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Challenges in working towards an internal Threshold of Toxicological Concern (iTTC) for use in the safety assessment of cosmetics: Discussions from the Cosmetics Europe iTTC Working Group workshop

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Target Journal: Regulatory Toxicology and Pharmacology

Manuscript type: workshop report

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Abstract

The Threshold of Toxicological Concern (TTC) is an important risk assessment tool, which has evolved over the last 50 years, and establishes acceptable low-level exposure values to be applied to chemicals with limited toxicological data. The data comprising existing TTC databases are from external exposures, and as such, the corresponding threshold limits are also representative of external exposures. With continued global interest in non-animal methods for human safety risk assessments, one of the logical next steps in the continued evolution of TTC is to develop this concept further so that it is representative of internal exposures (i.e. a TTC based on plasma concentration). An internal TTC (iTTC) would provide threshold values that could be utilized in exposure-based safety assessments. As part of the Cosmetics Europe (CosEu) Long Range Science Strategy (LRSS) research program, CosEu has initiated a project that is working towards the development of iTTCs that can be used for the human safety assessment. Knowing that the development of an iTTC is an ambitious and broad-spanning topic, CosEu organized a Working Group comprised a balance of multiple stakeholders (cosmetics and chemical industries, the EPA and JRC and academia) with relevant experience and expertise and workshop to critically evaluate the requirements to establish an iTTC. Outcomes from the workshop included an evaluation on the current state of the science for iTTC, the overall iTTC strategy, selection of chemical databases, capture and curation of chemical information, ADME information and repeat dose data, expected challenges, as well as next steps and ongoing work.

1. Background

Animal welfare concerns and regulatory restrictions on animal testing, along with the aspiration to develop methods more predictive of human biology than historical animal toxicology tests, have stimulated global interest in the development of alternative test methods. The European Union (EU) Cosmetics Regulation (Regulation 1223/2009) prohibits the marketing of finished products containing ingredients or combinations of ingredients that have been tested in animals. Testing bans on products (from September 11, 2004) and ingredients (from March 11, 2009) were followed by a marketing ban on all animal-tested ingredients and products (from March 11, 2013), irrespective of the availability of alternative non-animal tests. The testing and marketing bans mean that non-animal methods are needed for the safety assessment of cosmetics. To help advance the progression of non-animal methods, the Cosmetics Europe (CosEu) Long Range Science Strategy (LRSS) Research Program for 2016-2020 was established. The research program focuses on the development of non-animal testing strategies that can be utilized in a safety assessment paradigm that is applicable for both regulatory use and for substantiating safety of new ingredients when regulatory approvals are not required (Desprez et al., 2018). This alternative approach to animal testing program aims to enable the safety assessment of cosmetic ingredients without animal data in a manner that provides equal or better assurance of safety compared to historical animal testing paradigms. New methods focus on integrating both hazard characterization and improved exposure assessment into a probabilistic assessment at various levels of exposure. For systemic toxicity, there is a focus on the ability to link external exposure with internal exposure and kinetics and potential target organ toxicity.

The threshold of toxicological concern (TTC) is a safety assessment tool that involves establishing a low-level exposure value, from known chemicals with curated toxicity data, below which there is a low probability of adverse effects for chemicals lacking sufficient safety data. It is based on an analysis of the distribution of toxicity data for a large group of diverse chemicals and then assumes there is a negligible risk to health if exposure to an untested chemical is below the TTC. The TTC concept relies on knowledge of the range of toxicological hazard/potency for structurally diverse classes of chemicals for which good toxicity data exist. If human exposure to a substance is below the relevant TTC value, it can be judged “with reasonable confidence, to present a low probability of a risk” (Munro et al., 1996). It started in 1967 when Frawley proposed a Threshold of Regulation for chemicals intended for use in food-packaging materials (Frawley, 1967) and has evolved into a tiered risk assessment tool. Use of TTC for non-cancer endpoints involves categorizing chemicals into one of three Cramer Classes (Cramer et al., 1978; Munro et al., 1996): Class I, simple chemicals structures with known metabolism and innocuous

end products which suggest a low order of oral toxicity; Class II, complex structures which are less innocuous than Class I but lack the structural alerts linked to toxicity as in Class III; Class III, structures that permit no strong initial presumption of safety or may even suggest significant toxicity (Cramer et al., 1978). In 2004, guidance for the application of a tiered TTC approach for chemicals present in the diet was published by Kroes et al., (2004). Several classes of chemicals are excluded from TTC, including metals, proteins, inorganic substances, steroids, nanomaterials, organosilicon compounds, substances with endocrine activity, suspected bioaccumulation potential, and high potency carcinogens (Kroes et al., 2004; EFSA/WHO 2016; SCCSP, 2012). There have been multiple investigations (Lapenna and Worth, 2011; Tluczkiewicz et al., 2011; EFSA/WHO 2016) for potential revisions of the classification scheme; however, the decision tree has so far withstood these.

The Threshold of Regulation approach was originally intended for substances potentially migrating into foods from food packaging. The underlying toxicological datasets broadly were limited only by the availability of data and not by a consideration of the type of chemical or its intended use (first for carcinogens and later for non-cancer endpoints). More recent discussions have examined additional specific uses of TTC, including herbal products, pharmaceutical impurities, pesticides and water impurities (EFSA/WHO 2016). A paper by Kroes et al., (2007), which address the decision tree for alerts relating to genotoxic carcinogens, was the first explicit discussion on how to apply the decision tree to topically and intermittently applied cosmetic ingredients with respect to the chemical domain and the route-to-route extrapolation (oral-dermal). They concluded that TTC is a useful tool with applicability to cosmetic ingredients and impurities and proposed a default adjustment factor for the percent of dose that would be absorbed across the skin. In 2012, the Scientific Committee on Consumer Safety (SCCS, which provides opinions on health and safety risks of non-food consumer products in the EU) agreed that the TTC approach was scientifically acceptable for cosmetics, but they expressed concern that it was not generally applicable to all ingredients and exposure routes and should be considered on a case-by-case basis (SCCS/SCHER/SCENIHR, 2012). In 2016, a more favorable opinion of the SCCS was published (SCCS, 2016) based on work expanding the chemical domain by bringing in cosmetic associated chemicals (ingredients and contaminants) from the COSMOS database (Yang et al., 2017). This work was carried out by the EU and CosEU-funded COSMOS project (http://www.cosmostox.eu).

Since the TTC is based on toxicological data from oral exposure studies in animals expressed as an administered dose, the appropriate TTC level is compared to a human external exposure estimate. There are times in a safety assessment where the internal exposure is more meaningful, for instance linking the internal exposure to the cellular concentration leading to the occurrence of adverse effect. An ‘internal TTC’ (iTTC; i.e., a TTC based on plasma concentration) has been suggested by several groups (Bessems,

2009; Coecke et al., 2013; Hartung 2017; Partosh et al., 2015) as a possible evolution of the externally-based TTC that could be a useful approach in the development of non-animal methods and as a tool to further refine the use of TTC and expand its applicability. If an iTTC database could be established to derive iTTC thresholds, then human plasma concentrations (estimated or measured) for a given exposure scenario could be compared to these iTTC thresholds. The iTTC thresholds would provide conservative hazard-based values that could be utilized in exposure-based safety assessments in the context of: (1) refinement of de minimis exposure levels for dermal exposures; (2) metabolism-based structure-activity relationship (SAR) assessments; (3) low level aggregate exposures from different dose routes, or; (4) *in vitro* biological assays. Case study examples for some of these possible uses are presented in Supplementary File 1. One of the primary constraints to developing an iTTC database has been the significant resource investment that it would take to complete this work. Partosch et al., (2015) attempted to derive an iTTC by adjusting the external No Observable Adverse Effect Levels (NOAELs) of substances from three databases by *in silico* estimates. Limited details are provided regarding the calculations of internal exposure made in Partosch et al (2015); however, the authors do state that the NOAELs defined from administered dose were multiplied by *in silico* estimates for human oral bioavailability. The oral bioavailability prediction method used by Partosch et al., (2015) takes into account passive oral absorption but does not consider the possible impact of active transport (either influx or efflux), pre-systemic metabolism, systemic metabolism and clearance or any of the important factors that would impact internal exposure levels to target tissues after passive uptake from the GI tract. Animal-human differences in metabolism or other kinetic determinants for the chemicals were also not taken into account. While the TTC accounts for this based on default uncertainty factors, an iTTC may provide a means to account for interspecies toxicokinetics differences using more refined approaches. There was no attempt to include empirical data in the calculation of internal exposure. Lastly, the estimates provided in Partosch et al., (2015) were still based on external dose and not an internal exposure metric, such as plasma concentration. The work by Partosch et al., (2015) provided an initial view for what an iTTC could look like and was cited in the 9th Revision of the SCCS Notes of Guidance for the testing of cosmetics ingredient (SCCS, 2016), where it was proposed that an iTTC would be an improvement on the external dose TTC for the assessment of dermal exposures based on oral data (SCCS, 2016). However, as noted above (and discussed throughout this report), there are a number of further considerations that need to go into the development of an iTTC before it is ready to use in a safety assessment.

2. Cosmetics Europe Workshop

2.1 Purpose

Considering the recommendation from SCCS to utilize internal exposure more frequently in cosmetic safety assessment (SCCS, 2016), and the current lack of iTTCs that are ready to be used, the CosEu LRSS research program initiated a project dedicated to the development of iTTCs that could be used for human safety assessment. Since the iTTC objective is a broad-spanning topic, and to drive the development of the iTTC concept and engagement of regulators during the development of iTTC, CosEu set up a Working Group to help address some of the anticipated challenges. The Working Group (see author list for members) includes strategic partners from multiple stakeholders (from the cosmetics, fragrance and chemical industries and associations; the NIH, EPA, JRC and USP Standards Development; and academia) with varying expertise in areas relevant to the iTTC process e.g., ADME, Physiological Based Pharmacokinetic (PBPK) modeling, safety assessment, TTC, cheminformatics. The first Working Group workshop was held at CosEu on 6-7 April, 2017. Discussions focused on selection of the overall iTTC strategy, chemical databases, capture and curation of chemical information and repeat dose data, expected challenges, as well as the next steps.

2.2 iTTC preliminary framework

A proposed approach for deriving iTTCs based on the concentration of a chemical in blood is shown in Figure 1. The approach starts with identifying an existing TTC dataset which contains chemical specific NOAELs expressed as an external dose in mg/kg/day. For each of the chemicals within the dataset, it will be necessary to identify chemical-specific ADME data through a multi-tier approach of literature searching, using *in silico* estimation tools and generating empirical data. Chemical-specific PBPK modeling will then be conducted to convert the chemical-specific external exposures from the TTC dataset of chemicals to an internal blood concentration (Css, AUC, or C_{max}) for each chemical. Css has most commonly been used for comparison to exposure estimates of daily aggregated dose (Wetmore et al., 2012 and 2015), and is directly proportional to a 24-hour time integrated plasma concentration (AUC). However, PBPK modeling allows prediction of many possible dose metrics, such as C_{max}, which might also be used for comparing to exposure depending on the frequency of exposures and chemical half-life (Sipes et al., 2017). The PBPK modeling will be conducted using the appropriate species, dose, and route from the toxicity study. This will provide an estimate of the chemical-specific internal exposure associated with a given NOAEL from the toxicity study. The distribution of chemical-specific Css values will then be evaluated and an appropriately low (e.g. 5^{th} percentile) Css threshold will be identified for the group of chemicals and by Cramer Class. Uncertainty factors will also be applied to the Css threshold values to derive the iTTC values that can be applied to a human safety assessment. However, additional discussion will be needed to determine which values would be appropriate, given that a comparison of an

animal-derived iTTC value to a modeled human blood level for a specific exposure may help quantify extrapolation factors or uncertainty factors to account for interspecies toxicokinetics differences. Models to estimate the human internal exposure could potentially include factors that relate to the intra-species toxicokinetics uncertainty factors. Finally use of the iTTC for in vitro to in vivo extrapolation (IVIVE) comparisons from *in vitro* test results from human tissues/cells may address intraspecies TD questions. Overall, adequate uncertainty factors will need to be considered and applied, but there may be an opportunity to refine traditional default values.

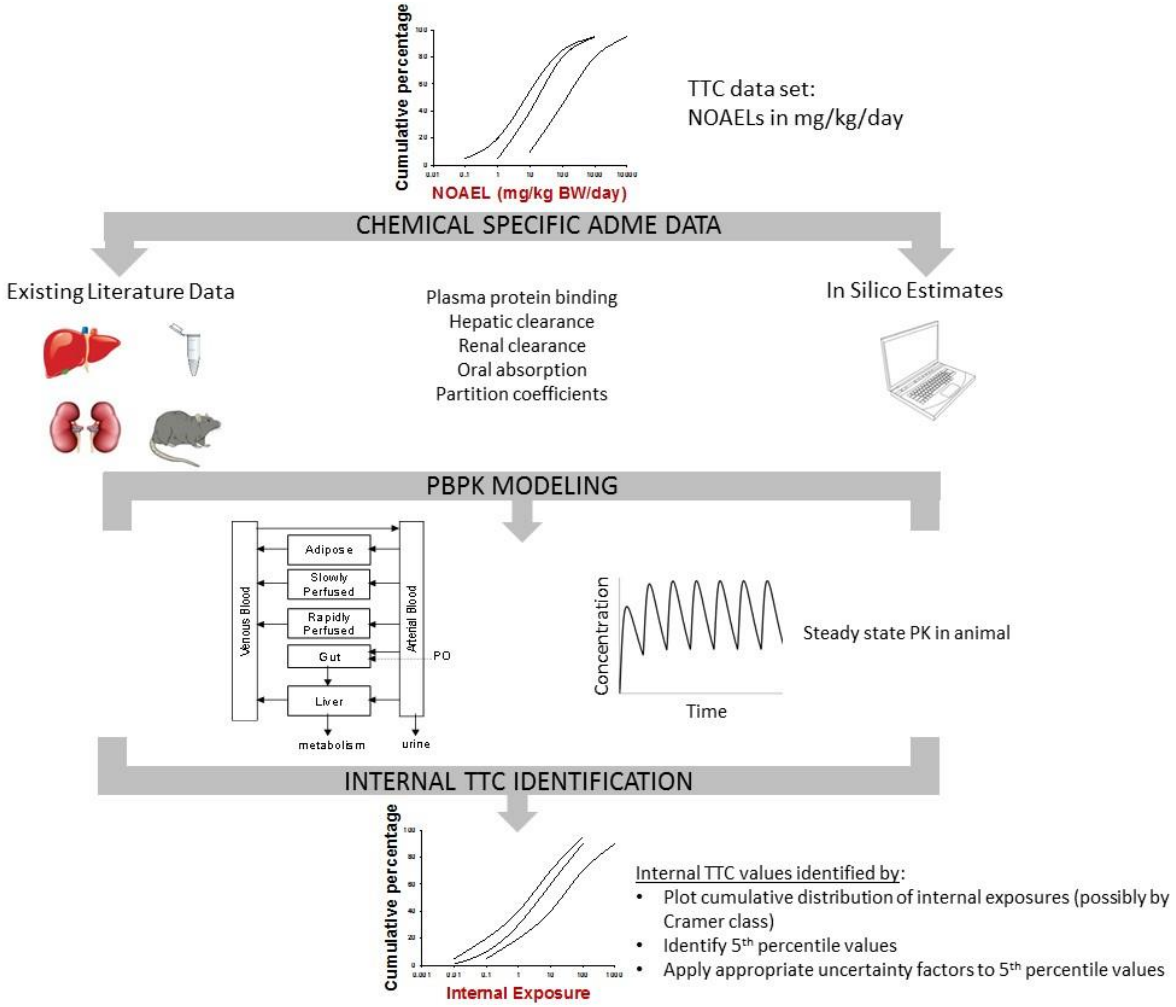

Figure 1 Proposed approach for deriving iTTC values

2.3 Chemical Databases for the iTTC project

The first step in the framework is to identify suitable TTC datasets on which the iTTC database would be based. Ideally, the data should be transparent and publicly available. Of the multiple databases that have been published, the Munro et al., (1996) non-cancer TTC database is one of the more widely known and

cited. This database consists of 613 chemicals, representing a range of industrial chemicals, pharmaceuticals, food substances and environmental, agricultural and consumer chemicals likely to be encountered in commerce. The database contains data from multiple species (rat, mouse, rabbit and hamster) and all data are derived from oral exposure studies. For each of the chemicals, the database identifies a sub-chronic or chronic NOAEL.

A second TTC database that was prioritized is the COSMOS database (553 chemicals), which was developed through a joint European Commission-CosEu funded project, "Integrated *in silico* models for the prediction of human repeated dose toxicity of cosmetics to optimise safety" "Safety Evaluation Ultimately Replacing Animal Testing" (SEURAT-1, COSMOS) (Cronin et al., 2012). The COSMOS TTC database is a non-cancer TTC dataset enhanced with cosmetic and packaging materials. It contains chemicals that have been tested in oral exposure studies in rat, mouse, dog, primate or rabbit and it contains NOAELs from studies of varying durations 28 days or longer (Yang et al., 2017). The Munro and COSMOS TTC databases have been combined, and the current version (version 2.0) is located on the COSMOS website at https://cosmosdb.eu.

The third relevant database that was selected by the Working Group is the Research Institute of Fragrance Materials (RIFM) database (1355 chemicals), which is composed of flavor and fragrance materials. RIFM follows the same approach as COSMOS and Munro and defines thresholds for each chemical included in the database. One of the unique features of the RIFM dataset is that it includes data from chronic, sub-chronic reproductive and developmental toxicity studies for a large number of chemicals in Cramer Class II, which is a Cramer Class that has historically been underrepresented in TTC databases. The combination of the RIFM dataset with the Munro and COSMOS databases results in more than 1000 unique chemicals.

The diverse chemical dataset that is derived by combining the Munro, COSMOS and RIFM databases introduces a practical challenge for the iTTC work related to the resources required to generate new *in vitro* ADME data and complete PBPK modeling for such a large set of chemicals. In some cases, it may be impractical to generate new *in vitro* ADME data for a chemical due to issues with chemical availability, cost, stability, solubility or toxicity. To narrow down the number of chemicals in the final iTTC workflow to a more manageable number that can be used in a strategic targeted assessment, the chemical space will be assessed according to descriptors related to chemical structure, as well as ADME and pharmacokinetics (PK) to ensure that the final iTTC chemical dataset contains a distribution of chemicals covering a broad chemical and PK space. (see Section 2.6.1).

2.4 Identifying and Addressing Challenges

Several challenges are anticipated for different aspects of the iTTC project. Workshop breakout sessions were held to address some of the top challenges and the discussions from these are described below.

2.4.1 Strategy for PBPK model simulation verification in absence of *in vivo* data

The iTTC project will utilize PBPK modeling to estimate the internal exposure that is associated with the external dose (NOAEL) from a toxicity study. Application of PBPK models typically involves a verification step which compares the simulated data from the PBPK model to *in vivo* data, so that the model accuracy can be evaluated (Clark et al., 2004; McLanahan et al., 2012). In the case of the iTTC project, many of the chemicals in the database will lack the existing PK data that would typically be used for model verification, and as such, the Working Group discussed possible strategies to evaluate the iTTC PBPK modeling workflow performance in the absence of PK data. One possible strategy for evaluating the accuracy and uncertainties in the PBPK modeling workflow is to utilize anchoring data similar to approaches published by Yoon et al., (2014), Wetmore et al., (2012, 2013, 2015) and Wambaugh et al., (2015). In such an approach, a PBPK modeling workflow is created that is based on *in vitro* and *in silico* ADME data. Then, the chemical-specific PBPK models are used to predict the plasma concentration (Css, AUC, or C_{max}) for a group of chemicals that have existing *in vivo* data so that the models and overall workflow can undergo an evaluation of accuracy and uncertainty. Once it is determined that the workflow can provide reasonable estimates of plasma concentrations, it can then be applied more broadly (with some level of confidence based on the initial evaluation of the workflow) to chemicals that lack *in vivo* data. This approach relies on a generic description of physiology, rather than a bespoke PBPK model that might include aspects of physiology only relevant to specific classes of chemicals. However, the data to develop such bespoke models are often unavailable and the models themselves are often difficult to reproduce (McLanahan et al., 2012). By assuming a more generic approach to physiology the possibility of problems with model implementation (i.e., computer coding) is reduced and the generic model can be verified by any data that are available.

Another strategy that was discussed as an alternative way to evaluate a model's accuracy involved the use of *in vitro* toxicity data (e.g. ToxCast, Tox21). In this scheme, the accuracy of the PBPK model is inferred by comparing the model estimate for the plasma concentration associated with the toxicity study NOAEL to the no effect concentrations seen *in vitro*, with the assumption that the two may be in comparable concentration ranges. One of several, inherent, challenges with this approach is whether the in vitro toxicity assays are relevant to the endpoints evaluated in the in vivo study. Another challenge comes from

the fact that many *in vitro* toxicity assays do not include metabolism or display tissue-specific kinetics which makes it difficult to compare to an *in vivo* NOAEL. Advances are occurring in this area, as exemplified by emerging *in vitro* models with improved tissue-like functionality, including transporters and metabolism (Bell et al., 2018; Ramaiahgari et al., 2017), however, more work is needed in this area. The last strategy that was discussed related to the possibility of utilizing an analog "read-across" approach, by identifying the key common PK characteristics to evaluate the relevance of analogues. Here, a 'PK analog' with existing *in vivo* PK data would be identified for a chemical that lacks *in vivo* PK data. In such a scheme, considerations and principles (potentially based on physicochemical properties) would need to be established to define what a suitable PK analog would be and then the PBPK model simulations for the chemical of interest would be compared to the *in vivo* data for the PK analog to evaluate model performance. There are currently no such criteria for establishing what a suitable PK analog would be, so use of this strategy would first require a significant amount of development.

2.4.2 Strategy for predicting if metabolism is neutral, activating or detoxifying (in the context of the effects identified in the repeat dose studies)

The safety assessment for industrial chemicals has historically focused on characterizing the external doses administered to animals during the toxicological evaluation to identify a NOAEL which would then be used for the safety assessment. In this paradigm, it is not necessary to know whether the toxicological effects observed in the animal are attributed to the parent chemical or a metabolite. However, when considering internal exposure as a dose metric for a safety assessment it becomes important to know if the toxicological effects are associated with the parent and/or metabolite(s) so that the internal exposure for the toxicant can be monitored or estimated. In the context of iTTC this becomes important because the chemical-specific NOAELs will be converted to an internal exposure and it will be necessary to know whether the parent or metabolite plasma concentration should be estimated.

Early in the discussion of this topic it was agreed by the workshop participants that it was important to distinguish reactive metabolites from stable circulating metabolites. Reactive metabolites are instable metabolites that form and react at the site of metabolism (they do not generally circulate the body) and can cause damage to the metabolizing tissue (Thompson et al., 2016). Stable circulating metabolites form at the site of metabolism and, unlike reactive metabolites, they can circulate away from the metabolizing organ and can distribute throughout the body. Stable circulating metabolites can be more or less active than the parent compound, and in cases where the metabolite is more active than the parent, metabolism is considered activating. A great deal of research has been conducted by the pharmaceutical industry to identify chemical features that are associated with chemicals that can form reactive metabolites (Stepan et

al., 2011; Bruns and Watson, 2012; Thompson et al., 2016) and it was agreed by the Working Group that this literature should be leveraged when reviewing the iTTC chemicals. Also, there are established *in vitro* assays that can be used for identifying when reactive metabolites form following metabolism (Huang et al., 2015; Erve et al., 2007) which could be used in cases where additional data are necessary to support *in silico* predictions. The *in vivo* toxicity data can also be informative since e.g., specific injury types are expected to largely occur in the major metabolizing organs for chemicals that form reactive metabolites (Zimmerman 1999; Thompson et al., 2016). With regards to predicting if a toxic metabolite may form, the Working Group discussed an *in silico* workflow that was presented which utilizes Meteor (Lhasa Limited, Leeds UK) metabolism prediction coupled with Derek Nexus (Lhasa Limited, Leeds UK) alerts for toxicity. Similarly, the OECD Toolbox can also simulate metabolites that can be coupled with profilers that flag for different endpoint specific toxicities or aspects of Toxtree (http://toxtree.sourceforge.net). In the workflow presented at the workshop, a parent chemical would be evaluated by Derek to determine what toxicity alerts are associated with it; the chemical would then be processed through Meteor to determine possible metabolites and then the metabolites would be run through Derek (or other platforms such as Sarah and Vitic Nexus) to determine what toxicity alerts are associated with them. A comparison of the alerts between the parent compound and metabolite would then, in theory, inform if metabolism was activating, detoxifying or neutral. The major limitations in this *in silico* workflow that prevent it from being actionable are that Meteor often overestimates the number of metabolites and often does not predict the metabolite that is responsible for toxicity and Derek alerts for toxicity can unknowingly be linked to a potential metabolite because metabolism is inherently included in the alert. A final strategy for predicting the impact of metabolism was not reached by the conclusion of the workshop but it was agreed that the most successful strategy will be an integration of more than one approach and will require expertise from multiple disciplines.

2.4.3 Strategy for handling factors such as renal clearance, transporters, extrahepatic metabolism

There are several examples where PBPK modeling has been used in a high-throughput context to predict plasma concentrations for a large number of chemicals (Wetmore et al., 2012; Wetmore et al., 2013; Wetmore et al., 2015; Wambaugh et al., 2015). Typically, these PBPK modeling approaches limited systemic metabolism to the liver and renal clearance to passive renal filtration in the kidney. These simplifications in the PBPK model are often justifiable when estimating human exposures to environmental chemicals, as was the case in the aforementioned examples, because it often results in a higher estimated systemic exposure compared to the inclusion of additional metabolism and/or clearance

pathways. However, with regards to the iTTC project, the internal exposure will be estimated for the animal toxicity study, and an approach that results in higher than expected internal exposures would be less conservative in the safety assessment, since higher estimates of NOAEL plasma concentrations lead to higher estimates of iTTC. As such, it is necessary to give some consideration to renal secretion, renal reabsorption, transporter effects and extrahepatic metabolism since these factors could impact the estimation of the plasma concentration.

The Working Group acknowledge the importance that renal secretion, renal reabsorption, transporter effects and extrahepatic metabolism could have on the PK of a chemical but also noted that it will be challenging to quantitatively address the impact of these factors across all chemicals; however, there was agreement that there may be qualitative approaches to help determine when these factors may have an impact on a chemical's PK e.g. rate-determining steps. Currently, *in vitro* models of renal transport using transfected cells are available; however, they express a very limited number of renal transporters and uncontrolled expression levels within de-differentiated cell types, and therefore do not accurately reflect the situation *in vivo*. Additionally, scaling of *in vitro* data appropriately in a PBPK model can be difficult and at times impossible due to data gaps for the necessary scaling factors and the need for *in vivo* data. It is not yet possible to use only *in vitro* derived kinetic data (Km and Vmax) as transporter input into a PBPK model (Jones et al., 2015; Scotcher 2016). The Km value available from an *in vitro* experiment can typically be used directly in a PBPK model; whereas, the Vmax that is derived for a transporter *in vitro* often poorly describes the kinetics when used directly in the PBPK model and it is necessary to use *in vivo* data to adjust the model to the correct Vmax (Scotcher 2016). For extrahepatic metabolism, a number of cellular and subcellular matrices other than liver exist, however, the liver is often the major metabolizing organ and the routine use of other matrices may result in added resources without much benefit. Also, there will be a need for good quality tissue specific scaling factors to appropriately conduct the IVIVE. If there are supporting data to indicate that extrahepatic metabolism is significant for a chemical, then it may be justifiable to generate additional data, albeit on a case-by-case basis.

Several *in silico* approaches were discussed as possible strategies that could provide qualitative estimates for when transporters or renal clearance could impact the PK of a chemical. One approach could include the utilization of a suite of existing QSARs that could predict when a chemical may be a substrate for a transporter. This approach has been used by others (Wambaugh 2015) to identify when an estimation of plasma concentration from a PBPK model not including transporter kinetics may be inaccurate. If a chemical is identified as being a substrate for a given transporter, it would be necessary to review the predicted Km values across transporters and their relative tissue specific expression levels to determine overall relevance. Additionally, data regarding the relevant tissues and chemical characteristics such as

permeability, metabolism and solubility could potentially help to evaluate the impact of the transporter on the overall PK of the chemical. Multiple papers have summarized the potential impact that a transporter may have following oral dosing for drugs belonging to different Biopharmaceutics Drug Disposition Classification System (BDDCS) classes, for example: transporters have minimal effect in the gut and liver for drugs with high solubility, permeability and metabolism (BDDCS class 1 chemicals); but chemicals with lower solubility, high permeability and metabolism (BDDCS class 2 chemicals), efflux transporter effects predominate in the gut and both uptake and efflux transporters can affect the liver (Shugarts and Benet 2009; Benet 2013; Benet et al., 2016). The Working Group also discussed the possibility of using *in silico* tools to predict the major elimination pathway for a chemical in order to better understand when renal clearance may be important. The discussion focused primarily on the possible use of the Extended Clearance Classification System (ECCS) (Varma et al., 2015) that was originally developed for drugs. The ECCS considers chemical ionization, molecular weight and permeability to infer if the major elimination route would be metabolism, renal or hepatic uptake. Application of the ECCS for chemicals other than drugs has been discussed as a screening process by others (Bell et al., 2018) and may be a helpful approach to apply to the iTTC chemicals. Overall, the Working Group agreed that the iTTC project should take a weight of evidence approach, where results from multiple strategies would be reviewed in the context of existing *in vitro* and *in vivo* data, as well as structurally similar chemicals, in order to estimate the likelihood and impact of transporters and renal clearance on the PK of a chemical.

2.5 Comparing human exposure back to the iTTC

The human safety assessment for a consumer product is typically based on external exposure and as such it would be necessary to convert the external dose to an internal exposure before it could be compared to the iTTC. *In silico* models such as mechanistic dermal penetration models (Kretsos et al., 2008; Polak et al., 2012; Dancik et al., 2013; Chen et al., 2015; Chen et al., 2016) and PBPK models as well as single PK equations for predicting Css (Wilkinson and Shand, 1975; Wetmore et al., 2012; Wetmore et al., 2013; Wetmore et al., 2015; Wambaugh et al., 2015) could all be potential options to convert the external dose from the human exposure assessment to an internal exposure. The use of PBPK models to convert dermal exposures to internal exposures has been previously reported (Dancik et al., 2015; Troutman et al., 2015; Bessems et al., 2017); whereas, the use of a PK equation for estimating Css has more frequently been utilized for oral exposures (Houston 1994; Obach et al., 1997; Wetmore et al., 2012; Wetmore et al., 2013; Wetmore et al., 2015). Wetmore et al., (2012; 2013; 2015) estimated internal exposure following an oral dose by using Equation 1:

$$C_{ss} = \left[\frac{k_0}{(GFR \times F_{up}) + \left[\frac{(Q_l \times F_{up} \times Cl_{int})}{(Q_l + F_{up} \times Cl_{int})} \right]} \right] X\ BW$$

Equation 1

where Css (mg/L) is estimated by considering the input rate (K_0, mg/kg/hr), glomerular filtration rate (GFR, L/hr), fraction unbound to plasma (F_{up}), liver blood flow (Q_l, L/hr), hepatic intrinsic metabolic clearance (Cl_{int}, L/hr) and bodyweight (BW, kg).

Equation 1 can be adapted to dermal exposure scenarios as described in equation 2:

$$C_{ss} = \left[\frac{J_{max} \times SA\ X\ D}{(GFR \times F_{up}) + \left[\frac{(Q_l \times F_{up} \times Cl_{int})}{(Q_l + F_{up} \times Cl_{int})} \right]} \right] X \frac{1}{24\ hrs}$$

Equation 2

where the daily Css (mg/L) is estimated by considering maximum dermal flux (J_{max}, mg/cm^2/hr), surface area of exposure (SA, cm^2), duration of exposure (D, hr) fraction unbound to plasma (F_{up}), glomerular filtration rate (GFR, L/hr), liver blood flow (Q_l, L/hr) and hepatic intrinsic metabolic clearance (Cl_{int}, L/hr). Equation 2 has the advantage that it enables estimation of internal exposure to a chemical in a relatively simple and straightforward manner. The data (i.e. dermal penetration rate, hepatic clearance and protein binding) needed for the Css estimation can all be collected through modern *in vitro* approaches. In some cases a parameter e.g., the fraction unbound, can be accurately predicted using *in silico* models (Bell et al., 2018). Additionally, the parameters in the equation enable possible consideration for various scenarios accounting for vehicle effects (J_{max}), product application, age (blood flow, GFR, SA, J_{max}) and health status (GFR, liver metabolism). One of the limitations for Equation 2 is that it does not account for all biological processes within a person, and as such, some processes that can affect systemic exposure are not accounted for, such as pre-systemic metabolism, non-hepatic metabolism, active renal clearance or reabsorption, or tissue-specific accumulation. However, in the context of estimating human exposure for a safety assessment, the absence of these processes (aside from renal reabsorption or metabolic activation) would generally result in an over estimate of human exposure (Wetmore et al., 2012; Wetmore et al., 2015), which would be protective for the safety assessment. While the use of a single PK equation may have some benefits for estimating the internal exposure for the human, it would be insufficient for deriving the iTTCs from the animal toxicity studies due to the fact that it would over-predict internal exposure (Wetmore et al., 2013) since it lacks some physiological processes, as mentioned above, which would result in less protective iTTC values.

2.6 Follow-up work

Since the iTTC workshop, there has been a series of follow-up calls with the Working Group to discuss the next steps. In parallel, several activities have been initiated, the current state of progress is described below.

2.6.1 Chemical space assessment for iTTC chemicals

Given the amount of resources it takes to model the plasma concentration for each chemical, it is desirable to make a targeted and strategic testing of chemicals while maintaining the structural and PK diversity. To accomplish this, two statistical approaches, k-means clustering and principal component analysis (PCA), were utilized, which considered structural and molecular descriptors, in addition to ADME properties as input parameters. The output from the k-means clustering and PCA analyses will help separate and group the chemicals into clusters so that representative chemicals can be selected, thus reducing the number of chemicals included in the PBPK modeling portion of the iTTC project to a more manageable number. Prior to conducting k-means clustering and PCA, structural accuracy of each chemical was determined by using SciFinder (American Chemical Society) and confirming concordance between the chemical name, CAS registration number and structure. Additionally, chemicals with incompletely defined structures or multiple components were excluded from the dataset since the *in silico* modeling approaches that will be utilized in the project can only be applied to chemicals with a single defined structure. Combining the Munro et al., (1996), COSMOS and RIFM databases and applying the exclusion criteria mentioned above (e.g. removing incompletely defined structures) resulted in approximately 1250 unique chemicals which could be included in the iTTC project. For each chemical, continuous molecular descriptors were calculated using the CORINA Symphony Descriptors Community Edition V2 (Molecular Networks GmbH, Germany and Altamira, LLC, USA) with SMILES strings as input; the presence or absence of relevant structural features was described using ToxPrint fingerprints (v2.0_r7.11) implemented in the ChemoTyper software Version 1.0 (Revision 14605M) (Molecular Networks GmbH, Germany and Altamira, LLC, USA); and physicochemical and ADME descriptors were estimated using ADMET Predictor 8.5 (Simulations Plus Inc., Lancaster CA), Advanced Chemistry Development, Inc. (ACD/Percepta, version 1.3, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2017) and OPERA (Mansouri et al., 2016 and 2018). An average value was used when an ADME descriptor could be estimated by more than one software package. Additional descriptors utilized in the assessment included Cramer Class (obtained from COSMOS website; for RIFM chemicals it was determined using Toxtree followed by expert review), ECCS class and expected

clearance pathway (obtained using Varma et al., (2015) approach and *in silico* inputs for permeability that were estimated from ADMET Predictor and ACD), passive permeability potential (obtained using Smith et al., (2014) approach) and likelihood for being a P-glycoprotein substrate (obtained using ADMET Predictor). Table 1 provides a summary of select descriptors for the iTTC chemical dataset, while Supplementary File 2 contains a list of all of the chemical specific descriptors, the k-means clustering group and the principal component (PC) values for PC 1 – PC 10. The freely available program, CheS-Mapper (v2.6.6) (Gutlein et al., 2012) was used to visualize the PCA.

The data matrix was cleaned to remove collinear (i.e. identical) or redundant (i.e. lacking relevant information) variables A PCA using the significant Corina Symphony, ToxPrint and *in silico* ADME descriptors was performed (Figure 2A) and will help with initial prioritization of the chemicals. The results of the PCA are displayed in Figure 2 in terms of a plot of the scores of the first three PCs. The plots in Figure are colored according to descriptors for k-means clustering (Figure 2B), Cramer Classification (Figure 2C) or expected clearance pathway (Figure 2D) to further distinguish the chemicals. A given cluster can be examined more closely, as illustrated in Figure 3, where the PCA is filtered to only display chemicals in one cluster (Figure 3A) and then the chemicals are classified by Cramer Classification (Figure 3B) or expected clearance pathway (Figure 3C). Taking such an approach will enable selection of representative chemicals for inclusion into the iTTC PBPK modeling work. It will be necessary to ensure that the final selected chemicals within the project still represent a distribution of NOAELs and that the 5th percentile of the NOAEL distribution is not significantly different from the distribution including all chemicals. Results from the literature search (see next section for details) will also be integrated into the chemical selection process so that the existing PK and ADME data can be maximized and to highlight where additional in vitro data may be required.

Table 1: Summary of select descriptors for the iTTC chemical dataset

Descriptor	Value[1]	Method
Discrete descriptors		
Cramer Class		Obtained from COSMOS Database; for RIFM chemicals it was determined using Toxtree followed by expert review
I	398	
II	95	
III	758	
Clearance Pathway		Estimated using Varma et al., (2015) approach
Metabolism	1026	
Renal	161	
Hepatic uptake	7	

Hepatic uptake or Renal	55	
Passive Permeability		Estimated using Smith et al., (2014) approach
Low	434	
High	817	
P-glycoprotein substrate		ADMET Predictor
Yes	129	
No	1122	
Continuous descriptors		
Molecular Weight (g/mol)	236 (30 - 1165)	ACD
Water Solubility @ pH 7.4 (g/L)	73 (1.8E-07 - 5.4E03)	Average of ADMET Predictor, ACD, OPERA
logP	2.3 (-9.2 - 12)	
logD @ pH 7.4	1.7 (-9.4 - 13)	Average of ADMET Predictor, ACD
MDCK (cm/s x 10^{-7})	7.7E02 (2.4 - 3.9E03)	ADMET Predictor
Rat plasma protein binding (% unbound)	34 (1.5 - 99)	
Rat volume of distribution (L/kg)	3.3 (0.14 - 41)	

[1] Discrete descriptors are reported as number of chemicals within a given category. Continuous descriptors are reported as mean (range). Descriptors calculated as explained in main text of paper.
MDCK = Madin-Darby Canine Kidney cells

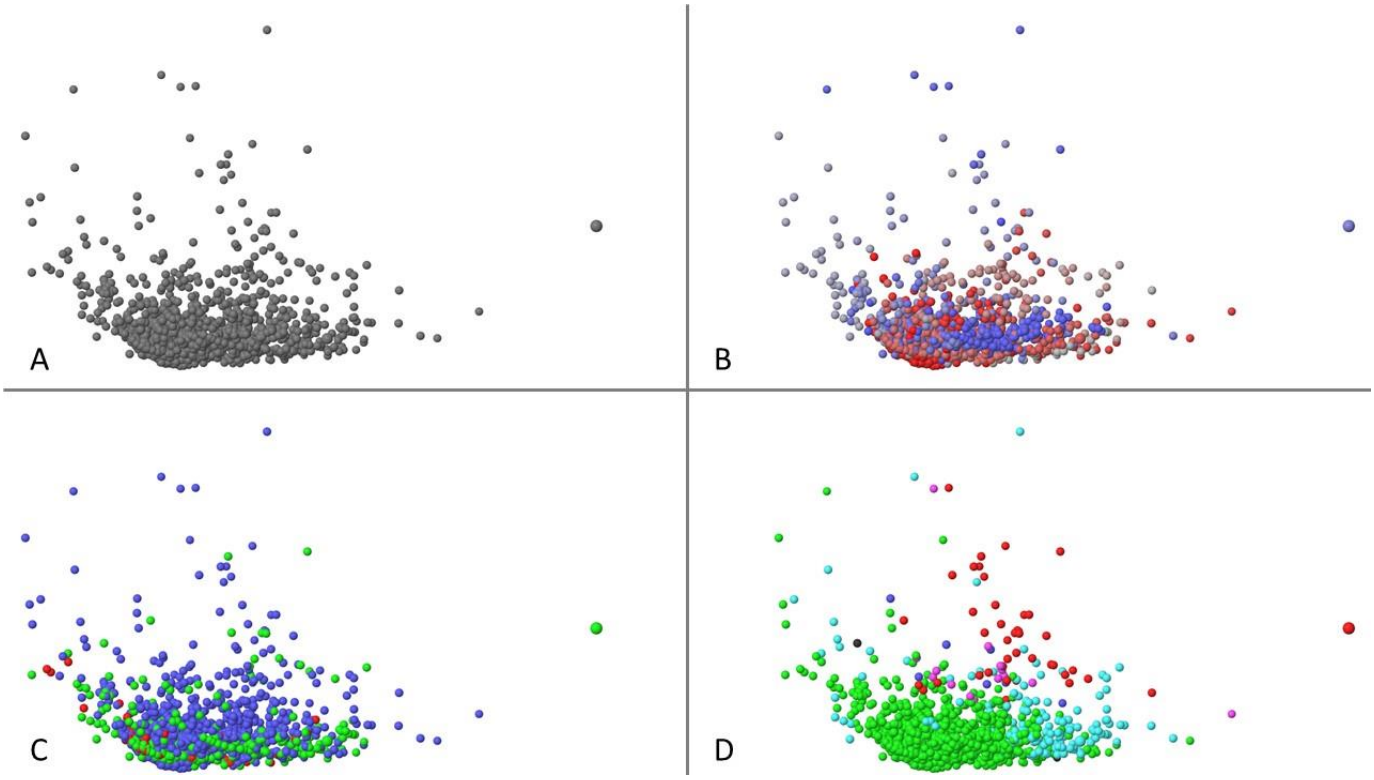

Figure 2: Principal component analysis (PCA) for the 1251 chemicals in the iTTC project when including molecular and ADME descriptors and Toxprint fingerprints. PCA when considering continuous descriptors and plotting PC1 vs PC2 vs PC3 (A). Discrete descriptors for k-means clustering (B), Cramer Classification (C) or expected clearance pathway (D) were integrated into the PCA to further classify the chemicals. The color code for Cramer classification is class I (green), class II (red) and class III (blue). The color code for clearance pathways is metabolism (green), renal (cyan), hepatic uptake (blue) and hepatic uptake or renal (red).

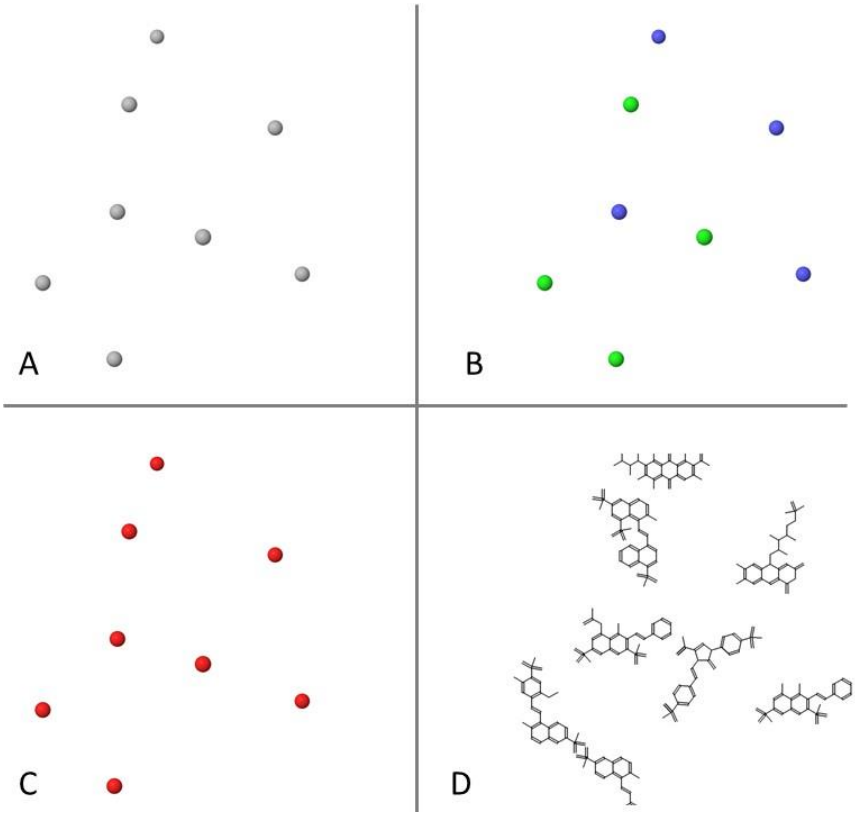

Figure 3: Principal component analysis (PCA) for the iTTC chemicals filtered so that it only displays chemicals from one cluster (cluster 21), from the k-means clustering assessment (A). Discrete descriptors for Cramer classification (B) and clearance pathway (C), as well as chemical structure (D) were integrated into the PCA to further classify the chemicals. The color code for Cramer classification is class I (green) and class III (blue). Only one clearance pathway (hepatic uptake or renal) was represented in the selected cluster.

2.6.2 Literature search

In the months following the iTTC workshop, an automated workflow was developed and is being utilized to locate relevant PK and ADME data for the iTTC chemicals. The workflow is implemented in Spyder using the Bio-python Entrez package to find articles with the specified compound, species, and either metabolism, pharmacokinetics, or clearance in PubMed. The search string that was used to search the title and abstract of articles via PubMed was: ({compound/synonym}) AND ("{species}" OR "-{species}" OR "{species_plural}") AND (metabolism OR pharmacokinetic* OR clearance). First, a list of synonyms was curated for each compound by searching the Medical Subject Heading (MeSH) database with its CASRN. Then, PubMed was searched iteratively using each compound's synonyms to attain the articles that matched the search string criteria. The search was performed with respect to a specific species in which

NOEL data was collected. A secondary search was then performed to create a term frequency matrix for each article using keywords geared towards prioritizing papers that have IVIVE and/or pharmacokinetics data. The hypothesis was that papers having a higher frequency of our specified terms would be more relevant for PBPK modeling. Thus, to prioritize the importance of an article, a score was calculated that summed the most important terms from the list. Manual review of the literature and extraction of the relevant data is ongoing. The literature search will: (1) provide an opportunity to identify existing PK and ADME data for chemicals within the TTC dataset; (2) help prioritize which chemicals need more data generated for modeling; (3) help identify existing *in vivo* data to support verification of PBPK models; (4) help determine if metabolism of a chemical is activating, detoxifying or neutral, (5) identify gaps in coverage of the toxicities in the database of chemicals based on metabolism and PK properties.

2.6.3 PBPK model platform review

An initial review was conducted to determine the most suitable PBPK modeling platform for the iTTC project. The iTTC project will require rapid PBPK modeling for hundreds of chemicals to predict the plasma concentration associated with the daily oral dose in an animal toxicity study. The toxicity studies for the iTTC chemicals were primarily conducted in rats, but also in mice, rabbits, dogs, hamster and monkey. The modeling approach will use IVIVE of metabolism and quantitative structure activity relationship (QSAR) prediction of tissue partitioning. As such, a platform is needed that includes a generic model for repeated oral dosing, capability to support metabolism IVIVE and QSAR prediction of partition coefficients, and a database of physiological parameters for multiple animal species. The ability for batch analysis is also necessary given the large number of chemicals included in the dataset. The PBPK model platform review first started with a broad review of the available PBPK model platforms followed by a filtering of the results to identify platforms most suitable for the current project. Two commercially available (GastroPlus (https://www.simulations-plus.com/software/gastroplus/) and Berkeley Madonna (https://berkeley-madonna.myshopify.com/)) and two open source (PLETHEM (http://www.scitovation.com/plethem.html) and PK-SIM (http://www.systems-biology.com/products/pk-sim.html)) PBPK model platforms were ultimately chosen for further review. These platforms were chosen for review for a number of different reasons including the fact that they had the relevant physiologies, can perform IVIVE and batch processing, and were accessible for use. The final PBPK modeling workflow for the iTTC project is still to be determined and will be guided by the outcomes from this review.

3. Conclusions and key decisions from the workshop

The TTC concept has evolved over the last 50 years and a logical next step in the continued evolution of TTC is to develop this concept further by converting the external NOAEL values from *in vivo* animal studies to internal plasma concentrations. The discussion held during the two-day iTTC workshop highlighted the complexity of developing an iTTC database and how significantly more research is required beyond current attempts where NOAELs were only adjusted for by applying an *in silico* oral absorption adjustment factor (Partosch et al., 2015). The broad-spanning complex nature of the project requires expertise from multiple disciplines, and therefore, an iTTC Working Group was formed. The iTTC Working Group developed strategies for a number of challenging aspects of the project while others, such as predicting the impact of metabolism, remain open for further discussion. The knowledge gained through this work will extend beyond the development of iTTC and will also be applicable to broader issues and will help advance non-animal approaches. A final summary of some of the key decisions made at the workshop include:

- The three main databases that will be included in the iTTC project are: Munro et. al. (1996), COSMOS and RIFM
- A chemical space analysis will be utilized to narrow down the number of chemicals in the final iTTC workflow to a more manageable number. An emphasis will be given to ensure that the final iTTC chemical dataset contains a distribution of chemicals covering a broad chemical and PK space.
- The existing chemical specific Cramer class (I, II, III) classifications for chemicals within the database will be accepted; however, it is premature to determine if the distributions of internal exposures should be separated by Cramer Class.
- The 5^{th} percentile will be utilized as a threshold selection level from the distribution of plasma concentrations (Css, AUC and C_{max}).
- Uncertainty factors will be applied to the 5^{th} percentile threshold level to derive the final iTTC values. The final determination of uncertainty factors will be at the end of the project once all relevant data is available.
- The iTTC project will: (1) base PBPK modeling on as much compound-specific data as possible; (2) use *in silico* tools to estimate parameters as necessary and deemed fit for purpose; (3) recommend experimental work only for key chemicals and key parameters; and (4) focus verification on chemicals that drive the iTTCs.
- There will be an effort to identify chemicals that might be problematic with respect to the involvement of renal clearance, transporters and extrahepatic metabolism in their clearance, and, when possible, estimate the impact of transporters to obtain the confidence in the prediction.

- An example of the use of iTTC in a human safety assessment is presented in Supplementary File 1.

Acknowledgments

The authors would like to acknowledge and thank several individuals for their help and support with different aspects of this work. Kamel Mansouri (formally at ScitoVation, currently at Integrated Laboratory Systems, Inc.) provided the estimates from OPERA. Joan Fisher, Greg Dameron and Cathy Lester (all at P&G) provided structure accuracy checks, ACD estimates and ECCS estimates, respectively. James Firman (Liverpool John Moores University) provided quality control analysis for the chemical space analysis.

Disclaimer

The United States Environmental Protection Agency, through its Office of Research and Development, funded and managed the research described here. However, it may not necessarily reflect official Agency policy, and reference to commercial products or services does not constitute endorsement.

Supplementary data

Supplementary File 1: Example use of iTTC in a human safety risk assessment

Supplementary File 2. Chemical specific input and output from the PCA and k-means clustering assessments

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