



In silico bioavailability triggers applied to direct and indirect thyroid hormone disruptors

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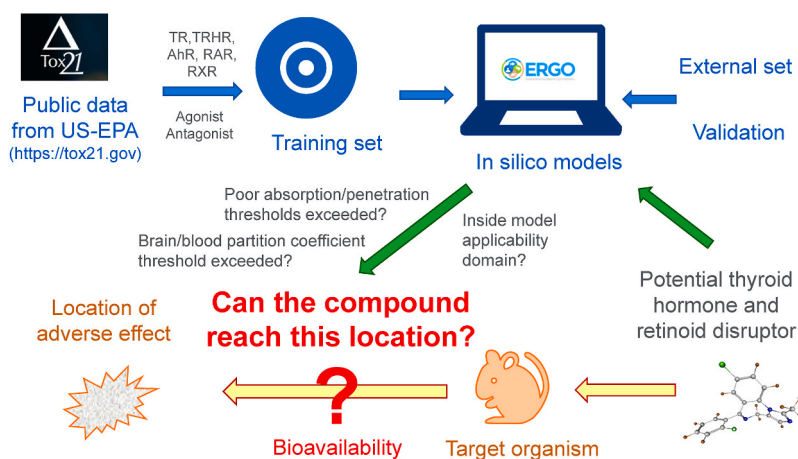
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HIGHLIGHTS

- Adverse effects of potential thyroid hormone disruptors require bioavailability.
- Internal bioavailability at locations where adverse effects may take place.
- New in silico estimation model to predict respective bioavailability presented.
- Molecular weight, lipophilicity, hydrogen bonding, blood/brain partitioning identified as suitable for this purpose.
- Model validated successfully, applicability domain characterized.

GRAPHICAL ABSTRACT



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ABSTRACT

Among endocrine disruption, interference with the thyroid hormone (TH) regulation is of increasing concern. Respective compounds encode through their structural features both the potential for TH disruption, and the bioavailability mitigating the toxicological effect. The aim of this study is to provide a substructure-based screening-level QSAR (quantitative structure-activity relationship) that discriminates bioavailable TH disruptors from not bioavailable counterparts, covering both direct and indirect (retinoid- and AhR-mediated) modes of action. The QSAR has been derived from literature data for 1642 compounds, and takes into account Lipinski's rule-of-five and the brain/blood partition coefficient $K_{\text{brain/blood}}$. For its validation, an external test set of 145 substances has been used. For 1787 compounds meeting the model application domain, the model yields only one false negative. The discussion addresses the mechanistic meaning of the bioavailability triggers molecular weight, H-bond donor and acceptor, hydrophobicity ($\log K_{ow}$), and the physicochemical properties

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underlying $\log K_{\text{brain/blood}}$. The model may serve as bioavailability-screening step within a decision support system for the predictive assessment of chemicals regarding their potential to disrupt thyroid hormone function in a direct or indirect manner.

Abbreviations

Abbreviation list

ACF -	atom-centred fragments	PAH -	polycyclic aromatic hydrocarbon
Aeid -	assays endpoint identification	PBDE -	polybrominated diphenyl ether
AhR -	aryl hydrocarbon receptor	PBPK models -	physiologically based pharmacokinetic models
AOP -	adverse outcome pathway	PCB -	polychlorinated biphenyl
ED -	endocrine disruptor	QSAR -	Quantitative Structure Activity Relationship
EU -	European Union	RAR -	retinoic acid receptor
ERGO project -	Endocrine Guideline Optimisation; EU Horizon 2020 project	RXR -	retinoid X receptor
HA -	hydrogen bond acceptors	SMILES -	Simplified Molecular Input Line Entry System
HD -	hydrogen bond donors	TH -	thyroid hormone
ChemProp -	Chemical Properties Estimation Software System	Tox21 program -	Toxicology in the 21st Century, US federal collaboration
LFER -	linear free energy relationship	TOX21_TR_LUC_GH3 -	cell-based assay for screening of TR-mediated effects
LSER -	linear solvation energy relationship	ToxCast -	Toxicity Forecaster, US EPA chemical prioritization research program
MM -	molecular mass	TR -	thyroid hormone receptor
		TRHR -	thyrotropin-releasing hormone receptor

1. Introduction

Endocrine disruption (ED) is a major concern for adverse effects of chemicals. It comprises a heterogeneous framework of several target organs, processes and modes of action with complex mutual interactions, and includes agonism and antagonism. Among various ED routes, thyroid hormone (TH) disruption (THD) is of particular concern (AOP Wiki, 2023; Reinwald et al., 2021; Duntas, 2015; Gutleb et al., 2016; Nugegoda and Kibria, 2016).

A considerable number of chemical classes with known THD has been examined and reviewed, including bisphenols (Rubin and Seebacher, 2022), bromophenols (Michalowicz et al., 2022 et al.), PAHs (Kim et al., 2021), perfluoroalkyl acids (Lee et al., 2020), PCBs (Takaguchi et al., 2019), perfluoroalkyl phosphinic acids (Liu et al., 2019), and PBDEs (Yu et al., 2019). In 2019, the EU Horizon 2020 project ERGO (Holbech et al., 2020) has been started with the aim of linking human and environmental health hazard and risks assessment of thyroid hormone disruptors.

Several in vitro test assays are available or under development to address different mechanisms of TH disruption. A currently ongoing project focusses on in vitro test systems, studying differences in THD across human-relevant test species (Ramhøj et al., 2023). The Tox21 program as a joint initiative of US governmental agencies (U.S. EPA., 2019) investigated ca. 8300 chemicals through several bioassays focusing on regulatory relevant THD endpoints including cross-talking. Accordingly, large THD data sets are available, keeping in mind the difficulty to discriminate direct THD from cross-talk pathways involving non-THD receptors (Paul-Friedman et al., 2019; Schmidt, 2020).

The present study focussed on the bioavailability of compounds interacting with the TH receptor, the thyrotropin-releasing hormone receptor (TRHR), the aryl hydrocarbon receptor (AhR) involved in hormone metabolism, the retinoic acid receptor (RAR), and the retinoid X receptor (RXR). The latter two have been included because of their potential cross-talk contribution to THD (Paul-Friedman et al., 2019; Schmidt, 2020). Note further that as summarized in the recently published AOP No. 458 in AOPwiki (<https://aopwiki.org/aops/458>), AhR receptor agonists may lead to neurodevelopmental changes in neonates through increasing the metabolic degradation of T4 that in turn

may decrease the T4 plasma level in rodents and possibly also human.

However, a principal short-coming of in vitro assays is their lack of mimicking the whole-organism bioavailability that concerns both absorption and the compound's fate from the initial organismic uptake to the site of action. The latter involves (at least two of) the following three steps: Transport across cellular membranes along through passive or facilitated diffusion or through some active (energy-consuming) mechanism, metabolism, and intracellular translocation to the site of action (receptor of TH protein or TH-related enzyme or non-TH cross-talk protein/enzyme). Here, the efficiency of all passive transport routes depends on molecular size, hydrophobicity, and on the rates of possibly available abiotic (e.g. hydrolytic) and metabolic degradation pathways. Note that compounds with a high in vitro THD potential may not reach their biological target(s) in human due to insufficient whole-organism bioavailability. In fact, even in vitro effects require the toxicant to successfully transfer cellular membranes and reach the intracellular site of action. From this viewpoint, separate information on the THD-relevant bioavailability would complement in vitro results, and thus support regulatory THD assessment.

Recently, passive sampling has been proposed to experimentally mimic passive uptake of waterborne xenobiotics (Kreutzer et al., 2022), and similarly desorption extraction was proposed to cover main aspects of xenobiotic uptake from soil (Posada-Baquero et al., 2022).

Chemoavailability is a concept complementing bioavailability for xenobiotics reacting with endogenous compounds (Böhme et al., 2016). It quantifies the trade-off between chemical reactivity (to covalently attack cytosolic compounds) and hydrophobicity (to accumulate in the membrane). Correspondingly, the opportunity for biotransformation is driven by an analogous competition between membrane affinity (driven by hydrophobicity) and the readiness to enter the cytosol (driven by water solubility). Note further that under thermodynamic conditions, intracellular aqueous concentration of xenobiotics equals the one in the external water such as the exposure concentration in aquatic bioassays (Blaschke et al., 2012; Freidig et al., 1999). While biotransformation can be assessed in vitro, such approaches do not capture the respective toxicokinetic phase in vivo, and are also limited to the metabolism-active components offered in the bioassay employed. Because biotransformation itself may differ significantly between in vitro and in vivo settings, explicit consideration of biotransformation

has been excluded from the present investigation.

The current study is confined to bioavailability at the cellular level, including cell interiors. In the *in vitro* assays, the test chemical is directly presented to cell lines containing the biological target, thus omitting the otherwise previous transport from the location of initial uptake and possibly associated metabolic processes. However, a chemical exposed *in vitro* still needs to enter the cell and then move through the cytosol to reach the site of action to exert TH disruption. Compounds exerting TH disruption *in vitro* in a direct or indirect (cross-talk) way are considered to be bioavailable in this study and are expected to be predicted as available for the cellular level by bioavailability models.

Basically, bioavailability results from physicochemical partitioning and transport processes. Accordingly, it is supposed to be related to physicochemical properties, which in turn depend on chemical structure. Therefore, QSAR (quantitative structure-activity relationship) models may be useful for predicting THD bioavailability, as had been shown previously with predicting bioavailability for bioconcentration (Nendza et al., 2018). To this end, existing QSARs for detecting poor absorption according to Lipinski's rule-of-five (Lipinski et al., 1997) and to predict the blood brain barrier (BBB) permeation (Abraham et al., 2006) have been included. BBB permeation governs the access to the central nervous system, and has already been addressed through respective property-specific thresholds (Kunwittaya et al., 2013). For the presently introduced scheme to predict THD bioavailability from chemical structure, only QSAR-calculated physicochemical properties have been used. Nevertheless, the derived screening-level scheme may also be applied with using experimental property data (if available in sufficient quality).

2. Material and methods

2.1. Construction of the training set

From the ToxCast database (U.S. EPA., 2019), all cell assay data on receptor-mediated activities relevant for thyroid hormone and retinoid disruption were used as starting basis for this study. Subsequently, known agonists or antagonist have been collected for the following THD-related receptors: thyroid hormone receptor (TR), thyrotropin-releasing hormone receptor (TRHR), retinoic acid receptor (RAR), retinoid X receptor (RXR), and the aryl hydrocarbon receptor (AhR, no antagonists). The two retinoid receptors have been included because of their potential cross-talk with the TH regulation, and AhR due to its role in TH metabolism. A list of the considered assays along with their endpoint identification (aeid) is given in Table 1, and further information on the assays can be found in the ToxCast database at the CompTox Chemicals Dashboard. However and as justified below, TR agonism and RXR antagonism have not been included in the primary data set building.

In order to examine the potential for predicting direct and indirect (cross-talk) THD-specific bioavailability, only compounds known to be

active as direct THD or retinoid disruptor or AhR ligand (facilitating T4 metabolism) could be taken into account. These direct and indirect THDs have been collected in our ERGO project (Holbech et al., 2020). Inactive chemicals may still be bioavailable in terms of reaching but not interfering with TH-related targets, whereas potentially active compounds will not trigger effects without sufficient bioavailability.

In many cases, data from more than one experimental study for the same compound and the same mode of action were available. These data were compared in terms of their classification as active or inactive, and in case of any ambiguities the respective compounds were excluded from further consideration for the particular mode of action.

The total set of active and thus – by definition - bioavailable compounds used for the present model development comprised 1761 different substances. Of these, 359 compounds consisted of more than one molecule and/or ions. Most of the latter were hydrates and hydrohalogenides (mostly hydrochlorides), halogenides, sulphates, and ammonium salts. For the subsequent model building, all such compound complexes or salts have been replaced by their main components that are supposed to trigger the TH disrupting effect.

Subsequently, duplicates resulting from the same main component were eliminated, reducing the data set to 1698 chemicals. Then, 12 stereoisomers of other data set compounds were removed, because the bioavailability-relevant physicochemical properties do not depend on stereochemistry (in possible contrast to the TH-disrupting effect). Finally, 43 inorganic or metalorganic chemicals were omitted because they are outside the domain of the QSARs used for predicting bioavailability-related physicochemical properties, and because their bioavailability regime most likely differs from the one applicable for organic compounds. This last step reduced the training set to finally 1642 organic compounds. Accordingly, inorganic and metalorganic compounds are outside the applicability domain of the presently introduced bioavailability model.

2.2. Biological domain of the training set

Removal of one of two duplicates included also cases where only one of the two substance mixtures was THD-active. In this sense, also some of the removed duplicate main components contributed to the total number of different modes of action (MOAs).

One chemical (proflavin) of the final set is active regarding all five THD-related targets (TR, TRHR, RAR, RXR, AhR; see above), there are 32 compounds interfering with four targets, 219 addressing three targets, 592 chemicals affecting two targets, and (the remaining) 798 substances active concerning one target. However, the absence of an activity towards a target does not necessarily mean respective inactivity, because not all compounds extracted from the Tox21 database were tested for each MOA. Note further that in the present *in vitro* data set of the training set compounds, the potential impact of cytotoxicity has not been reported and is thus not accounted for.

With regard to the individual MOAs, 788 compounds would act as TR

Table 1

Assays considered in this study: Name, ToxCast database (U.S. EPA., 2019) end point identification (aeid) together with mode and purpose of assay.

Assay name	Assay aeid	mode	organism	tissue	Assay Design Type Sub	Assay Design Type (reporter)	Cell Short Name
TOX21_TR_LUC_GH3_Agonist	803	Agonist	rat	pituitary gland	luciferase induction	inducible	GH3
TOX21_TR_LUC_GH3_Antagonist	804	Antagonist	rat	pituitary gland	luciferase induction	inducible	GH3
TOX21_AhR_LUC_Agonist	806	Agonist	human	liver	luciferase induction	inducible	HepG2
TOX21_RAR_LUC_Agonist	1659	Agonist	mouse	embryo	luciferase induction	inducible	C3H10T1/2
TOX21_RAR_LUC_Antagonist	1839	Antagonist	mouse	embryo	luciferase induction	inducible	C3H10T1/2
TOX21_RXR_BLA_Agonist_ratio	1820	Agonist	human	kidney	beta lactamase induction	inducible	HEK293T
TOX21_TR_RXR_BLA_Antagonist_Followup_ratio	2257	Antagonist	human	kidney	beta lactamase induction	inducible	RXRa-UAS-bla HEK293T
TOX21_TRHR_HEK293_Agonist	2364	Agonist	human	kidney	intracellular calcium	biochemical	HEK293
TOX21_TRHR_HEK293_Antagonist	2365	Antagonist	human	kidney	intracellular calcium	biochemical	HEK293

Note: GH3: rat pituitary cell line, HEK 293T: human embryonic kidney cell line, HepG2: hepatoblastoma cell line, LUC: luciferase, RAR: Retinoic acid receptor, RXR: retinoid X receptor, TR: thyroid hormone receptor alpha, TRHR: thyrotropin-releasing hormone receptor.

antagonists, 493 as AhR receptor (human liver) agonists, 260 as TRHR disruptors, 964 as RAR disruptors, and 267 as RXR signalling pathway agonists. Concerning the criticism regarding the mechanistic interpretation of TR assay positives (Paul-Friedman et al., 2019; Schmidt, 2020), this assay was the sole positive test for only 91 of the 788 identified antagonist.

As noted above, TR agonism and RXR antagonism were not included in the training set because of the very low number of respective positive (active) assay results. From the 31 data items for TR agonism, five of them were genuine duplicates, and another two were two E/Z isomers. In addition, with methylphenol there was actually one UVCB (Unknown or Variable composition, Complex reaction product or Biological material) representing three different isomers. Moreover, seven of the TR agonists were already in the training set because of other agonistic MOAs: Two of them were RXR-active, one active towards AhR, RAR and RXR, one regarding AhR and RAR, one concerning TR and AhR and RAR, and one active towards TR and TRHR and RAR. The remaining 17 compounds of these TR agonists together with the three methylphenol isomers have been included in the external prediction set below.

In the original Tox21 dataset, 19 compounds are reported as RXR signalling pathway antagonists. These include four duplicates, one inorganic compound, and one mixture. From the remaining 13 structures, 12 were already in the training set because of other antagonistic activities. The latter covered one antagonist of TR, one of RAR, two regarding AhR, one interfering with TR and AhR, six acting against TR and RAR, and one antagonist of TR, TRHR and RAR. The finally remaining chemical pralatrexate has also been added to the prediction set.

Table S1 of the SI includes all training set compounds together with their MOAs and the chemical structures used for calculating the bioavailability-related physicochemical properties (see below), and the associated SMILES codes (Daylight, 2019a) are listed in Table S2 of the SI. Note finally that the performance of the bioavailability model presented below did not show any MOA-specific dependence. Accordingly, MOAs were not included in the subsequent model presentation and analysis.

2.3. Chemical domain of the training set

The training set of 1642 organic compounds includes 25 hydrocarbons, 30 halogenated hydrocarbons, 412 molecules containing also oxygen atoms (possibly besides halogen), 217 compounds where C, H and possibly halogen is augmented by nitrogen atoms, and 602 compounds composed of C, H, possibly halogen and both O and N. Moreover, 349 compounds contain sulfur or phosphorus or both, and in seven molecules have a silicon atom.

Regarding structural complexity, besides the 25 hydrocarbons without functional groups there are 265 compounds with one single functional group, 171 chemicals with multiple occurrences of one type of functional groups, 567 compounds with two different types of functional groups, and 614 chemicals with more than two types of functional groups.

In terms of polarity (see Ebert et al., 2023 for a respective detailed explanation), 55 chemicals are classified as nonpolar or weakly polar. Moreover, 988 compounds are polar with HBD groups (and typically but not necessarily also HBA groups), 559 polar compounds are HBAs but without HBD groups, and there are 40 polar chemicals without HBD and HBA groups.

The chemical domain is further characterised by an atom-centred fragments (ACF) approach (Kühne et al., 2009; Ebert et al., 2023). Basically, from each non-H atom in the molecule one ACF of a certain order (see below) is created that includes the following structural properties: atom type, aromaticity state, number of attached hydrogens, presence/absence of ring closures, and bonded neighbour atoms including the respective bond order/type. First-order ACFs include only the directly bonded neighbour atoms, whereas second-order ACFs also

enclose the neighbours attached to the bonded neighbour atoms. The chemical domain membership of a particular compound is characterized by comparing the ACFs of this compound in terms of occurrence and frequency to the ACF pool built from the training set. There are 2105 first-order and 9340 s-order ACFs. All calculations were carried out with the software system ChemProp (UFZ Department of Ecological Chemistry, 2021).

2.4. External test set

To validate the derived model for predicting TH-related bioavailability, an external test set of 145 direct and indirect THDs has been set up. Accordingly, the validation would be successful if at least most of the test set compounds would be classified as being bioavailable.

Among the 145 test set compounds, 17 and the three methylphenol isomers are TR agonists, and pralatrexate is the only RXR signalling pathway antagonist. For the remaining 124 chemicals, the same five MOAs apply as for the training set. Among these, 21 chemicals are active towards two different targets, with the remaining 103 substances triggering only one target. More specifically, 105 compounds act as AhR receptor (human liver) agonists, seven as TRHR disruptors, and 33 as RAR agonist. The TH-related activities and SMILES codes are given in Tabs. S3 and S4 of the SI.

Regarding the atomic composition, the test set includes only one hydrocarbon and one halogenated hydrocarbon, 23 molecules containing C, H, possibly halogen and oxygen atoms, 13 corresponding compounds with N instead of O, and 80 respective chemicals with both N and O. Moreover, 26 compounds have S or P atoms or both S and P. However, there is no compound with Si, and – as with the training set – no compound with any other heteroatom. Other atom types do not occur.

Concerning structural complexity, besides the hydrocarbon and the halogenated hydrocarbon there are 14 compounds with one single functional group, four chemicals with multiple occurrences of one type of functional groups, 42 compounds with two different types of functional groups, and 84 chemicals with more than two types of functional groups.

With respect to polarity, two chemicals are nonpolar or weakly polar, and 115 compounds are polar with HBD groups. Another 27 polar compounds are HBAs without HBD groups, and there is one polar chemical with no HBD and no HBA group (see Ebert et al., 2023 for a detailed explanation of our approach regarding polarity).

Application of our ACF approach (Kühne et al., 2009) yields 626 first-order and 1713 s-order ACFs. Interestingly, only 27 of the 145 test set compounds are fully within the ACF training set domain. Moreover, another 11 test set compounds are borderline inside the training set (nine of them with 1 s-order ACF outside the training set ACF pool, one with higher numbers of second-order ACF occurrences than in the training set, and one compound showing both of these mismatches, 56 are borderline outside the training set (with more than 1 s-order ACF not in the training set), and 51 fully outside the ACF training set domain (with first-order ACF mismatches).

Thus, successful validation of the model would enlarge its chemical domain, whereas a respective validation failure would require a further model refinement regarding either model scheme modifications or respective restrictions of its applicability domain.

2.5. Models

2.5.1. The rule-of-five

The rule-of-five model (Lipinski et al., 1997) has been examined for its suitability to characterise the bioavailability for direct and indirect THD. This model enables the identification of compounds expected to show low bioavailability in terms of poor absorption or membrane permeation. In this context, the following five structural features trigger an enhanced probability for poor absorption/permeation: more than

five hydrogen bond donors HBD, more than ten hydrogen bond acceptors (HBA), a molecular weight MW above 500 Da, and a logarithmic octanol/water partition coefficient $\log K_{ow}$ larger than five. According to Lipinski, poor absorption or membrane permeation is predicted if at least two of these four criteria are fulfilled. Note that the “5” in “rule-of-5” results from the fact that the numerical values of all four thresholds are 5 (#HBD, $\log K_{ow}$) or a multiple of 5 (#HBA, MW).

In the ChemProp (UFZ Department of Ecological Chemistry, 2021) implementation, hydroxy and amino groups serve as HBDs, and any oxygen and nitrogen atoms as HBAs (both of which correspond to the original Lipinski scheme). Regarding $\log K_{ow}$, ChemProp employs a consensus model based on several fragment methods from literature (UFZ Department of Ecological Chemistry, 2021). Calculation of the molecular weight MW is straight-forward.

2.5.2. The blood/brain model

The ability to penetrate the blood-brain barrier (BBB) is an important property for drugs designed to reach the central nervous system. The blood/brain partition coefficient provides a quantitative measure for it. In this study, its inverse - the brain/blood partition coefficient $K_{brain/blood}$ - has been applied. Here, $\log K_{brain/blood} \geq -0.52$ is assumed to indicate good BBB penetration (Kunwittaya et al., 2013). This $\log K_{brain/blood}$ threshold corresponds to an equilibrium concentration of the compound in the whole brain of at least 30 % of the compound concentration in blood. More conservative with respect to brain safety, also cutoff values around $\log K_{brain/blood} = -1$ are discussed (Kunwittaya et al., 2013). This latter threshold implies that at thermodynamic equilibrium, the compound concentration in the brain is 10 % of the one in the blood.

The estimation of the brain/blood partition coefficient employs an LSER (linear solvation energy relationship, Abraham et al., 2006), also known as Abraham equation or pp-LFER (polyparameter linear free energy relationship):

$$\log K_{brain/blood} = -0.088 - 0.185 E + 0.596 S + 0.623 A + 0.630 B - 0.630 V + 1.210 I_c \quad (1)$$

Here, the excess molar refractivity E is related to the softness of the molecular electronic structure, S characterizes the solute polarity and polarizability, A and B quantify the H-bond acidity (HBD capability) and H-bond basicity (HBA capability), respectively, and V is a characteristic volume (Abraham and McGowan, 1987; Platts et al., 1999). I_c is 1 for

carboxylic acids and 0 otherwise. The constant of -0.088 combines the original model constant (-0.526) with an indicator value for an in vitro setup (0.438).

3. Results and discussion

3.1. Results and model adaption

3.1.1. Rule-of-five model

The results of the rule-of-five model for the 1642 TH-active chemicals are given in Table S5 of the SI. Because no K_{ow} could be estimated for 11 compounds, the latter are assumed to be bioavailable following a conservative approach.

Interestingly, 92 of the 1642 compounds TH-active in vitro are predicted to be poorly bioavailable, which corresponds to a moderate error rate of 5.6%. In 91 of these cases, MW was one of the triggers for poor absorption/permeation. Only carmin acid ($C_{22}H_{20}O_{13}$) is predicted to be poorly bioavailable because of too many HBDs (nine) and HBAs (13), with an MW of 492 Da still slightly below the threshold. Moreover, 19 of these 92 substances (1.2 % of all) fulfilled two additional conditions besides MW.

Figs. 1 and 2 visualize the number of compounds exceeding the original HBD, HBA, MW and $\log K_{ow}$ triggers in terms of cumulative distributions. For 12 (0.7%) and 47 (2.9%) compounds, the numbers of HBD and HBA groups exceed 5 and 10, respectively (Fig. 1). The MW threshold of 500 Da is exceeded by 122 (7.4%) chemicals, and for 292 (18%) substances $\log K_{ow}$ is above 5 (Fig. 2). Regarding hydrophobicity, however, only 61 (3.7%) of the latter 292 compounds exceed also another threshold and are thus predicted to be poorly bioavailable.

Clearly, the MW limit of 500 Da is the worst-performing threshold with respect to TH-related bioavailability. Increasing this threshold to 600 Da already reduces the number of wrong predictions from 92 to 63 (i.e. from 5.6 % to 3.8 % of all). A threshold of 750 Da reduces the error rate to 32 (1.6%) cases.

Increasing the $\log K_{ow}$ limit does not appear to be reasonable. Besides a rather moderate effect on decreasing the error rate, a significantly higher cutoff for the hydrophobicity appears to be mechanistically not meaningful in the context of membrane permeability.

Increasing the #HBD or #HBA thresholds would also not significantly enhance the performance.

Interestingly, requiring the exceedance of three instead of two

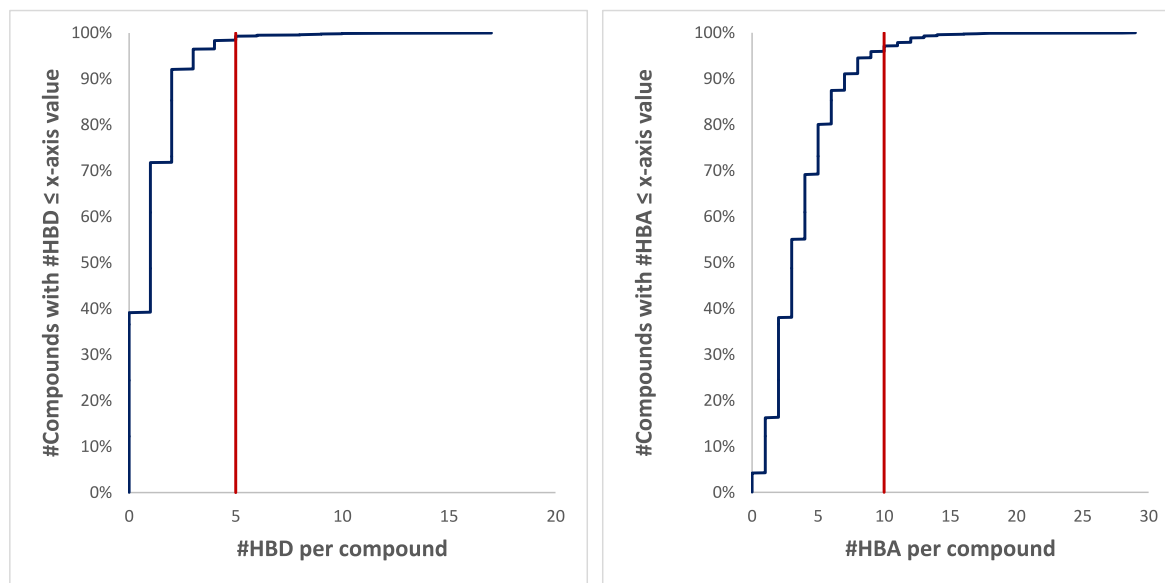


Fig. 1. Cumulative distribution of the number of H-bond donor groups (#HBDs, left) and H-bond acceptor groups (#HBAs, right) of the 1642 training set compounds. The vertical lines denote the suggested bioavailability thresholds of 5 HBDs and 10 HBAs, respectively.

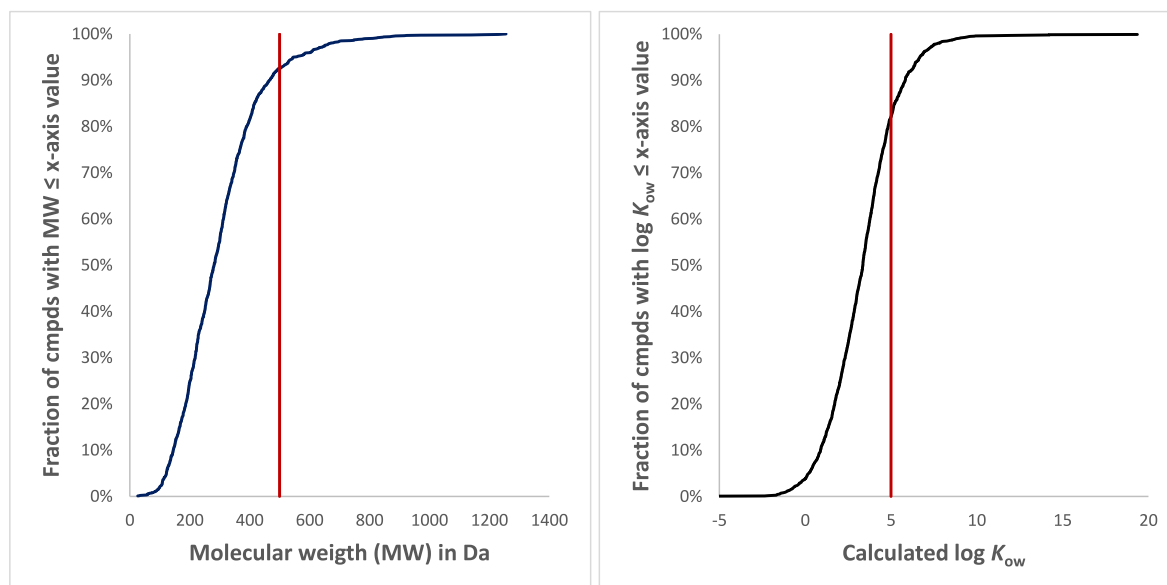


Fig. 2. Cumulative distributions of the molecular weight MW (left) and the logarithmic octanol/water partition coefficient $\log K_{ow}$ (right) of the 1642 training compounds. The vertical lines denote the suggested bioavailability thresholds of 500 Da and 5, respectively.

thresholds significantly decreases the number of wrong predictions to only 19 (1.2%). Combined with an MW threshold increased to 600 Da yields only 16 wrongly classified chemicals (1.0 %), and with a still higher MW threshold of 750 Da threshold this number decreases further to nine compounds (0.5 %).

Coming back to the original model, some of the calculated property values appear to be extremely high and may exceed the – however unknown – applicability domain of this approach. Although respective compounds are typically quite large and would be expected to have a low probability for passive diffusion across membranes, they might still profit from active transport mechanisms that are not addressed by the rule-of-five model. Note further that with increasing molecular size, fragment models tend to overestimate $\log K_{ow}$ for two reasons: Technically, because small errors associated with a given fragment value will add up linearly to an overall large error, and physically, because molecular folding to reduce the solute-solvent interface is not accounted for. For these reasons, the following upper limits for applying the rule-of-five model have been imposed: $\#HBD \leq 15$, $\#HBA \leq 20$, $MW \leq 1000$ Da, and $\log K_{ow} \leq 10$. Eight chemicals (0.5 % of the compounds) exceed one or more of these limits, and omission of this subset reduces the number of misclassifications (actually bioavailable in vitro vs rule-of-5-predicted to be not bioavailable) from originally 92 (see above) to 88 (5.4%). When imposing to exceed three (rather than two) thresholds with an MW cutoff of 750 Da as most conservative approach, only 7 chemicals (0.4 %) remain as false negatives.

3.1.2. Brain/blood partitioning model

The BBB permeability in terms of $\log K_{brain/blood}$ (see above) could be calculated through eq. (1) for 1620 of the 1642 training-set compounds. The resultant cumulative distribution is shown in Fig. 3, and the numerical results are listed Table S5 of the SI. Taking a conservative approach as with the $\log K_{ow}$ consensus model described above, the 22 compounds without a predicted $\log K_{brain/blood}$ value were assumed to be bioavailable. With the above-mentioned $\log K_{brain/blood}$ threshold of -0.52 applied as lower limit for reaching the brain, 147 chemicals (8.9 % of all) could not be operative in the brain, with still 40 of them (2.4% of all) yielding $\log K_{brain/blood} < -1$. When applying a $\log K_{brain/blood}$ cutoff of -1.3 where under thermodynamic equilibrium the compound concentration in the brain is 5% of the one in the blood, still 22 in vitro TH-active compounds (1.3%) would be predicted to not reach the brain. From this viewpoint, the 2.4% error rate when sticking to -0.52 as log

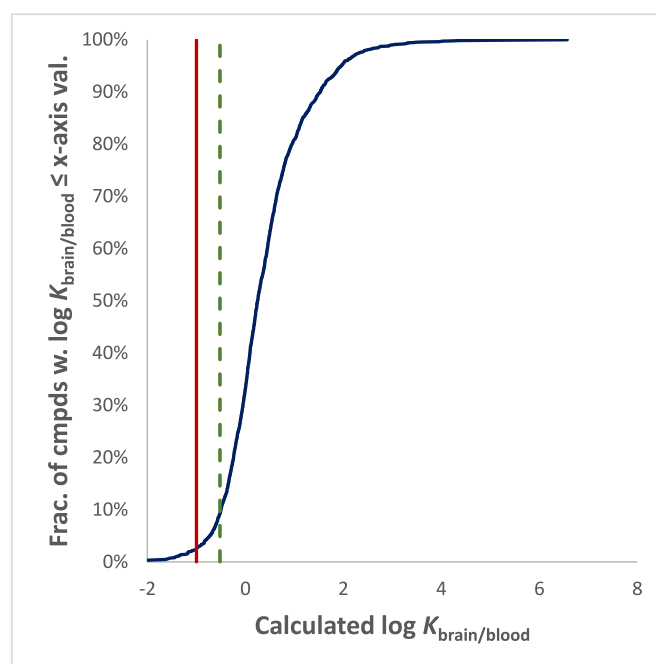


Fig. 3. Cumulative distribution of the logarithmic brain/blood partition coefficient $\log K_{brain/blood}$ across 1620 training-set compounds. The dashed right vertical line denotes the originally suggested bioavailability threshold of -0.52 . The left vertical line gives a more conservative threshold of -1 .

$K_{brain/blood}$ threshold appears acceptable for addressing the general TH-related bioavailability. For brain-specific TH regulation endpoints, however, the lower $\log K_{brain/blood}$ threshold of -1 may be applied.

3.1.3. Combined model

Since the rule-of-five model and $\log K_{brain/blood}$ (at least partly) differ in their bioavailability misclassifications, the following approach appears promising: Only if both models predict a compound to be not bioavailable for direct or indirect THD, the respective classification holds. Conversely, a chemical is considered as bioavailable if this is predicted by at least one of the two models.

The respective model combination employing the original rule-of-5 scheme (without domain limitations) and the $\log K_{\text{brain/blood}}$ model (eq. (1)) with threshold -0.52 (brain-to-blood compound concentration ratio of at least 3:10) yields only five (0.3%) misclassifications (false negatives). With $\log K_{\text{brain/blood}} \geq -1$ (brain: blood compound concentration ratio of at least 1:10), only two compounds (0.1%) are misclassified as being not bioavailable.

When combining the rule-of-five model with the original criteria but augmented by the domain limitations and $\log K_{\text{brain/blood}}$ (eq. (1)) with threshold -0.5 , only one false negative remains, minopafant $\text{C}_{46}\text{H}_{73}\text{N}_4\text{O}_9$. Here, the prediction of $\log K_{\text{ow}} = 9.5$ mainly results from a long aliphatic side chain that also contributes most to the large MW of 826 Da. Moreover, minopafant has 13 H-bond acceptor groups and thus exceeds also the #HBA threshold. Regarding $\log K_{\text{brain/blood}}$, the respective minopafant value of -1.33 is below the thresholds of -0.5

and -1 , respectively. Accordingly, the misclassification of minopafant as not bioavailable remains when raising the rule-of-five MW threshold to 750 Da, and also when employing the lower $\log K_{\text{brain/blood}}$ cutoff.

3.2. External validation

The number of external test compounds with #HBD and #HBA values below or equal certain respective values in terms of cumulative distributions is shown in Figs. 4 and 5. For easy comparison with the training set results, the respective training set scales have been used. The associated numerical values are given in Table S6 of the SI.

The original Lipinski rules classify five chemicals (4%) as not being bioavailable. Of these, two have more than ten HBA groups with molecular weights above 600 Da but below 750 Da. The other three compounds exceed the $\log K_{\text{ow}}$ limit in addition to the MW limit.

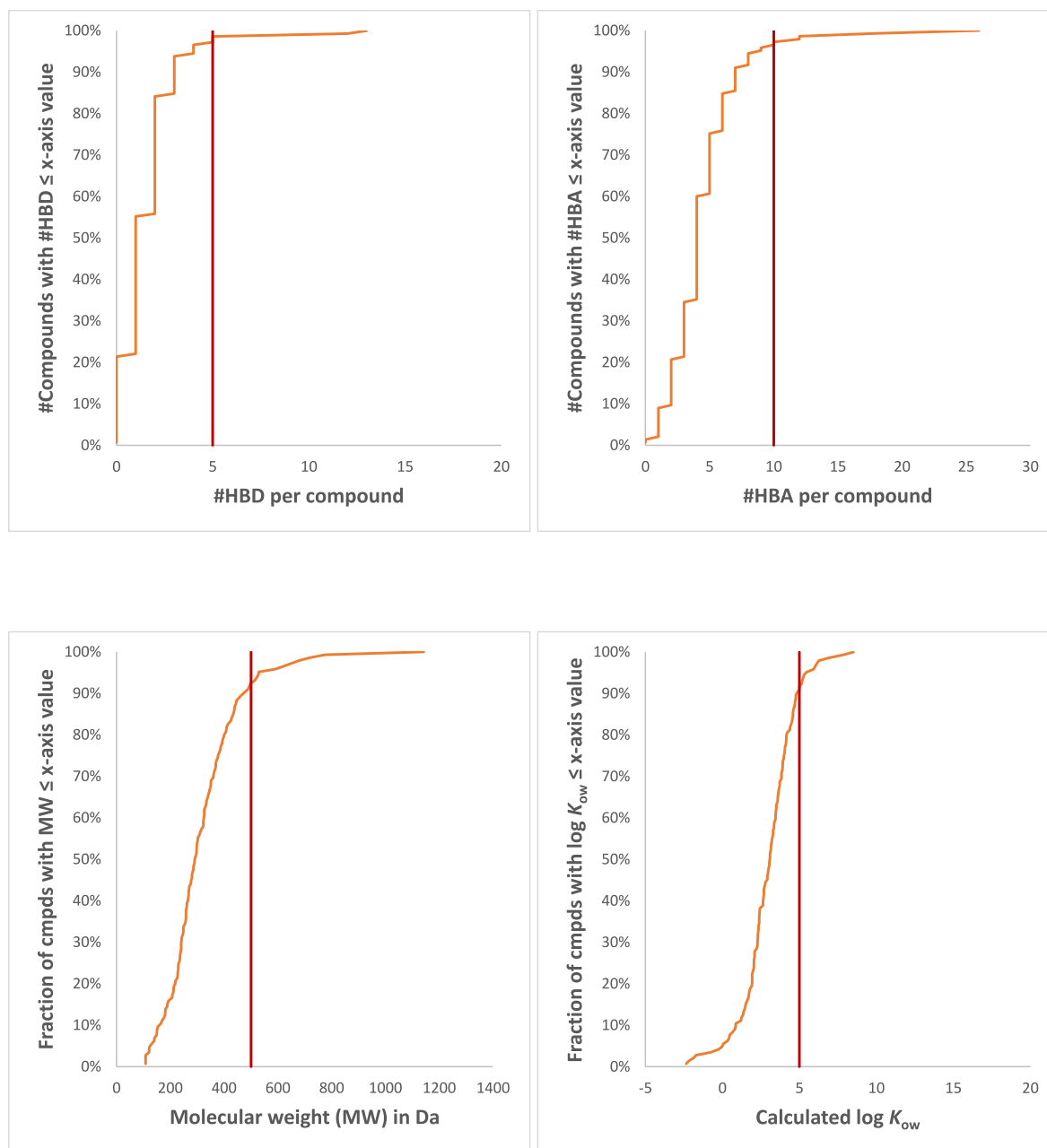


Fig. 4. Cumulative distributions of the number of compounds concerning #HBD (upper left), #HBA (upper right), molecular weight (MW, lower left), and $\log K_{\text{ow}}$ (lower right) for the 145 external test set compounds. The vertical lines denote the suggested bioavailability thresholds of 5, 10, 500 Da, and 5 (original rule-of-five), respectively.

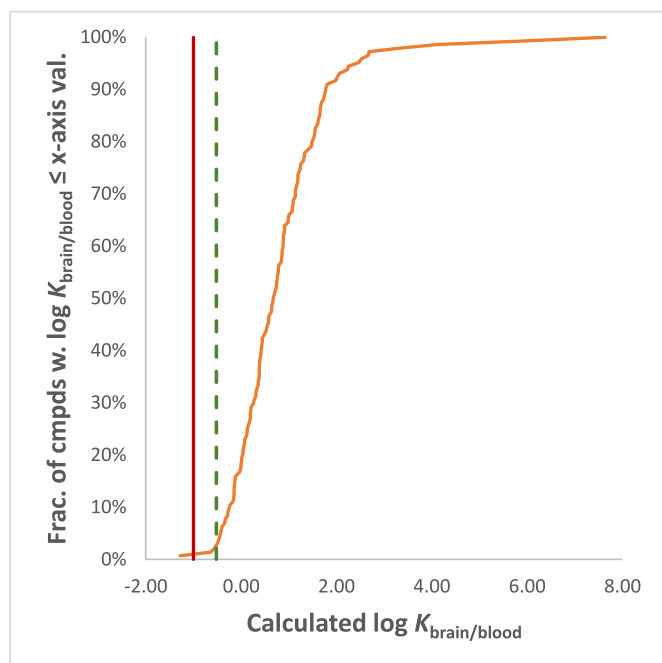


Fig. 5. Cumulative distribution of $\log K_{\text{brain/blood}}$ for the 145 external test set compounds. The dashed right vertical line denotes the originally suggested bioavailability threshold (lower limit) of -0.52 . The left vertical line gives a more conservative threshold (lower limit) of -1 .

Another three compounds have a $\log K_{\text{brain/blood}}$ below the lower limit of -0.52 (brain/blood compound concentration ratio of at least 30%), with one compound showing a $\log K_{\text{brain/blood}}$ even below -1 (brain/blood compound concentration ratio $\leq 10\%$). However, these three compounds do not trigger the rule-of-five alert, despite a $\log K_{\text{ow}}$ above five (reflecting the low $K_{\text{brain/blood}}$). For one other compound neither $\log K_{\text{ow}}$ nor $\log K_{\text{brain/blood}}$ could be predicted, thus assuming bioavailability according to our conservative approach.

Overall, external validation confirms the presently introduced bioavailability model. Moreover, because of various properly predicted test set compounds outside the training set ACF domain the latter is now extended as indicated above. For that reason, the domain characterisation given in the SI covers all 1787 properly predicted training and test set compounds. The respective ACF pool is available in ChemProp and listed in terms of SMARTS codes (Daylight, 2019b) in Tabs. S7 and S8 of the SI, featuring 2185 first-order and 10031 s-order ACFs extracted from 1787 training and prediction set compounds.

3.3. Discussion

The present approach is confined to the passive-diffusive component of molecular bioavailability with respect to disrupting the TH regulation in a direct or indirect manner. To this end, Lipinski's rule-of-five properties #HBD, #HBA, MW and $\log K_{\text{ow}}$ alone (without and with a modified MW threshold) and in combination with $\log K_{\text{brain/blood}}$ are highly successful in predicting the bioavailability of (direct and indirect) TH disruptors in vitro. Note, however, that the presently introduced approach ignores active transport pathways to TH-related targets if present. Moreover, the database of in-vitro test results restricts the model applicability to predicting the in-vitro bioavailability of potential THDs. In the following, the mechanistic basis underlying the five physicochemical properties used for our model is highlighted.

The rule-of-five MW impact on the TH-related bioavailability is straightforward. Increasing MW typically (although not necessarily) correlates with increasing molecular size, translating into a decreasing ability to passively diffuse across biological membranes. Our

modification of Lipinski's MW threshold from 500 to 750 Da as maximum suggests that for interfering directly or indirectly with the TH regulation, the THD concentration at the target may be smaller than for drugs affecting many other MOAs.

Regarding $\log K_{\text{ow}}$, its rule-of-five impact on bioavailability can be explained as follows. Above a certain hydrophobicity threshold (here: $\log K_{\text{ow}} > 5$), membrane accumulation rather than membrane permeation prevails (see, e.g., Böhme et al., 2016), reducing the intracellular compound concentration below the minimum value required to exert a biological effect.

The final two rule-of-five parameters #HBD (≤ 10) and #HBA (≤ 10) serve as upper limits for good bioavailability. This probably reflects the fact that bulk water (including the cytosol and its water-rich sites of proteins) favours H-bonding over water-poor media such as protein-interior sites (see, however, below).

The model for predicting $\log K_{\text{brain/blood}}$ contains the characteristic volume V that also reflects molecular size. As can be seen from the negative regression coefficient of V in eq. (1) (see section 2.5.2), increasing V drives increasing partitioning into blood because of an increasing steric hindrance for passing the BBB. The few exceptions concern compounds containing aromatic rings with three to four iodine substituents (in one case Br instead of I), thus showing some similarity to the thyroid hormone T3. In these cases, however, the high MW is dominated by the I atoms, whereas V is only moderate with values of 3–4. In fact, only 14 of the 1642 dataset compounds have V values above 6 and at the same time MW values above 800 Da, with all of them featuring at least 45 carbon atoms and a number of $-O-$ or $O=$ groups acting as hydrogen-bond acceptors.

Interestingly, both the H-bond acidity A (HBD capability) and basicity B (HBA capability) in eq. (1) support compound partitioning from blood to brain. On the one hand, this contrasts with the above-discussed rule-of-five upper limits #HBD ≤ 10 and #HBA ≤ 10 for good compound bioavailability. On the other hand, the positive impact of A and B on TH-related bioavailability would be in accord with the notion that H-bonding is important for TR agonists and antagonists (Paul-Friedman et al., 2019). From a physicochemical viewpoint, however, increasing H-bonding would increasingly favour aqueous solutions such as blood (as opposed to brain) and the cytoplasm (as opposed to water-poor protein-interior sites; see above). The opposing impact of H-bonding on the rule-of-five vs $\log K_{\text{brain/blood}}$ prediction of bioavailability may thus reflect the following trade-off: As long as #HBD and #HBA are below a certain threshold (above which aqueous solution is strongly preferred), they may support triggering certain MOAs such as TH-related disruption as in the present case (particularly when H-bonding is involved in the bioactivity mechanism).

Note that the regression coefficients of parameters E and S also used for predicting $\log K_{\text{brain/blood}}$ have opposite signs, which is driven by their partial linear dependence (because molecular softness and polarizability represent similar molecular aspects). Taking both together, an overall positive impact on $\log K_{\text{brain/blood}}$ remains, possibly indicating that increasing polarity/polarizability facilitates crossing the BBB.

As can finally be seen from eq. (1), the presence of carboxylic acid groups strongly favours partitioning from blood to brain. First, this is in conflict with simple solution chemistry that would predict $-\text{COO}^-$ to strongly prefer aqueous solution. Second, however, according to Paul-Friedman et al. (2019) an acidic group is required for true TR antagonists. In the training set, 110 chemicals contain carboxylic groups. Whereas 47 of them were detected as TR antagonist, 44 of these 47 compounds exerted also other MOAs. Coming back to the rule-of-five, 76 of the 110 carboxylic acids do not exceed any threshold, and another 19 exceed only one. 13 carboxylic compounds exceed two even three thresholds and another two even three thresholds. However, one chemical of each of the latter two groups is outside the presently defined chemical domain of the model. Overall, it appears that despite solution thermodynamics (favouring aqueous solution for $-\text{COOH}/-\text{COO}^-$), carboxylic acid groups may still be involved or even strongly support

certain MOAs such as TR antagonism, which is accounted for in the present model through including $\log K_{\text{brain/blood}}$.

Note finally that $\log K_{\text{brain/blood}} \geq -0.52$ holds for in vitro bioavailability. Regarding the in vivo situation, the minimum $\log K_{\text{brain/blood}}$ value in accord with sufficient brain bioavailability should be decreased by ca. 0.4 log units (from -0.52 to -1). The latter implies that for in vitro settings a larger minimum compound concentration is required to exert bioactivity in the brain. Most likely, this – at first sight – puzzling issue reflects the fact that in vitro tests are often employed using plastic vials that absorb part of the compound concentration, and thus may reduce the nominal compound concentration significantly. Accordingly, it appears that the apparently lower bioavailability in vitro as compared to its in vivo counterpart results from plastic wall absorption rather than from biochemistry.

3.4. Software implementation

The presently introduced QSAR for predicting bioavailability of potential direct and indirect THDs has been implemented in our ChemProp software (UFZ Department of Ecological Chemistry, 2021). This software can be used free-of-charge but subject to a licence agreement.

4. Conclusions

The presently introduced QSAR may augment current and future in vitro screens for identifying direct and indirect THDs. More specifically, compounds predictively classified as not THD-bioavailable have a low priority for respective in vitro testing. Accordingly, a correspondingly informed in vitro test strategy may allocate its resources to focusing on those THD-suspect compounds that are classified as correspondingly bioavailable. Future investigations may address potential extensions of the bioavailability model to other in vitro assays as well as to in vivo test results. Moreover, the currently derived structure-inherent bioavailability triggers may support the mechanistic interpretation of existing and new experimental THD findings.

CRedit authorship contribution statement

Ralph Kühne: Methodology, Investigation, Formal analysis, Software, Writing – original draft. **Klára Hilscherová:** Investigation, Formal analysis, Writing – review & editing. **Marie Smutna:** Investigation, Formal analysis. **Friederike Leßmöllmann:** Formal analysis, Data curation. **Gerrit Schüürmann:** Funding acquisition, Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2023.140611>.

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