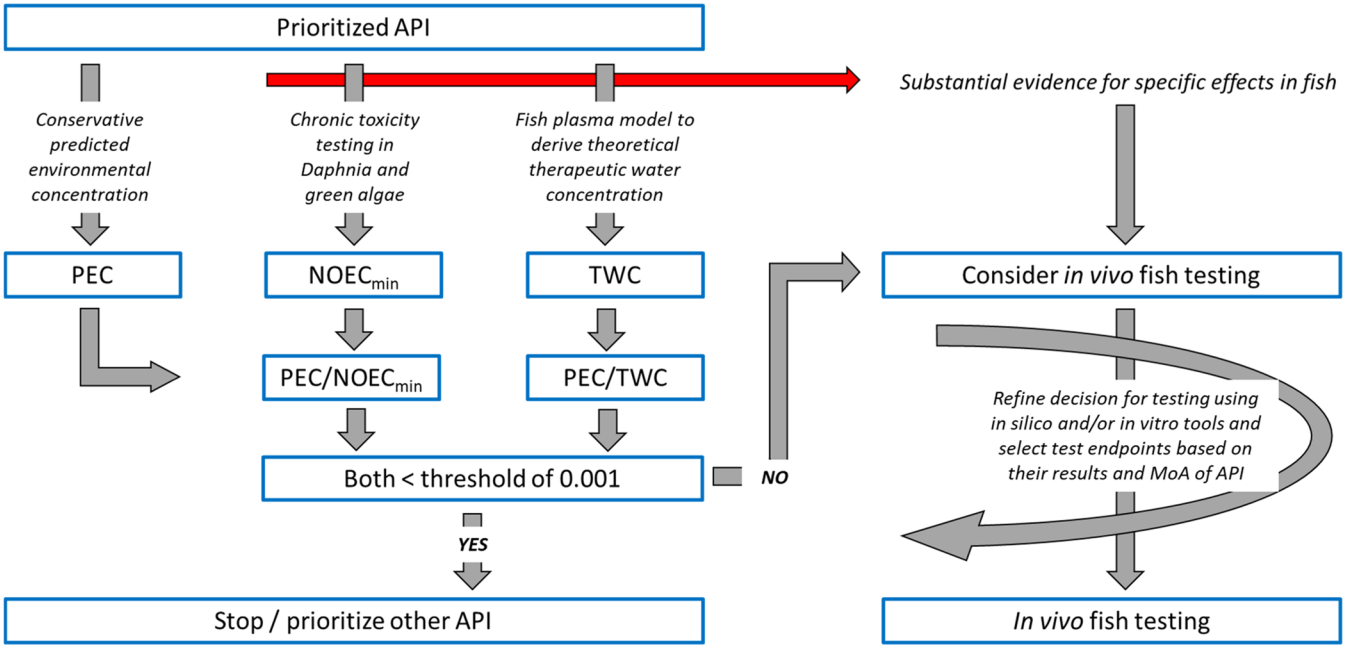

Figure 1. Scheme of the proposed decision tree. API: active pharmaceutical ingredient, PEC: predicted environmental concentration, NOEC$_{min}$: the lowest no observed effect concentration among those derived with *Daphnia* and algae, TWC: theoretical therapeutic water concentration, and MoA: mode of action.

Here, we first describe the proposed decision tree and discuss scientific evidence supporting its synthesis. It is important to recognize that the present study intends to establish a decision process for reducing animal testing for legacy pharmaceuticals. Although the scheme employs risk ratios, it is not the intention to offer an alternative ERA approach. The assessment of a validation data set in the present study aims to verify that substituting chronic fish ecotoxicity data according to the proposed decision tree does not result in a lower level of environmental protection compared with an existing standard ERA using chronic fish data. For this purpose, we relied on the ERAs and underlying ecotoxicity and exposure data published by ref 3 that represent an up-to-date collection of regulatory data and that are fully and freely accessible (as Supplements of ref 3). We did not reassess, extend, or modify this very recent data collection to keep our study transparent and consistent.

We have excluded two groups of APIs, namely, antibiotics and APIs that target human sex steroid receptors (as indicated in ref 3), as out of the scope of the proposed decision tree. For antibiotics, algae or cyanobacteria are considered to be the most relevant and sensitive taxa for assessing ecotoxic effects,[11] and fish testing will likely no longer be required for regulatory assessments of antibiotics.[12,13] In the case of APIs that interact with human sex steroid receptors, a tailored risk assessment focusing on fish is required[6] based on substantial evidence for specific, receptor-related effects in fish at low exposure concentrations.[14−16] This renders the avoidance of in vivo fish testing by substitution with other endpoints inappropriate for such APIs. Gunnarsson et al.[3] demonstrated that when the pharmacological target of an API was presumably present in fish, but not in *Daphnia* or algae, then the no observed effect concentration (NOEC) for fish was lower than that for algae and *Daphnia* for about 70% of APIs. This association was strongly driven by APIs with an endocrine mode of action, which supports the requirement for a tailored fish testing strategy according to the EMA guideline for such compounds. If, however, the pharmacological target was also present in *Daphnia* or algae, these taxa were equally likely as fish to provide the lowest NOEC.

PROPOSED DECISION TREE

The proposed decision tree (shown in Figure 1) is designed to process APIs in an identical way, regardless of how they may, or may not, have been prioritized beforehand. If substantial evidence already exists that a given API can exhibit specific effects in fish, then that API is considered directly for in vivo testing in fish following the path indicated by the red arrow in Figure 1. Following this path does not necessarily exclude the need for testing other taxa. The critical question of what is considered "substantial evidence" is discussed below. If no substantial evidence for specific effects in fish is available, the API enters the risk-based component of the decision tree (shown on the left-hand side of Figure 1). Here, two different proxies are used to characterize hazard, and both are compared to the predicted environmental concentration (PEC) as a conservative estimate of environmental exposure.

The first proxy for characterizing hazard is the minimum no observed effect concentration (NOEC$_{min}$) derived from standard chronic toxicity tests with *Daphnia magna* and green algae according to OECD technical guidelines 211 and 201, respectively (i.e., nonvertebrate chronic in vivo testing). From the experimentally derived NOECs (or, alternatively, the concentration with a 10% effect, EC$_{10}$) from these two taxa, the lowest NOEC is selected to provide NOEC$_{min}$. The values used for deriving NOEC$_{min}$ should not be modeled or extrapolated from acute toxicity estimates because such estimates may be misleading due to the wide range of acute-to-chronic ratios observed specifically for pharmaceuticals[17,18] and the difficulty of predicting the chronic toxicity of ionizable organic chemicals,[19] which many pharmaceuticals are.

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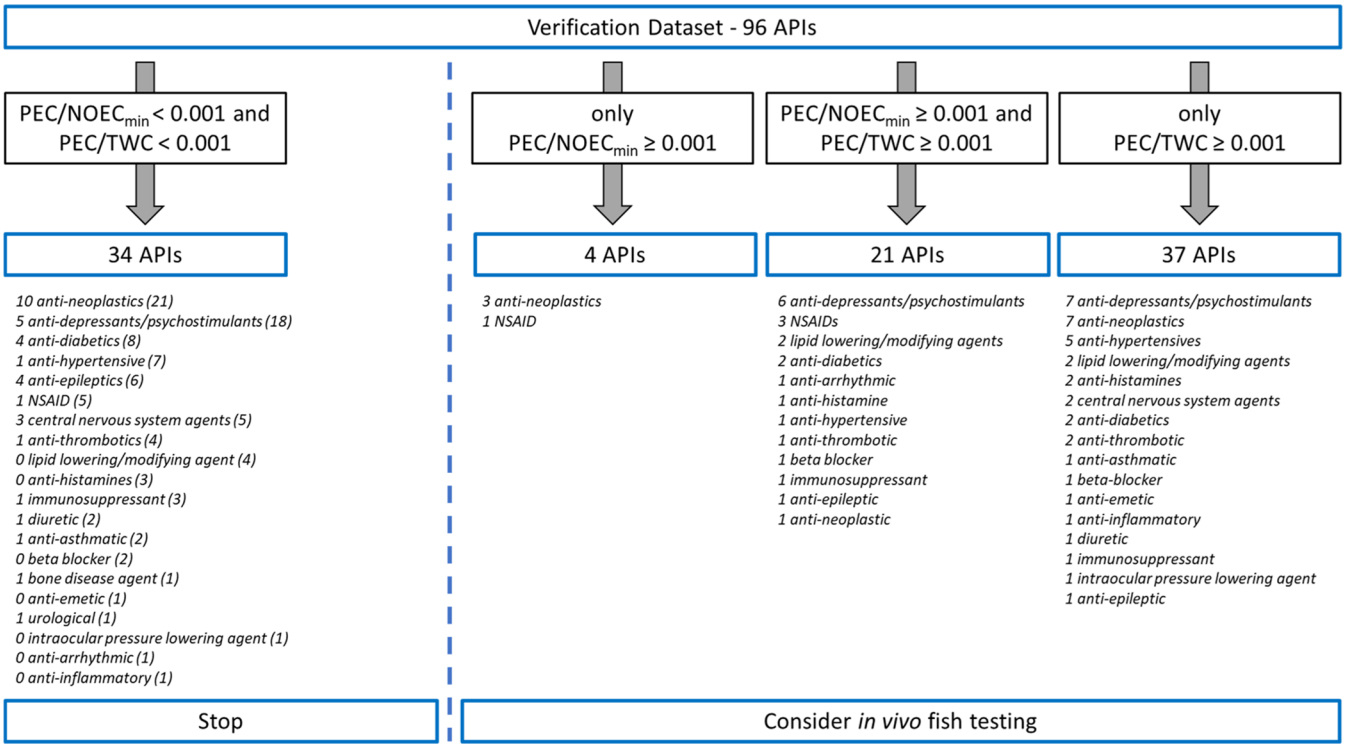

Figure 2. Verification data set taken through the decision tree. The left panel shows the 34 APIs excluded from in vivo fish testing and their distribution to the 20 broadly defined mode-of-action groups. The total number of verification data set APIs per group is given in brackets to illustrate the proportion of APIs per group that would be excluded from fish testing (e.g., 10 of in total 21 antineoplastics). The right panel summarized the groups of the 62 APIs considered for in vivo fish testing, separated by the criteria that supported the respective decision.

The second proposed proxy for hazard characterization is derived by the fish plasma model (FPM). The water concentration causing a blood plasma concentration in fish similar to the human therapeutic plasma concentration has been referred to as the critical environmental concentration (CEC[20]) or theoretical therapeutic water concentration (TWC[3]). The FPM postulates that toxic effects in fish (including the impairment of growth, development, and/or reproduction) are unlikely to occur below this concentration.[21] In the FPM, the octanol−water partition coefficient (K_{ow}), a measure of a chemical's lipophilicity, determines its level of passive uptake into fish and, consequently, the blood plasma concentration.[22] For the case of ionizable APIs, the pH-corrected octanol−water distribution coefficient ($\log D_{ow}$) has been used as a substitute for $\log K_{ow}$.[3] Applying the FPM as an additional in silico tool alongside NOEC$_{min}$ serves as a safety net for APIs that may specifically affect fish (as opposed to other taxa, such as algae or crustaceans) as a consequence of the evolutionary conservation of pharmacological targets that are exclusive to vertebrates,[3,23] which may result in effects that are not captured by NOEC$_{min}$.

In the next step of the decision tree, both of these proxies for hazard (NOEC$_{min}$ and TWC) are related to the API's PEC in surface water. The PEC employed in the decision tree should preferably be a conservative estimate, i.e., not considering metabolism in the patient, removal in sewage treatment, and/or degradation in the receiving environment. A more refined PEC, taking the above aspects into account, could be used if robust data for the above aspects are available. However, using refined PECs may impact the level of protection provided by the decision tree (i.e., increase the likelihood of deprioritizing fish testing), and it was beyond the scope of this study to evaluate such an impact.

The two derived risk quotients (PEC/NOEC$_{min}$ and PEC/TWC) are then compared to a threshold value, which is set to provide high certainty for avoiding risk for adverse effects in fish. If at least one of the two risk quotients is greater than the threshold value, the API should be considered for in vivo testing in fish. If both quotients are below the threshold value, the API does not need to be considered further for in vivo fish testing. We propose a threshold value of 0.001 for both risk quotients. With regard to PEC/NOEC$_{min}$, the proposed threshold of 0.001 includes an assessment factor of 10, as used in the EMA guideline,[6] to derive the predicted no-effect concentration (PNEC) from the lowest NOEC (from a complete standard data set) for comparison to PEC. An additional factor of 100 is included as a safety margin, assuming that NOEC$_{fish}$ is no more than 100-fold lower than NOEC$_{min}$. PEC/TWC is intended to capture the exceptions to this rule (i.e., for APIs where NOEC$_{fish}$ is more than 100-fold lower than NOEC$_{min}$). The threshold of 0.001 for PEC/TWC also aligns with a safety factor of 1000 suggested by ref 21 for the usage of FPM in initial risk assessments. The threshold value applied for these risk quotients in the decision tree may be adapted in the future, pending new data and supporting information that would warrant this.

PROPOSED DECISION TREE APPLIED TO A VERIFICATION DATA SET

Critical aspects of the decision tree are whether data on algae and *Daphnia* together with the chosen threshold value of 0.001 can indeed safely substitute for NOEC$_{fish}$, and whether the TWC branch of the tree can reliably capture the cases where

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the $NOEC_{min}$ approach fails. To evaluate these aspects, we generated a verification data set that we took through the decision tree. This verification data set was compiled from all APIs assessed by ref[3] excluding antibiotics, APIs that are intentionally designed to target human sex steroid receptors, APIs that lacked a full complement of NOECs (fish, daphnia, and algae), APIs that lacked a TWC, and APIs that lacked a consumption-based PEC. We choose consistently for all APIs in the present study the PECs from ref 3 (Supplement 2) that had been calculated using the maximum of country-specific consumption data within the EU together with 10-fold dilution in receiving surface waters and assuming no metabolism and no removal by the sewage treatment process because this calculation resembles most the conservative standard approach according to EMA.[6] The TWC values (calculated using $\log K_{ow}$ or $\log D_{7-7.4}$ and the minimum human therapeutic plasma concentration, min C_{max}) were also taken from ref 3 (TWC 3 in the Supplementary Material). In the resulting verification data set of 96 APIs, the lowest endpoint was $NOEC_{fish}$ for 47 APIs, $NOEC_{daphnia}$ for 26 APIs, and $NOEC_{algae}$ for 19 APIs. For the remaining 4 APIs, $NOEC_{daphnia}$ and $NOEC_{fish}$ were equal to or lower than $NOEC_{algae}$. All data (provided in Table S1) were taken without modifications (except the correction of one transfer error, as indicated in Supporting Information S1) from the Supplementary Material in ref 3 derived originally from publicly available European Public Assessment Reports (EPARs) and/or entries in the Swedish database FASS (fass.se). While these data represent a high-quality and up-to-date compilation, they may not necessarily include for all APIs the endpoints that have been or would be used for a regulatory ERA in some countries. Attempting this would be impossible, however, because data used by national authorities are not publicly available,[13] in contrast to data collected from public EPARs and the Swedish FASS database. In a recent publication on data approved for regulatory usage by the German Environment Agency,[13] 73 of the APIs they assessed were in our verification data set, and for 65.8% of them, the NOECs for all three trophic levels were the same as in ref 3. Furthermore, for 80% of the APIs common to both data resources, the difference between NOECs was less than 3.2-fold, as stated by ref 13. Hence, the regulatory data set used by Schwarz et al.[13] was apparently largely similar to that used by Gunnarsson et al.[3] Given that the actual data are not provided in Schwarz et al.[13] due to confidentiality reasons, we resorted to using the data set of Gunnarsson et al.[3]

The 96 APIs in our verification data set were assigned into 20 broadly defined therapeutic mode of action groups (see Figure 2) based on the target gene analyses in ref 3. These groups are broadly defined and do not fully reflect a purely therapeutic categorization (such as the Anatomical Therapeutic Chemical, ATC, classification), nor are they a purely target-gene-based pharmacological classification because many APIs would fall into several groups or the number of groups would be too large. Antineoplastics (many of those in the data set that inhibit a subset of kinases) and antidepressants/psychostimulants (almost exclusively targeting receptors relating to dopamine or serotonin) were very well represented in the verification data set, while several other groups were poorly represented and contained just one or two APIs (see Figure 2). Various groups of APIs are not represented at all in the verification data set, for example, antimycotics, muscle relaxants, or diagnostic agents. Hence, the verification data set is not fully representative of the universe of marketed pharmaceuticals. Nevertheless, the data set contains APIs from numerous therapeutic classes, often targeting different molecular receptors within the broadly defined groups.

The 96 APIs in the verification data set were taken through the decision tree to evaluate its "reliability", measured by the proportion of APIs that are erroneously excluded from in vivo fish testing (i.e., the false negative rate). An API is considered a false negative if its $NOEC_{fish}$ is more than 100-fold lower than $NOEC_{min}$ and is also not captured by the PEC/TWC safety net. We evaluated the "effectiveness" of the decision tree based on the proportion of APIs that were excluded from in vivo fish testing, i.e., how much reduction of fish testing could be achieved by applying the decision tree.

Over one-third (34 of 96) of the APIs in the verification data set would be excluded from in vivo fish testing by the decision tree, as both risk quotients ($PEC/NOEC_{min}$ and PEC/TWC) were below the threshold of 0.001 (Figure 2). Assuming that a similar proportion would be deprioritized for fish testing from a list of legacy APIs, applying the decision tree would enable a substantial reduction (i.e., by about one-third) of in vivo fish testing, highlighting the "effectiveness". Among these 34 APIs in the verification data set that were excluded from fish testing by the decision tree, none had $NOEC_{fish}$ more than 100-fold lower than $NOEC_{min}$. In other words, there were no false negatives among the 96 APIs in the verification data set, i.e., there were no APIs for which the application of the decision tree and applying an assessment factor of 1000 (equivalent to using 0.001 as threshold) to $NOEC_{min}$ in a subsequent risk assessment would have resulted in a lower protection level for the environment. Only one API (betamethasone) in the verification data set has $NOEC_{fish}$ more than 100-fold lower than $NOEC_{min}$, demonstrating the conservatism of the threshold chosen in this decision tree for the $NOEC_{min}$ criterion.

Among the 62 APIs that were considered for in vivo fish testing (Figure 2), this decision was supported by both risk quotients ($PEC/NOEC_{min}$ and PEC/TWC) for 21 APIs. For the other 41 of these 62 (i.e., about 66%), only one of these two quotients supported the decision, which demonstrates that they are not redundant but rather complementary warranting the inclusion of both in the decision tree. In fact, PEC/TWC highlighted many more APIs for consideration of in vivo fish testing than those identified with $PEC/NOEC_{min}$, illustrating the usefulness of PEC/TWC as a conservative safety net against erroneous exclusion of APIs from fish testing. Across all 96 APIs (and treating censored (unbound) NOECs as if they were definitive values, i.e., 10 mg/L for NOECs given as $\geq$10 mg/L), TWC was significantly lower than $NOEC_{fish}$ (t-test, $p < 0.0001$), but the two estimates were not significantly associated (pairwise Pearson correlation, $p > 0.05$). There were only 16 cases among the 96 APIs where $NOEC_{fish}$ values were lower than TWC. These 16 APIs belonged to the groups of antineoplastics, antiepileptics, antidepressants, or NSAIDs and included the only bone disease agent in the verification data set. Thus, using the FPM in parallel to $NOEC_{min}$ adds not only an additional margin of safety but also a layer of confidence that may be particularly warranted due to concerns of modes of action being specifically relevant for fish (see the below discussion on substantial evidence for specific effects in fish). TWC was also significantly lower than $NOEC_{min}$ (t-test, $p < 0.0001$); $NOEC_{min}$ represented for only 15 APIs the more conservative hazard estimate.

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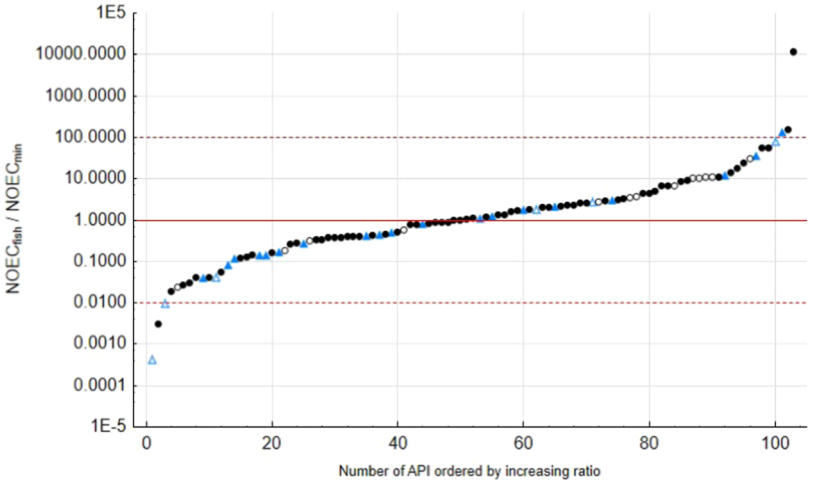

Figure 3. Ratio of NOEC for fish to a minimum of NOECs for *Daphnia* and algae ($NOEC_{min}$) for 103 APIs taken from ref 3. Censored ratios (at least ≥1 or up to ≤1) are indicated by empty symbols. Blue triangles represent APIs that are not included in the verification data set.

If a threshold value of 0.01 (instead of 0.001) was used in the decision tree for both risk quotients (i.e., applying only an additional safety factor of only 10 instead of 100 to $NOEC_{min}$ for any subsequent risk assessment), this would lead to exclusion of 57 out of 96 APIs from in vivo fish testing and thereby reduce vertebrate animal use by more than 50%. However, adopting this less conservative threshold would also lead to nine false negatives. In other words, using a threshold of 0.01 results in exclusion of 10% of APIs from in vivo fish testing for which $NOEC_{fish}$ is more than 10-fold lower than $NOEC_{min}$, which were not captured by the PEC/TWC criterion. For these APIs, the use of fish testing to generate $NOEC_{fish}$ followed by the application of the standard assessment factor of 10 would result in a lower PNEC for fish compared to that resulting from $NOEC_{min}$ with an assessment factor of 100 (standard 10 + additional 10 for not testing fish). We conclude, therefore, that the proposed threshold of 0.001 ensures an appropriate balance for assuring the reliability of the decision tree (no impact on how protective a subsequent risk assessment would be when using an assessment factor of 1000 on $NOEC_{min}$) and its 'effectiveness' for minimizing in vivo fish testing (substantial reduction in fish testing).

FURTHER EVIDENCE SUPPORTING THE PROTECTIVENESS OF $NOEC_{MIN}$ FOR $NOEC_{FISH}$

Due to the lack of TWC or consumption-based PEC, 30 APIs with a full chronic ecotoxicity data set[3] were not included in the verification data set. These APIs, however, provide further information on how protective $NOEC_{min}$ is for $NOEC_{fish}$. Therefore, an additional analysis was undertaken using the maximum amount of suitable ecotoxicity data to directly compare $NOEC_{fish}$ to $NOEC_{min}$. We included in this analysis APIs with NOECs given as greater-than or less-than values (i.e., censored NOECs, also called unbound NOECs), as long as it can be concluded whether $NOEC_{fish}$ was either clearly lower or higher than $NOEC_{min}$. Specifically, an API was included if the ratio of $NOEC_{fish}$ to $NOEC_{min}$ was given as $>x$ with x being any value greater than 1 or if the ratio was given as $<y$ with y being any value smaller than 1. These $NOEC_{fish}$-to-$NOEC_{min}$ ratios are provided in Table S1. Two examples illustrating the handling of censored NOECs are as follows: Celecoxib was not included because it cannot be concluded that $NOEC_{min}$ (≥11 μg/L) is lower than $NOEC_{fish}$ (≥230 μg/L). Aprepitant was included because it can be concluded that $NOEC_{fish}$ (≥195 μg/L) is greater than $NOEC_{min}$ (18 μg/L). In fact, the $NOEC_{min}$ of aprepitant is at least 10-fold, and potentially more than 100-fold, lower than $NOEC_{fish}$. For all APIs included in this additional analysis with a censored ratio of $NOEC_{fish}$ to $NOEC_{min}$, it can be stated whether $NOEC_{fish}$ is potentially more than 100-fold lower (or higher) than $NOEC_{min}$. For 20 APIs in the verification data set, such a conclusion was not possible, resulting in a data set of 103 APIs for this additional analysis.

The distribution of the $NOEC_{fish}$-to-$NOEC_{min}$ ratios for these 103 APIs is shown in Figure 3, and the actual values are provided in Table S1. There were three APIs for which $NOEC_{fish}$ values were definitely more than 100-fold lower than $NOEC_{min}$ values (symbols below the 0.01 reference line in Figure 3). Namely, these were the two glucocorticoids in the data set (mometasone and betamethasone) and the tyrosine kinase inhibitor (antineoplastic) lapatinib. Neither mometasone nor lapatinib was included in the verification data set due to a lack of availability of TWC. There were five more APIs where $NOEC_{fish}$ values were potentially more than 100-fold lower than $NOEC_{min}$ values (empagliflozin, ezetimibe, iobitridol, nilotinib, and sorafenib; empty symbols between the 0.01 and 1.0 reference lines). All five had $NOEC_{min}$ values given as greater-than values and defined $NOEC_{fish}$ values. In two cases (empagliflozin and iobitridol), $NOEC_{min}$ values were ≥100 mg/L and $NOEC_{fish}$ values were above 1 mg/L, which means that a more than 100-fold difference is unlikely (and ultimately not relevant). This leaves six APIs in total (mometasone, betamethasone, lapatinib, ezetimibe, nilotinib, and sorafenib) that could erroneously be excluded from in vivo fish testing when using only the PEC/$NOEC_{min}$ criterion. Four of these six APIs had an available TWC: two out of the four were correctly identified for in vivo fish testing only by the PEC/TWC criterion, one only by the PEC/$NOEC_{min}$ criterion, and one by both criteria. This underlines the importance of applying the FPM in parallel with the PEC/$NOEC_{min}$ approach.

Among the 103 APIs, three had $NOEC_{min}$ values more than 100-fold lower than $NOEC_{fish}$ values (everolimus, fluorouracil, and vorinostat; symbols above the 100 reference line) and another 12 had $NOEC_{min}$ values potentially 100-fold lower

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than $NOEC_{fish}$ values (empty symbols between the 1 and 100 reference lines). Hence, the distribution of $NOEC_{fish}$-to-$NOEC_{min}$ ratios (Figure 3) was not biased toward a higher frequency of low $NOEC_{fish}$-to-$NOEC_{min}$ ratios. Overall, the majority of APIs (80 of the 103) showed less than a 100-fold difference between the $NOEC_{fish}$ and $NOEC_{min}$ values, and 64 APIs showed less than a 10-fold difference. This provides further evidence that an additional safety factor of 100 to $NOEC_{min}$ would be protective for $NOEC_{fish}$, with such a substitution rendering in vivo testing of fish in many cases unnecessary.

SUBSTANTIAL EVIDENCE FOR SPECIFIC EFFECTS ON FISH

There are several lines of reasoning that may justify in vivo fish testing of APIs independent of the risk-based part of the decision tree (i.e., along the red arrow shown in Figure 1). The evidence needed to justify this, however, needs to be both scientifically sound and sufficiently strong, given the ethical obligation to minimize vertebrate animal usage. In such a case, although the respective API would take a shortcut in the decision tree, it would not necessarily be omitted from the testing on *Daphnia* and algae. What is deemed substantial evidence to justify directly going to in vivo fish testing will need to be judged on a case-by-case basis and will likely change as the associated science develops. There is not sufficient knowledge yet available to provide comprehensive guidance on this, but we address some relevant considerations in the following discussion.

One line of reasoning could be a finding that certain APIs or groups of APIs are systematically erroneously excluded from fish testing, i.e., they are not reliably covered by the risk-based part of the decision tree. Based on the available data, however, there seems to be no such systematic bias, given that no false negatives were found in the verification data set and there were only a few potential false negatives in the additional analysis of $NOEC_{fish}$-to-$NOEC_{min}$ ratios.

The second line of reasoning could be that available experimental data from the literature or modeled acute toxicity estimates for fish indicate specific effects or high toxicity of a given API in fish, but these data are perhaps considered insufficiently conclusive or consistent or standardized to be used in an ERA. Such data would need to be evaluated critically case by case to decide whether the evidence is sufficiently robust to justify (further) fish testing.

The third line of reasoning could be that certain APIs or groups of APIs consistently have $NOEC_{fish}$ values much lower than $NOEC_{min}$ values, which may point to specific effects in fish related to the pharmacology of these APIs. No obvious patterns were found in the analysis of the verification data set, as most of the broadly defined groups contained APIs that were excluded from fish testing as well as APIs that were considered for fish testing (see Figure 2). The three APIs clearly showing more than 100-fold lower $NOEC_{fish}$ values than $NOEC_{min}$ values belonged to two different therapeutic groups: glucocorticoid anti-inflammatory (betamethasone and mometasone) and antineoplastics (lapatinib). About half of the antineoplastics were excluded from fish testing (Figure 2) and the $NOEC_{fish}$-to-$NOEC_{min}$ ratios of antineoplastics (spanning from ≤0.009 for lapatinib to 11 428 for fluorouracil) covered the full range and represented the extremes of the distribution (Figure 3). Hence, there is no substantial evidence that would suggest that all antineoplastics should directly be considered for in vivo fish testing. Even for tyrokinase inhibitors (TKIs) as the subgroup of antineoplastics dominating the verification data set (16 out of 21 antineoplastics), there was no evidence that $NOEC_{fish}$ was consistently lower than $NOEC_{min}$. Hence, the finding that among all TKIs, only lapatinib has a more than 100-fold lower $NOEC_{fish}$ value than $NOEC_{min}$ does not justify that TKIs in general should be considered directly for in vivo fish testing independent of the risk-based part of the decision tree (i.e., along the pathway indicated by the red arrow in Figure 1). However, it could be argued that TKIs that specifically target the same receptors as lapatinib (HER2 and EGFR2) may be considered for in vivo fish testing directly, i.e., independent of the risk-based part of the decision tree. Betamethasone and mometasone were the only glucocorticoids (the latter one was not in the verification data set). For both, $NOEC_{fish}$ was based on effects on body weight or length observed in a standard early life stage test (OECD 210). Glucocorticoids are known to specifically interact with the neuroendocrine systems of vertebrates, including those in fish.[24−27] Based on the finding here that $NOEC_{fish}$ values are more than 100-fold lower than $NOEC_{min}$ values for the two glucocorticoids and knowledge on the highly complex and specific functioning of glucocorticoids in vertebrates, it can be argued that data on chronic toxicity in fish need to be generated for a glucocorticoid independent of the risk-based part of the decision tree to enable a reliable risk assessment.

REFINING THE DECISION USING IN SILICO TOOLS OR IN VITRO MODELS

In silico and/or in vitro data and tools are proposed for use in the next step of the decision tree (Figure 1) to review whether in vivo testing on fish is really warranted and justified for an API that has not been filtered out from fish testing in the first steps. Such tools and models are often developed and proposed as substitutes for in vivo testing in the context of the 3Rs approach, and they may also aid in prioritization approaches and provide insights into the mechanisms of toxicity.[28] Numerous methods have been proposed as alternatives to fish in vivo testing, but they are mostly not accepted as regulatory substitutes because they often lack validation of quantitative relationships required to enable extrapolation to adverse effects or lack clearly defined applicability domains.[28,29] Hence, in the context of the decision tree proposed here, results obtained with such methods should serve as supporting evidence only to help decide on the need for in vivo fish testing.

Among the APIs in the verification data set, the PEC/TWC criterion filtered out about twice as many APIs for in vivo fish testing compared with the PEC/$NOEC_{min}$ criterion (Figure 2), demonstrating that it is the more conservative of the two criteria. This is supported by the finding that TWC was the more conservative hazard estimate than $NOEC_{min}$ for 84% (81 of 96) of the APIs. For 10 of the 37 APIs considered for in vivo fish testing exclusively due to the PEC/TWC criterion, the $NOEC_{fish}$ values were higher than $NOEC_{min}$ values. Hence, in hindsight, in vivo fish testing would not be necessary, as no lower endpoint was derived by testing fish. This indicates that a less conservative threshold for the PEC/TWC criterion could avoid even more in vivo fish tests. However, greater trust in a (potentially improved) FPM model would be required to defend a threshold of, e.g., 0.01 instead of 0.001 for PEC/TWC. Experimental studies have demonstrated that the FPM (an in silico tool) provides reasonably reliable predictions of

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Table 1. Ratio of $NOEC_{fish}$ to $NOEC_{min}$ (as Shown in Figure 2) and Values for the Hazard Ratios Used in the Decision Tree (Values Above the Threshold of 0.001 Indicated in Bold) for a Set of 12 APIs for Which $M_{tox}PC$ was Collected and Compared to $FPC_{PE}C$[a]

API	$NOEC_{fish}/NOEC_{min}$	PEC/TWC	$PEC/NOEC_{min}$	FPC_{PEC} ($\mu g/L$)[b]	$M_{tox}PC$ ($\mu g/L$)[c]	$FPC_{PEC}/M_{tox}PC$
ezetimibe	<0.18	**9.992**	0.0006	33.97	266	0.13
propranolol	55.0	**0.0319**	**0.1514**	0.59	10	0.06
atorvastatin	3.21	**0.3047**	**0.0053**	1.07	22	0.05
dasatinib	0.26	**0.00199**	0.00002	0.08	2	0.04
everolimus	150.0	**0.0097**	**0.0190**	0.03	0.76	0.04
aripiprazole	2.22	**0.0105**	**0.0059**	0.29	14	0.02
rosuvastatin	55.6	**0.2835**	**0.0129**	0.18	18	0.01
montelukast	0.77	**0.1397**	0.0004	6.98	3330	0.002
ceritinib	6.62	**0.0142**	0.00004	0.18	155.8	0.001
dabrafenib	14.0	0.00004	0.00001	0.06	342	0.0002
zoledronic acid	0.82	0.0000001	0.0000001	0.00002	0.6	0.00003
desloratadine	1.33	**0.0212**	0.00011	0.04	900	0.00004

[a]LO(A)EL: lowest observed adverse effect level (in mammalian species), FPC_{PEC}: fish plasma concentration at predicted environmental concentration; $M_{tox}PC$: mammalian plasma concentration at LO(A)EL. [b]Predicted fish plasma concentration at the PEC, calculated according to Gunnarsson et al.[3] [c]Plasma concentration at LO(A)EL measured in the species providing the lowest LO(A)EL among available repeat-dose studies.

blood plasma concentrations of APIs in exposed fish,[30,31] even for small cationic molecules that fall outside the applicability domain of the original model.[32] Data derived from in vitro models, e.g., cultured fish gill cells[33] or in silico tools modeling passive transport across membranes,[34] may help to confirm or rebut the assumption of relevant uptake into fish and thereby inform on the reliability and plausibility of a predicted TWC. In silico prediction models of bioconcentration in fish e.g, refs 35−37, eventually including consideration of metabolism,[38] may also be useful as supporting evidence for TWC. Another option is the use of in silico tools that extrapolate chronic fish toxicity from mammalian toxicity[39] or from acute or chronic *Daphnia* toxicity.[40−42] While these models do not necessarily provide (precise) quantitative estimates for $NOEC_{fish}$, they may nevertheless serve as supporting evidence to check the plausibility of an FPM prediction.

Analogous to the FPM, blood plasma concentrations that result in toxicological effects in mammals may be useful to support a final decision on whether to conduct in vivo fish testing. Mammalian toxicity data are generally available for authorized APIs from preclinical studies, often together with measured blood plasma concentrations of the API in dosed animals. To explore the usefulness of such data as additional supporting evidence within the decision tree, preclinical data for a subset of APIs were compiled from the website of the U.S. Food and Drug Administration (Drugs@FDA). The lowest plasma concentration related to the lowest observed (adverse) effect level (LOE(A)L) was selected as $M_{tox}PC$. Details on data compilation and derivation of $M_{tox}PC$ are provided in the Supplements. The $M_{tox}PC$s were compared to theoretical blood plasma concentrations in fish exposed at PEC (FPC_{PEC}) as calculated by the FPM (Table 1). The higher the ratio of FPC_{PEC} to $M_{tox}PC$, the higher the likelihood for toxic effects in fish, assuming positively correlated susceptibility of fish and mammals in response to internal blood plasma concentration. The ratio of FPC_{PEC} to $M_{tox}PC$ did not exceed 1 for any of the 12 evaluated APIs (Table 1), i.e., the predicted internal concentrations of fish exposed to environmental concentrations were in all cases lower than plasma concentrations known to be toxic in preclinical test species. The ratio of FPC_{PEC} to $M_{tox}PC$ exceeded 0.1 for only one API (the lipid-lowering agent ezetimibe), which also had the lowest $NOEC_{fish}$-to-$NOEC_{min}$ ratio among these 14 APIs. Ezetimibe had been considered for in vivo fish testing by the decision tree based on the very high PEC/TWC ratio (exceeding 1); $PEC/NOEC_{min}$ was just below the threshold of 0.001. The less than 10-fold difference between FPC_{PEC} and $M_{tox}PC$ for ezetimibe is therefore deemed to support a decision for in vivo fish testing. There were nine APIs with an FPC_{PEC}-to-$M_{tox}PC$ ratio at or above 0.0002 (Table 1). $NOEC_{fish}$ for this majority of APIs was slightly below or (in some cases considerably) above the respective $NOEC_{min}$, which indicates that $M_{tox}PC$ provides no consistent support for a decision for or against in vivo fish testing. Among the 12 APIs, there were two (zoledronic acid and desloratadine) with very low FPC_{PEC}-to-$M_{tox}PC$ ratios (below 0.00001). For both, $NOEC_{fish}$ is similar to $NOEC_{min}$. While zoledronic acid was excluded from in vivo fish testing by the decision tree, desloratadine was considered for fish testing based on the PEC/TWC criterion. In the case of desloratadine, this decision could be revised based on the very low FPC_{PEC}-to-$M_{tox}PC$ ratio as a supporting argument. Overall, this initial analysis based on a small example set of 12 APIs with $M_{tox}PC$ data indicates that information on mammalian toxicity could help, in principle, in reaching a final decision on the necessity of in vivo fish testing for APIs not excluded already by the first steps of the decision tree. However, only extreme ratios of FPC_{PEC} to $M_{tox}PC$ (above 0.1 or below 0.0002) appear to provide conclusive support. In addition to challenging these initial findings by repeating the analysis with a larger set of APIs (including ideally more with $NOEC_{fish}$ being smaller than $NOEC_{min}$), this approach may also be improved by further exploring the relationship between blood plasma concentrations in mammals and in fish that relate to toxicological/biological effects.

The decision tree proposed in this paper provides a strong basis for significantly reducing in vivo testing on fish for legacy APIs. Based on the data set used here, we advocate that a threshold of 0.001 applied to the risk quotients $PEC/NOEC_{min}$ and PEC/TWC offers a reasonable balance between being sufficiently protective and being overly conservative, i.e., avoiding in vivo fish testing of APIs as much as possible, without compromising the level of environmental protection in a subsequent risk assessment. The PEC/TWC quotient was lower than the $PEC/NOEC_{min}$ quotient for 83% of APIs from

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the verification data set, and for 38% of the APIs, only the PEC/TWC quotient triggered in vivo fish testing. Hence, including the FPM provided an additional layer of conservatism and safety to protect against erroneous exclusion of APIs from in vivo fish testing that could occur when using only $NOEC_{min}$. The applied threshold of 0.001 is currently considered appropriate, as it ensured that none of the 96 APIs in the verification data set was erroneously excluded from consideration for fish testing. Since the threshold of 0.001 is deemed fairly conservative (equivalent to applying an assessment factor of 1000 instead of 10 to the lowest available NOEC), recalibration may be warranted in the future to obtain an even greater efficiency of the decision tree, i.e., avoid in vivo fish testing for a greater number than about one-third of APIs. This may be possible if, for example, more data become available that allow for a robust read-across from chronic endpoints in *Daphnia* and algae to chronic endpoints in fish. Further improvements and validation of the FPM may particularly help to establish a less conservative threshold value. The risk-based component of the decision tree is complemented by a shortcut to take APIs with substantial evidence, e.g., for specific effects in fish related to the pharmacological mode of action, directly to consideration for in vivo fish testing.

While the current version of the decision tree can be applied immediately to avoid unnecessary testing on fish for legacy APIs, new data and evidence as they emerge can (and should) be used for its further development, refinement, and validation. Because the decision tree aims to be at least as protective as a standard ERA according to the guideline,[6] the tree would also require updating if the data requirements for the standard ERA changed (e.g., requests for data on other species or other endpoints). The decision tree could also be applied as a screening tool when generating an ERA for a new API during market authorization or as a guiding tool in the development of more environmentally benign APIs. With regard to immediate action, the proposed decision tree will be put into practice in the Innovative Medicines Initiative project PREMIER (https://imi-premier.eu/). Within PREMIER, ecotoxicological data for around 25 legacy APIs will be generated, while also seeking to minimize in vivo fish testing as much as reasonably possible. The ecotoxicological data generated within, or otherwise made available through PREMIER, together with further improvements of the FPM, will be used to further assess the reliability and suitability of the decision tree, notably for APIs lacking any ecotoxicity data. Similarly, as further in silico and in vitro tools become available in the future, these will be incorporated where possible to develop and refine the decision tree. Ultimately, our intention is that the decision tree will enable better and more integrative use of all available (eco)toxicity data and minimize fish usage in testing pharmaceuticals, without compromising environmental protection.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c07222.

Data on individual pharmaceuticals included in the study (Table S1) and text describing how mammalian toxicity data were derived (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Anja Coors − *ECT Oekotoxikologie GmbH, 65439 Flörsheim/Main, Germany;* orcid.org/0000-0002-9224-2917; Email: a.coors@ect.de

Authors

A. Ross Brown − *Biosciences, University of Exeter, Exeter EX4 4QD Devon, U.K.;* orcid.org/0000-0002-3892-8993

Samuel K. Maynard − *Global Sustainability, AstraZeneca, Cambridge CB2 8DU, U.K.*

Alison Nimrod Perkins − *Eli Lilly and Company, Indianapolis, Indiana 46285, United States*

Stewart Owen − *Global Sustainability, AstraZeneca, Cambridge CB2 8DU, U.K.*

Charles R. Tyler − *Biosciences, University of Exeter, Exeter EX4 4QD Devon, U.K.*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.2c07222

Notes

The authors declare the following competing financial interest(s): S.K.M., A.N.P., and S.O. are employees of pharmaceutical companies, which market some of the here included pharmaceuticals. All other authors claim no conflict of interest.

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