Developing non-animal methods for thyroid toxicity testing – Validation of a novel *in vitro* assay investigating Deiodinase I inhibition

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By

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The work described here was performed from February 2019 until April 2022 at BASF SE in Ludwigshafen am Rhein. It was supervised by PD Dr. Robert Landsiedel, Vice President of Special Toxicology at the department of Experimental Toxicology and Ecology of BASF SE, and Prof. Dr. Gerhard Wolber, Head of the Molecular Design Lab at the Institute of Pharmacy of the Freie Universität Berlin.

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DECLARATION OF INDEPENDENCE

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me.

This dissertation was neither accepted nor rejected in a previous doctoral process and was not submitted to any other institution.

ABBREVIATIONS

6PTU	6-Propyl-2-thiouracil
AOP	Adverse outcome pathway
ATG	Aurothioglucose
BAT	Brown adipose tissue
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
DIO	Iodothyronine deiodinase
DIP	Data interpretation procedure
DIT	3,5-Diiodo-L-tyrosine, diiodotyrosine
DMSO	Dimethyl sulfoxide
DNT	Developmental neurotoxicity
DTT	Dithiothreitol
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EU-NETVAL	European Union Network of Laboratories for the Validation of Alternative methods
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
GIVIMP	Guidance Document on Good In Vitro Method Practices
GLP	Good laboratory practice
IATA	Integrated approach to testing and assessment
IC_{50}	derived 50% inhibition concentration
IVIVE	*In vitro* to *in vivo* extrapolation
IYD	Iodotyrosine deiodinases
JRC	Joint Research Centre
KE	Key event
LAT	L-type amino acid transporter
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MCT	Monocarboxylate transporter
MIE	Molecular initiating event
MIT	3-Iodo-L-tyrosine, monoiodotyrosine
NAM	Non-animal method
NIS	Sodium/iodide symporter
OATP	Organic-anion transporting polypeptides
PBPK	Physiologically based pharmacokinetic modelling

QSAR	Quantitative structure-activity relationships
rT3	3,3’-5’-Triiodo-L-thyronine, reverse T3
SK	Sandell-Kolthoff
SULT	Sulfotransferase
T3	3,3',5-Triiodo-L-thyronine
T4	3,3',5,5'-Tetraiodo-L-thyronine, L-Thyroxine
TBBPA	Tetrabromobisphenol A
TBG	Thyroxin-binding globulin
Tetrac	3,3',5,5'-tetraiodothyroacetic acid
TH	Thyroid hormone
THBP	Thyroid hormone binding protein
THR	Thyroid hormone receptor
THS	Thyroid hormone system
TPO	Thyroid peroxidase
TRH	Thyrotropin-releasing hormone
Triac	3',3,5-Triiodothyroacetic acid
TSH	Thyroid-stimulating hormone
TTR	Transthyretin
UDGPT	UDP-glucuronosyl transferase

LIST OF PUBLICATIONS

Main author publications

1. Weber, A. G., Birk, B., Müller, C., Schneider, S., van Ravenzwaay, B., Funk-Weyer, D., & Landsiedel, R. (2022). The thyroid hormone converting enzyme human deiodinase 1 is inhibited by gold ions from inorganic salts, organic substances, and by small-size nanoparticles. *Chemico-Biological Interactions*, *351*, 109709.

2. Weber, A. G., Birk, B., Herrmann, C., Huener, H. A., Renko, K., Coecke, Schneider, S., Funk-Weyer, D., & Landsiedel, R. (2022). A New Approach Method to Study Thyroid Hormone Disruption: Optimization and Standardization of an Assay to Assess the Inhibition of DIO1 Enzyme in Human Liver Microsomes. *Applied In Vitro Toxicology*, *8*(3), 67-82.

3. Weber, A. G., Birk, B., Giri, V., Hoffmann, S., Renko, K., Coecke, S., Schneider, S., Funk-Weyer, D. & Landsiedel, R. (2023). Assessment of the Predictivity of DIO1-SK Assay to Investigate DIO1 Inhibition in Human Liver Microsomes. *Applied In Vitro Toxicology*, *9*(2), 44-59.

Further publications (not part of this thesis)

1. Garcia de Lomana, M., Weber, A. G., Birk, B., Landsiedel, R., Achenbach, J., Schleifer, K. J., Mathea, M., & Kirchmair, J. (2020). In silico models to predict the perturbation of molecular initiating events related to thyroid hormone homeostasis. *Chemical Research in Toxicology*, *34*(2), 396-411.

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1. INTRODUCTION

1.1 Thyroid and thyroid active substances (endocrine disruptors)

Over the past decades, there has been a massive increase in scientific and public awareness of man-made substances that may have adverse effects on humans. This includes substances that can potentially influence endocrine signalling pathways and thereby cause adverse health effects (1, 2). One possible affected signalling pathway is the thyroid hormone system (THS). The THS is a complex endocrine system, consisting of metabolizing enzymes, transporters, and receptors responsible for synthesis, distribution, signalling, metabolism, and elimination of thyroid hormone (TH). TH is produced in the thyroid and its signalling effects regulates energy metabolism, breathing and heart rate in adults. The thyroid itself is a butterfly-like shaped organ consisting of two connected lobes, located below the Adam's apple (*laryngeal prominence*) in the neck. The foetal thyroid gland is the first forming functional endocrine structure. Initially, supply of the foetal brain with maternal thyroid hormone is required for proper development. After three months of development, the follicular structures are formed, the thyroid becomes functional and maternal TH supply gradually decreases (3).

Thyroid hormone signalling controls important functions in growth, differentiation, and metabolism. The TH-mediated action involves the expression and activation of many genes and pathways that control general metabolism. This includes the regulation of basal metabolic rate and oxygen consumption (4, 5), activity of mitochondria including size, number and intramitochondrial genes (6). The importance of TH on basal metabolic rate regulation is evident in the thermoregulation of infants. As infants are unable to produce heat by shivering and are susceptible to hypothermia, TH-regulated, non-shivering thermogenesis in brown adipose tissue is an important way for sufficient heat production (5, 7). Also, TH signalling regulates respiration rate (8), cardiovascular functions like heart rate or blood flow and volume (9), gastrointestinal activity, and liver function including gene expression responsible for lipogenesis and oxidative processes (10). In addition, physiological TH concentrations are needed for neurogenesis, neuronal migration and myelination, as well as neural connectivity of the foetal brain (11, 12).

1.1.1 Alterations of the thyroid hormone system

Fundamentally, alterations or defects in adult THS homeostasis can lead to a decreased thyroid function, hypothyroidism, or an increased thyroid function, hyperthyroidism.

Hypothyroidism describes the state in which the organism cannot provide enough TH to ensure a normal physiological state. Symptoms include cold intolerance, fatigue, weight gain and anorexia (13). Iodine deficiency is the most common cause for hypothyroidism. The effects of low iodide intake have been observed for thousands of years, although the cause has been unclear. An undersupply of iodide leads to hyperplasia as well as hypertrophy of thyrocytes, which becomes macroscopically visible through the formation of goitres. Adverse effects range from mild symptoms like goitre formation to altered growth and development of children. Insufficient TH supply during pregnancy or later stages of child development, normally induced by insufficient dietary intake of iodine, can lead to conditions like the congenital iodine deficiency syndrome (formerly known as “Cretinism”). Patients can be affected by mental retardation, short stature, deaf-mutism, and spasticity (14). Also, iodine deficiency is the main cause of other brain alterations in childhood (15). The early foetus is dependent on maternal TH, crossing the placenta until thyroid function is fully developed. In humans, it is now broadly accepted that a reduction of free T4 levels in serum of pregnant women in the first trimester might cause neurodevelopmental changes in the offspring including decreased intelligence coefficients (IQ) and cognitive disorders (16-18). As such, the early foetus is susceptible towards small alterations in TH supply leading to neurodevelopmental effects, also called developmental neurotoxicity (DNT). Effects on structure and function of the developing brain depend on magnitude, duration, and timing of THS disruption (19, 20). Severe iodine deficiency can lead to abortions or stillbirths (21). Treatment of iodine deficient, pregnant women decreases foetal and perinatal mortality and improves cognitive performance of new-born children (22). While severe iodine deficiency has pronounced effects, the adverse effects of mild or moderate iodine deficiency are less clear. Randomised studies investigated the effect of iodine supplementation in mild to moderately iodine-deficient pregnant women (23). Although iodine supplementation mitigated maternal and thyroid size of the infant, no effects on free TH concentration or adverse, clinical outcomes like child development or maternal goitre could be shown.

Temporary Iodine deficiency is a condition to which humans have adapted through evolutionary development. As such, a human functional thyroid can store thyroid hormone for about three months, compensating for phases of reduced iodine intake. Zimmermann *et al.* claim that, in the modern world, iodine deficiency is the most common cause for mental impairment that is preventable by supplementation of iodine; most commonly done by salt iodization (15). As such, iodine supplementation eliminated the occurrence of new iodine

deficiency syndrome cases in previously iodine-deficient countries like Switzerland, but isolated regions of western China suffer from increased cretinism cases (15, 24).

The physiological state of oversupply of the organism with TH is called hyperthyroidism. In contrast to hypothyroidism, typical symptoms are heat intolerance, restlessness, or weight loss. The most common causes of hyperthyroidism in the US are toxic adenomas, toxic multinodular goitre and autoimmune diseases like Graves' disease or chronic thyroiditis (25). Treatment usually involves thyroidectomy and/or administration of radioactive iodine or inhibitors of TH synthesis (26, 27). Compared to the prevalence of iodine deficiency, patients with excess intake of iodine are rarely reported (28). Clinical symptoms include nausea, gastrointestinal upset, and diarrhoea. Affected patients often suffer from thyroid-related diseases or belong to a group with specific risk factors including elderly, neonates or pregnant women (29).

Thyroid cancers can be categorized into four different types: papillary, follicular, medullary, and anaplastic. Risk and survival prognosis vary greatly over the different forms with papillary thyroid cancer having best prognosis and follicular thyroid cancer, poorly differentiated tumours and anaplastic thyroid carcinomas having high mortality. The incidence of thyroid cancer is rising worldwide, which can be mostly attributed to the use of ultrasound examination (30). Already small, papillary, mostly benign tumours can be detected, likely leading to overdiagnosis and overtreatment (31). As such, incidence rates are higher in more developed countries like the Netherlands, although mortality rates are comparable. Nonetheless, better detection systems led to the increased detection of early stage tumours (31). So far, ionizing radiation is the only known human thyroid carcinogen (32). The occurrence of thyroid cancer in hyperthyroid patients is rare (33). Patients with subclinical hypothyroidism are associated with increased incidence and mortality for thyroid and colorectal cancer (34). There may also be links between thyroid status and other cancer types such as breast cancer (35). The role of high iodine intake on thyroid cancer was investigated, showing that iodine deficiency is a risk factor for thyroid cancer development, especially follicular thyroid cancers (36). Due to the different types of thyroid tumours, treatment also varies greatly. Thyroid cancers are usually treated by surgical approaches, administration of radioactive iodine or administration of multikinase inhibitors (37).

1.1.2 Endocrine disruption

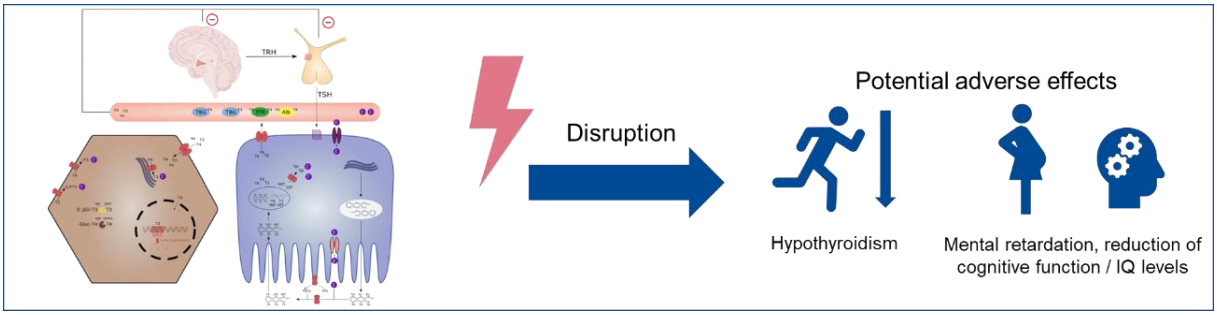


Figure 1: Disruption of the thyroid hormone system can lead to potential adverse effects like hypothyroidism in adults and mental impairments in the offspring including retardation or reduction of cognitive function.

The THS can also be targeted by man-made and natural occurring chemicals, potentially leading to disruption of THS function (2, 38) (Figure 1). Substances that can interfere with the endocrine systems are called endocrine active substances and can finally be assessed as endocrine disruptors. In a guidance document for the identification of endocrine disruptors published by the European Chemicals Agency (ECHA), European Food Safety Authority (EFSA) and the European Commission's Joint Research Centre (JRC), an endocrine disruptor must meet the following requirements: (i) it shows an adverse effect in an intact organism or its progeny, (ii) it has an endocrine mode of action, i.e., it alters the function(s) of the endocrine system and (iii) the adverse effects is a consequence of the endocrine mode of action (39). The first article mentioning the term "endocrine disruptor" was published by Colborn *et al.* in 1992, bringing together scientific results from various fields and the establishment of a new toxic mode of action (40). However, studies on adverse effects of substances on the endocrine system have been around for much longer. The use and abuse of steroids to enhance reproduction and growth began as early as the 1940s. Steroidal substances such as ethinyl estradiol have also been used as contraceptives. Scientific research on the safety of the substances used began at the same time (2).

In addition to humans, endocrine effects on the environment are known for long. The organochloride DDT was used as an insecticide for decades, to prevent the spread of malaria and typhoid (41). DDT is one of the largest reported examples of endocrine, environmental disruption. In humans, DDT is a likely endocrine disruptor and a suspected carcinogen, as classified by IARC in group 2A, "probably carcinogenic to humans" (42).

1.1.3 The thyroid system and its known inhibitors

The THS is complex with multiple regulation steps and a negative feedback loop that regulates the production and secretion of regulatory hormones. Figure 2 summarizes the THS and known potential targets for substance-induced disruption; important targets are linked to Figure 2 by numbers in square brackets, e.g. “TRH receptor [1]”.

TRH receptor [1] and TSH receptor [2]: The thyrotropin-releasing hormone (TRH) is synthesized in the Hypothalamus and, upon release, stimulates the anterior pituitary gland to secrete thyroid-stimulating hormone (TSH). The TSH receptor localizes on the surface of thyrocytes, as well as extra-thyroidal tissue like fibroblasts or adipocytes (43). Upon binding of TSH to the TSH receptor, an intracellular signalling cascade is initiated, leading to increased cAMP generation and PI3 kinase activation. This leads to growth and differentiation of thyrocytes as well as increased TH synthesis and release of stored TH into the blood stream. Interactions of substances with the TRH or the TSH receptor are rare (44), but only limited assays and research are available (45).

NIS [3]: TSH receptor activation also leads to increased uptake of iodide by the sodium/iodide symporter (NIS). The NIS localizes on the basolateral membrane of thyrocytes and mediates the active transport of iodide from the bloodstream into the thyrocyte cells. This occurs through a symport of one I^- and two Na^+ ions in the opposite direction to the sodium gradient, up concentrating iodide 20 to 40 fold compared to serum concentrations. Other expression sites besides the thyroid are intestinal enterocytes or breast tissue during lactation to ensure iodide supply in the foetus. NIS expression is regulated via TSH stimulation (46). The NIS is competently inhibited by a variety of anions with similar ion volumes as iodide. These include perchlorates, thiocyanates, hexafluorophosphates, selenocyanates, bromides, as well as nitrate (47). A medical application of NIS inhibitors is not described. Inhibitors of other iodide efflux transporters may be used in cancer treatment. As such, imidazothiazoles are likely to inhibit the efflux of iodide from thyrocytes and could be used in thyroid cancer treatment by increased retention of therapeutically administered radioactive iodide in cancer cells (48).

TSH also regulates the expression of the protein thyroglobulin, a 660 kDa precursor macromolecule of TH. One thyroglobulin molecule consists of up to 120 tyrosine residues, linked by a protein backbone. It is of ribosomal origin and is transported into the lumen by exocytosis, forming the colloid. So far, there is no evidence that thyroglobulin production is a specific target for toxicants (3).

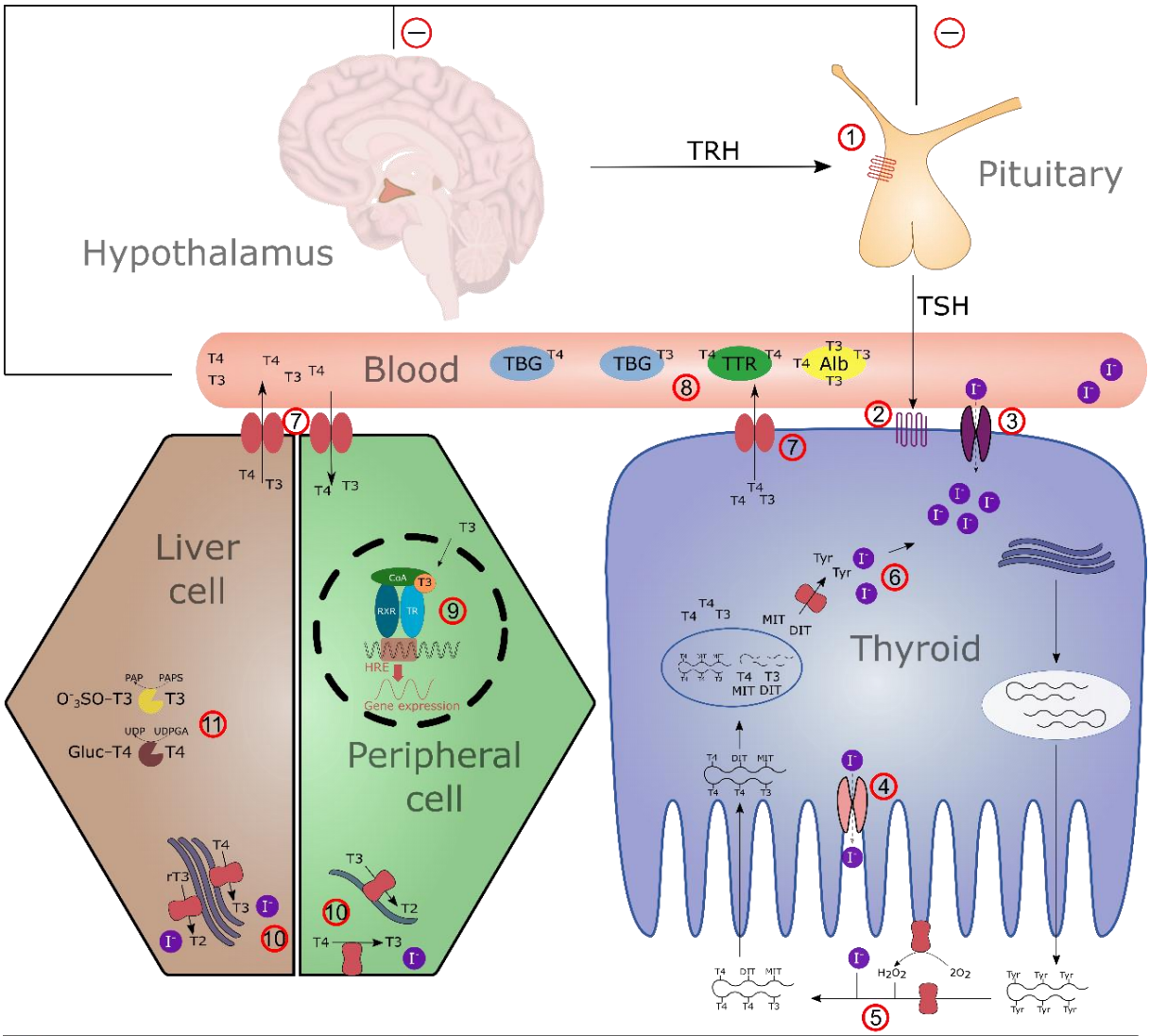


Figure 2: The thyroid hormone system and known molecular targets for substance-induced disruption. The hypothalamus produces thyrotropin-releasing hormone (TRH) which stimulates the anterior pituitary by activating the TRH receptor. This leads to the release of thyroid-stimulating hormone (TSH) into the blood stream. Thyroid activity, differentiation, and growth depends activation of the TSH receptor leading to increased iodide uptake into thyrocytes and production of thyroid hormone (TH) by incorporation of iodide into tyrosine (Tyr) residues of thyroglobulin by the thyroid peroxidase (TPO) in the lumen. Bound TH is taken up into the cells by endocytosis and TH (Triiodothyronine, T3 and thyroxine, T4) as well as mono- and diiodo tyrosine (MIT and DIT) is liberated by lysosomal proteolysis. The iodide-containing precursor molecules MIT and DIT are deiodinated by iodotyrosone deiodinases (IYD) and iodide is recycled. The THs are released into the blood stream by specific transporters. The thyroid hormone binding proteins Thyroxine-Binding-Globuline (TBG), Transthyretin (TTR) and Albumine (Alb) bind TH in serum and distribute TH to peripheral cells. TH are taken up into cells by specific transporters and mediate their effect by binding to thyroid hormone receptors, ultimatevily triggering gene expression of regulated genes. Elimination of THs in the liver occur mainly by sulfation and glucuronidation. Another step of metabolization is the deiodination of TH by enzymatic removal of iodide atoms leading to more active TH metabolites like T4 and T3 or less active ones like reverse T3 (rT3) or Diiodothyronine (T2) (indicated by red box). Finally, TH levels regulate TRH and TSH release via a negative feedback loop.

Pendrin [4]: The anion exchange transporter Pendrin mediates the iodide transport from the inner cell compartments across the apical cell membrane into the lumen of the thyrocytes. In addition, pendrin also transports bases like bicarbonate in exchange for chloride, likely explaining its expression in the inner ear to regulate acid-base balance. Loss of functional pendrin due to mutations is associated with prelingual deafness and the pendred syndrome (49). The pendred syndrome is characterized with deafness, often present from birth and worsening during development, and formation of goitres. Even though pendrin might be a molecular target for substance-induced inhibition, only few inhibitors are described (50).

TPO [5]: The thyroid peroxidase (TPO) catalyses the stepwise (i) oxidation of iodide to iodine resulting in iodinated tyrosine molecules bound to thyroglobulin and (ii) coupling of iodinated tyrosine residues, forming the THs 3,3',5-Triiodo-L-thyronine (T3) and 3,3',5,5'-Tetraiodo-L-thyronine (L-Thyroxine, T4) [5]. TPO expression occurs exclusively in the thyroid. The oxidation reaction requires H_2O_2, which is provided by the Dual Oxidase 2. The production of TH by TPO is a sensitive and important step in the THS since substance-induced inhibition of TPO is well documented and used for the medical treatment of hypothyroidism (51). Inhibitors of TPO like Methimazol can lead to hypothyroidism *in vivo* and can be used to medicinally treat hyperthyroidism (51). Perinatal exposure of rats with the TPO inhibitor Amitrole reduced pup serum T4 concentrations and leads to formation of heterotopias, a brain malformation based on misplaced neurons in the white matter (52).

The storage capacity of the colloid for normal adults is several months. In contrast, the thyroidal storage capacity of rats is only a few days. Upon TSH regulated endocytosis of iodinated thyroglobulin into the thyrocytes, TH and iodinated tyrosine residues are liberated from the thyroglobulin backbone by lysosomal proteolysis in the thyrocytes.

Iodotyrosine Deiodinases [6]: Iodotyrosine deiodinases (IYD) can cleave of iodide from iodinated tyrosine residues, recycling the by-products of TH synthesis, 3-Iodo-L-tyrosine (Monoiodotyrosine, MIT) and 3,5-Diiodo-L-tyrosine (Diiodotyrosine, DIT). IYDs prevent excess loss of iodide by recycling MIT and DIT molecules. The biological relevance of IYD is well documented as genetic loss of DEHAL1 (one isoform of IYDs) leads to hypothyroidism, goitre formation, and maternal retardation by excessive excretion of MIT/DIT (53, 54). Bromated and nitro tyrosines are potent inhibitor of IYD activity *in vitro* (55) and treatment of rodents with 3-nitro-L-tyrosine led to decreased serum-bound iodide, increased TSH levels as well as goitre formation, all reversable by iodide supplementation (56).

TH transporter [7]: To reach the inner cell, T3 and T4 must be transported across membranes. Although it has long been assumed that hormones enter the cell via diffusion (57), membrane transport via specific transport is now well established. However, the molecular transport of TH via a transporter was only described ~25 years ago (58). Transporter of TH include the solute

carrier family SLC10A1, multidrug resistance-associated proteins, the L-type amino acid transporters (LAT) LAT1 and LAT2, various organic-anion transporting polypeptides (OATP) and the specific monocarboxylate transporters (MCT) MCT8 and MCT10 (59). The biological relevance of MCT8 for TH transport and signalling is well known. Symptoms of individuals, predominantly male, with mutations of MCT8 include neurological abnormalities, severe axial hypertonia, muscle hypoplasia, and absent speech development (60). The MCT8 is expressed in numerous tissues like brain, liver, kidney, as well as adrenal and thyroid gland (61) and functions as the main TH transporter, including TH across the blood-brain barrier in brain (62). The homologous transporter MCT10 is also ubiquitously expressed and preferably transports T3 (63). Few substances have been identified interfering with TH uptake via MCT8 *in vitro* like the antidepressant desipramine or the natural substance Silichristin from milk thistle (64, 65). *Ex vivo* experiments in human term placentas showed reduced maternal to foetal T4 transfer in the presence of Silychristin (66).

TH binding proteins [8]: Thyroid hormones are hydrophobic, so most of the circulating TH is bound to plasma proteins. 0.03% of T4 and 0.3% of T3 are present in serum in their unbound form. The binding of TH to TH binding proteins (THBP) fulfils important functions. The half-life of TH is lengthened by binding of TH by reducing catalytic elimination. The thyroid-binding globulin (TBG) binds 75% of the circulating T4 as well as 75% of T3 in human serum (67). Transthyretin (TTR), albumin and to a lesser extent apolipoprotein B 100 are other important THBP. The different THBPs are believed to serve different functions. T3 and T4 have a strong affinity to TBG, resulting in strong binding and low dissociation of TH (68). This leads to a TH reservoir formation on the serum due to low elimination. The binding of TH to albumin is weak with constant association and dissociation, which is why the binding is assumed to of lesser specificity. TTR is considered to be the important mediator of TH transport since the affinity of TH to TTR is intermediate between TBG and albumin, sufficient to transport TH as well as releasing the TH (69). TTR is also an important transporter of T4 across blood-placental (70), and blood-brain or cerebrospinal fluid-border barrier (71). Displacement of TH from THBP by binding of a substance may increase free TH concentrations, leading to decreased TH levels by increased TH clearance. Displacement of T4 from TBG or TTR could be shown for several substances *in vitro* (72, 73). The human relevance of this molecular endpoint is difficult to assess. Most of the studies on THBP are performed in rodents. Adult rodents do not express the high affinity THBP TBG (74), and rodents are likely not the most suitable animal model for the human assessment. Still, substance-induced displacement of T4 at THBP might be a relevant for mammals. *In vitro studies* on plasma samples from polar bears located in Svalbard, a species with high fat content and high storage capacity for lipophilic substances, could show that binding sites of TTR were completely saturated with persistent chemicals (75), potentially contributing to observed TH hormone changes (76). Some populations like Inuit people form

Nunavik are highly exposed to persistent substances through their traditional diet. Still, levels of OH-PCBs, chlorophenols and PFOS measured in serum of Inuit women did not correlate with measured serum TH parameters, as the concentration of T4-displacing substances was likely not high enough (77).

TH receptor [9[: Thyroid hormones exert their effect by binding to nuclear receptors, the thyroid hormone receptors (THR). THRs possess binding domains for TH and the DNA. Both T3 and T4 can bind to the domain, but T3 binds with higher affinity. THR form homo- and heterodimer complexes with other nuclear receptors, particularly the retinoid-X receptor. The complex can interact with transcriptional response elements on the DNA by binding of coactivators and corepressors and thus controls the expression of TH regulated genes. Binding of TH shifts the affinity of the complex further towards coactivator recruitment (78). Several functional THRs are known, originating from two THR genes: *TRα* and *TRβ*. The isoform TRα1 is especially expressed in neurons during foetal development, heart, and muscle; TRβ1 is expressed in most tissues; TRβ2 is highly expressed in the anterior pituitary, suggesting a pivotal role in the feedback mechanism, and TRβ3 in kidney lung and liver (10, 79, 80). T3 binds to all isoforms in similar affinity, but the affinity of T3 is about 50-fold higher than the affinity of T4. Substances that can interact with the THR could either lead to a functional response (agonist) or block the functional response (antagonist), e.g., by blocking the binding of TH to the THR. Numerous THR agonists were discovered and may potentially be used as a treatment for a variety of TH-related diseases (81). Some environmental chemicals like the halogenated bisphenols Tetrabromobisphenol A or Tetrachlorobisphenol A were described as weak THR antagonists (82, 83). Several screening assay testing thousands of substances only identified few THR agonists and antagonists (44, 84) and Paul-Friedman *et al.* concluded that the endpoint of THR agonism/antagonism is likely not relevant for most environmental chemicals (84).

Metabolization of TH can occur by multiple pathways within the human body, summarized in Figure 3.

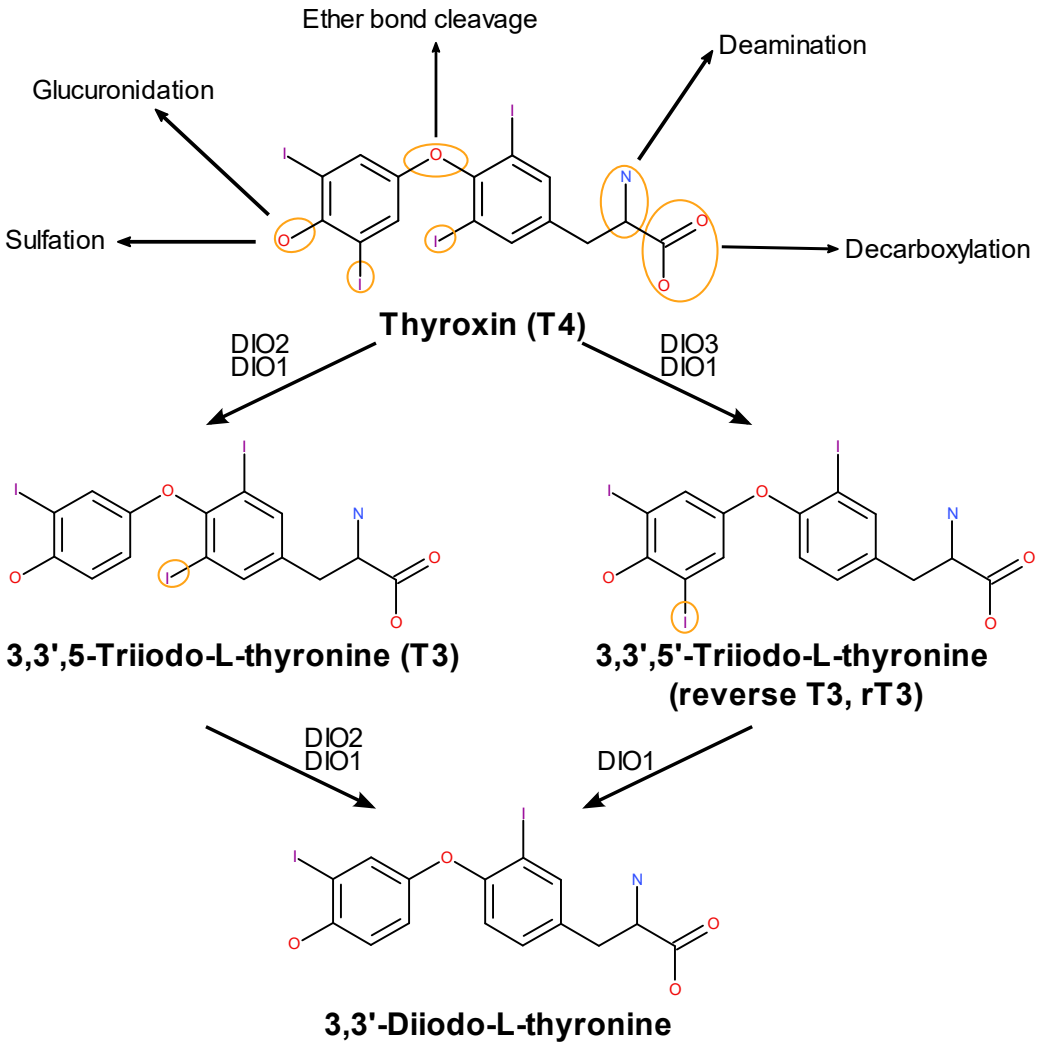


Figure 3: Metabolism of the thyroid hormone Thyroxine (T4). T4 can be metabolized at various positions including sulfation, glucuronidation, cleavage of the ether bond, deamination, and decarboxylation. In addition, thyroxine can be deiodinated by the isoforms of Deiodinases (DIO1, DIO2, DIO3) forming the metabolites 3,3'-5-Triiodo-L-thyronine (T3) or 3,3'-5'-Triiodo-L-thyronine (reverse T3, rT3). They can be further metabolized by deiodination forming multiple variations of Diiodo-L-thyronine (3,3'-Diiodo-L-thyronine is shown exemplary).
The figure was reproduced from (85) and further expanded. Positions within the molecule that are predominantly targeted by DIO catalysed metabolization are marked with orange circles.

Iodothyronine Deiodinases [10]: The most prominent step of TH metabolization in humans is the deiodination by iodothyronine deiodinases (DIO). DIOs play an important role in regulating cellular T3 levels by activation or inactivation of T3 and T4 and are crucial in providing the cytoplasmic T3 pool (86). In humans, three isoforms, DIO1, DIO2 and DIO3, with differing substrate preference, reaction kinetics, cellular and subcellular function as well as physiological function are described (Table 1). All DIO isoenzymes have a selenocysteine residue in the catalytic centre that is essential for deiodination of TH. The DIO catalysed reaction is assumed to follow ping-pong type reaction kinetics forming an iodide-enzyme complex by iodide transfer from the TH (87).

Table 1: Parameters of human deiodinase isoenzymes. Adapted according to (86, 88, 89)
6PTU, 6-Propyl-2-thiouracil, BAT: brown adipose tissue, CNS: central nervous system, DTT: dithiothreitol, rT3: reverse T3, TH: thyroid hormone

Parameter	DIO1	DIO2	DIO3
Tissue expression	Liver, kidney, thyroid, pituitary	CNS, pituitary, BAT, placenta	CNS, placenta
Subcellular location	Plasma membrane	Endoplasmatic reticulum	Plasma membrane
Cofactor	unknown (endogenous), DTT (artificial)	unknown (endogenous), DTT (artificial)	unknown (endogenous), DTT (artificial)
Preferred substrate	rT3, T3S	T4, rT3	T3, T4
Physiological function	degradation of rT3 and sulfated TH and recycling of iodide	production of plasma T3 as well as intracellular T3, thermogenesis	clearance of TH
Known inhibitors	6PTU, aurothioglucose, iopanoic acid	iopanoic acid, aurothioglucose (less)	iopanoic acid, aurothioglucose (less)
Susceptibility to 6PTU	High	Low	Low

DIO1 deiodinates predominantly on the outer ring of TH. DIO1 expression is especially high in liver and kidney and is localized to the inner surface of the cell membrane; it also expressed in the thyroid and the pituitary gland. In principle, DIO1 can have an inactivating as well as activating effect on TH action. Hepatic and renal DIO1 activity are believed as the major source of circulating T3, while liver DIO1 predominantly facilitates the breakdown of inactive 3,3'-5'-Triiodo-L-thyronine (reverse T3, rT3) (90).

DIO2 catalyses outer ring deiodination and is the major source of systemic T3 by peripheral T3 production (86). Conversion of T4 to T3 by DIO2 is increased in hypothyroid patients while hyperthyroidism leads to inactivation by ubiquitination. The ubiquitination can be split off again via the Hippel-Lindau protein leading to reactivation of DIO2 (91).

DIO3 deiodinates the inner ring of iodothyronine molecules, catalysing the inactivation of T4 and T3 to inactive TH metabolites. DIO3 is mainly expressed in the uteroplacental region and in the foetus itself where it controls the influence of maternal TH concentrations on foetal development (92). Despite the reduced expression in adults, the DIO3 pathway accounts for 80% of daily TH inactivation (93). DIO3 expression is increased in hyperthyroidism (92). Interestingly, *DIO3* is also considered as an oncofoetal gene as DIO3 expression is increased in many tumours during their differentiation process (94).

The biological relevance of DIOs as a molecular target for substance-induced inhibition is ambiguous, as there are only few known genetic alterations connected to functional changes yet (92). Genetic analysis in humans identified two families with altered DIO1 function, leading to relatively mild clinical changes including elevated serum rT3 and rT3/T3 ratio. Similar changes in TH metabolism were found after investigating a mouse model with inactivated DIO1 (95). DIO2 knock-out studies in mammalians could show inner ear effects or impaired

immunological functions (96, 97). Changes in DIO expression are also well documented in cancer and in the low-T3 syndrome during critical illness (98). Also, the role of DIOs in critical illness is well known, in which altered DIO expression and activity are linked to changes in TH metabolism (99).

A variety of substances are known that can inhibit DIO activity *in vitro*: structural analogues of TH metabolites (100); halogenated, often iodinated or brominated, polycyclic dyes like the food dye erythrosine B (101); thiouracils like 6-Propyl-2-thiouracil (6PTU) (102) or thioamides like methimazole (103); gold-containing substances including the organic gold compounds aurothioglucose (104-106), sodium aurothiomalate and auranofin (107) as well as inorganic gold compounds and small gold nanoparticles (107); iodinated aromatic compounds like the radiocontrast agent iopanoic acid (108), the cholecystographic agent iopanoate and the antiarrhythmic drug amiodarone (109). Various *in vitro* screenings using recombinant DIO enzyme of all three isoforms expanded the list of potential DIO inhibitors (106, 110, 111). Studies on *in vivo* DIO inhibitors remain scarce. Thiouracils including 6PTU are the most prominent *in vivo* inhibitors since 6PTU has a long history in the treatment of hyperthyroidism (112). 6PTU specifically inhibits DIO1 with negligible effect on the other isoenzymes (113). Amiodarone and iopanoate were sometimes used to treat hyperthyroidism based on its inhibition of DIO1 and DIO2. The treatment resulted in high iodide loading and accumulation in adipose tissue and was therefore no longer used for treatment of hyperthyroidism (114).

Secondary metabolizing enzymes [11]: The phenolic hydroxyl group of TH can be conjugated by the phase II excretion pathways glucuronidation and sulfation, ultimately leading to increased renal and biliary excretion by increasing polarity. The UDP-glucuronosyl transferases (UDPGTs) are microsomal enzymes responsible for glucuronidation and glucuronidation is the major metabolic pathway for TH in rats. In humans, only up to 15% of TH is excreted in bile as glucuronidated TH (115), and can be induced by medication like phenobarbital (116). Upon glucuronidation, TH is excreted from the body, but hydrolysation by intestinal glucuronidases can lead to reuptake via the enterohepatic circulation (117). The main phase II excretion pathway in humans is sulfation of TH, catalysed by sulfotransferases (SULTs). Sulfation of T4 and T3 leads to increased deiodination by DIO1 (85). Both sulfotransferases and DIO1 are prominently expressed during foetal development (118). Sulphated TH can be reactivated by removal of the sulphate group, potentially giving sulphated TH a reservoir function in foetal development (119). Depending on DIO1 expression, a balance between sulfation, reactivation and degradation is established. In rodents, some chemicals like sodium phenobarbitone or pregnenolone-16-a-carbonitrile induced the activity of UDGPTs and SULTs, resulting in increased elimination of TH (120). The increased turnover results in a compensatory feedback that leads to higher thyroid activity via increased TSH release, hyperplasia and hypertrophy of thyrocytes and ultimately to thyroid follicular neoplasia (115,

121). The increased phase II enzyme activity in rodents as well as other phenotypical differences like the loss of TBG expression in adult rats will contribute to the shortened half-life of T4 in rats of about 1 day as compared to humans of 5 to 9 days (122). Therefore, assumptions can be made that substance-induced induction of phase II enzymes involved in TH metabolism might play a minor role in humans (122).

The alanine side chain of TH can be modified by decarboxylation (-COOH) or deamination (-NH2). Deamination results in 3,3',5,5'-tetraiodothyroacetic acid (Tetrac) or 3',3,5-Triiodothyroacetic acid (Triac), both can bind to the THR (123) and can have similar activity on TSH secretion as TH in patients with thyroid hormone resistance (124). In humans, tetrac and triac are mainly deiodinated, with high affinity to DIO1 (125). This makes them a treatment alternative for patients with resistance to TH and need for TH supplementation. Decarboxylation of T4 and further deiodination leads to iodothyronamines like 3'-iodothyronamine or the fully deiodinated p-(p-(2-Aminoethyl) phenoxy) phenol (126). Both are endogenously present and have acute regulatory effects on cardiac function and body temperature (127).

A minor pathway in TH metabolism is the cleavage of the ether-link of TH, forming iodinated tyrosines. It is catalysed by peroxidases and accounts for up to 5% of TH elimination (125).

In addition to regulation steps of the THS in the expression, activity and degradation of involved enzymes, receptors and transporters, TRH and TSH release in the hypothalamus is regulated by a negative feedback dependant on TH concentration in plasma.

1.2 Regulatory testing for endocrine disruption

The Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/ 2009 was published by EFSA, ECHA and the JRC to provide a basis for the assessment of endocrine disruptors (39). It also includes testing schemes to determine TH levels, liver enzyme activity and guidance on how to exclude specific mode of actions. In April 2023, the European Commission expanded the existing CLP regulation and, in addition to new hazard classes regarding highly toxic and persistent substances, introduced classes on endocrine disruptors for human health and the environment (128). Substances that are classified as endocrine disruptors cannot be registered in the EU, unless exposure to the environment is negligible. In the US, endocrine disruptors can be used if risk assessment is applied and a sufficient margin of exposure can be demonstrated (129).

Adverse outcome pathways (AOPs) describe the casual connection of events at a biological level leading to an adverse effect. AOPs generally start with a molecular initiating event (MIE). In response, one or multiple cellular key events (KE) are triggered that are connected by biological or chemical relationships, ultimately leading to an adverse outcome in the organism. AOPs can be used in testing and regulatory assessment strategies. The linear structure of the AOPs is a simplification of the biological situation, since regulatory mechanisms such as feedback loops or converging KEs additionally influence the development of potential adversities (130). In an effort to collect qualitative information on AOP development and to provide a database for already existing AOPs, the AOP wiki was published (131). Multiple AOPs covering the THS are published (131). Exemplary, an AOP describing the connection of TPO inhibition and neurodevelopmental adversities are shown in Figure 4. Multiple AOPs that share MIEs, KEs or adverse outcomes can be grouped into AOP networks. This allows display and integration of complex biological relationships such as feedback loops. For the thyroid, several AOP networks have been published (45, 130, 132).

AOPs can be used to develop an integrated approach to testing and assessment (IATA). IATAs are structured approaches to integrate, evaluate and weigh data from non-animal methods as well as animal experiments to perform hazard identification and characterization and/or safety assessments (133). AOPs can help the development of IATAs in multiple ways: AOPs can help (i) to identify targets for testing and thus support assay development, (ii) structuring and grouping of gathered information and (iii) to support the evaluation of existing data and identify data gaps (134).IATAs and/or individual methods include data interpretation procedures (DIP) to translate the read-outs of assays into an overall assessment.

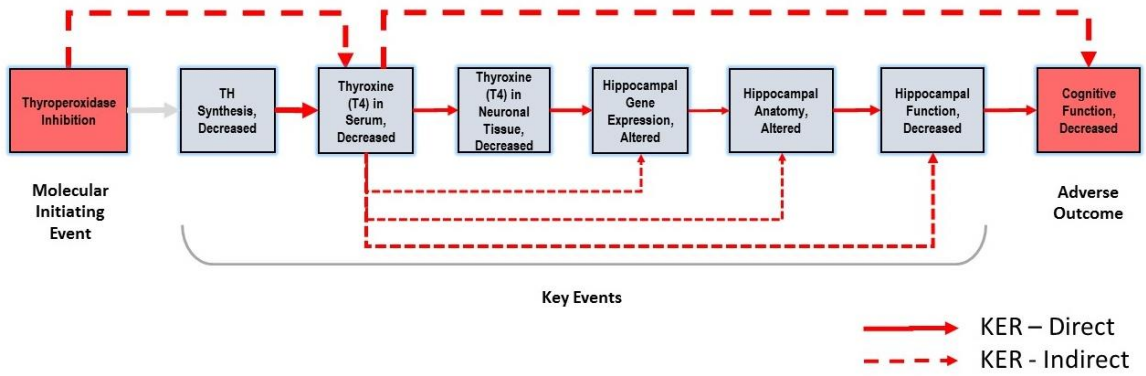


Figure 4: Adverse outcome pathway (AOP) 42: Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals.
Taken from (131). KER: Key event relationship

1.2.1 *In vivo* thyroid testing

Testing for DNT or more specifically for changes in THS parameters, is routinely done in animal experiments. The Guidance Document for the identification of endocrine disruptors by EFSA (39) considers the thyroid endpoint to be sufficiently met for mammals if thyroid parameters were examined in OECD test guidelines no. 407 (Repeated Dose 28-day Oral Toxicity Study in Rodents), 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents), 409 (Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents; if available), 416 (Two-Generation Reproduction Toxicity), 451-3 (Combined Chronic Toxicity/Carcinogenicity Studies) or, if available, 443 (Extended One-Generation Reproductive Toxicity Study). T3, T4 and TSH levels are also measured in OECD test guideline 414, 421, 422 or 443 (see Table 2 for measured thyroid parameters in animal studies and Table 3 for more information about measured parameters and timepoint of measuring).

Table 2: Thyroid parameters that are routinely tested in animal OECD test guidelines. Adapted from (39). DNT: development neurotoxicity, TH: thyroid hormone, OECD: Organization for Economic Cooperation and Development

OECD test guideline no.	Study type	Thyroid weight / Thyroid histopathology	T3/T4/TSH levels (for further information see Table 3)	Other
407	Repeated Dose 28-day Oral Toxicity Study	Optional / Yes	Optional	
408	Repeated dose 90-day oral toxicity study	Yes / Yes	Mandatory[a]	
451/452/453	Chronic/cancer studies	Yes / Yes	-	
414	Prenatal developmental toxicity study	- / -	Mandatory in dams[a]	
421/422	Reproduction/developmental screening study or combined	Yes / Yes	Yes	
416	Two-generation toxicity study	Yes / Yes	-	
443	Extended One generation toxicity study	Yes / Yes	Yes	also DNT parameters[b]
426	DNT study	- / -	-	DNT parameters

[a]since guideline from 2018

[b]in DNT cohort 2A and 2B

Table 3: thyroid hormone parameters that are tested in rodent OECD guidelines. GD: gestation day; PND: postnatal day; T3: 3,3′ ,5-Triiodo-L-thyronine; T4: 3,3′ ,5,5′ -Tetraiodo-L-thyronine; TSH: Thyroid-stimulating hormone. Taken and adapted from (135).

OECD test guideline no.	Total T3	Total T4	TSH	Assessments of the thyroid gland
408	At study termination			
414	GD: 20/21: Adults			
421	-	Adults; PND 4 pups optional; PND 13 pups; PND 13 dams optional		Adults; PND 13 pups optional
422	-	Adults; PND 4 pups optional; PND 13 pups; PND 13 dams optional		Adults (n=5); pups and remaining adults optional
443	-	P_0/F_1 adults at termination; PND 4 pups optional; PND 22 pups	P_0/F_1 adults at termination; PND 22 pups	P_0/F_1 adults at termination; PND 22 pups

Interpretation of TH parameters measured in animal experiments in a regulatory context proves to be difficult. Appendix A of the EFSA guidance states that circulating serum T3 and/or T4 changes already presents a concern for neurodevelopment, without histopathological findings (39). Also, humans and rodents are treated with equal sensitivity if substance-specific data is not available. Rodent testing to predict DNT effects in humans is subject of criticism, as rodents have multiple interspecies differences compared to humans. Exemplary, the rat has a more active thyroid, characterized by increased TH production, higher turnover rate of TH, different metabolization pathways and enzyme expression as well as different expression of TH binding proteins (THBP). This leads to different half-lives of TH, especially noticeably for T4 (human: 5-9 days, rats: 0.5 to 1 day) and increased TSH levels in rats (136, 137). Also, adverse outcomes by chronic thyroid stimulation differ: humans usually suffer from goitre formation and rats develop thyroid cancer (136). The brain development during pre-, peri- and postnatal periods also differs between rats and humans. With respect to stages of pregnancy, the neocorticogenesis takes longer in humans than rats. Also postnatal development as well as maturation of the CNS takes longer in humans than in rats (138). As such, rodents might not be the ideal model to investigate thyroid toxicity.

1.2.2 *In vitro* thyroid testing

Russel and Burch introduced the principle of the **3Rs** in 1959 raising awareness to implement the (i) **R**eduction of used animals to a minimum, (ii) **R**efinement of existing animal models to **R**educe animal harm and (iii) replacement of animal methods if equivalent non-animal methods are available (139). In 2021, the European Parliament published a resolution on plans and actions to accelerate the transition to innovation without the use of animals in research, regulatory testing and education (140). The transition from animal to non-animal methods (NAM) is also part of the EU's green deal, ultimately fully replacing the use of animals. The term "NAMs" includes all methods, technologies, approaches, or combinations that provide information on the hazard of a substance or on risk assessment without using animals. These include *in vitro*, *in silico*, *in chemico* and *ex vivo* approaches (141, 142).

Even though demand for NAMs is constantly rising, especially for regulatory requirements, there is a lack of standards for the development and provision of information in NAMs, especially *in vitro* methods. As such, published *in vitro* assays often lack documentation and reproducibility. Numerous assays to investigate TPO or NIS inhibition are available while only few were extensively characterized or reproduced in more than one laboratory. This hinders their applicability as well as use for regulatory applications. Applied concepts such as Good Laboratory Practice (GLP) include the organizational process and the general conditions under which health and environmentally relevant safety tests are planned, carried out and monitored (143). GLP conform studies are preferred in the generation of *in vivo* and *in vitro* studies for the registration of pharmaceuticals and (agro-)chemicals to ensure the complete documentation of planning, implementation, and evaluation of the study. In a collaborative effort to develop a guidance for the development of standardized and reproducible *in vitro* methods, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and a group of international experts published a Guidance Document on Good In Vitro Method Practices (GIVIMP) (144). The document provides guidance on critical aspects for the development of reliable and reproducible i*n vitro* methods. It covers aspects like facilities and workflow, material and reagents, test systems, the experimental design, control and reference items, or performance. It also gives guidance on the generation of Standard Operation Procedures (SOPs) that should include all critical tasks and information about the method. GIVIMP was adopted by the Organization for Economic Cooperation and Development (OECD) in 2018. The implementation of GIVIMP in the development of *in vitro* methods is time-consuming and is associated with increased efforts and costs. However, standardized, and reproducible *in vitro* methods are essential prerequisites for the developments of methods are to be used for regulatory purposes (145).

1.2.3 Key events vs holistic models

The goal of thyroid toxicity testing is to replace animal studies with NAMs. In animal experiments, the influence of the entire organism on a test substance, including uptake, distribution, metabolism, and elimination, as well as the effects of the substance on other organ systems, can be shown. Animal studies need extrapolation of the generated animal data to the human situation. *In vitro* assays often use human material to prevent needed extrapolation for interspecies differences. They are often protein or cell assays that can only provide information about the specific interaction with the tested target. The rat cell line FRTL-5 can take up iodide upon TSH exposure via the NIS and can be used as a model to investigate substance-induced inhibition of iodide uptake (146, 147). The immortalized human cell line Nthy-ori 3-1 expresses the TH-synthesizing enzyme TPO and can investigate inhibition of substances on TPO activity in cell lysate (148, 149). These assays can answer questions specific to the investigated KE in the THS. Used cell systems can also have functional limitations. Cytochemical analysis showed that the majority of TPO is localized in the cytoplasm of Nthy-ori cells and is not incorporated into the cell membrane (150). Using Nthy-ori cells, TPO inhibition can therefore not be studied in living cells, but in dead, lysed cells. As such, multiple *in vitro* studies must be combined to give a comparable result as *in vivo* studies.

Another possibility is the creation of higher tier of *in vitro* test systems that can map multiple interactions following a holistic approach. In the case of the thyroid, a cell system could be created that can map the entire synthesis pathway of TH, according to the AOP methodology (45). Thus, the KEs of TSH receptor activation, uptake of iodide by NIS, synthesis of TH by TPO, recycling of iodide by IYD, and transport of TH from the cell to the surrounding compartment might be mapped. Using primary human thyroid tissue and 3D cell culture, thyroid-specific markers and cell morphology as well as the production of TH could be demonstrated in *an in vitro* environment (151). However, primary cells are susceptible towards loss of differentiation or senescence, especially in extended cultivation conditions. In addition, primary, human thyroid tissue is expensive and only available to a limited extent. Continuous, often immortalized cell lines are cheaper, but often suffer from functional limitations. New immortalization methods randomly insert genes associated with cell apoptosis, cell cycle control and stem cell properties into genome of target cells, enabling functional immortalization of cells (152). Using this technique, an immortalized human thyroid cell line was created with morphological and functional properties of thyroid cells (153). Using suitable media and 3D culture the cells formed follicular structures and produced thyroglobulin, which is essential for TH synthesis. However, the cells were unable to produce significant levels of TH, showing the need for further adjustments of culture conditions.

Creating a holistic model by including other organs involved in the THS in addition to the thyroid can further increase the significance of the model. Organ-on-a-chip models are cell culture models connected by microfluidic channels that can combine the function and activity of multiple organ systems. A first organ-on-a-chip model combining primary human thyroid cells and human liver organoids was generated (154, 155). The thyroid cells have thyroid-typical markers and produce T3 for several weeks of cultivation; the liver organoids produce TH binding proteins and can form T4 glucuronidates and sulfates. The combined system is stable for several weeks and TH synthesis can be inhibited by the TPO inhibitor methimazole. However, complex test systems like organ-on-a-chip models are expensive and difficult to handle.

Despite promising advantages, further efforts regarding cultural conditions, the origin of thyroid cells as well as implementation of additional organ systems must be made to create holistic models of the THS that are reproducible and affordable.

1.2.4 Computational models

The OECD has published a Revised Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption describing the use of a conceptual framework for testing and assessment of Endocrine Disruptors (156) The framework is categorized in different levels of assay data: level 3 to 5 comprises of *in vivo* assays which can provide information on selective endocrine mechanisms or extensive data on adverse, endocrine effects, even over several generations; level 2 lists *in vitro* assays that can mostly provide information on selected mechanisms; and level 1 includes both physical and chemical properties of the substance, read-across as well as *in silico* methods. *In silico* methods that are often used in toxicology are quantitative structure-activity relationships (QSAR), physiologically based pharmacokinetic modelling (PBPK) modelling or usage of in vitro-to-in vivo extrapolation (IVIVE).

QSARs predict a biological effect based on the chemical structure of a substance. They require a large data set to correctly predict the outcome. Ideally data is available for various substance groups. If there is only limited data available, QSARs can be developed considering a certain substance group. There are numerous QSAR models addressing different KEs of the THS available (157-159)

PBPK models describe the pharmacokinetics of a substance within the organism using mathematical equations (160), divided into different compartments. These compartments are often describing organs or tissues that are connected via a circulatory blood compartment. PBPK modelling is routinely used in drug development and health risk assessment.

In vitro data can provide a good indication of the inherent hazard of a substance. The determined nominal assay concentration of an endpoint in an *in vitro* assay like the derived 50% inhibition concentration (IC_{50}) is different than the actual concentration that causes an effect on the target *in vitro*. Differences can result from binding of the substance to assay material like plastic or to proteins, especially to added serum in cell assays. In addition, evaporation and spontaneous as well as enzymatic degradation of the substance can lead to a reduction in substance concentration (161). The integration of *in vitro* to *in vivo* extrapolation (IVIVE) can help to determine the actual substance concentration and improve *in vitro* assays (162). So far, IVIVE is rarely used for regulatory applications *of in vitro* data. For a complete shift from *in vivo* to *in vitro* testing, the application of IVIVE will be essential.

A novel concept that integrates data generated from *in silico*, *in chemico* and *in vitro* testing is the next generation risk assessment approach. The goal is human safety risk assessment that is exposure-led, hypothesis-driven and designed to prevent harm (163). This approach is especially important for the cosmetic industry, which is not allowed to use data from animal testing (164).

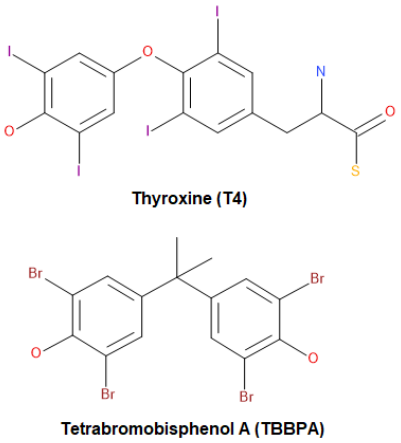
1.2.5 Substances that interfere with the THS

Test substances that can specifically inhibit KEs in the thyroid hormone system are available for most KEs. Exemplary, the natural substance Silychristin is described as a specific inhibitor of the thyroid hormone transporter MCT8 (65). However, Silychristin was not tested in most other targets. Therefore, no final assessment can be made whether the substance interact with other thyroid targets. The emergence of multiple *in vitro* screenings with thyroid endpoints in the recent years may help in demonstrating specific effects.

Many substances that are reported inhibitors of KEs of the thyroid hormone system are inhibitors of multiple KEs in the THS. As such, 6-propyl-2-thiouracil (6PTU) is a described inhibitor of deiodinases, especially DIO1, but also a potent inhibitor of TPO (103, 165). Exemplary, the activity of Tetrabromobisphenol A (TBBPA), a halogenated bisphenol, in multiple KEs of the THS is shown in Table 4. *In vitro*, effects of TBBPA on DIOs, IYDs and the THBP TTR could be shown, likely based on the structural similarity of TBBPA to T4. Interpretation of *in vitro* data and correlation to *in vivo* and human data is particularly challenging for these substances. In addition, *in vivo* thyroid data are lacking for most substances that were identified as active in *in vitro* screenings. There is corresponding *in vivo* data for pharmaceuticals like 6PTU, pesticides or chemicals that are produced in large tonnages. However, correlation to *in vivo* is not possible for most *in vitro* thyroid-active substances.

Table 4: Thyroid related activity of Tetrabromobisphenol A (TBBPA) in the screening assays by the US EPA. The right picture illustrates the structural similarity between the thyroid hormone Thyroxine (T4) and TBBPA.
DIO: deiodinases, KE: Key event, NIS: sodium-iodide symporter, TPO: thyroid peroxidase, IYD: iodotyrosine deiodinases, THR, thyroid hormone receptor, TTR: transthyretin

KE	TBBPA
DIO (110)	Full dose response for all isoforms IC_{50} DIO1: 37.4 µM IC_{50} DIO2: 49.5 µM IC_{50} DIO3: 38.4 µM
NIS (166)	No inhibition
TPO (167)	Non-specific activity based on low selectivity score
IYD (55)	Active, but no full dose response IC_{50}: 59.8 µM
THR (168)	Non-specific activity based on cytotoxicity
TTR[a] (169)	Full dose response IC_{50}: 0.22 µM[a]



[a] the screening data from the TTR binding assay by the US EPA is not yet performed/published. Hence, the data from a T4-TTR binding assay was shown, that used fluorescent-labelled T4 to show displacement of T4 from TTR (169).

1.3 The Deiodinase I (DIO1)-Sandell-Kolthoff (SK) assay

1.3.1 Background

Inhibition of DIO is one KE (Event 1002 – AOP Wiki (131)) that is linked to multiple thyroid-related AOPs including Adverse Neurodevelopmental Outcomes in Mammals (AOP8). Testing for DIO inhibition *in vivo* is difficult since enzyme-rich tissue is often not available, and expression of DIO isoenzymes overlap in tissues. Historically, *in vivo* testing uses radioactively labelled tracer substrate and selective DIO inhibitors to analyse individual DIO activity in target tissue after treatment (104, 170-172). Also, liquid chromatography-tandem mass spectrometry can be used to quantify TH metabolites, before and after DIO catalysed deiodination (126, 173). Instead of quantifying the TH metabolites, the iodide released during enzymatic deiodination can also be measured. Renko *et al.* used the Sandell-Kolthoff (SK) reaction to determine DIO activity in mice liver microsomes and investigate substance-induced inhibition (174). The SK reaction uses the reduction of yellow-coloured cerium(IV) to colourless cerium(III) by arsenite, catalysed by the released, available iodide concentration (175). The SK reaction is a widely used method for iodide quantification including urinary iodide concentration (176), and different THS KEs including inhibition of NIS (147, 166, 177), cellular TH uptake by TH transporter (178), and IYDs (55, 179). Originally, the assay used readily available liver homogenate, allowing for studies on inhibition of DIO1. By using recombinant enzyme as enzyme source, investigation DIO2 and DIO3 inhibition is also possible (106). The US EPA

used a comparable method to screen portions of the ToxCast database, a comprehensive chemical database, and selectively investigate DIO inhibition of all isoforms (55, 111).

In 2014, the DIO inhibition using the SK reaction and mouse liver microsomes as enzyme source was listed by the OECD scoping document that summarized available *in vitro* and *ex vivo* assays that are able to show inhibition on the THS (180). Based on this document and available literature data, the assay was selected to be part of a validation study of *in vitro* methods covering different endpoints in the inhibition of the THS. The project is led by the European Union Network of Laboratories for the Validation of Alternative methods (EU-NETVAL) and EURL ECVAM. The validation team of one method usually consists of a coordinator at EURL ECVAM, a member of EU-NETVAL as executing laboratory, and, if available, the original developer of the method. The method validation is consisting of two parts: the reproducibility of the method is assessed (part 1), and the method is checked for its reliability and relevance (part 2).

The Tracking System for Alternative methods towards Regulatory acceptance by EURL ECVAM currently lists 18 *in vitro* methods covering multiple endpoints in the THS as part of the validation program (date of retrieval: 10.01.2023) (181). The DIO1 assay using the SK reaction as quantification readout and human liver microsomes as enzyme source is part of the validation program as “Thyroid method 4a: Deiodinase 1 activity based on Sandell-Kolthoff reaction” (182). The validation of the DIO1-SK assay consists of coordinators at EURL-ECVAM, the BASF as EU-NETVAL member, and Kostja Renko as the original method developer (Figure 5).

Figure 5: Involved partners for the validation of the DIO1-SK assay in the context of the thyroid *in vitro* validation program.

1.3.2 The DIO1-SK assay

The method used in the DIO1-SK assay adheres to the method from Renko *et al.* that used mouse liver microsomes as enzyme source (174). Here, human liver microsomes are used as a source for DIO1 enzyme (Figure 6). Human liver microsomes are broken down parts of the endoplasmatic reticulum containing many metabolic enzymes, including DIO1 (183). They are available from different suppliers with differing properties like sex, number of donors and enzyme activity. Enzymatic deiodination activity varies greatly between microsome batches, even from the same supplier. Therefore, measures to standardize microsomal activity were integrated into the method.

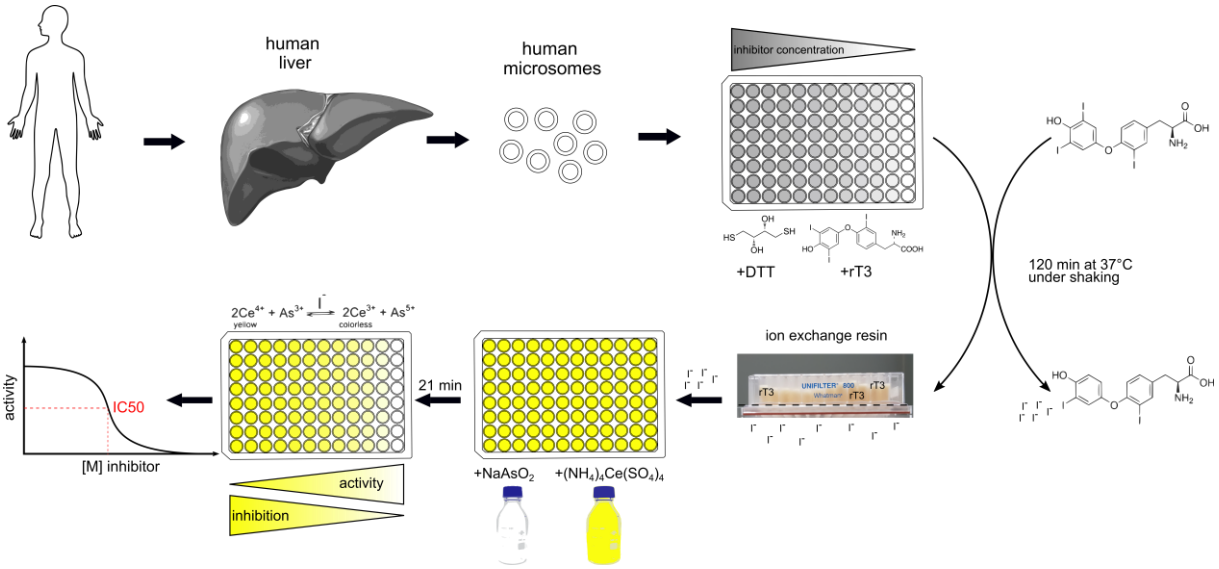


Figure 6: Schematic procedure of the DIO1-SK assay. Human liver microsomes are incubated under pH-buffered conditions with the substrate reverse T3 (rT3), an artificial enzyme-regenerating cofactor Dithiothreitol (DTT), and the test and control items. The assay plates are incubated for 2 hours at 37°C under constant shaking. The enzymatically released iodide is separated from the remaining assay constituents including substrate by an ion exchange resin. The iodide concentration of the eluted sample is then quantified by the Sandell-Kolthoff (SK) reaction by formation of colourless Ce^{3+} from yellow Ce^{4+} using arsenite. The reaction is catalysed by the available iodide concentration. Non-linear curve fitting is used to derive inhibition values for the test items.

2. MOTIVATION AND OBJECTIVES OF THE WORK

In recent decades, the effects of substances on the THS and resulting adverse effects have become an increasing focus of science. The current gold standard for the investigation of endocrine-mediated effects on the thyroid gland represents the evaluation of histopathological and clinical parameters (e.g., T3 and T4 levels in blood plasma) from *in vivo* experiments in laboratory animals. Following the 3R principle and the EU's green deal, changing regulatory testing of substances from animal testing towards the usage of NAMs has high priority. OECD approved *in vitro* guidelines are needed to promote the regular use of *in vitro* methods worldwide and to initiate a change from *in vivo* to *in vitro*. GIVIMP provides a framework for establishing, standardizing, optimizing, documenting *in vitro* methods as well as creating SOPs. Adhering to the GIVIMP standards should lead to more uniform, transparent methods and increase the reliability and robustness of methods, e.g., by reducing uncertainties of *in vitro* methods, and hereby increase the acceptance of the developed method.

There is a "crisis of reproducibility" from scientific studies (184) and a framework for establishing scientific confidence in new approach methodologies has been proposed (185, 186). The aim of this work was to develop and prevalidate NAMs that are robust, standardized, well-described, and provide relevant information on different thyroid KEs. The DIO1-SK assay was the primary focus of the EURL ECVAM thyroid validation program and of this thesis. The optimization and standardization of the assay should adhere to the GIVIMP concept to enable or accelerate a potential OECD validation. Multiple changes were introduced to the method compared to the original method from *Renko et al.* using mice liver microsomes to increase robustness and reproducibility according to GIVIMP (144).

Three studies have been published for the DIO1-SK assay, covering different aspects of the assay development.

2.1 Testing gold-containing substances as a case study to show the usability of the DIO1-SK assay

In this study, the DIO1-SK assay was used to explore the assays predictivity regarding a described class of DIO inhibitors, organic gold substances. In addition, inorganic and gold nanoparticles were tested to show the screening potential of the assay. A strategic comparison using substances containing the gold ion and substances lacking the gold moiety was introduced to verify the gold mediated DIO1 inhibition. The applicability of the solubility method towards poorly soluble substances was challenged by testing inorganic gold salts (107).

Publication: Weber, Andreas Georg, et al. "The thyroid hormone converting enzyme human deiodinase 1 is inhibited by gold ions from inorganic salts, organic substances, and by small-size nanoparticles." Chemico-Biological Interactions 351 (2022): 109709.

Published conference contributions in the context of this work:

- 5th German Pharm Tox Summit, 2020, poster presentation: Inhibition of human thyroid hormone converting Deiodinase 1 by gold nanoparticles
- 6th German Pharm Tox Summit, 2021, oral presentation: Inhibition of human Deiodinase 1 by gold containing organic substances, inorganic salts and nanoparticles
- 10th International Conference on Nanotoxicology, 2021, poster presentation: Inhibition of human thyroid hormone converting Deiodinase 1 by gold nanoparticles
- 11th World Congress on Alternatives and Animal Use in the Life Sciences (WC11), 2021, poster presentation: Deiodinase 1 in human liver microsomes is inhibited by organic and inorganic gold compounds and gold nanoparticles

2.2 Standardization, optimization, and showing reproducibility of the DIO1-SK assay

The aim of this work was to show the standardization and optimization efforts that were added to the method during the development of the assay. Different specificity testing strategies to further strengthen the trust in generated results were introduced and integrated into the method. The resulting SOPs, which includes the entire methodology of the DIO1-SK assay, should then be tested with six substances in five independent assay runs each to demonstrate the reproducibility of the assay. This approach should represent the first part of the validation efforts as part of the *in vitro* thyroid validation from EURL ECVAM (183). So that other laboratories can also establish the assay, possible pitfalls should be described and necessary measures to establish the method should be shown.

Publication: Weber, Andreas Georg, et al. "A New Approach Method to Study Thyroid Hormone Disruption: Optimization and Standardization of an Assay to Assess the Inhibition of DIO1 Enzyme in Human Liver Microsomes." Applied In Vitro Toxicology 8.3 (2022): 67-82.

Published conference contributions in the context of this work:

- 57th congress of the European societies of toxicology (EUROTOX 2021), 2021, poster presentation: Validation and specificity testing of an in vitro method assessing substance-induced DIO1 inhibition in human liver microsomes
- 58th Annual Meeting & TOXEXPO (SOT2019), 2019, poster presentation: Optimization and standardization of a human microsomes based DIO inhibition in vitro assay

2.3 Assessing the predictivity of the DIO1-SK assay

The aim of this study was to show the predictivity of the assay in a second part of the thyroid validation study. For this purpose, a blinded set of substances shall be tested in the method including solubility and specificity testing. After deblinding, the data shall be evaluated and compared with *in vitro* and *in silico* literature data to show accordance to previously generated results (187). Data generated from *in vitro* assays must be evaluated and classified to be usable for regulatory purposes. This requires data interpretation procedures (DIP) that enable the data to be categorized. As such, a DIP should be generated to enable interpretation of results.

Publication: Weber, Andreas Georg, et al. "Assessment of the Predictivity of DIO1-SK Assay to Investigate DIO1 Inhibition in Human Liver Microsomes." Applied In Vitro Toxicology 9.2 (2023): 44-59.

Published conference contributions in the context of this work:

- 7th German Pharm Tox Summit, 2022, oral presentation: Assessing reproducibility, robustness and predictivity of an in vitro method to assess DIO1 inhibition in human liver microsomes
- 11th World Congress on Alternatives and Animal Use in the Life Sciences (WC11), 2021, poster presentation: Assessing reproducibility, robustness and predictivity of an in vitro method to assess DIO1 inhibition in human liver microsomes
- 61st Annual Meeting & TOXEXPO (SOT2022), 2020, poster presentation: Validation of a human microsome based DIO1 inhibition in vitro assay

3. RESULTS

3.1 The thyroid hormone converting enzyme human deiodinase 1 is inhibited by gold ions from inorganic salts, organic substances, and by small-size nanoparticles

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Author contribution: In this work, the author was responsible for data curation, formal analysis, investigation, methodology, validation, visualization and writing of the original draft.

Abstract

The selenocysteine-containing enzyme class deiodinases (DIO) consists of three isoforms. DIOs play a role in regulation of thyroid hormone (TH) signaling through the removal of iodide from TH leading to TH that interact with the hypothalamic-pituitary-thyroid (HPT) axis with differing potency. Some gold-containing organic substances are known to inhibit many selenoenzymes, including DIOs. It is, however, unclear whether the Au-containing substances or the Au ions are causing the inhibition. In this study, five organic and inorganic gold substances as well as three gold nanoparticles (AuNPs) were tested for their potential to inhibit DIO1. The enzyme activity was tested using human liver microsomes as an enzyme source and reverse T3 as a substrate; iodide release was measured by the Sandell-Kolthoff method. The three organic gold substances aurothioglucose, auranofin and sodium aurothiomalate inhibited DIO1 with IC_{50} between 0.38 and 0.7 µM while their structural analogues lacking the gold ion did not. Likewise, the two tested gold salts, Au(I) and Au(III) chloride, showed a concentration-dependent inhibition of the DIO1 with IC_{50} values of 0.95 and 0.57 µM. Further, AuNPs of different sizes (100, 30 and 5 nm diameter) were tested with only the 5 nm AuNPs leading to inhibition with an IC_{50} of $8x10^{14}$ AuNP/L. This inhibition was not caused by the Au ions released by the AuNP into the incubation media. The exact mechanism of inhibition of DIO1 by 5 nm AuNPs should be further examined. In conclusion, the microsomal DIO1 assay demonstrated the inhibition of DIO1 by gold ions originating from different gold-containing substances, but not by Au released from AuNPs; rather DIO1 is inhibited by 5 nm, but not larger, AuNPs.

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The thyroid hormone converting enzyme human deiodinase 1 is inhibited by gold ions from inorganic salts, organic substances, and by small-size nanoparticles

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ABSTRACT

The selenocysteine-containing enzyme class deiodinases (DIO) consists of three isoforms. DIOs play a role in regulation of thyroid hormone (TH) signaling through the removal of iodide from TH leading to TH that interact with the hypothalamic-pituitary-thyroid (HPT) axis with differing potency. Some gold-containing organic substances are known to inhibit many selenoenzymes, including DIOs. It is, however, unclear whether the Au-containing substances or the Au ions are causing the inhibition. In this study, five organic and inorganic gold substances as well as three gold nanoparticles (AuNPs) were tested for their potential to inhibit DIO1. The enzyme activity was tested using human liver microsomes as an enzyme source and reverse T3 as a substrate; iodide release was measured by the Sandell-Kolthoff method. The three organic gold substances aurothioglucose, auranofin and sodium aurothiomalate inhibited DIO1 with IC_{50} between 0.38 and 0.75 μM while their structural analogues lacking the gold ion did not. Likewise, the two tested gold salts, Au(I) and Au(III) chloride, showed a concentration-dependent inhibition of the DIO1 with IC_{50} values of 0.95 and 0.57 μM. Further, AuNPs of different sizes (100, 30 and 5 nm diameter) were tested with only the 5 nm AuNPs leading to inhibition with an IC_{50} of 8×10^{14} AuNP/L. This inhibition was not caused by the Au ions released by the AuNP into the incubation media. The exact mechanism of inhibition of DIO1 by 5 nm AuNPs should be further examined. In conclusion, the microsomal DIO1 assay demonstrated the inhibition of DIO1 by gold ions originating from different gold-containing substances, but not by Au released from AuNPs; rather DIO1 is inhibited by 5 nm, but not larger, AuNPs.

1. Introduction

Numerous substances of natural or man-made origin can interfere with molecular targets, receptors, transporters, and enzymes, engaged in hormonal regulation leading to potential adverse health effects in humans. Some substances are suspected to specifically target the HPT axis; a complex, endocrine system consisting of TH synthesis in the thyroid, transport in the blood, different activating and inactivating metabolization steps as well as a feedback mechanism between the brain and the thyroid. THs are important regulators of basal metabolism and cardiac function [1]. In addition, sufficient TH levels are essential during fetal development, *inter alia* for brain development. Perturbations can lead to neurodevelopmental deficits like mental retardation [2] or reduction of cognitive functions [3,4].

Many substances interfering with the HPT axis exert their effect via inhibition of deiodinases (DIOs), a class of selenocysteine-containing enzymes. DIOs are regulators of systemic as well as peripheral TH levels through the deiodination of TH, generating TH metabolites with differing activity. The expression of the three known human isoforms, DIO1, DIO2 and DIO3, is dependent on the tissue and time during embryonic and fetal development [5]. DIO1 is mainly expressed in the liver and kidney; here, the enzymes inactivate TH metabolites to prevent the excretion of excess iodide. In the thyroid gland, DIO1 is producing

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Abbreviations

AuNPs	Gold nanoparticles
diH_2O	deionized water
DIO	Deiodinases
DIO1	Deiodinase 1
DMARD	Disease-modifying antirheumatic drug
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GIVIMP	Guidance Document on Good In Vitro Method Practices
HPT	Hypothalamic-pituitary-thyroid
PTU	6-Propyl-2-thiouracil
rT3	reverse T3, 3,3′,5′-Triiodothyronine
TH	Thyroid hormone
T3	3,3′,5-Triiodo-L-thyronine
T4	3,3′,5,5′-Tetraiodo-L-thyronine, thyroxin

systemically available T3 from T4 [6]. The selenocysteine-residue in the catalytic center of the DIO1 is essential for the TH deiodination. The reaction is assumed to follow a ping-pong type reaction kinetic with the formation of an iodide-enzyme intermediate (enzyme-Se-I) by iodide transfer from TH metabolites catalyzed by DIO1 [7]. The physiological importance of the DIOs can be demonstrated from mammalian knock-out studies resulting in inner ear defects [8] or impaired immunological function [9] for *Dio2*. Changes in expression are known for several types of cancer or during the development of low-T3 syndrome in critical illness [10]. The effect of substance-induced inhibition of deiodinases on humans is difficult to interpret (e.g., due to multiple thyroid-mediated modes of actions of known and well-studied DIO inhibitors) regarding its impact on adversity in an intact organ. Though, there are increasing concerns that thyroid-disrupting substances might exert their effect through inhibition of the DIO pathway [11].

Selenoenzymes like the DIOs, but also glutathione peroxidase or thioredoxin reductases are known to be inhibited by organic gold substances [6,12,13].

The disease-modifying antirheumatic drug (DMARD) aurothioglucose (ATG) was formerly used to treat rheumatoid arthritis [14] and has known DIO inhibiting effects on all three DIO isoforms with suggested competitive inhibition [15–17]. The potency of DIO inhibition differs among the three isoforms, with DIO1 being strongly inhibited and less inhibition occurred with the DIO3 enzyme [16]. Two additional gold containing DMARDs are still used for the treatment of rheumatoid arthritis: sodium aurothiomalate (SATM) and auranofin (AUF). Both ATG and SATM are potent inhibitors of the glutathione peroxidase with a K_i of ATG of 2.3 μM at pH 7.6 and 37 °C [18]. The mechanism of organic gold(I) derivatives on glutathione peroxidases is based on high affinity of the gold towards the selenolate forming a glutathionate-gold (I)-selenocysteine glutathione complex [19]. The selenoenzyme thioredoxin reductase is inhibited by AUF with a Ki of 4 nM [13] as well as ATG, *in vivo* and *in vitro* [20,21].

Human liver microsomes contain a variety of metabolizing enzymes including DIO1 and can be used as an enzyme source for the testing of DIO1 inhibition [22,23]. Released iodide from the deiodination can be quantified using the Sandell-Kolthoff reaction [24] which uses the reduction of yellow Ce^{4+} to Ce^{3+} and oxidation of As^{3+} to As^{5+} with a catalyzing function of iodide as a colorimetric readout.

This study aimed at exploring the DIO1 inhibition by gold-containing substances and to identify the gold ion or the whole gold-containing substances as the active inhibitor. Therefore, organic, and inorganic gold substances, their analogues lacking the gold moiety as well as different sizes of gold nanoparticles (AuNPs) were tested regarding their DIO1 inhibition in human liver microsomes.

2. Material and methods

2.1. Chemical and reagents

Unless specified otherwise, all used chemicals and reagents were obtained from Sigma-Aldrich. Human liver microsomes were purchased from Thermo-Fisher or Sigma-Aldrich. The 30 and 5 nm AuNPs were obtained from nanoComposix (San Diego, USA). Further information on the used AuNPs is specified in Supplementary table 2. Batch RRR0013 of the 5 nm AuNPs was used for liver microsome experiments and batch IAD0060 was used for the 5 nm AuNP centrifugation experiment. Water was purified using a Milli-Q Advantage A10 Water Purification System (Burlington, Massachusetts) and termed "diH_2O" after purification.

2.2. Selection of test items

Chemical suppliers were searched for organic and inorganic gold-containing substances for which a structural analog lacking the gold moiety was also available. The obtained test items that were used in this study are specified in Table 1 and Fig. 1 and visualized using BIOVIA Draw 2020 (BIOVIA, Dassault Systèmes, BIOVIA Draw 2020, Version 20.1, San Diego).

2.3. Solubility testing of the used test items

The solubility of the test items in an appropriate solvent (DMSO is preferred) was determined prior to the method to define the highest soluble concentration of a test item in the respective solvent with a highest solved concentration of 100 mM of the test item stock solution. Solubility of the test item was checked in pure solvent, the subsequent dilutions (10% solvent) in diH_2O and under final method conditions (1% solvent) by microscopical inspection. If needed, heat and/or ultrasonic was applied to aid solubility. If the test item was not fully dissolved under any of the conditions, subsequent dilution steps of the test item in the respective solvent were performed and the solubility in pure solvent, subsequent dilutions and under final method conditions was checked again; if the substance was still not soluble, a different solvent was used. The AuNPs were supplied in 2 mM citrate buffer in a homogenous suspension and not subject of solubility testing.

All tested organic and inorganic substances could be dissolved in DMSO but NaCl which was solved in diH_2O (Supplementary table 1).

2.4. Test item preparation

The highest soluble concentration of the test item in DMSO was determined; typically, this ranged between 10 and 100 mM

Table 1
Used organic and inorganic substances as well gold nanoparticles (AuNPs) in this study.

Chemical name	Acronym/identifier
Organic substances	
Aurothioglucose	ATG
1-Thio-β-D-glucose sodium salt	TGSS
Sodium aurothiomalate	SATM
Mercaptosuccinic acid	MSA
Auranofin	AUF
1-Thio-β-D-glucose tetraacetate	TGTA
Inorganic substances	
Gold(I) chloride	Au(I)Cl
Gold(III) chloride	Au(III)Cl_3
Sodium chloride	NaCl
Silver nitrate	$AgNO_3$
Copper(I) chloride	Cu(I)Cl
Gold nanoparticles (AuNPs)	
100 nm gold nanoparticles	100 nm AuNPs
30 nm gold nanoparticles	30 nm AuNPs
5 nm gold nanoparticles	5 nm AuNPs

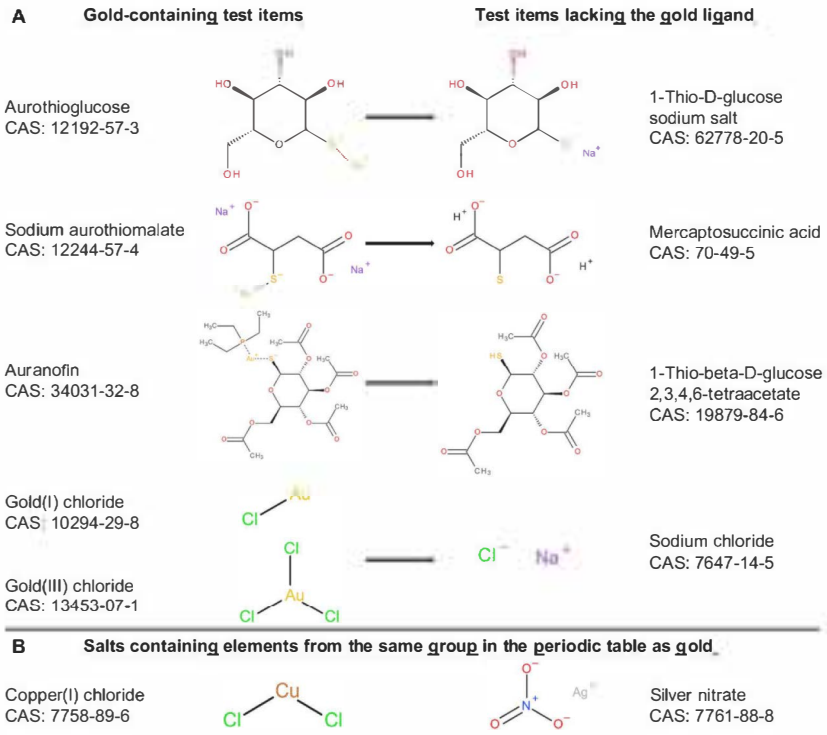

Fig. 1. Tested substances for DIO1 inhibition containing gold and substances lacking the gold ligand (A) as well as substances containing an element from the same group in the periodic table as gold (B).

(Supplementary table 1). Sodium chloride (NaCl) was solved in diH_2O and the tested AuNPs were provided in a citrate buffer (2 mM) by the supplier. On the day of analysis, test item dilutions were prepared freshly while keeping a final solvent concentration of DMSO or diH_2O of 1% in the method since 1% did not influence iodide release activity of human liver microsomes. For the testing of AuNPs, 10% citrate buffer filtrate had no effect on the iodide release activity of human liver microsomes (Supplementary figure 1) and a final method concentration of 10% citrate buffer filtrate was used for the testing of AuNPs.

2.5. Quality control measures

In accordance to the Guidance Document on Good In Vitro Method Practices (GIVIMP) [25]), a well characterized reference item with well-known response was used to grade the response of the test system. 6-Propyl-2-thiouracil (PTU) is a thioamide and a well-known specific inhibitor of DIO1 [26] as well as the thyroid peroxidase [27]. It has a long history in the treatment of hyperthyroidism [28] and was used as the reference item in this method. On the day of analysis, a stock solution of 100 mM PTU in DMSO was freshly prepared and a final method concentration of 1 mM of the reference item PTU was conducted on every plate in triplicates.

Respective solvent controls with 100% diH_2O or 1% DMSO in diH_2O were also conducted in triplicates on every plate. A citrate buffer filtrate was prepared via filtration of 100 nm AuNPs with 0.02 μM Syringe filters and used as the solvent control (final method concentration: 10%) for testing of the AuNPs and as a diluent for the dilutions of the AuNPs.

2.6. Iodide release incubation

The iodide release method using human liver microsomes was done in a 96-well format in triplicates and a total volume of 100 μl. The microsomes were carefully thawed on ice and diluted in diH_2O resulting in a solution of 5–20 μg enzyme per reaction well, depending on the iodide release activity of the used microsome batch. Microsome-batch specific iodide release activity was determined via a microsome-response screening.

10 μl of test item dilutions, 40 μl diH_2O diluted human liver microsomes and 50 μl of a potassium phosphate (0.2 M, pH 6.8)/EDTA (2 mM) buffer, supplemented with reverse T3 (rT3, final method concentration: 5 μM) and dithiothreitol (DTT, final method concentration: 0.04 M), were added to a 96-well microtiter plate (polystyrene, 360 μl well volume, TPP, Trasadingen, Switzerland), sealed with an impermeable cover sheet and incubated for 2 h under constant shaking at 37 °C. After the incubation period, the released iodide ions were separated from the residual components of the samples via ion exchange using a DOWEX resin (W50-XS, 100–200 mesh, Acros Organics) covered 96-well filter plate (800 μl well volume, filter bottom with longdrip, Sigma-Aldrich). 75 μl of the sample solutions and 100 μl 10% acetic acid were added to the columns of the Dowex resin filled 96-well filter plate with subsequent elution via vacuum into a 96-deep square well collection plate (polypropylene, well volume 2 mL, Supelco, Bellefone, Pennsylvania). Depending on the iodide concentration in the samples, undiluted sample, or dilutions in 10% acetic acid were prepared and used in the Sandell-Kolthoff reaction to quantify the containing iodide concentration.

2.7. Sandell-Kolthoff reaction

The Sandell-Kolthoff reaction is used to measure free iodide concentrations via the reduction of yellow Ce^{4+} to Ce^{3+} and oxidation of As^{3+} to As^{5+} with a catalyzing function of iodide leading to fading of the yellow color.

50 µl of diluted (using 10% acetic acid) or undiluted eluted samples from the 96-deep square collection plate was added to a microtiter plate with subsequent addition of 50 µl of cerium solution [25 mM $(NH_4)_4Ce(SO_4)_4*2H_2O$, 0.5 M H_2SO_4] to all samples of the plate. The reaction was initiated via addition of 50 µl arsenic solution [25 mM $NaAsO_2$, 0.8 M NaCl, 0.5 M H_2SO_4] using an electronic multichannel pipette allowing close to simultaneous start of the reaction between the samples of the assay plate. Immediately after, the samples were shaken for 2 s and absorption was measured at 415 nm at the start of the reaction and after 21 min of incubation using a Sunrise™ Absorbance Reader (Tecan Trading AG, Männedorf, Switzerland).

2.8. Centrifugation of AuNPs for removal of AuNPs, subsequent testing and quantification of gold content using ICP-MS

5 nm AuNP samples as obtained from the supplier ($3.8*10^{16}$ particles/L) as well as serial dilutions in citrate buffer filtrate were incubated with 50% phosphate buffer, 40% diH_2O and 10% of the 5 nm AuNP samples (final volume per dilution: 6 mL) for 2 h to mimic assay conditions. 5 mL of the incubated samples were transferred to centrifugation tubes and the remaining volume was kept for later testing in the method. The centrifugation tubes were centrifuged at 100′000*g for 30 min and the supernatant was carefully removed to not disturb the pelleted AuNPs. 1 mL of the centrifugated supernatant was kept for later testing while the remaining volume was centrifugated again using the same procedure. After a total of three centrifugation steps and subsequent removal of the supernatant, all the taken supernatant samples were tested regarding their potency to inhibit iodide release in the assay.

Additionally, 5 nm AuNP samples as obtained from the supplier ($3.8*10^{16}$ particles/L), samples of the highest concentration of the non-centrifugated 5 nm AuNPs that mimic assay conditions ($3.8*10^{15}$ particles/L) and the centrifugated supernatant samples of the 5 nm AuNP samples were analyzed regarding their gold content. The samples were acidified, and the gold content was measured via inductively coupled plasma-mass spectrometry (ICP-MS; limit of detection: 0.1 mg/L).

2.9. Data processing

Three independent assay runs were performed for each test item. Data was processed and analyzed using Microsoft Excel; visualization and the 50% inhibition concentration (IC_{50}) calculations were done in GraphPad Prism 8 (Graph Pad Software, San Diego, California).

Absorption data was processed in the following steps: (1) determination of the change in absorbance for each well between initial values and values after 21 min; (2) determination of the change in absorbance via subtraction of the inhibited background reaction using the reference item (PTU) control values; (3) normalization to the mean of the respective solvent controls resulting in normalized (%) iodide release activity values for each well.

All three replicates per test item concentration were plotted with normalized iodide release activity values on y-axis (linear) and test item concentration on x-axis (logarithmic) in GraphPad Prism. The sigmoidal concentration-response model "[Inhibitor] vs. response – Variable slope (four parameters)" was used to generate a function reflecting assay characteristics, visualize a concentration-response relationship and, if possible, calculate the IC_{50}:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + (IC_{50} / X)^{\text{HillSlope}})$$

where "Top" represents the maximal response, "Bottom" represents the lowest response, and "HillSlope" describes the steepness of the curve.

3. Results and discussion

Selenoenzymes like the DIOs are known to be inhibited by gold-containing organic substances like ATG, which has been used as an antirheumatic agent [12]. The three tested gold-containing DMARDs, ATG, SATM and AUR, inhibited the iodide release activity in human liver microsomes, with similar IC_{50} (Fig. 2, A; Table 2). The derived IC_{50} value for ATG of 0.64 µM in this study differs from a previously reported IC_{50} of 0.02 µM ATG in native liver homogenate as well as recombinant protein in pigs [29]. Additionally, to its inhibitory effect on DIO1, ATG also inhibits DIO2 and DIO3. Data from experiments using recombinant enzyme of all three DIOs have shown that 1 mM as well as 200 µM is sufficient to fully inhibit deiodinase activity [30,31]. Further, the potency of ATG on the different DIOs was studied in native and recombinant porcine enzymes indicating a 20-fold higher susceptibility of DIO1 towards gold-induced inhibition compared to DIO2 and DIO3 [29]. It can therefore be assumed that the tested gold substances are also able to inhibit human DIO2 and DIO3, presumably in reduced potency, which would need further investigation. To our knowledge, SATM and AUR were not tested regarding their potential to inhibit DIO1 prior to this study and no inhibitory potency is reported.

In addition, three substances with the DMARD's structure but lacking the gold cation were investigated without showing inhibition of iodide release activity (Fig. 2, B) indicating gold as essential to the inhibition of DIO1. This is in line with reports of 5 µM thioglucose having no effect on enzyme activity of transiently expressed rat DIO1 [15]. Likewise, thiomalate as well as thioglucose had low to no effect on selenocysteine-catalyzed oxidation of cysteine in an *in chemico* cysteine oxidation model while the gold-containing substances inhibited 50% of selenocysteine-catalyzed oxidation at 1.2 µM ATG and 1.8 µM aurothiomalate [32]. The tested AUF analogue lacking the gold cation additionally lacked the triethylphospine group connected to the gold ion in the AUF molecule. A structural analogue, triphenylphospine, was tested in a screening approach using human recombinant DIO enzyme of all three isoforms at a single high concentration of 200 µM triphenylphosphine and did not show signs of DIO inhibition in either of the isoforms [31].

To investigate the inhibiting potential of non-organic gold substances, the two gold salts Au(I)Cl and Au(III)Cl_3 were tested for inhibiting iodide release activity in human liver microsomes. Both gold salts completely inhibited iodide release activity and the IC_{50} values of 0.95 µM for Au(I)Cl and 0.57 µM for Au(III)Cl_3 are in the same range as those of organic gold substances (Fig. 3, A; Table 2). DTT was used in the DIO1 assay as an enzyme regenerating agent. DTT may reduce Au(III) to Au(I); the reduction of gold(III) by thiols and thioethers like cysteine and methionine has been observed earlier [33,34]. NaCl had no effect on the iodide release activity in human liver microsomes, indicating that the gold rather than chloride ions are the cause for DIO1 inhibition observed with Au(I)Cl and Au(III)Cl_3 (Fig. 3, B).

Gold is a member of the eleventh group of the periodic table (the "copper family"). Since elements in a group have similar chemical characteristics, two other elements of this group, silver, and copper, were tested regarding their ability to inhibit DIO1. Silver ions can interact with selenium substances [32] and are known inhibitors of the selenium-glutathione peroxidase [35]. In this study, silver nitrate ($AgNO_3$) as well as copper chloride (Cu(I)Cl) were tested and displayed a non-sigmoidal concentration-response correlation of the measured iodide release following incubation at the tested concentrations, which might indicate a non-specific effect on the DIOs (Fig. 3, C). The possible inhibition by $AgNO_3$ could either be triggered by the silver or nitrate ions. Inhibition of the nitrate ion seems unlikely since a different nitrate salt, potassium nitrate, was tested negative regarding DIO inhibition in human recombinant enzyme of all three isoforms tested at 200 µM [31].

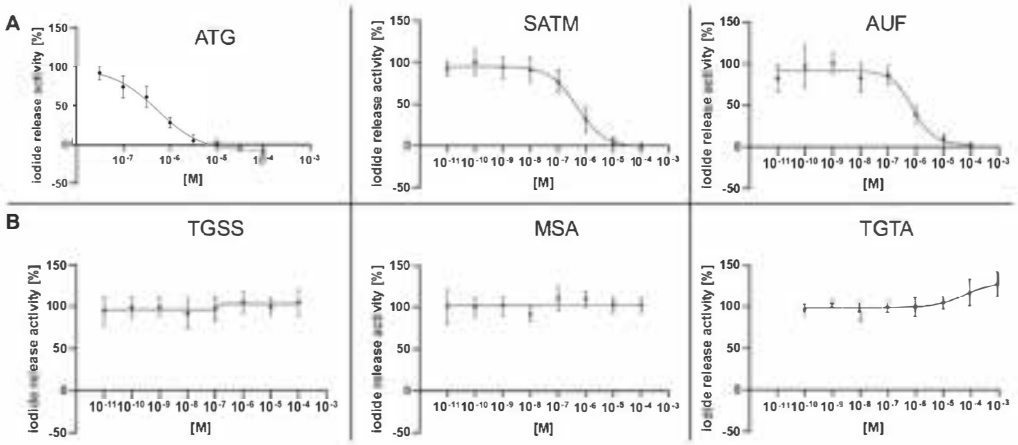

Fig. 2. Concentration response curves for iodide release inhibition by three gold-containing, organic substances (A) and structural analogues lacking the gold ligand (B) in human liver microsomes. Results are presented as relative percentage of the maximal iodide release activity of the solvent control ±SD and normalized to the reference item 6-Propyl-2-thiouracil (three replicates per concentration, three independent repeats for each substance).
ATG = aurothioglucose, SATM = sodium aurothiomalate, AUF = Auranofin, TGSS = 1-Thio-β-D-glucose sodium salt, MSA = mercaptosuccinic acid, TGTA = 1-Thio-β-D-glucose tetraacetate

Table 2
Summary of the concentration-response for the tested organic and inorganic substances as well as gold nanoparticles (AuNPs) regarding iodide release inhibition in this DIO inhibition method using human liver microsomes. Data is shown as derived value and as the 95% confidence interval (95% CI).

Acronym/Identifier	Tested concentrations [μM]	IC_{50} [μM]		HillSlope		IC_{20} [μM] [a]
		Derived value	95% CI	Derived value	95% CI	
SATM	0.00001–100	0.48	0.31–0.77	0.88	0.62–1.25	0.1
MSA	0.00001–100	–	–	–		–
ATG	0.0316–100	0.64	0.35–0.73	0.93	0.65–1.28	0.14
TGSS	0.00001–100	–		–		–
AUR	0.00001–100	0.75	0.46–1.32	1.15	0.66-n.d.	0.22
TGTA	0.0001–1000	–		–0.49		–
Au(I)Cl	0.00001–100	0.95	0.65–1.47	0.77	0.60–1	0.16
$Au(III)Cl_3$	0.00001–100	0.57	0.33–1.04	0.72	0.52–0.98	0.08
NaCl	0.0001–1000	–	–	–		–
$AgNO_3$	0.0001–1000	[b]	–	–		–
Cu(I)Cl	0.0001–1000	[b]	–	–		–

Acronym/ Identifier	Tested concentrations [particles/L]	IC_{50} [particles/L]	IC_{50} - 95% CI [particles/L]	HillSlope	HillSlope – 95% CI	IC_{20} [particles/L] [a]
100 nm AuNP	$1.21*10^{8}$ - $3.84*10^{11}$	–	–	–		–
30 nm AuNP	$6.01*10^{9}$–$1.9*10^{13}$	–	–	–		–
5 nm AuNP	$1.96*10^{12}$–$6.2*10^{15}$	$8.0*10^{14}$	n.d.	1.07	0.96–1.13	$2.2*10^{14}$

n.d.: not determinable.
Information on additional dose metrices of the tested substances are presented in Supplementary table 3.
[a] Calculated using ECanything 2 from GraphPad, San Diego, California, https://www.graphpad.com/quickcalcs/ECanything2/.
[b] Not determinable based on non-sigmoidal shape of the curve at the tested concentrations resulting in flawed fitting of the bottom of the derived curves.

The observed decrease of iodide release activity by $AgNO_3$ might be an artifact due to silver ions forming insoluble silver phosphate precipitates when combined with potassium phosphate which is a constituent of the buffer used by this method. Further, silver ions and the released iodide might precipitate as silver iodide which might also explain the non-sigmoidal decrease of activity of $AgNO_3$. Likewise, Cu(I)Cl was shown to precipitate as cuprous iodide in the presence of iodide [36]. Precipitation of iodide as cuprous iodide would decrease both free copper ions and released iodide in the samples and ultimately result in the observed loss of iodide release activity for Cu(I)Cl.

In addition to organic and inorganic gold substances, three AuNPs with spherical shape and different diameters were tested regarding their potency to inhibit DIO1 in human liver microsomes. The larger AuNPs samples of 100 and 30 nm diameter did not decrease iodide release activity while the 5 nm AuNPs sample caused a decrease with a sigmoidal concentration-response and an IC_{50} of 8.0×10^{14} particles/L (Fig. 4, A). Compared to the IC_{50} values that were derived from testing of the organic and inorganic gold-containing substances ranging from 0.48 to 0.95 μM (corresponds to 2.9 to 5.7×10^{17} Au^{+} ions/L), the derived IC_{50} for the 5 nm AuNPs is lower. The trigger for the AuNP-induced DIO1 inhibition could potentially result from multiple mechanisms: (a) dissolved gold(I) cations in the sample solutions, (b) through local release of gold(I) ions in close proximity to the AuNPs further induced by shaking conditions in the method, (c) prosthetic binding to the enzyme resulting in confirmational changes or (d) inhibition at the catalytic center of the enzyme by the AuNPs themselves. To be able to exclude the gold ions dissolved from the 5 nm AuNP as the cause of the observed DIO1 inhibition, 5 nm AuNPs were incubated mimicking the assay

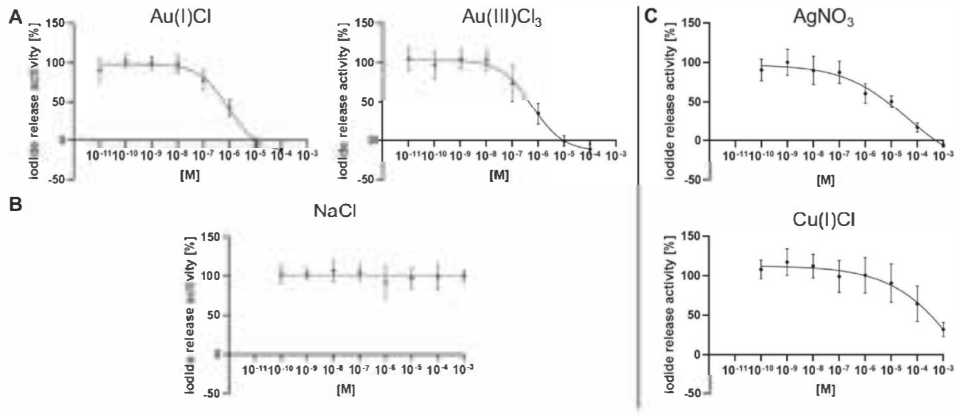

Fig. 3. Concentration response curves displaying iodide release inhibition for (A) two inorganic gold salts Gold(I) and Gold(III) chloride, (B) sodium chloride as a structural analogue lacking the gold ligand and (C) two inorganic substances of the same group as gold, silver nitrate and copper chloride. Results are presented as relative percentage of the maximal iodide release activity of the solvent control and normalized to the reference item 6-Propyl-2-thiouracil (three replicates per concentration, three independent repeats for each substance, concentration-response for NaCl was only performed once in triplicates per concentration).
Au(I)Cl = gold(I) chloride, $Au(III)Cl_3$ = gold(III) chloride, NaCl = sodium chloride, AgNO3 = silver nitrate, Cu(I)Cl = copper(I) chloride

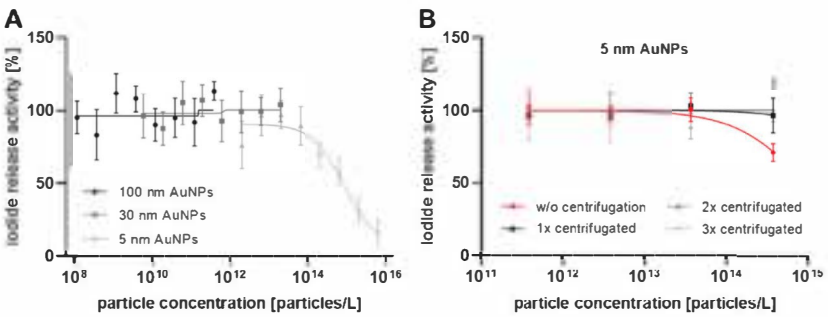

Fig. 4. Concentration response curves displaying iodide release inhibition for gold nanoparticles (AuNPs) with three different diameter sizes (100, 30 and 5 nm) using human liver microsomes (A). To elucidate the observed inhibition with 5 nm AuNPs, 5 nm AuNP dilutions were incubated under assay conditions, ultra-centrifugated three times and the removed supernatant was tested in the method regarding its potency to inhibit iodide release (B). Results are presented as relative percentage of the maximal iodide release activity of the solvent control ±SD and normalized to the reference item 6-Propyl-2-thiouracil (for A: three replicates per concentration, three independent repeats for each AuNP, for B: three replicates per concentration).

conditions. The AuNPs were then removed via ultracentrifugation and the supernatants were subsequently tested for DIO1 inhibition. No inhibition of iodide release was detected with the supernatant of the 5 nm AuNP (Fig. 4, B) indicating that dissolved gold ions are not the mediator of the observed DIO1 inhibition but rather DIO1 inhibition is mediated by the presence of 5 nm AuNPs. In addition, the 5 nm samples as provided by the supplier and the centrifuged 5 nm AuNP supernatant samples were analyzed for their gold content by ICP-MS. The gold content of the supernatant of the centrifugated 5 nm AuNP samples was below the limit of detection of 0.1 mg/L, already after the first centrifugation step. The centrifugation of the 5 nm AuNP samples removed at least 99% (Supplementary table 2) of the total measured gold content in the 5 nm AuNP samples further indicating that the centrifugation is sufficient to remove total gold content.

The highest tested particle concentration of 100 and 30 nm AuNPs was 300 to 15′000 lower than the tested particle concentration of the 5 nm AuNPs (Table 2). The absence of DIO1 inhibition by the larger AuNPs may be the result of the lower particle number concentration and/or the larger particle size. AuNPs with a diameter of 5 nM present in a higher number concentration inhibited DIO1. Interestingly, inhibition of the selenocysteine enzymes thioredoxin reductase and glutathione peroxidase by 5 nm polyallylamine-coated AuNPs was observed at a particle concentration similar to our findings of 3.0×10^{14} particles/L in three cell lines by Daems and coworkers [37].

While the relevance of gold induced DIO1 inhibition for the *in vivo* situation is unclear, there are few studies that have looked at the distribution and even adverse thyroid effects of gold cations. Blocka et al. measured gold plasma concentrations of patients after intramuscular administration of a single dose of 50 mg SATM. The highest plasma concentrations of 4–8 mg Au/L, corresponding to 20–40 μmol Au/L, were detected 2–6 h after the application [38], indicating that gold might reach hepatocytes in sufficient quantities to inhibit DIO1 activity. In the liver however, which predominantly expresses DIO1, gold accumulates mainly in the Kupffer cells and only a smaller part reaches the hepatocytes [39]. Our presented results in human liver microsomes may therefore be over-predictive for the expected *in vivo* situation. Additionally, DIO1 inhibition in the liver may have a rather local effect as it is debatable whether it would also affect thyroid hormone parameters in the blood [40]. A retrospective study involving 11 patients that were given 25–50 mg ATG or SATM weekly or bimonthly was unable to show a correlation between the cumulative gold dose and thyroid parameters in the blood (total T4, T3 and rT3 as well as TSH concentrations) [41]. AUF was cytotoxic in B16 mouse melanoma cell and human ovarian cancer cell lines with IC_{50} of 1.5 μM and 1.06 μM, respectively [42,43]. Hence, cytotoxicity may occur at concentrations that are lower than concentrations needed for DIO1 inhibition.

Some information for the *in vivo* distribution of AuNPs is available. Distribution of AuNPs is highly dependent on size but also shape and coating of the particles. In rats, injected or intravenously administered 10 nm AuNPs distribute to various organs including liver, spleen, blood, kidney, and thymus while bigger AuNPs accumulate only in the blood, liver, and spleen [44,45]. A study using smaller AuNPs injected 2 nm AuNPs intravenously in female adult mice and traced the distribution by autometallography [46]. In all the examined animals, the administered gold could be found in lysosomes of macrophages, especially in Kupffer cells in the liver [47]. Based on these findings, distribution of a larger fraction of the particles into hepatocytes seems unlikely, even with smaller AuNPs.

4. Conclusions

The pharmaceutical ATG is a known inhibitor of selenocysteine-containing enzymes including the DIOs. Our results have shown that the gold(I) ions, rather than the entire gold-containing molecules are mediating the inhibitory effect. Au(III) was as effective as an inhibitor as Au(I) either because it is directly acting or readily reduced to Au(I). This inhibition may be specific to gold, as we could not observe DIO1 inhibition with other cations of the eleventh group of the periodic table. Potential inhibition by Cu- and Ag-ions may, however, have been disguised by precipitation of these ions with media constituents or released iodide. AuNPs did not release enough free gold-ions to inhibit DIO1. Small AuNPs of 5 nm, but not larger particles of 30 or 100 nm, could inhibit the enzyme in human microsomes. The exact mechanism of DIO1 inhibition by NP should be explored in future studies, e.g. by testing the mechanism in human hepatocytes. Likewise, the significance of the findings of this study in human liver microsomes regarding human adversity needs to be further explored in more complex test systems.

Author statement

Andreas Weber: Investigation, Writing original draft, Editing; Barbara Birk: Methodology, Project management; Chantal Müller: Investigation; Steffen Schneider: Funding acquisition, Review; Ben van Ravenzwaay and Dorothe Funk-Weyer: Resources, Review; Robert Landsiedel: Conceptualization, Review, Supervision.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Robert Landsiedel reports financial support was provided by BASF SE. Robert Landsiedel reports a relationship with BASF SE that includes: employment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2021.109709.

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3.2 A New Approach Method to Study Thyroid Hormone Disruption: Optimization and Standardization of an Assay to Assess the Inhibition of DIO1 Enzyme in Human Liver Microsomes

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Author contribution: In this work, the author was responsible for conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization and writing of the original draft.

Abstract

Identifying substances which can disturb thyroid hormone (TH) signaling pathways is gaining importance and is soon to be a regulatory requirement in the EU. The European Commission Joint Research Centre's (EC JRC) European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) is coordinating the validation of multiple *in vitro* methods focusing on different thyroid modes of action (MoA) resulting in an *in vitro* test battery. Deiodinases (DIO) are regulators of TH signaling by activating or inactivating TH via deiodination. Inhibiting DIO activity is one potential MoA of substance-induced TH signaling disruption. Here, the DIO1-SK assay using human liver microsomes as a DIO1 enzyme source and the Sandell-Kolthoff (SK) reaction as a colorimetric readout to quantify released iodide was standardized and optimized according to GIVIMP (Good *In Vitro* Method Practices) (145, 180). Potential pitfalls are described, and possible solutions are provided. The reproducibility of the method was demonstrated by testing a set of six described DIO1 inhibitors in five independent assay runs. The results of this study were used to amend the testing protocol: a set of acceptance criteria was defined to assess the validity of assay runs and strategies to verify the specificity of observed DIO1 interaction were implemented. This work completes the pre-validation of the DIO-SK assay and defines a robust assay which provides results that can be used with confidence.

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A New Approach Method to Study Thyroid Hormone Disruption: Optimization and Standardization of an Assay to Assess the Inhibition of DIO1 Enzyme in Human Liver Microsomes

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Abstract

Introduction: Identifying substances which can disturb thyroid hormone (TH) signaling pathways is gaining importance and is soon to be a regulatory requirement in the EU. The European Commission Joint Research Centre's (EC JRC) European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) is coordinating the validation of multiple *in vitro* methods focusing on different thyroid modes of action (MoA) resulting in an *in vitro* test battery. Deiodinases (DIO) are regulators of TH signaling by activating or inactivating TH via deiodination. Inhibiting DIO activity is one potential MoA of substance-induced TH signaling disruption.
Objectives and Results: Here, the DIO1-SK assay using human liver microsomes as a DIO1 enzyme source and the Sandell-Kolthoff (SK) reaction as a colorimetric readout to quantify released iodide was standardized and optimized according to Good *In Vitro* Method Practices (GIVIMP). Potential pitfalls are described, and possible solutions are provided. The reproducibility of the method was demonstrated by testing a set of six described DIO1 inhibitors in five independent assay runs. The results of this study were used to amend the testing protocol: a set of acceptance criteria was defined to assess the validity of assay runs and strategies to verify the specificity of observed DIO1 interaction were implemented.
Conclusion: This work completes the first part of the DIO1-SK assay validation and defines a robust assay which provides results that can be used with confidence.

Keywords: deiodinase, liver microsomes, reproducibility, Sandell-Kolthoff, standardization

Introduction

The impairment of thyroid hormone (TH) homeostasis has been associated with several adverse outcomes: that is, hypothyreosis of mothers can impair the neuronal development of their children.[1–3] Xenobiotic substances can disturb the different functions of the hypothalamic–pituitary–thyroid (HPT) axis. Such disturbances are key events (KE) that can be linked to adverse outcomes in adverse outcome pathways (AOPs).[4,5] Several thyroid-related AOP networks have been published during the last years.[6–8]

Inhibition of the deiodinases (DIOs) is one KE (Event: 1002—AOP Wiki[5]) linked to several thyroid-related AOPs, i.e., Adverse Neurodevelopmental Outcomes in Mammals (AOP8). The DIOs, a family of selenocysteine-containing enzymes, consist of three isoforms that regulate TH signaling

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through the deiodination of iodothyronines, resulting in the formation of metabolites with differing activity. DIO1 contributes to the triiodothyronine (T3) production in the thyroid and facilitates the recycling of iodide from TH metabolites in excreting organs such as the liver and the kidney. DIO2 and DIO3 regulate local TH signaling in peripheral tissue by activation of thyroxine (T4) to T3 (DIO2) or inactivation (DIO3) of THs. The expression of DIO enzymes varies in different tissues and in different stages of fetal development.[9]

The DIO activity can be measured by quantifying TH and its metabolites in DIO-containing incubations using liquid chromatography–tandem mass spectrometry.[10,11] This is, however, a demanding procedure that often provides more information than needed. Classically, DIO activity was investigated by using a radioactively labeled tracer substrate and quantifying the released radioactive iodide.[12–15] Instead of using a radiolabeled substrate, the released iodide can be quantified by the Sandell-Kolthoff (SK) reaction. The reduction of yellow-colored cerium(IV) to colorless cerium(III) by arsenite is dependent on the concentration of iodide that is released from the substrate. The color change is quantified by light absorption at 415 nm.[16]

The SK reaction has been used to quantify iodide for various investigations, such as the determination of urinary iodide concentration,[17] the uptake of iodide through the sodium–iodide symporter,[18,19] cellular TH uptake by the TH transporter,[20] and released iodide from iodotyrosine DIOs[21,22] as well as DIOs.[23–25] The SK method is universally accessible and rather simple to perform, but is susceptible to interferences. Several ions and molecules are known to directly affect the reaction including iodide containing compounds,[26] nitrite and ferrous ions,[16] copper, chromium, Ni^{2+}, Hg^{2+}, Al^{2+}, or thiocyanate.[27]

The method to measure the effect of a test substance on DIO1 activity in murine liver microsomes was originally published by Renko et al.[23] This method was listed by the OECD scoping document that summarized available *in vitro* and *ex vivo* thyroid assays.[28] The method was further developed and evaluated as part of a validation study for 18 *in vitro* methods covering different AOPs of the HPT axis by the European Union Network of Laboratories for the Validation of Alternative methods (EU-NETVAL) coordinated by the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM). The method validation is conducted in two parts: assessing the method's reproducibility in part 1 and reliability and relevance in part 2. High standards regarding reproducibility, standardization, and documentation are essential to use methods with confidence—especially those being proposed for regulatory application.[29]

The development and validation of this method adhered to the "Guidance Document on Good *In Vitro* Method Practices (GIVIMP)",[29,30] to define a standardized *in vitro* method that can reproducibly show DIO1 inhibition in human liver microsomes. In this study, we report the results from part 1: further development and assessment of transferability and reproducibility ("Reproducibility Assessment Study"). The method of Renko et al was adapted to human liver microsomes, standardized, and optimized for robust and reproducible performance (Fig. 1). The method was tested regarding its reproducibility and performance (Reproducibility Assessment) by testing six described DIO1 inhibitors in five valid assay runs.

Testing strategies were introduced to exclude nonspecific effects of the test substance: test items were tested without microsomes to reveal potential interference in the SK reaction, and inhibition of a secondary microsomal protein, the

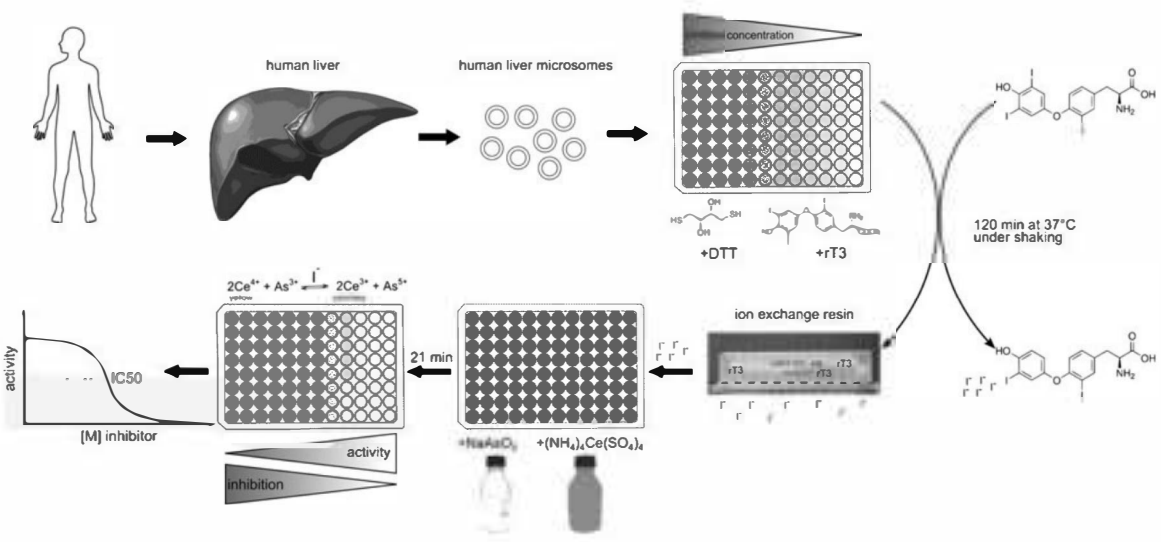

FIG. 1. Schematic procedure of the DIO1-SK assay. Human liver microsomes are incubated with the substrate rT3, an artificial enzyme-regenerating cofactor DTT, and the test items under pH-buffered conditions. The released iodide is separated from the remaining assay constituents including substrate by an ion exchange resin. The iodide concentration of the eluted sample is then quantified by the SK reaction using the formation of colorless Ce^{3+} from yellow Ce^{4+} by arsenite that is catalyzed by the available iodide concentration. Curve fitting is used to derive inhibition values for tested items. DIO1, deiodinase I; DTT, dithiothreitol; rT3, reverse triiodothyronine; SK, Sandell-Kolthoff.

alkaline phosphatase (ALP), was addressed. This strategy follows the concept of cell viability readouts as an indication of nonspecific effects in cell-based *in vitro* methods. A detailed description of the optimized and standardized method including its challenges is provided and will enable other laboratories to obtain reliable data on DIO1 inhibition using human microsomal preparations.

Materials and Methods

Standard operation procedures

The method standardization and optimization led to two standard operation procedures (SOPs) providing information on the test system, material, method, and data evaluation. The first SOP "SOP: DIO1-SK assay" addresses the DIO1-SK assay and was approved by the EURL ECVAM (Ispra, Italy) as well as the original method developer, Renko, on September 29, 2020 (Supplementary Pdf S1). This SOP was used during this thyroid validation study. The second SOP "SOP: ALP testing" provides a method to assess another enzyme activity in microsomes, which is independent of DIO1 activity and can hence be used to identify nonspecific DIO1 inhibition relying on general denaturation or protein binding (Supplementary Pdf S2). This SOP was developed in parallel to the thyroid validation study.

Material

The chemicals and materials used in the DIO1-SK assay are listed in the "SOP: DIO1-SK assay" (Supplementary Pdf S1). Chemicals and material that were obtained from other sources or used in addition to those listed in the SOP are explicitly stated in this article. Reverse T3 (rT3) was ordered from Cayman (Ann Arbor), Santa Cruz Biotechnology (Dallas) and Sigma-Aldrich. Ninety-six-well filter plates were purchased from VWR (microplates, 96-well, clear polystyrene, 800 μL, DNA Binding; Whatman) or Sigma-Aldrich (800 μL, clear polystyrene, filter bottom with long drip; Whatman). DOWEX ion exchange resin was purchased from Thermo-Fisher (50WX2-200; ACROS Organics) or Merck (AmberChrom® 50WX2 hydrogen form, 100–200 mesh).

Human liver microsomes were purchased from Sigma-Aldrich (pooled), Thermo-Fisher (pooled, 50 donors) or BioIVT (Westbury, NY). The used batch #QQY of human liver microsomes for the Reproducibility Assessment was distributed by the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) and originally ordered from BioIVT; the batch was derived from 150 donors of mixed gender. The other used microsome batches met the minimum requirements that were set in the SOP (Supplementary Pdf S1).

Control and test item preparation

The control and test item preparation is described in the SOP (Supplementary Pdf S1, SOP: DIO1-SK assay). In accordance with GIVIMP, positive, negative, and solvent controls as well as a reference item were used (Table 1). 6-Propyl-2-thiouracil (6PTU), a specific DIO1 inhibitor, was used as a reference item; aurothioglucose (ATG), an inhibitor of all DIO isoforms, was used as a positive control; a structural analog of ATG lacking the gold moiety, TGSS (1-thio-β-D-glucose sodium salt), does not inhibit DIO1 activity[31,32] and was used as a negative control. The solubility of used test and control items was verified according to SOP: DIO1-SK assay.

Control item and test item stock solutions as well as their respective predilutions were prepared according to SOP: DIO1-SK assay.

DIO1-SK assay

The incubation of human microsomes with the test and reference items and the positive, negative, and solvent control, as well as the separation by ion exchange and the SK reaction was performed according to SOP: DIO1-SK assay (Supplementary Pdf S1); plate layouts are also presented in this SOP. In short, human liver microsomes were incubated with the test item, the substrate rT3, the cofactor dithiothreitol (DTT), and a phosphate/diethylene diamine tetraacetic acid (EDTA) buffer. The final concentrations were as follows: 0.1 M H_2KPO_4/HK_2PO_4, 40 mM DTT, 1 mM EDTA,

TABLE 1. USED CONTROL SETUP AND CONTROL ITEMS IN THE DEIODINASE I-SANDELL-KOLTHOFF ASSAY IN ACCORDANCE WITH GOOD *IN VITRO* METHOD PRACTICES

Control item	*Function*
Reference item	Quantitative control. The inhibition observed with the test item is normalized to maximum inhibition obtained with the highest concentration of the reference item. The highest concentration of the reference item is tested on each assay plate and a concentration–response curve of the reference item is performed on each assay day. Used in this method: 6PTU at a maximum assay concentration of 10^{-3} M. Concentration–response curves of 6PTU ranged from 10^{-3} to 10^{-8} M.
Positive control	Qualitative control. The highest concentration of the positive control is tested on each assay plate. Used in this method: ATG at a maximum assay concentration of 10^{-4} M.
Negative control	Control not inhibiting DIO1 activity. The highest concentration of the negative control is tested on each assay plate. Used in this method: TGSS at a maximum assay concentration of 10^{-4} M.
Solvent control	Blank control. The solvent control ensures that the response does not originate from the applied solvent and shows no DIO1 inhibition. Solvent controls are used to normalize the inhibition observed with the test items to the maximum possible DIO1 activity. Used in this method: DMSO at a concentration of 1%.

6PTU, 6-propyl-2-thiouracil; ATG, aurothioglucose; DIO1, deiodinase I; DMSO, dimethyl sulfoxide; TGSS, 1-thio-β-D-glucose sodium salt.

5 μM rT3, and 1% dimethyl sulfoxide (DMSO). The reactions were constantly shaken for 2 hours at 37°C. Released iodide was separated using ion exchange resin-filtered plates and subsequently quantified using the SK reaction.

Data processing

Data were processed according to "SOP: DIO1-SK assay" (Supplementary Pdf S1) deriving multiple values describing the result of the assay (Table 2). The sigmoidal dose–response model "log(Inhibitor) versus response – Variable slope (four parameters)" (GraphPad Prism version 9.3.1 for Windows; GraphPad Software, San Diego, CA), was used to visualize a concentration–response relationship of calculated iodide release activities (IRAs) and used test item concentrations [Eq. (1)]. This was used to estimate the concentration causing 50% inhibition (IC_{50}) of the test item.

Equation 1: The sigmoidal dose–response model "log(Inhibitor) versus response – Variable slope (four parameters)" was used to visualize concentration–response relationships of test items. "Top" represents the maximal response, "Bottom" represents the lowest response, and "HillSlope" describes the steepness of the curve.

$$Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogIC}_{50} - x) * \text{HillSlope}}} \quad (1)$$

Assessing assay performance

Various control parameters were used to check the proper function of the assay: the IRA of the positive and negative control, the coefficient of variation (CV) of the log IC_{50} estimate for the reference item, and the z'-factor that is widely used to assess quality of assays.[33] A z'-factor above 0.5 is considered sufficient to demonstrate the significance of an assay.[34] The z'-factor was calculated using the standard deviation and mean of $\Delta OD_{21\ minutes}$ of the nine replicates of the reference item and the solvent control on each assay plate [Eq. (2)].

Equation 2: calculation of the z'-factor. "RI" represents the reference item 6PTU, "SC" represents the solvent control, "σ" represents the standard deviation, and "μ" represents the mean.

$$z' - \text{factor} = 1 - \frac{3(\sigma_{RI} + \sigma_{SC})}{|\mu_{RI} - \mu_{SC}|} \quad (2)$$

TABLE 2. CALCULATED VALUES IN THE DATA PROCESSING OF THE DEIODINASE I-SANDELL-KOLTHOFF ASSAY

Measured and calculated numbers	*Description*
OD_{415nm}	Optical density at a wavelength of 415 nm
ΔOD_{21min}	Delta of measured optical density at a wavelength of 415 nm after initial measurement (0 minutes) and after 21 minutes; typically, the measured reaction progressed linearly during this interval
ΔOD-BG	The BG (ΔOD_{21min}) obtained with the highest concentration of the reference item (6PTU) causing full inhibition is subtracted from the ΔOD_{21min}
IRA	ΔOD-BG, normalized to the ΔOD-BG of the solvent control (≙ maximum IRA activity); IRA is presented in %

BG, background; IRA, iodide release activity.

Microsome batch-specific IRA

For each microsome batch, a microsome batch-specific IRA of the solvent controls was determined according to "SOP: DIO1-SK assay" (Supplementary Pdf S1) and the defined microsome concentration, as well as the defined dilution factor of samples in the SK reaction, was used for testing of this microsome batch in the DIO1-SK assay.

Reproducibility assessment

The literature was screened for potential DIO1 inhibitors as potential test items. Six test items were chosen based on their inhibitory activity and tested in the DIO1-SK assay (Table 3).

6PTU and ATG were used as a reference item and as a positive control, respectively. Both substances were also used as test items. On each plate, the reference item and the solvent controls were tested in nine replicates, and the positive and negative controls in three replicates. Each test item was tested in eight concentrations in triplicates. For each test item, five valid assay runs were performed. The assay runs were performed by three different laboratory staff members to check the intralaboratory reproducibility.

ALP activity testing

The ALP is located in human microsomes, and its activity is independent of DIO1 activity. In this study, ALP activity is used to identify nonspecific inhibition of DIO1. Testing for ALP activity was performed according to "SOP: ALP activity testing" (Supplementary Pdf S2) with one exception: only the highest concentration of each test item was used for ALP activity testing.

ALP activity testing is based on the formation of yellow (405 nm) *para*-nitrophenol from *para*-nitrophenyl phosphate. The tissue-nonspecific alkaline phosphatase (TNAP) inhibitor [2,5-dimethoxy-*N*-(quinolin-3-yl) benzene sulfonamide, CAS No. 496014-13-2] is described as a specific TNAP inhibitor[35] and was used as the reference item for ALP activity testing.

Number of repetitions

All experiments were carried out in at least three technical replicates; if more replicates were conducted, the number of technical replicates was specified for this experiment.

Experiments that were carried out for the standardization and characterization of the method were usually performed as one biological replicate. While independent biological replication is preferred, assay optimization decision points can be derived from a single experiment.

TABLE 3. SUMMARIZED INFORMATION ABOUT THE USED BATCHES OF THE TEST ITEMS 6-PROPYL-2-THIORURACIL, 5-PROPYL-2-THIOURACIL, AUROTHIOGLUCOSE, GENISTEIN, 3,3′,5,5′-TETRABROMOBISPHENOL A, AND 2-CHLORO-*N*-PHENYLACETAMIDE

Name	*6PTU*	*5PTU*	*ATG hydrate*	*GEN*	*3,3′,5,5′-TBBPA*	*2CPA*
Acronym	6PTU	5PTU	ATG	GEN	TBBPA	2CPA
CAS No.	51-52-5	2954-52-1	12192-57-3	446-72-0	79-94-7	587-65-5
Supplier	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich
Batch No.	BCBX0879	SLBL3627V	0000054738	SLBW0900	MKCB9769	MKCC3899
Purity, %	99.6	99	96.1	99	99.3	99.9
Molecular weight, g/mol	170.23	170.23	218.20	270.24	543.87	169.61
DIO1 inhibition described in literature	[14,23,25,32,38]	[39]	[15,32,38]	[25,38]	[11,25]	[25]
Stock solution concentration, M, and solvent	10^{-1} in DMSO	10^{-1} in DMSO	10^{-2} in DMSO	3.16×10^{-4} in DMSO	10^{-3} in DMSO	10^{-2} in DMSO
Highest concentration tested, M	10^{-3}	10^{-3}	10^{-4}	3.16×10^{-6}	10^{-5}	10^{-4}

2CPA, 2-chloro-N-phenylacetamide; 5PTU, 5-propyl-2-thiouracil; 6PTU, 6-propyl-2-thioruracil; ATG, aurothioglucose; GEN, genistein; TBBPA, 3,3′,5,5′-tetrabromobisphenol A.

Concentration–response testing and specificity testing of test items were performed in at least three biological replicates.

Results

This section describes the results of the method development, modifications, and improvements, the results of the Reproducibility Assessment Study concerning performance and reproducibility, as well as the test results obtained with the six test items.

Initial method development

To confidently identify DIO1 inhibitors among the test items, a high resolution of the response, characterized by the calculated ΔOD-BG (BG: background), is essential. The response depends particularly on (i) the maximum SK-reaction obtained with the solvent controls and (ii) the minimum background SK-reaction obtained with the maximum concentration of the reference item.

The maximum SK-reaction (i) may be influenced by a variety of factors, mainly the IRA of the used microsome batch and the microsome concentration, incubation time, and used concentrations of reagents. The background reaction (ii) should be kept at a minimum. Increases in background could either result from assay constituents or impurities that are active in the SK reaction itself; this can be outlined by the $\Delta OD_{21\ minutes}$ values of the reference item. Precipitations can be formed from the test item or reactions of the test item with assay constituents; these precipitations can influence the initial measured OD_{415nm}.

Role of assay constituents in the method. The DIO1 activity of murine liver microsomes is known to be inhibited by the reference item of the method, 6PTU, as well as heat inactivation.[23] In the present study, the DIO1-SK assay was performed with untreated, heat-inactivated, 6PTU-inhibited, or heat-inactivated as well as 6PTU-inhibited human liver microsomes. 6PTU only or in combination with heat-inactivated microsomes led to $\Delta OD_{21\ minutes}$ comparable with those of heat-inactivated microsomes. This is indicating full inhibition of DIO1 activity by the reference item 6PTU in human liver microsomes (Fig. 2A).

Multiple batches of different suppliers of the substrate, rT3, were compared regarding their activity. Concentration–response curves with the reference item 6PTU were derived for each substrate batch to exclude potential impact of the substrate, for example, through impurities. All rT3 batches produced curves that produced comparable 6PTU concentration–response curves (Fig. 2B).

This method uses ion exchange resin to separate organic-bound iodide molecules from the released iodide products. The ion exchange resin needs to be prepared as an aqueous suspension where it forms a reddish, cloudy, and viscous suspension. Before filling into filter plates, the resin suspension is washed several times with 10% acetic acid for 10 minutes to remove present dyes and exclude interference in the SK reaction. Samples of the supernatant of each washing step were tested in the SK reaction. The supernatants of the first washing steps led to a decrease of the OD_{415nm} after an initial measurement (data not shown) and an increase of the background reaction. After five washing steps, these effects reached a plateau; additional washing steps did not further reduce the background reaction (Fig. 2C).

Due to the physical properties of the resin, some separation of the aqueous part from the resin cannot be fully avoided, even under constant stirring. Resin suspensions with different viscosities were prepared and casted into a filter plate. The wells that were filled with viscous resin suspension resulted in overall higher reaction values compared with the samples that were prepared with more liquid resin suspension (Supplementary Fig. S1), further showing the importance of uniform resin distribution over the plates.

In addition to separating substrate and free iodide, the resin filtration step may also retain other assay constituents that would drive the SK reaction. Microsome incubations with solvent control and reference item were filled into a filter plate that was prepared with different volumes of resin suspension. After the ion exchange, optical densities (ODs) were measured in the SK reaction. ODs of the solvent control

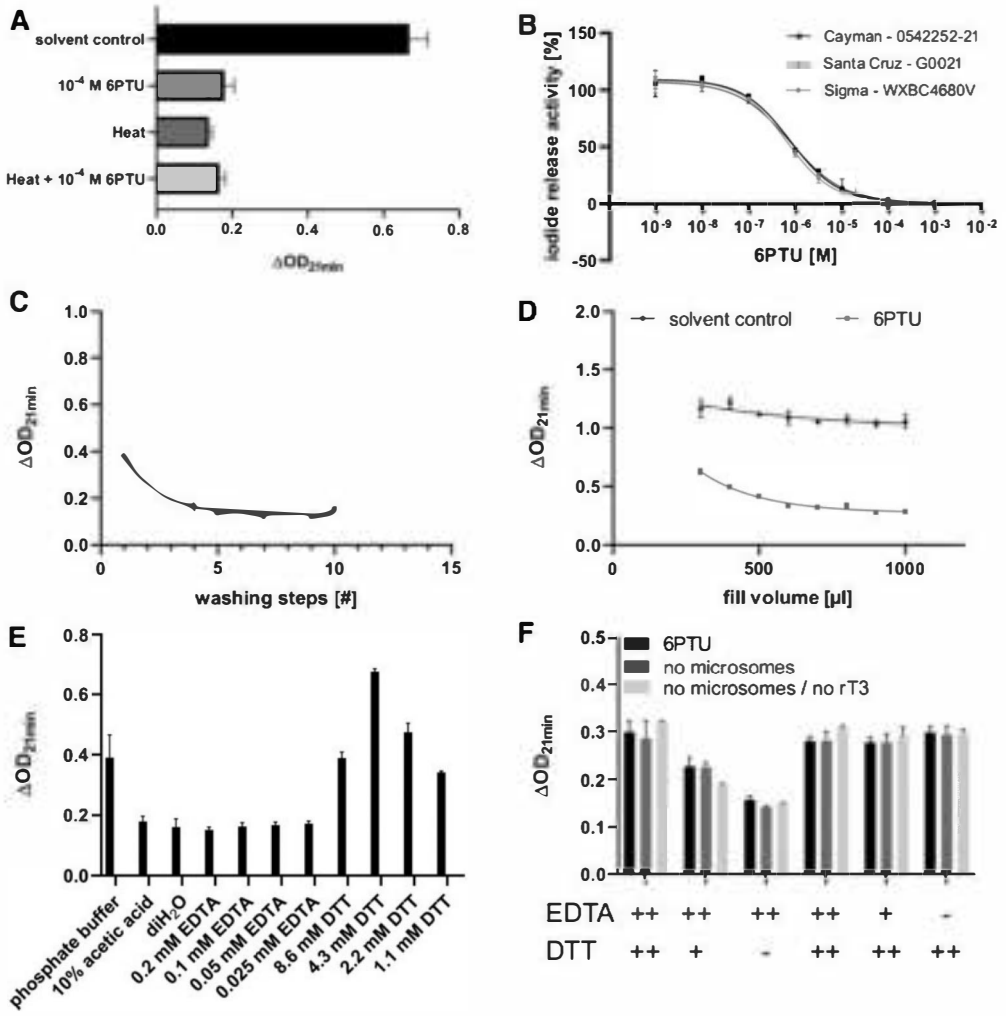

FIG. 2. Characterization of assay constituents and ion exchange separation. **(A)** Microsomal incubations with solvent control, reference item (6PTU), heat inactivated (95°C for 30 minutes), and reference item treated, as well as heat-inactivated microsomal incubations, were compared regarding their iodide release. Results are shown as mean of $\Delta OD_{21\ minutes}$ value ± SD. **(B)** rT3 batches (presented as "supplier—batch number") of three different suppliers were compared regarding their concentration–response curves with the reference item 6PTU. Results are shown as mean iodide release activity value ± SD and a sigmoidal concentration–response curve was derived. **(C)** Ion exchange resin was washed with 10% acetic acid, incubated until the resin settled, and the resulting supernatant was tested in the SK reaction. Results are shown as mean of $\Delta OD_{21\ minutes}$ value ± SD. **(D)** A 96-deepwell plate with different filling volumes of ion exchange resin was prepared and tested using solvent control and reference item-treated samples. Results are shown as mean of $\Delta OD_{21\ minutes}$ value ± SD. **(E)** Different assay constituents were tested directly in the SK reaction without prior microsome incubation and ion exchange separation (three to six technical replicates per condition). Results are shown as mean of $\Delta OD_{21\ minutes}$ value ± SD. **(F)** different EDTA and DTT concentrations were tested in microsomal preparations (assay concentration EDTA: ++, 2 mM; +, 1 mM; −, no EDTA; assay concentration DTT: ++, 80 mM; +. 40 mM, −, no DTT) using controls that prevent iodide release activity and were tested in the SK reaction. Results are shown as mean of $\Delta OD_{21\ minutes}$ value ± SD. 6PTU, 6-propyl-2-thiouracil; DTT, dithiothreitol; EDTA, diethylene diamine tetraacetic acid; OD, optical density; rT3, reverse T3; SD, standard deviation.

as well as reference item were dependent on the resin volume. A minimum volume of 600 μL of resin suspension kept the influence of assay components in the assay to a minimum (Fig. 2D). Since the resin itself had no effect on the SK reaction, an increased leakage of assay constituents from the incubation step through the ion exchange separation into the SK reaction was assumed as the cause for background increases. The individual constituents of the assay were tested in the SK reaction without prior ion exchange.

The cofactor DTT increased the background reaction as well as the initial measured OD. Increasing concentrations of EDTA led to a decrease of initial measured OD while

having no effect on the background reaction. Interestingly, phosphate buffer at the used concentration also influenced both the initial measured OD and the overall reaction (Fig. 2E and Supplementary Fig. S2A). The ion exchange separation step likely acts as a barrier preventing leakage of assay constituents into the SK reaction. Different concentrations of EDTA and DTT were tested in the DIO1-SK setup, including microsome incubation and ion exchange separation, with additional controls lacking rT3 or microsomes or both. Still, DTT was able to drive the SK reaction in a concentration-dependent manner in all tested controls, while EDTA had no effect on the initial measured OD anymore (Fig. 2F); the ion exchange separation step likely prevented EDTA from leaking into the eluted sample.

Different concentrations of phosphate buffer and the HEPES buffer as a replacement for the phosphate buffer[24] were tested regarding potential background increases. Phosphate buffer showed little reduction of initial measured OD at used assay concentrations (0.1 M) in both solvent controls and reference item samples. A reduced concentration of the phosphate buffer (0.025 M) or replacement of the phosphate buffer by the HEPES buffer negated the observed effect on an initial OD parameter (Supplementary Fig. S2C). To ensure that the reduced phosphate buffer concentration is not affecting substance-induced DIO1 inhibition, concentration–response curves with the reference item, 6PTU, were performed using 0.1, 0.05, 0.025, and 0.01 M phosphate buffer concentrations. 6PTU response curves were comparable with different phosphate buffer concentrations (Supplementary Fig. S2D).

In summary, DTT itself is active in the SK reaction, it is not retained in the ion exchange separation, and is the main driver of the observed background in the SK reaction.

Microsome activity. Human liver microsome batches show differences in their deiodination activity of rT3. This is resulting in different maximal ΔOD-BG values (as a measure of enzyme activity). A microsome–response curve with the batch QQY from BioIVT was performed in the DIO1-SK assay, and different dilutions of eluted samples after the ion exchange separation step were tested in the SK reaction (Fig. 3). Increasing microsome concentrations led to a higher overall reaction in the solvent controls up to 10 μg of protein per reaction volume (Fig. 3A1). Concentrations higher than 10 μg led to increased background reaction as observed in the reference item-treated samples (Fig. 3A2). Higher dilution of samples in the SK reaction overall led to a decrease of both solvent controls and reference item-treated samples and prevented higher achievable ΔOD-BG.

Based on background increases at high microsome concentrations observed in several microsome batches, 20 μg was defined as the highest microsome concentration for future testing of individual microsome batches.

The generation of a microsome batch-specific enzyme activity curve is used in this method to assess the IRA and to determine a microsome batch-specific protein concentration to be used for assay runs. The aim is to determine the microsome concentration per reaction volume and the 10% acetic acid dilution in the SK reaction that offers the highest OD

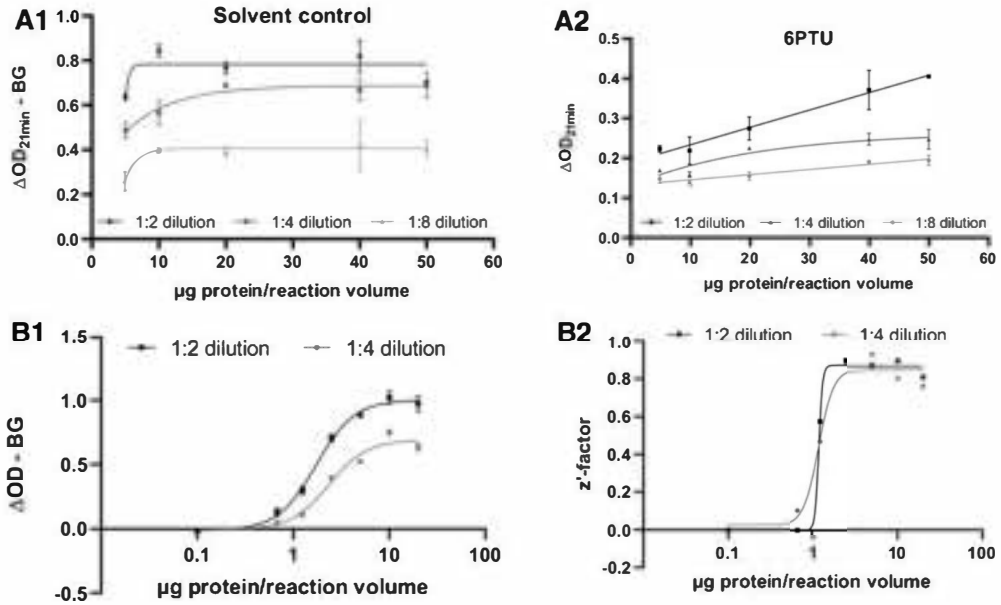

FIG. 3. characterization of microsome activity and sample dilution in the SK reaction. **(A)** Microsomal preparations with different microsome concentrations were prepared and tested in the SK reaction using different dilutions in 10% acetic acid (microsome batch #QQY from BioIVT (Westbury, NY); two technical replicates per microsome concentration). Shown are the mean ΔOD-BG value ± SD for the solvent control **(A1)** and the mean $\Delta OD_{21\ minutes}$ value ± SD for reference item-treated (6PTU) samples **(A2)**. **(B)** A microsome–response curve using different microsome concentrations for the human liver microsome batch #QQY from BioIVT was performed and measured in 1:2 and 1:4 10% acetic acid dilution in the SK reaction. Results are shown as ΔOD-BG value ± SD **(B1)**. In addition, the z'-factor for each microsome concentration and 10% acetic acid dilution was derived **(B2)**. 6PTU, 6-propyl-2-thiouracil; BG, background; OD, optical density.

resolution between solvent controls and the reference item. As an example, a concentration–response curve was derived using different concentrations of the microsome batch QQY with solvent controls and reference item samples for each microsome concentration. The respective z'-factors were derived (Fig. 3B). The ΔOD-BG obtained in the SK reaction after incubation with different microsome concentrations reached a plateau at a protein concentration of 10 μg of protein per reaction volume (Fig. 3B1). The calculated z'-factors plateaued at 2.5 μg of protein (Fig. 3B2).

For this microsome batch, 5 μg of microsome per incubation volume and a subsequent 1:2 dilution offered a high OD difference (represented by a high z'-factor).

Iodide quantification using the SK reaction. Increasing the cerium(IV) ion concentration might be one tool to compensate for increased background activity, provided the photometer used is still able to linearly quantify the resulting OD. In addition, increasing the incubation time might facilitate the maximum difference between solvent control and reference item. To check this, solvent control and reference item-treated samples were incubated from 1 to 4 hours and the released iodide content was measured in the SK reaction using solutions with increasing cerium concentrations. Both the increase of cerium concentration and the increased incubation times led to higher ΔOD-BG values in the solvent controls (Fig. 4A). Reference item samples only increased with higher cerium concentrations and were not affected by longer incubation times.

A concentration–response curve of the reference item was derived using 2 hours of incubation time, and different cerium concentrations were used in the SK reaction (Fig. 4B). The increase of cerium in the SK reaction had no effect on the reference item concentration–response curve, but increased the z'-factor.

Additional assay procedures were addressed to further optimize the method. Initially, released-iodide containing samples were eluted in the ion exchange separation step by the application of vacuum to ion exchange resin-filled filter plates. This elution process led to differences in the signal of wells as a function of their distance to the vacuum source, and sometimes failed to fully elute samples that were far away from the vacuum source. As an adaption, plates were eluted using centrifugation with free swinging buckets, which resulted in a more uniform elution. The centrifugation speed that was needed to fully elute the entire volume of samples varies between different filter plates; 150 g was generally sufficient to elute all resin-filled filter plates.

Based on the characterization and standardization efforts, the following setup was defined for the Reproducibility Assessment: The highest possible filling volume of 600 μL of ion exchange resin was used for the preparation of filter plates; the incubation was prepared with the defined microsome-batch-specific concentration using a 0.1 M phosphate buffer and was incubated for 2 hours; the SK reaction was performed with the defined microsome-batch-specific dilution factor in 10% acetic acid and 25 mM cerium(IV) ion solution.

Reproducibility assessment

The six test items in this Reproducibility Assessment were tested regarding their solubility in the stock solution in DMSO, the predilution in deionized water, and their final concentration in the incubation. The highest soluble concentrations in the stock solution and the final concentrations are shown in Table 3; detailed information about the solubility of each test item is shown in Supplementary Figure S3.

Five assay runs were performed for each test item in eight concentrations in the DIO1-SK assay, using information of pretests for the determination of final concentrations. 6PTU, 5PTU, ATG, and 2-chloro-*N*-phenylacetamid (2CPA) fully inhibited DIO1 activity and showed sigmoidal concentration–response curves with IC_{50} values of 4.12, 1.52, 0.50, and 7.39 μM, respectively (Fig. 5A–C, E and Table 4). Tetrabromobisphenol A (TBBPA) and genistein (GEN) only showed inhibition up to about 50% at the highest tested concentration and did not produce full concentration–response curves (Fig. 5D, E). No discerning IC_{50} values could be derived.

Performance of the method. The performance of control items was monitored for each assay plate (Fig. 6). The IRAs

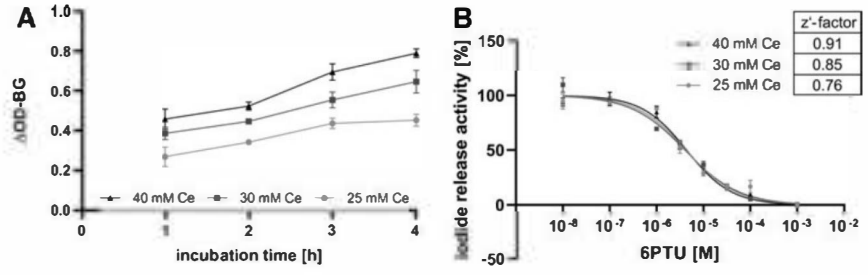

FIG. 4. Impact of microsome incubation time and cerium concentration in the SK reaction on the performance of the method. **(A)** Microsomal preparations with increasing incubation time were performed and tested with varying cerium concentrations in the SK reaction. Shown are mean ΔOD-BG values ± SD for the solvent control. **(B)** A concentration–response curve of the reference item 6PTU was performed, tested in the SK reaction using different cerium concentrations and the z'-factors were calculated (2 hours of microsome incubation). Results are shown as mean iodide release activity value ± SD, and a sigmoidal concentration–response curve was derived using the function "Inhibitor versus response – Variable slope (four parameters)."

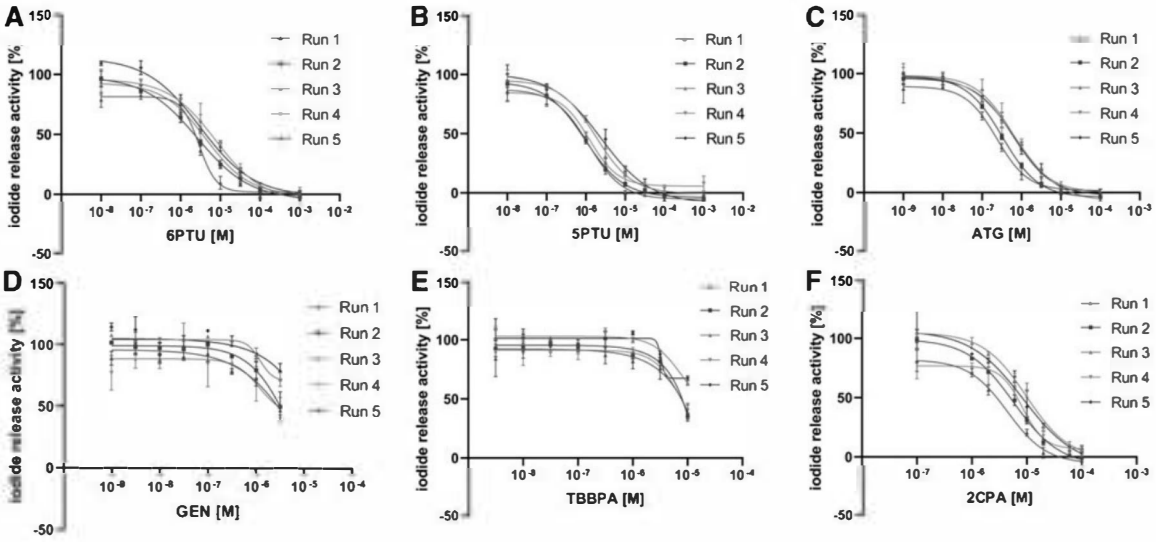

FIG. 5. Reproducibility Assessment of the DIO1-SK assay. Iodide release activity in human liver microsomes after incubation with six known DIO1 inhibitors was determined (microsome batch #QQY, 5 μg protein/reaction volume, incubated for 2 hours at 37°C). Five independent assay runs with three technical replicates per concentration and test item were performed by three different laboratory staff members (data shown as mean iodide release activity ± SD of three technical replicates per concentration). A sigmoidal concentration–response curve was derived using the function "log(Inhibitor) versus response – Variable slope (four parameters)." 6PTU **(A)**, 5PTU **(B)**, ATG **(C)**, GEN **(D)**, TBBPA **(E)**, 2CPA **(F)**. 2CPA, 2-chloro-*N*-phenylacetamide; 5PTU, 5-propyl-2-thiouracil; 6PTU, 6-propyl-2-thiouracil; ATG, aurothioglucose; GEN, genistein; TBBPA, tetrabromobisphenol A.

TABLE 4. SUMMARY OF THE CONCENTRATION–RESPONSE SCREENINGS OF SIX TEST ITEMS IN THE REPRODUCIBILITY ASSESSMENT AND AVAILABLE LITERATURE DATA ON INHIBITION VALUES OF EACH TEST ITEM

			Max. inhibition		IC_{50}		
Chemical	*CAS*	*Max. tested concentration, µM*	*Mean, %*	*SD, %*	*Mean, µM*	*SD, µM*	*Literature*
6PTU	51-52-5	1000	100.6	2.1	4.1	2.1	5.4 μM[25] *Recombinant human DIO1 enzyme, SK reaction* 1.7 μM[45] *Murine liver homogenate, radioactive release assay* 1.3 μM[23] *Murine liver homogenate, SK reaction*
5PTU	2954-52-1	1000	102.1	5.7	1.5	0.6	Twofold higher relative inhibitory activity than 6PTU[39] *Rat liver microsomes, radioactive immunoassay*
ATG	12192-57-3	100	101.5	2.1	0.5	0.2	0.02 μM[46] *Pig liver microsomes, radioactive release assay*
GEN	446-72-0	3.16	40.9	14.6	—	—	3 μM[38] *Recombinant human DIO1 enzyme, SK reaction* 2.6 μM[25] *Recombinant human DIO1 enzyme, SK reaction*
TBBPA	79-94-7	10	51.1	15.2	—	—	37.4 μM[25] *Recombinant human DIO1 enzyme, SK reaction*
2CPA	587-65-5	100	96.4	5	7.4	2.2	18.8 μM[25] *Recombinant human DIO1 enzyme, SK reaction*

The model "log(Inhibitor) versus response – Variable slope (four parameters)" was used to derive inhibition curves and concentrations causing 50% inhibition (IC_{50}) values for the six tested items.

2CPA, 2-chloro-N-phenylacetamide; 5PTU, 5-propyl-2-thiouracil; 6PTU, 6-propyl-2-thiouracil; ATG, aurothioglucose; GEN, genistein; SD, standard deviation; SK, Sandell-Kolthoff; TBBPA, tetrabromobisphenol A.

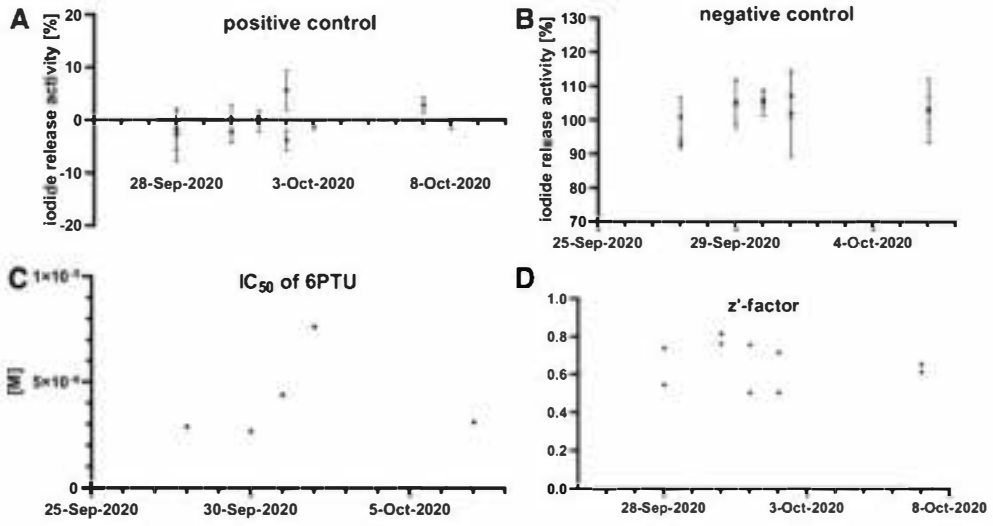

FIG. 6. Performance of control values in the Reproducibility Assessment. The iodide release activity values for the positive control, ATG (**A**), and the negative control, TGSS (**B**), were monitored for each assay plate. Data are shown as mean iodide release activity ± SD. The concentrations causing 50% inhibition (IC_{50}) of the reference item 6PTU were derived for each performed concentration–response curve using the function "Inhibitor versus response – Variable slope (four parameters)." (**C**) In addition, the z'-factor was derived for each assay run using the $\Delta OD_{21\ minutes}$ values of the nine replicates of the reference item, 6PTU, and the solvent control for each assay plate (**D**). ATG, aurothioglucose; TGSS, 1-thio-β-D-glucose sodium salt.

of the negative control and positive control were close to 100% and 0%, with low standard deviation (Fig. 6A, B). The IC_{50} values of the reference item, 6PTU, ranged from 2.65 to 7.60 μM (Fig. 6C) and the CV of the log IC_{50} estimate was always below 2%. z'-Factors were consistently over 0.5 (Fig. 6D); one run with a z'-factor below 0.5 had an apparent outlier in the reference item replicates, determined using boxplot outlier testing. After removal of this outlier, the run met the acceptance criterion.

To assess the variability between assay plates, the number of triplicates with a CV of 20% was determined. Among all triplicates of the 10 assay runs, only four triplicates showed a CV above 20%.

The results of the control items in the Reproducibility Assessment resulted in the selection of acceptance criteria as well as quantitative descriptors to assess the validity of assay runs (Table 5).

Assessing specificity of DIO1 inhibition

In vitro assays often lack controls to account for potential nonspecific interactions. Many ions or iodide-containing

TABLE 5. PROPOSED ACCEPTANCE CRITERIA FOR THE DEIODINASE I-SANDELL-KOLTHOFF ASSAY

Acceptance criteria	*Measured values*	*Suggested cutoff value*
Numeric	mean ± SD	
IC_{50} of reference item 6PTU, μM	4.12 ± 2.06	$1 < x < 10$
CV of log IC_{50} estimate of reference item, %	1.28 ± 0.47	$x < 3$
IRA of negative control, %	100.28 ± 2.84	$80 < x < 120$
IRA of positive control, %	−2.97 ± 4.04	$x < 20$
z-Factor	0.65 ± 0.14	$x > 0.5$
Binary	yes/no	
Shape of reference item (sigmoidal?)	Yes (In all valid assay plates)	x = yes
The final concentration–response curve of the reference item is composed of minimum six concentrations from three replicates	Yes (In all valid assay plates)	x = yes
The final concentration–response curve of the test item is composed of minimum six concentrations from three replicates	Yes (In all valid assay plates	x = yes

Shown are measured values of derived acceptance criteria in the Reproducibility Assessment and suggested cutoff values for future runs of the DIO1-SK assay. Mean and SD were calculated from the corresponding values of the 10 valid assay runs; criteria assessing reference item–response curve are only based on the 5 performed runs.

6PTU, 6-propyl-2-thiouracil; CV, coefficient of variation; IRA, iodide release activity.

substances are known to drive the SK reaction. To identify potential interference, the highest tested concentration of each test item and reference item, as well as positive and negative control of the reproducibility assessment, was incubated with and without a microsome. In the absence of microsomes, none of the tested items was active in the SK reaction (Fig. 7A).

The DIO1-SK assay is a cell-free assay using microsomes, and cell viability testing as an indication of nonspecific effects cannot be performed. Hence, the TNAP was used as an indicator of nonspecific inhibition, for example, due to nonspecific protein binding and modification, leading to structural denaturation. TNAP is one of the many isoenzymes of the ALP family. It has a main function in bone mineralization and is expressed in various tissues, including skeletal tissues, kidney, gut, and the liver.[36] A protein–response curve using human liver microsomes was linear up to 20 μg of protein per incubation volume (Supplementary Fig. S4A).

A specific TNAP inhibitor, 2,5-dimethoxy-*N*-(quinolin-3-yl) benzene sulfonamide (CAS No. 496014-13-2),[35] was used as a reference item to show ALP inhibition. TNAP inhibitor concentration–response curves were reproducible and produced comparable ALP inhibition curves in three independent runs with IC_{50} values of 0.19, 0.12, and 0.08 μM (Supplementary Fig. S4B). The six test items of the reproducibility assessment as well as the positive and negative control and the reference item of the DIO-SK assay did not inhibit ALP activity in human liver microsomes (Fig. 7B).

Discussion

Method development, optimization, and standardization

The influence of the assay conditions on the outcome of the assay was investigated and described in detail. The results were used to define the optimized assay conditions and to give advice to laboratories establishing the DIO1-SK assay. A process to establish the method is shown in Table 6.

The SK reaction has been used by different groups to investigate iodide release as a function of DIO1 activity.[23,24] Little information on the influence of the materials and assay conditions on the results has yet been investigated and published.

Human liver microsomes. Higher microsome concentrations up until 20 μg of protein per incubation volume led to a better resolution in the assay and increased the z'-factor; even higher microsome concentrations had no benefit. The microsome concentration that offers the highest OD difference between the reference item and solvent control that did not plateau in the microsome–response curve should be used.

Other materials used in the SK. It is highly recommended that batches of the substrate rT3 are tested once supplied to prove their performance and, upon availability of respective methods, identity. One out of the five batches used in this study did not show activity (data not shown).

The OD should linearly increase with the cerium(IV) ion concentration. Some cerium(IV) ion solution batches caused higher background in the SK reaction, likely due to iodide contaminations (Supplementary Fig. S5). These batches should be excluded.

The cofactor DTT can drive the SK reaction without iodide. DTT is used in the method to regenerate the DIO1 enzyme from its oxidized nonfunctional form.[37] The ion exchange resin can retain DTT and prevent it from leaking into the final SK reaction solution. A minimum fill volume of 600 μL of resin suspension per well is advised to significantly reduce the observed background reaction of DTT.

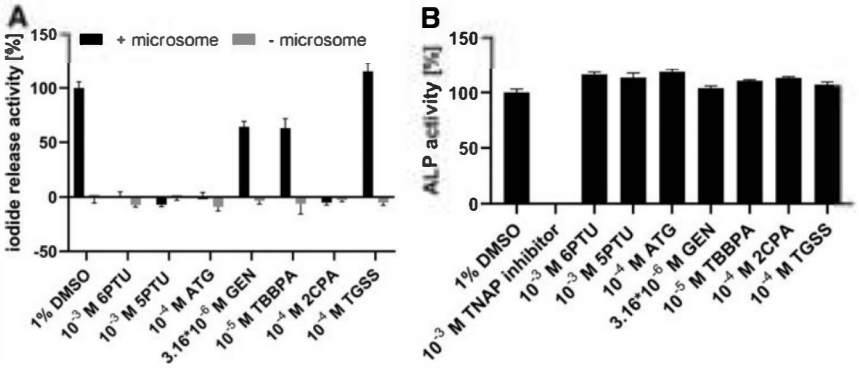

FIG. 7. Specificity testing of control and test items of part 1. **(A)** Test items from the reproducibility assessment, as well as the negative (TGSS) and solvent control (1% DMSO), were tested with microsome (batch #QQY, 5 μg/reaction volume) and without (addition of 40 μL diH_2O only) in the incubation phase of the DIO1-SK assay, ion exchange separation, and the SK reaction (1:2 dilution in 10% acetic acid, 25 mM cerium solution). **(B)** Control and test items of the reproducibility assessment, as well as the TNAP inhibitor, were tested for potential inhibition of ALP activity (batch #QQY, 5 μg of protein/reaction volume, incubated for 1 hour at 37°C). For both figures, data are shown as the mean ± SD of three biological replicates using three technical replicates for each tested substance. ALP, alkaline phosphatase; diH_2O, deionized water; DMSO, dimethyl sulfoxide; TNAP, tissue-nonspecific alkaline phosphatase.

Table 6. Proposed Actions for Establishing the Deiodinase I-Sandell-Kolthoff Assay to Another Laboratory

	What?	*When?*	*Why?*
Initial considerations using the SK reaction only			
Work safety	Due to the use of arsenite and acid, precaution should be taken to apply obligatory and facultative protective measures. Working under a dedicated fume hood is recommended. A proper waste management needs to be applied	Before establishing the assay	The frequent use of arsenite as a potent carcinogen in combination with sulfuric acid needs awareness to protect employees
Photometer	Testing of cerium concentrations (e.g., final cerium concentrations in the SK reaction of 20–1 mM Ce) directly in the SK reaction and analyze linearity of OD_{415nm} measurement	Before establishing the assay or switch to a new photometer	Measurements that are not in the linear range of OD_{415nm} quantification might influence testing of test items
Cerium and arsenic solution	Testing of prepared solutions directly in the SK reaction, comparing with diH_2O and 10% acetic acid controls	Before establishing the assay or ordering of a new cerium or arsenic batch	Potential background contamination, especially in cerium batches, might affect the SK reaction
Iodide–response curve in the SK reaction	Performance of an iodide–response curve in the SK reaction (e.g., final iodide concentration in the assay of 500–0.1 nM)	Before testing of the DIO1-SK assay run	Identification of background contaminants is essential to ensure maximum resolution in the method
Characterization of the DIO1-SK assay			
rT3	Testing in the DIO1-SK assay, ideally vs. rT3 batch with known activity	Before establishing assay or ordering of new batch	Some rT3 batches showed low or no activity in the assay
Liver microsomes	Microsome–response curve in the DIO1-SK assay, also measuring different 10% acetic acid dilutions in the SK reaction	Before establishing assay or when switching to a new microsome batch	Microsome batches show differing activity and microsome-batch-specific assay conditions need to be defined
Background reaction in DIO1-SK assay	Conducting a DIO1-SK assay with reference item and solvent controls in triplicates all over the plate; compare controls, especially background values, and derive the z'-factor. Also check for drifts of response over the whole plate	Before performance of first DIO1-SK assay run with test items	Assay material similar to the used ion exchange resin can have an influence on the background reaction during the SK reaction
Monitoring assay performance	Monitor OD values of all controls (solvent, positive and negative controls, as well as reference item) and the assessment criteria over time	After completion of assay plates	Assay performance might differ over time and monitoring will help to identify potential causes
Before testing of an unknown test item			
Solubility of the test item	Define the highest soluble concentration of the test item in pure DMSO, 10% test item/DMSO dilution in diH_2O, and under final assay conditions (50% potassium phosphate/EDTA buffer, 40% diH_2O, and 10% of the 10% test item/DMSO dilution)	Before testing of an unknown test item	The solubility of a test item defines the available concentration for DIO1 inhibition. Undissolved test item solutions might lead to inaccurate inhibition values or artifacts in the assay due to precipitations.

diH_2O, deionized water; DMSO, dimethyl sulfoxide; EDTA, diethylene diamine tetraacetic acid; OD, optical density; rT3, reverse triiodothyronine.

Phosphate buffer and EDTA decreased the measured OD parameters when added directly to the SK reaction solution. This is most likely caused by precipitations. Again, this is avoided by passing the solution through the ion exchange resin. Other groups have successfully replaced phosphate buffer by HEPES buffer.[24]

Once the ion exchange resin is in aqueous suspension, it forms a reddish cloudy suspension that affects the measured OD. In addition, water-soluble components of the resin were shown to be active in the SK reaction. These observations varied among resin batches. The standardized washing procedure is sufficient to avoid this (Fig. 2). When filling plates with resin, the viscosity of the resin suspension might influence the amount of resin per well. This will impact the assay results.

Reading the OD of the SK reaction solution is time critical. Hence, the order and speed of measurements might influence the results. A plate reader that can simultaneously measure all wells of the plate is preferred. Otherwise, the time lag between measurements of different wells of a plate must be accounted in the study protocol.

Method improvements. The SK reaction was generally performed using a 25 mM cerium(IV) ion solution. Increasing the cerium concentration improved the z'-factors by increasing the $\Delta OD_{21\ minutes}$ of all samples, while only marginally increasing standard deviation. The use of 40 mM cerium(IV) ion solutions in the SK reaction might be a valuable adaption of the method leading to more robust results, especially under conditions with increased background reaction.

Increasing the time of microsome incubation might increase the dynamic range of the method. Two hours of incubation was sufficient to release enough iodide.

During assay development, additional assay procedures were addressed to further improve reproducibility of the method. Centrifugation is preferred over suction to elute samples from the ion exchange resin. The rapid addition of arsenite solution is crucial for a simultaneous start of the SK reaction. Arsenite should be added to all wells of a plate within a maximum of 15 seconds. Using pipetting tools such as a liquidator pipette (Steinbrenner, Germany) or an automated multichannel pipette is advisable.

Reproducibility assessment

Six test items were investigated in 10 assay runs to estimate the reproducibility of their concentration–response curves. A standardized control setup according to GIVIMP, using reference and control items and a set of acceptance criteria, was used to monitor the performance of the DIO1-SK assay and confirm the validity of results. Inhibition values of the respective controls and reference item were reproducible. Derived z'-factors generally met the acceptance criterion of 0.5. Future data evaluation might include outlier testing or robust statistics such as mean absolute deviation or the robust z'-factor. The reproducibility assessment was carried out by three different laboratory coworkers and no variation by the performing personnel was observed. In addition to this intralaboratory reproducibility study, an interlaboratory ring-trial should be performed to assess the reproducibility across different laboratories.

The results of the Reproducibility Assessment led to the adjustment of the initial acceptance criteria. There may be a need for further adaptations after gaining experience with the routine use of the assay in different laboratories.

The reference item, 6PTU, has shown full inhibition of IRA, comparable with the heat-inactivated microsome protein. As 6PTU is a specific DIO1 inhibitor[38] and no activity was observed in 6PTU inhibited samples, DIO2 or DIO3 activity contributing to the observed IRA can be ruled out, with the used substrate rT3. Concentration–response curves were reproducible in multiple biologically independent runs and comparable with those previously published (Table 4). 6PTU is a suitable reference item for this method. Little is known about the inhibition of DIO1 by the structural analog of 6PTU, 5PTU. Visser et al investigated the inhibitory potency of thiouracil analogs and found 5PTU to be a twice as potent as 6PTU.[39] This is consistent with our result indicating a 2.7-fold higher potency of 5PTU compared with 6PTU.

The positive control ATG is known to fully inhibit DIO isoforms, including DIO1[25,38] based on the affinity of its gold ligand to the selenocysteine-containing catalytic center of DIO.[40] Inhibition curves for ATG were reproducible, and the IC_{50} value of 0.5 μM was similar to the previous results of 0.64 μM.[32]

The test item 2CPA was identified as an inhibitor of recombinant human DIO1 and showed only marginal DIO2 and 3 inhibition.[25] The DIO1 IC_{50} in this study, using human liver microsomes, was 7.4 μM. The IC_{50} determined in recombinant enzymes was 2.5-fold higher. Liver microsomes possess xenobiotic-metabolizing enzymes that may either inactivate or attenuate substrates of DIO1, or generate metabolites that are more or less active DIO1 inhibitors.[41] Many xenobiotic-metabolizing enzymes such as cytochromes P450 or uridine glucuronyl transferases require cofactors, whereas hydrolases such as esterases or epoxide hydrolases catalyze their reaction independent of cofactors.

Various aromatic amines, including 2CPA, were shown to be hydrolyzed *in vitro* when incubated with human liver microsomes,[42] potentially leading to more potent DIO1 inhibiting metabolites. Also, a different disposition of the test item in the *in vitro* systems (recombinant enzyme vs. microsomes) might lead to observed differences in inhibition potency.[43] The isoflavone GEN and flame retardant TBBPA were described as potent substances fully inhibiting DIO1.[25,38] In these studies, GEN and TBBPA were tested up to 200 μM and 190 μM, respectively. In this work, a strong focus was placed on fully dissolving tested substances. This might result in lower concentrations tested for poorly soluble substances compared with other publications and might impact derived inhibition values. As such, the highest tested concentrations of GEN and TBBPA were 3.16 and 10 μM, respectively. Observed DIO1 inhibition of these concentrations was not high enough to derive reliable IC_{50} values.

Testing substances above the solubility limit can result in higher inhibitory potency if the insoluble components form a depot that provides a steady supply of disposable molecules. However, testing of precipitated substances could also introduce artifacts that are not related to the disposable concentrations. For further tests, the testing of concentrations that lead to homogeneous solutions might be included.

Improving specificity of the assay

The readout of this method is based on the SK reaction and a colorimetric readout of the redox reaction: the loss of absorption at 415 nm. Such loss-of-signal assays are susceptible to nonspecific interactions[44] that could be caused by various conceivable effects such as test items that are active in the SK reaction itself, interaction of the test item with the substrate or the cofactor, spontaneous iodide release, or nonspecific protein binding/modification. Testing of test items with and without an iodide-releasing enzyme can reveal test items that are able to release iodide without DIO1 activity. It can also reveal test items that are active in the SK reaction, provided they pass the ion exchange separation.

Both modes of interference would *per se* lead to a virtual change in iodide release. Testing for interference of the test item with the cofactor DTT was not further investigated. It was suggested to replace DTT with weaker reducing agents such as cysteine or glutathione since DTT was shown to be active in the SK reaction.

The testing of another enzyme present in liver microsomes independent of DIO1 activity provides information about the functionality of the microsomes. Inhibition of ALP and DIO activity in microsomes can indicate interactions of the test item with microsomes, which are not specific to the DIO1 activity. If the secondary analyzed enzyme is affected in addition to the DIO1 readout, this hints toward nonspecific interaction of the test item with the microsomes, ultimately leading to an inhibition of the enzyme activities. Obviously, this does not generally rule out the DIO inhibiting effects. The homodimeric protein ALP was used since its activity can be quantified by light absorption, and a photometer can be used for ALP and DIO1 testing.

In comparison with the DIOs, the ALP uses Zn^{2+} and Mg^{2+} as cofactors and more alkaline conditions are needed for enzymatic activity.[36] Neither of the six test items of the Reproducibility Assessment led to changes in absorbance without microsomes present nor inhibited ALP activity in human liver microsomes, indicating that their effects on DIO1 activity are specific. The ALP specificity testing approach increases the specificity of the DIO1-SK assay and will be included and challenged in part 2 of the assay validation.

Conclusion

In this work, the DIO1-SK assay was amended and improved making it a robust and standardized assay. SOPs were issued and are available as supplements, and further improvements are proposed. The different steps and conditions of the assays were investigated, and the effect of variations is described to inform the assay user and to ensure the robust performance of the assay. The reproducibility of the DIO1-SK assay was tested using a set of known DIO1 inhibitors. In addition, ALP activity tests were introduced to exclude nonspecific interactions of the test items. The DIO1-SK assay proved to be a reliable method in this intralaboratory Reproducibility Assessment Study (part 1). Xenobiotic-metabolizing competence of the method could be introduced by the addition of necessary cofactors. The results of the relevance study (part 2) will be published before long.

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Author Disclosure Statement

The authors A.G.W., B.B., C.H., H-A.H., S.S., D.F.-W., and R.L. are employees of BASF SE, a chemical company, which may use the DIO1 assay to develop and register commercial products in the future. S.C. and K.R. declare no conflict of interest.

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Supplementary Material

Supplementary Pdf S1
Supplementary Pdf S2
Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5

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3.3 Assessment of the predictivity of the DIO1-SK assay to investigate DIO1 inhibition in human liver microsomes

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Author contribution: In this work the author was responsible for conceptualization, data curation, formal analysis, investigation, methodology, validation and writing of the original draft.

Abstract

Introduction

The European Union Reference Laboratory for alternatives to animal testing (EU RL ECVAM) coordinates the validation of multiple thyroid *in vitro* assays aiming to set up an *in vitro* test battery. The validation consists of two independent parts: part 1 (Reproducibility assessment) in which the reproducibility of the method is evaluated by testing a small number of described inhibitors and part 2 (Predictivity assessment) where the predictivity of the method is investigated by testing a blinded set of substances. The Deiodinases (DIO) are important regulators of thyroid hormone metabolism and potential targets for substance-induced thyroid disruption. We previously reported the part 1 results for the Sandell-Kolthoff-reaction-based type 1 deiodinase (DIO1-SK) assay and an extensive description of the method. Here, we report the results of the part 2 testing in the DIO1-SK assay.

Methods

A set of 22 test substances consisting of known DIO inhibitors *in vitro* as well as substances otherwise interfering with the thyroid hormone system and substances with no such activities tested in the standardized DIO1-SK assay. Experiments were performed on blinded substances, which were deblinded after experimental completion, statistically evaluated and compared to literature data. Finally, an *in vitro* data interpretation procedure (DIP) was generated.

Results and discussion

Seven test substances produced a maximum DIO1 inhibition greater than 90% and eleven test substances below 20%; they were regarded as inhibitors and non-inhibiting substances, respectively. Two test substances, Ketoconazole and Silichristin, were found to be not applicable based on assay interference. Inhibition data were consistent with results of relevant *in vitro* and computational models. Using the variation of control data, an *in vitro* DIP was

defined categorizing test substances (i) by efficacy using the maximum inhibition data and (ii) by potency using the IC_{50} data of the test substance.

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Assessment of the Predictivity of DIO1-SK Assay to Investigate DIO1 Inhibition in Human Liver Microsomes

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Abstract

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Keywords: deiodinase, liver microsomes, predictivity, Sandell-Kolthoff, validation

Introduction

THE THYROID HORMONE SYSTEM (THS) can be affected by many substances, potentially leading to endocrine disruption, due to complex neuronal, humoral, and intracellular signaling. Function and regulation of the THS, comprising the hypothalamus, pituitary, and thyroid as well as target organs of thyroid hormone (TH) action, are dependent on numerous specific molecular functions, including various catalytic enzymes, several transmembrane transporters for hormones as well as iodide, humoral transporters/binding proteins for hormones, and hormone receptors (Fig. 1). In

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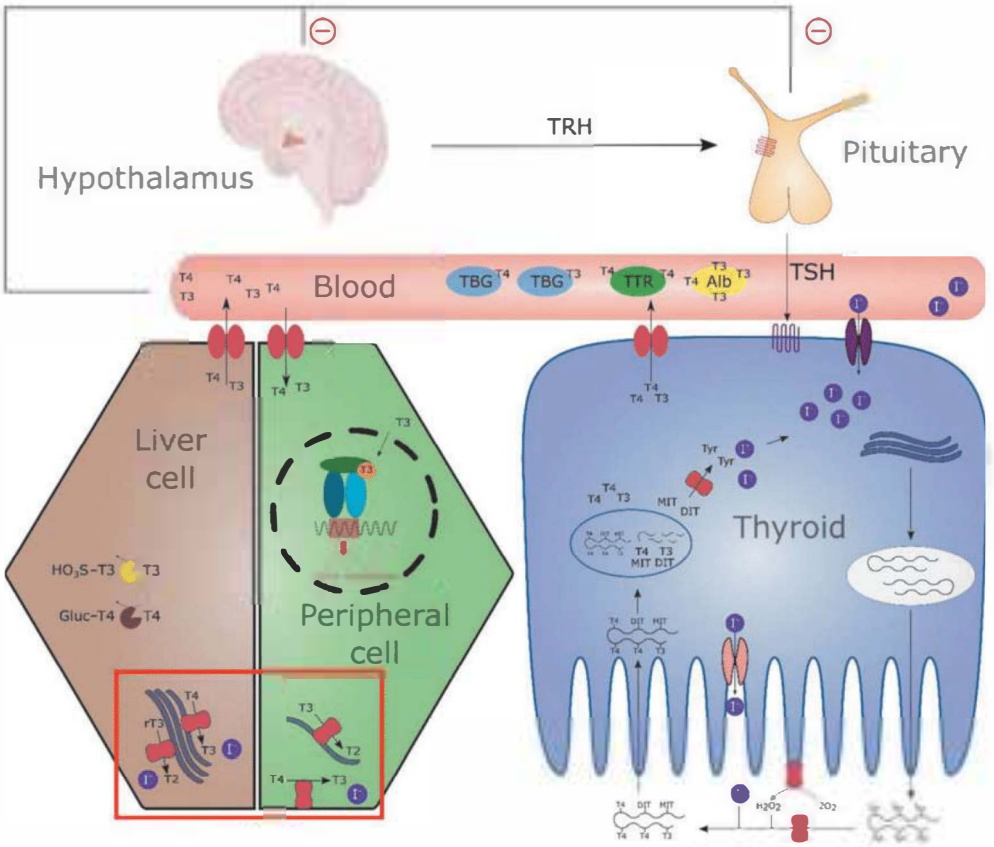

FIG. 1. The TH system. The hypothalamus produces TRH that stimulates the anterior pituitary to release TSH. Thyroid activity and growth depend on activation of the TSH receptor, leading to stimulation of TH biosynthesis, including, for example, increased iodide uptake into thyrocytes and production of TH by binding of iodide to Tyr residues of thyroglobulin in the lumen. Bound TH is taken up into the cells by endocytosis and TH (T3 and T4) as well as MIT and DIT is liberated by lysosomal proteolysis. The iodine-containing byproducts of TH biosynthesis, MIT and DIT, are deiodinated by the iodotyrosine deiodinase to retain the released iodine. The THs are released into the blood stream by specific transporters. TH binding proteins (Alb, TBG, and TTR) distribute TH to peripheral cells where they are taken up into the cell by specific transmembrane transporters. In this study, depending on the cell type-specific expression pattern, the three iodothyronine deiodinases exert their function of pre-receptor control of TH action by activation and inactivation through TH deiodination. Enzymatic, selective removal of iodine atoms from, for example, the pre-hormone T4 will lead to the active TH T3 or less active ones like rT3 or T2 (indicated by red box). Finally, TH mediate their effect by binding to the nuclear TH receptors, triggering gene expression of TH-regulated genes. In the sense of homeostasis, TH levels regulate TRH and TSH release through a negative feedback loop. Elimination of THs occurs mainly in the liver by sulfation and glucuronidation. Alb, albumin; DIT, diiodotyrosine; MIT, monoiodotyrosine; rT3, reverse T3; T2, diiodothyronine; T3, 3,3′,5-triiodo-L.thyronine; T4, thyroxine; TBG, thyroid-binding globulin; TH, thyroid hormone; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; TTR, transthyretin; Tyr, tyrosine.

particular, the enzymatic deiodination of TH, thyroxine (T4), and triiodothyronine (T3) to more active or less active TH metabolites is a crucial mechanism of pre-receptor of TH action, enabling cell-specific fine tuning of their intracellular fate and potency. This deiodination is catalyzed by the deiodinase (DIO) enzyme family; in humans, three isoenzymes are known.[1] They belong to the family of selenoproteins, having a selenocysteine residue in the catalytic center, which is crucial for DIO function.

Despite their structural similarity, the isoforms differ in substrate preference, position of deiodination (5- and 5′-deiodination), reaction kinetics, and cellular and subcellular localization, and their physiological function (Table 1). Only a few DIO-inhibiting substances are known. *In vitro*, various inhibitors were identified,[2–4] but *in vivo* data in animals or humans remain scarce. 6-Propyl-2-thiouracil (6PTU) has been used as a treatment for hyperthyroidism since at least 1946.[5] 6PTU specifically inhibits type 1 deiodinase (DIO1), while having little effect on DIO2 and DIO3.[6] Like 6PTU, other thiouracils inhibit DIO1 competitively to the cofactor.[7]

In addition, 6PTU inhibits the thyroid peroxidase (TPO), which is a key enzyme of TH biosynthesis within the thyroid gland, responsible for iodide oxidation and coupling. It is

TABLE 1. PARAMETERS OF HUMAN DEIODINASE ISOENZYMES

Parameter	*DIO1*	*DIO2*	*DIO3*
Tissue expression	Liver, kidney, thyroid, pituitary	CNS, pituitary, BAT, placenta	CNS, placenta
Subcellular location	Plasma membrane	Endoplasmic reticulum	Plasma membrane
Cofactor	Unknown (endogenous), DTT (artificial)	Unknown (endogenous), DTT (artificial)	Unknown (endogenous), DTT (artificial)
Preferred substrate	rT3, T3S	T4, rT3	T3, T4
Physiological function	Degradation of rT3 and sulfated TH and recycling of iodide	Production of plasma T3 as well as intracellular T3, thermogenesis	Clearance of TH
Known inhibitors	6PTU, ATG, iopanoic acid	Iopanoic acid, aurothioglucose (less)	Iopanoic acid, aurothioglucose (less)
Susceptibility to 6PTU	High	Low	Low

Adapted according to Refs.[10–12]

6PTU, 6-propyl-2-thiouracil; ATG, aurothioglucose; BAT, brown adipose tissue; CNS, central nervous system; DTT, dithiothreitol; rT3, reverse 3,3′,5-triiodo-L.thyronine; T3, 3,3′,5-triiodo-L.thyronine; T4, thyroxine; TH, thyroid hormone.

unclear which inhibition is responsible for the treatment of hypothyroidism. It was postulated that the therapeutic effect of 6PTU is due to the inhibition of TPO rather than DIO1[6] since the thiouracil methimazole inhibits TPO, but not DIO1 and shows similar therapeutic effects.[8] Nevertheless, DIO1 inhibition is a potential mechanism to cause disturbance of the THS. Robust and reproducible methods are required to investigate DIO1 inhibition *in vitro*.

We reported the standardization of an *in vitro* assay to investigate DIO1 inhibition.[9] This method uses human liver microsomes as a source for DIO1 and the Sandell-Kolthoff (SK) reaction to quantify released iodide. This method was originally published by Renko et al. using murine liver microsomes.[13] The method is part of a validation program led by the European Commission's Joint Research Centre (EC JRC) EU Reference Laboratory for alternatives to animal testing (EURL ECVAM).[14] In cooperation with the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) and the respective method developer of each method, an *in vitro* test battery consisting of different THS disrupting methods, should be generated.

This method validation comprises two parts, both adhering to the "Guidance Document on Good *In Vitro* Method Practices (GIVIMP)"[15]: (1) testing known DIO1 inhibitors in five independent runs to validate the reproducibility of the method (reproducibility assessment, part 1)[9] and (2) testing a set of blinded test substances, including known DIO1 inhibitors as well as negative substances to validate the predictivity (predictivity assessment, part 2). The reproducibility of the DIO1-SK assay has been demonstrated already.[9] In this study, we report the results of the predictivity assessment (part 2) for the DIO1-SK assay.

A set of 22 test substances was blinded (the chemical identity and DIO1 inhibition of the test substances was unknown to the test laboratory) and tested with the DIO1-SK assay. The test substance set included known *in vitro* inhibitors of DIO, as well as THS-interfering substances with other targets and substances not active in this respect. The set was tested in two additional assays to assess the specificity of observed DIO1 inhibition in the DIO1-SK assay, as described in Weber et al.[9]: (i) testing of the substance in the DIO1-SK assay without microsomes can identify substances that lead to spontaneous iodide release or substances that interfere with the SK reaction and (ii) testing the effect on alkaline phosphatase (ALP), another microsomal enzyme that can indicate global enzyme inhibiting activity unspecific to DIO1.

After completion of the experimental phase and transfer of data to EURL ECVAM, blinded test substances were deblinded. The DIO1 inhibition data were then compared to relevant *in vitro* and computational data in the literature. Finally, a data interpretation procedure (DIP) was developed based on the distribution of the generated *in vitro* data to classify the test substances into suggested categories of DIO1 inhibition.

Materials and Methods

Standard operation procedure

Standard operation procedures (SOPs) for the DIO1-SK assay and the ALP specificity testing approach were published.[9] These SOPs were adjusted and revised for this part 2 study to allow more freedom in the choice of material. The SOP:DIO1-SK assay contains information about material, performance, and analysis for the DIO1-SK assay and testing for specificity with and without microsomes during the incubation phase (Supplementary Pdf S1). The SOP:ALP activity testing contains information on how to perform the specificity testing approach that addresses the enzyme ALP (Supplementary Pdf S2). All experimental data of this study were generated using these SOPs.

Material

The required material was previously described[9] and is specified in detail in both SOPs. Materials like the batches of microsomes (provided by EURL ECVAM) or control items remained the same compared to part 1 testing.

Test substances

EC JRC EURL ECVAM selected test substances for the validation of several New Approach Methodologies (NAMs) addressing different THS modes of action. A hybrid approach was used combining different criteria: literature data and expert knowledge on THS disruption covering *in vitro*, *in vivo*, and epidemiological expertise as well as information on the availability of substances. Among the eligible substances, test substances that cover the largest possible chemical space were selected. For DIO1 specifically, an

appropriate balance between assumed positive and negative substances was aimed.

For the validation of the DIO1-SK assay, EURL ECVAM supplied 22 blinded test substances in brown glass vials. The vials were stored according to their specified storage conditions. The chemical identity of test substances was masked, but relevant safety information and molecular weight were reported for each substance (rounded to 25 Dalton). After completion of the experimental part and providing the generated data to EURL ECVAM, the 22 test substances were deblinded by disclosing the chemical name and CAS number to the testing facility, without providing further information about the expected result in the DIO1-SK assay (Table 3).

DIO1-SK assay

The method is described in detail in the SOP:DIO1-SK assay and Weber et al.[9] and shown in Figure 2. Briefly, test substances were incubated in a 96-well format with human liver microsomes, the substrate reverse (r)T3, the cofactor dithiothreitol (DTT), and phosphate+EDTA buffer for 2 hours at 37°C under constant shaking (assay concentrations: 0.1 M H_2KPO_4/HK_2PO_4, 40 mM DTT, 1 mM EDTA, 5 μM rT3, and 1% dimethyl sulfoxide). Ion-exchange resin-filled filter plates were used to separate released iodide from remaining substrate by application of vacuum to elute the samples. The released and eluted iodide was quantified using the SK reaction.

The SK reaction describes the formation of colorless Ce^{3+} from yellow Ce^{4+} using arsenite that is catalyzed by the available iodide concentration.[16] A set of control items was previously described[9] and was used to control and monitor assay performance (Table 2). Using the control data for each assay plate, the iodide release activity (IRA) is determined as a normalized value. The IRA is indicative of the amount of iodide released by DIO1 catalysis. A value of 100% IRA is the maximum possible DIO1 activity, while 0% DIO1 activity represents full inhibition by 6PTU. DIO1 inhibition [%] is calculated as 100 − IRA [%].

Before testing, the solubility of the test substances was characterized (Table 3); extensive information about the solubility results is given in Supplementary Table S1. Most of the test substances were soluble at assay concentrations between 10^{-3} and 10^{-4} M with some of the test substances being less soluble (as low 10^{-6} M). Stock solutions were prepared on the day of assay performance.

All test substances were tested in three different systems: (i) the DIO1-SK assay, (ii) in DIO1-SK testing without microsomes, and (iii) ALP activity testing.

At least three valid, independent assay runs were performed for each test substance. Testing was performed in three technical replicates for each concentration, resulting in at least nine inhibition values for each concentration. Initially, test substances were tested in a range-finding assay run. In this study, the determined highest soluble concentration and consecutive 10-fold (v/v) dilutions of each test substance were tested.

If any concentration tested inhibited IRA by more than 20%, tested concentrations of following assay runs were adapted, including more concentrations in this concentration range. For these substances, a final set of at least three

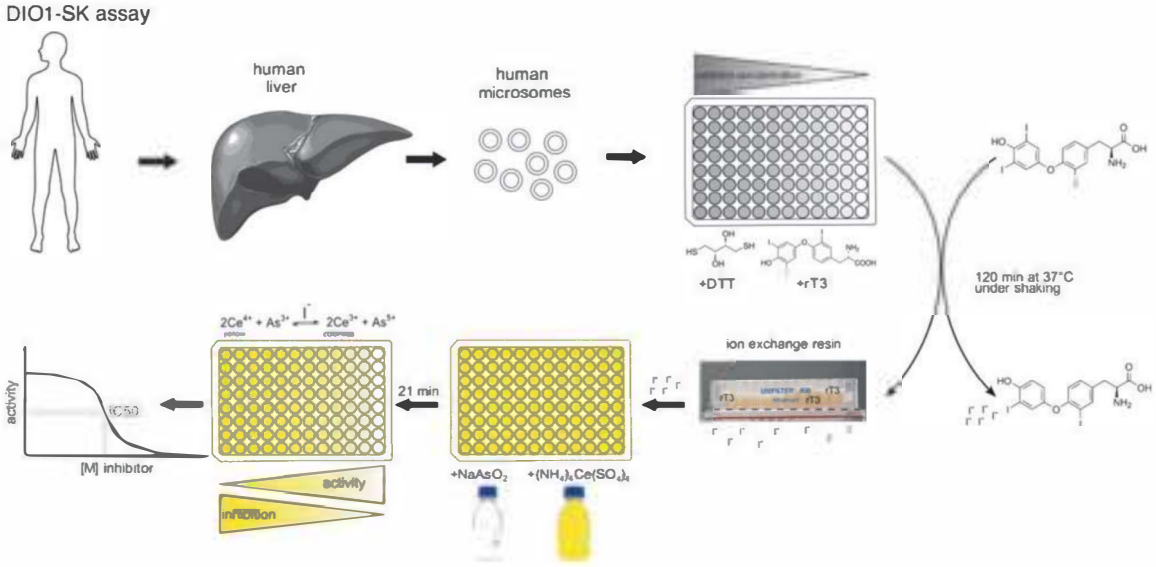

FIG. 2. Schematic procedure of the DIO1-SK assay. Human liver microsomes are incubated under pH-buffered conditions with the substrate rT3, an artificial enzyme-regenerating cofactor DTT, and the test and control items. The assay plates are incubated for 2 hours at 37°C under constant shaking. The enzymatically released iodide is separated from the remaining assay constituents, including substrate, by an ion-exchange resin. The iodide concentration of the eluted sample is then quantified by the SK reaction by formation of colorless Ce^{3+} from yellow Ce^{4+} using arsenite. The reaction is catalyzed by the available iodide concentration. Nonlinear curve fitting is used to derive inhibition values for the test items. DIO1-SK, Sandell–Kolthoff reaction-based type 1 deiodinase; DTT, dithiothreitol. Figure was adapted from Weber et al.[9]

TABLE 2. USED CONTROL ITEMS IN THE DIO1-SK ASSAY ACCORDING TO THE CONCEPT OF GOOD *IN VITRO* METHOD PRACTICES[15]

Control items	*Function*
RI	6PTU at a maximum assay concentration of 10^{-3} M. Concentration–response curves of 6PTU ranged from 10^{-3} to 10^{-8} M.
PC	ATG at a maximum assay concentration of 10^{-4} M.
NC	TGSS at a maximum assay concentration of 10^{-4} M.
SC	DMSO at a concentration of 1%.

6PTU, 6-Propyl-2-thiouracil; ATG, aurothioglucose; DIO1-SK, Sandell–Kolthoff reaction-based type 1 deiodinase; DMSO, dimethyl sulfoxide; NC, negative control; PC, positive control; RI, reference item; SC, solvent control; TGSS, 1-thio-β-D-glucose sodium salt.

independent assay runs with adapted concentrations was performed and the range-finding assay run was not used for the final assay run set used for maximum inhibition and IC_{50} derivation. If the test substance did not show inhibition of >20% in the range-finding assay run, this assay run was included in the final set of at least three valid assay runs.

It was ensured that every test substance was tested in at least two of the three possible positions of the assay plate layout to account for a potential influence of the plate position. Three different laboratory co-workers performed the experiments to ensure intralaboratory reproducibility.

Testing for assay interference without microsomes

Incubations without microsomes were performed according to the DIO1-SK assay SOP. Testing was performed like the DIO1-SK assay, but the microsomal fraction was replaced with deionized water. One initial assay run was performed for all test substances using the highest concentration only. If an increase of IRA >10% was observed in the initial assay run, two additional, independent assay runs were performed for the respective test substances.

ALP inhibition assay

The colorimetric ALP assay is well established and described in the literature.[17,18] Testing for ALP inhibition was performed according to SOP:ALP activity. The assay is based on the colorimetric quantification of a metabolite formed by the dephosphorylation activity of ALP in human liver microsomes. One initial assay run was performed for all test substances using the highest concentrations only. Two additional assay runs were performed for test substances that inhibited ALP by more than 15%.

Comparison of data to existing literature data

The data from this study were compared to published *in vitro* or *in silico* inhibition data on DIO1 inhibition: (i) the ToxCast database of the U.S. EPA with more than 1800 substances was investigated for DIO inhibition using a comparable method. The method also uses ion-exchange resin to separate released iodide from assay components and uses the SK reaction to quantify iodine, yet recombinant DIO enzymes were used instead of human liver microsomes.[2] (ii) Garcia de Lomana et al. developed a battery of *in silico* models for different targets of the THS, including DIO1.[19] The models were based on the *in vitro* screenings of the ToxCast database by the U.S. EPA.

Statistical approaches, machine learning methods, data balancing techniques, and neural networks were used to derive the models. The *in silico* model uses *in vitro* data from the U.S. EPA DIO screening and generated predictions are

TABLE 3. USED TEST SUBSTANCES AND THEIR HIGHEST TEST CONCENTRATION, DEFINED BY SOLUBILITY TESTING

Identifier	*CAS number*	*Stock concentration, mM*	*Max. tested concentration, μM*
6PTU	51-52-5	100	1000.0
Ampicillin	69-53-4	10	100.0
Aspirin	50-78-2	100	1000.0
Bisphenol A diglycidyl ether	1675-54-3	100	1000.0
Ethylene thiourea	96-45-7	100	1000.0
Fipronil	120068-37-3	1	1.0
Fipronil sulfone	120068-36-2	31.6	316.0
Hexadecyltrimethylammonium bromide	57-09-0	10	100.0
Ketoconazole	65277-42-1	100	1000.0
Linoleic acid	60-33-3	100	1000.0
Linolenic acid	463-40-1	3.16	31.6
Mefenamic acid	61-68-7	0.1	1.0
Morin hydrate xH_2O	654055-01-3	10	100.0
N,N,N′,N′-tetramethyl thiourea	2782-91-4	100	1000.0
Nordihydroguaiaretic acid	500-38-9	31.6	316.0
Octyl methoxycinnamate/2-ethylhexyl 4-methoxycinnamate	5466-77-3	31.6	316.0
Pentachlorophenol	87-86-5	1	10.0
Resorcinol	108-46-3	100	1000.0
Salsalate	552-94-3	31.6	316.0
Silichristin	33889-69-9	100	1000.0
Sodium perchlorate	7601-89-0	100	1000.0
Tannic acid	1401-55-4	100	1000.0

thus not independent. (iii) The U.S. EPA ToxCast screening of the enzyme class iodotyrosine deiodinases (IYDs) uses recombinant IYD to depict inhibitory effects. Compared to DIOs, IYDs represent a different class of enzymes with different inhibitors. However, the method also uses ion-exchange resin to separate released iodide and the SK reaction to quantify iodine.[20,21] The IYD inhibition data were used to obtain information on the specificity of DIO1 inhibition by the test substances.

Statistical analysis

R statistical software[22] was used to evaluate inhibition and control data by generating IRA values. GraphPad Prism (version 9.3.1 for Windows; GraphPad Software, San Diego, CA) was used for the graphical representation of data. The DRC package[23] in R was used to fit a four-parameter log-logistic dose–response model (LL.4) and to derive IC_{50} values [Equation (1)]. The models used in GraphPad Prism and R use the same parameters and function. Only the final set of assay runs was used for curve and IC_{50} derivation and range-finding assay runs in which the tested concentrations of subsequent runs were changed due to observed inhibition in the range-finding assay run were not used.

Equation (1): the four-parameter log-logistic function "LL.4" was used to derive curve parameters for the test substance and, if possible, to derive IC_{50} values. "c" represents the lowest response, "d" the maximal response, "b" the slope of the curve, and "e" the IC_{50} value.

$$f(x) = c + \frac{d - c}{1 + \exp(b(\log(x) - \log(e)))}$$

The z'-factor was derived according to SOP:DIO1-SK assay and was previously described.[9] If an assay run did not meet the acceptance criterion of a z'-factor above 0.5, box plot outlier testing was performed. The respective lower limit, defined as lower quartile $-1.5 \times$ interquartile range (IQR), and upper limit, defined as upper quartile $+1.5 \times$IQR, were calculated for the replicates that were used for z'-factor derivation (solvent control and reference item) for mentioned assay runs. If replicates were below the lower limit or above the upper limit of the respective control/item, the replicate was defined as an outlier; no more than two outliers were excluded per control/item set. If the assay run did meet a z'-factor of 0.5 after outlier removal, it was considered a valid assay run; otherwise, the assay run was removed from evaluation.

Results

Assay performance, quality control, and comparison of assessment criteria in the DIO1-SK assay

The performance of DIO1-SK assay runs was monitored with a set of acceptance criteria (Table 4); variation of control items per assay run and over time is shown in Supplementary Figures S1 and S2. The variation of control items was low and set acceptance criteria were mostly met. The separation between reference item and solvent control was high, as indicated by a z'-factor, usually above 0.7. Some runs did not meet the acceptance criterion of a z'-factor above 0.5. This might be due to rare cases of the ion-exchange resin becoming impermeable and resulting in high standard deviation (SD) in the controls.

TABLE 4. USED ACCEPTANCE CRITERIA IN THE DIO1-SK ASSAY AND THE DETERMINED MEAN AND VARIATION DATA IN THIS VALIDATION STUDY

Acceptance criteria		*Data of this study*	
Criterion	*Cut-off value*	*Mean*	*SD*
IRA of solvent control, %	—	100.0	6.9
IRA of reference item, %	—	0.0	3.1
IRA of negative control, %	$80 < x < 120$	99.9	9.1
IRA of positive control, %	$x < 20$	1.0	4.1
IC_{50} of the reference item, μM	$1 < x < 10$	3.0	1.3
CV of log IC_{50} estimate of reference item, %	$x < 3$	1.1	0.6
z'-Factor	$x > 0.5$	0.7	—

CV, coefficient of variation; IRA, iodide release activity; SD, standard deviation.

In this study, outliers were identified by box plot outlier testing and excluded for these cases. Alternatively, the robust z'-factor could be derived and used instead of the z'-factor. The robust z'-factor is less vulnerable to outliers since the mean is substituted for the median and the SD for the median absolute deviation.[24] As such, only 3 assay runs did not meet a robust z'-factor of 0.5 before outlier exclusion (compared to 11 assay runs if the z'-factor is used). After outlier removal, all assay runs reached a z'-factor of 0.5, but two assay runs did not reach a robust z'-factor of 0.5.

Concentration range finding

Every test substance was first tested in a range-finding experiment in the DIO1-SK assay. If any of the tested concentrations led to an IRA decrease of more than 20%, the following assay runs were adapted, including more test substance concentrations in the range of inhibitory concentrations. In total, the concentrations of 11 of the 22 test substances had to be adapted. If no IRA decrease >20% was found in the range-finding experiment, concentrations were not changed and the range-finding experiment was considered the first of the required three biologically independent assay runs. For the final assessment, a cutoff of 25% was selected; 20% as a cutoff for the range finding was selected to not miss concentrations due to variability of range-finding results. The maximum concentrations used to determine DIO1 inhibition are listed in Table 3.

DIO1 inhibition

The results of the DIO1-SK assay for the test substances are shown in Table 5 and exemplary graphs are shown in Figure 3. The results for all test substances are summarized in Supplementary Figure S3. Seven test substances produced a maximum inhibition ≥90%. Derived IC_{50} values ranged from 2.0 μM for tannic acid and 85.4 μM for bisphenol A diglycidyl ether (BADGE). Eleven test substances produced inhibition below 20% at the highest concentration.

TABLE 5. SUMMARIZED RESULTS OF THE 22 TEST SUBSTANCES IN THE DIO1-SK ASSAY

Identifier	CAS number	Max. tested concentration, M	Max. inhibition, %		IC_{50}, M	
			Mean	SD	Mean	SD
6PTU	51-52-5	1.00E-03	107.1	5.7	2.7	0.6
Tannic acid	1401-55-4	1.00E-03	105.4	2.8	2.0	0.3
Linoleic acid	60-33-3	1.00E-03	104.9	9.7	66.6	19.7
Nordihydroguaiaretic acid	500-38-9	3.16E-04	104.3	2.3	4.7	1.7
Bisphenol A diglycidyl ether	1675-54-3	1.00E-03	101.3	1.0	85.4	42.4
Morin hydrate xH_2O	654055-01-3	1.00E-04	99.0	2.1	8.4	3.3
Hexadecyltrimethylammonium bromide	57-09-0	1.00E-04	97.2	4.2	44.0	48.6
Silichristin[a]	*33889-69-9*	*1.00E-03*	*55.1*	14.1	–	
Pentachlorophenol	87-86-5	1.00E-05	43.5	7.2	–	
Linolenic acid	463-40-1	3.16E-05	41.7	41.7	–	
Ketoconazole[a]	*65277-42-1*	*1.00E-03*	*28.0*	20.0	–	
Salsalate	552-94-3	3.16E-04	19.6	2.9	–	
Ethylene thiourea	96-45-7	1.00E-03	19.4	2.1	–	
Resorcinol	108-46-3	1.00E-03	16.2	3.2	–	
Fipronil sulfone	120068-36-2	3.16E-04	12.8	8.7	–	
Ampicillin	69-53-4	1.00E-04	12.3	5.2	–	
Octyl methoxycinnamate/2-ethylhexyl 4-methoxycinnamate	5466-77-3	3.16E-04	11.9	4.7	–	
Fipronil	120068-37-3	1.00E-06	11.2	5.4	–	
N,N,N′,N′-tetramethyl thiourea	2782-91-4	1.00E-03	8.7	2.8	–	
Aspirin	50-78-2	1.00E-03	6.2	7.9	–	
Mefenamic acid	61-68-7	1.00E-06	6.2	2.1	–	
Sodium perchlorate	7601-89-0	1.00E-03	4.4	2.4	–	

IRA of the used control and reference items, the derived 50% inhibition concentration (IC_{50}) of the reference item 6PTU, derived CV of the estimate of the reference item, and *z′*-factor of each assay run in the part 2 of the DIO1-SK assay over all performed assay runs.
- : IC_{50} could not be derived because the inhibition was too low or only ambiguous IC_{50} values could be derived.
[a]Silichristin and ketoconazole were determined as not applicable in the DIO1-SK assay based on specificity testing.

Results of specificity testing

Two additional assays, (i) the DIO1-SK without microsomes and the (ii) ALP inhibition assay, were performed with each test substance to identify nonspecific interactions with the test system. Results for selected test substances are shown in Figure 4; results for all test substances of specificity testing without microsomes and ALP testing are shown in Supplementary Tables S2 and S3, respectively.

(i) Testing in the DIO1-SK assay without microsomes was used to identify test substances that can drive the SK reaction without release of iodide from the substrate by DIO1 and/or lead to spontaneous iodide release not catalyzed by DIO1. Silichristin produced an IRA activity around 50% in the presence of microsomes. This IRA activity was still present in the absence of microsomes, indicating interference of silichristin with the assay (Fig. 4A). Silichristin is hence not applicable to the DIO1-SK assay. The results of silichristin do not reflect its DIO1 inhibition and are excluded from further analysis. No other test substance showed considerable IRA increases in the absence of microsome.

(ii) Testing the activity of ALP as a second microsomal enzyme was used to identify inhibition of microsomal enzyme activity, which is not specific to DIO1. ALP inhibition greater than 20% after 60 minutes of incubation was regarded as ALP inhibiting. Silichristin was again active in inhibiting ALP activity. In addition, ketoconazole inhibited ALP activity by 59% (Fig. 4B). This may indicate nonspecific interactions of Ketoconazole with the microsomes. Ketoconazole was thus regarded as not applicable to the DIO1-SK assay. Results of the DIO1-SK assay with Ketoconazole were not considered for further analysis regarding DIO1 inhibition.

Some test substances (BADGE, morin hydrate, nordihydroguaiaretic acid, octyl methoxycinnamate, and tannic acid) led to abnormal reaction kinetics in ALP activity testing (Supplementary Table S3). This is likely due to test substance-induced, nonspecific conversion of the substrate, *para*-nitrophenyl phosphate, to its yellow, quantifiable metabolite, which was also visible in the increase of an initially measured optical density (OD) in the ALP inhibition assay ($OD_{0\ minute,415nm}$). The test substances that had an $OD_{0\ minute,415nm}$ of 0.15 or above were excluded from ALP analysis. Still, nordihydroguaiaretic acid showed abnormal reaction kinetics and was removed from evaluation. These test substances were removed from ALP analysis (Table 7), but were not excluded from the DIO1-SK assay. Yet, their applicability to the DIO1-SK assay could only be checked by one of the two specificity assays.

Data interpretation

As there are only little data about *in vivo* DIO1 inhibition available, we approached data interpreting by defining a procedure to identify substances that are not active in the assay, that is, have IRA responses like the negative and solvent control. The distribution of the IRA values of controls that can

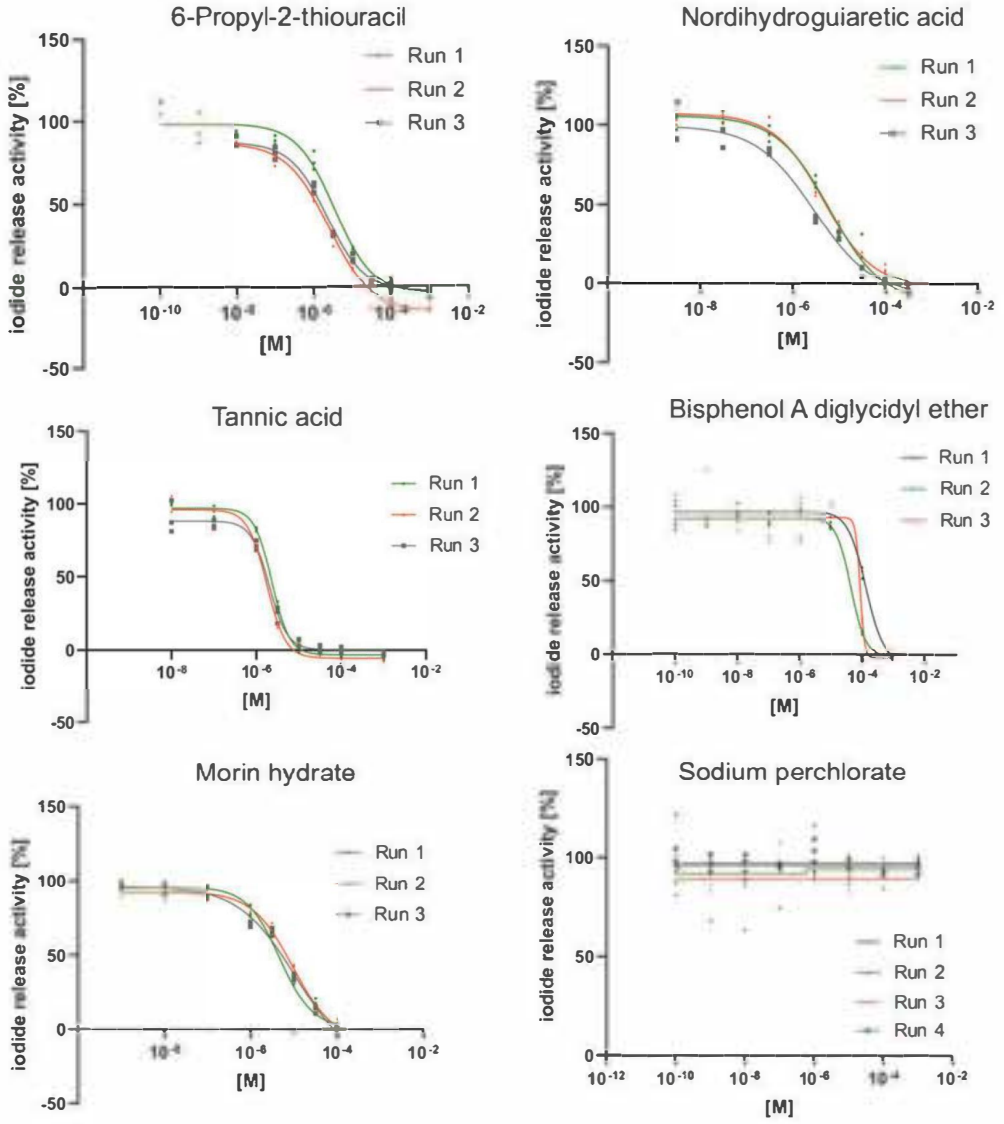

FIG. 3. Exemplary DIO1-SK assay results for six test substances. Data are shown as mean iodide release ±SD of three technical replicates per concentration of at least three assay runs. The sigmoidal concentration–response curves were derived using the function "[Inhibitor] versus response—Variable slope (four parameters)" in GraphPad Prism. Range-finding assay runs that led to changed concentrations were not used for the derivation of IC_{50} values and are not shown. SD, standard deviation.

inhibit DIO1 (reference item and positive control) and of controls that have no effect on DIO1 (solvent control and negative control) is shown in Table 6. The data had an approximately normal distribution (Supplementary Fig. S4).

The ranges of the mean +/−3-fold SD of the control items were determined (Table 6). Based on the lower range of the solvent control (79.2% IRA) and negative control, 1-thio-β-D-glucose sodium salt (TGSS; 72.6% IRA), a threshold of 75% IRA (≙25% DIO1 inhibition) was defined. Results of test substances at and above this cutoff (>75%) are assessed to be not DIO1 inhibiting, whereas results below this cutoff are assessed to be DIO1 inhibiting.

A second threshold was defined using the upper range of the DIO1-inhibiting controls. A threshold of 10% IRA (≙90% DIO1 inhibition) was defined, below which a test substance has complete inhibitory activity similar to the reference item, 6PTU, at a concentration of 10^{-3} M, and the positive control, aurothioglucose (ATG). By applying these two thresholds, assay results can be divided into three efficacy categories: (i) category 1: test substances leading to complete inhibition

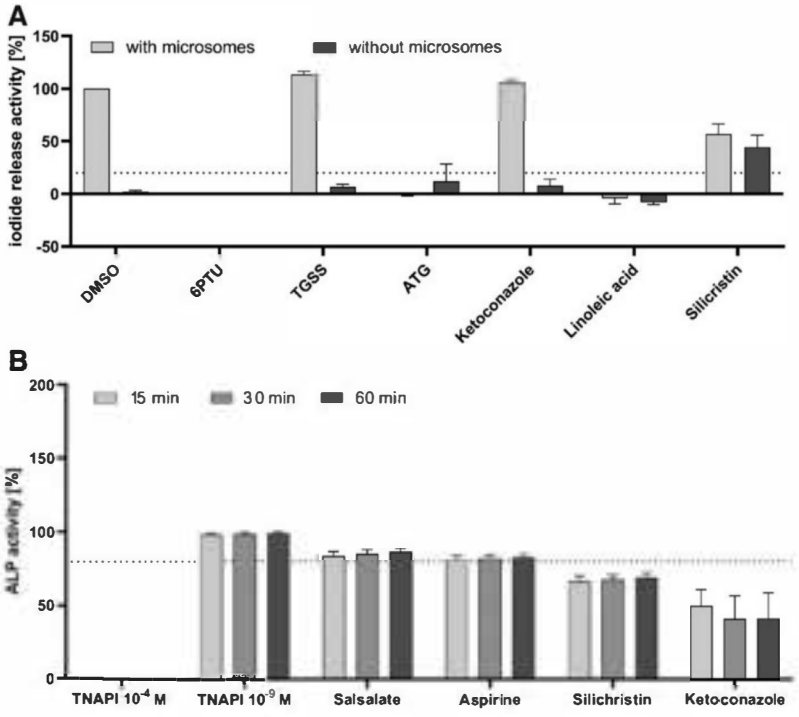

FIG. 4. Specificity testing results to identify nonspecific test substances in the DIO1-SK assay for selected test substances. **(A)** Data are shown as mean IRA values ±SD of three technical replicates per test substance and positive (ATG) as well as negative control (TGSS), nine replicates for the reference item (6PTU) and the solvent control (DMSO). Three independent assay runs with and without microsome were performed for each test substance that exceeded a threshold of 10% in an initial assay run. For these substances, data are shown as the mean of the three assay run IRA values ±SD. The dashed line represents the 20% IRA threshold in samples without microsomes above, which the test substance was defined as "not applicable in DIO1-SK assay" since the test substance itself was active in the SK reaction. **(B)** ALP activity of test substances as well as the reference item of ALP testing, the TNAPI, was tested at the highest concentration after 15, 30, and 60 minutes of incubation. Only the ALP applicable test substances are shown that led to inhibition greater than 15% in any on the three time points. Data are shown as the mean of three assay runs ±SD for each time point. The dashed line represents the 80% ALP activity (≙20% inhibition) threshold, below which test substances were defined as ALP inhibiting. 6PTU, 6-propyl-2-thiouracil; ALP, alkaline phosphatase; ATG, aurothioglucose; DMSO, dimethyl sulfoxide; IRA, iodide release activity; TGSS, 1-thio-β-D-glucose sodium salt; TNAPI, tissue-nonspecific alkaline phosphatase inhibitor.

of DIO1 comparable to 6PTU (>90% inhibition), (ii) category 2: test substances with inhibitory activity not resulting in complete DIO1 inhibition (between 90% and 25% inhibition), and (iii) category 3: test substances that do not inhibit DIO1 (<25% inhibition). Using this classification system, 7 test substances were categorized in category 1, 2 test substances in category 2, and 11 test substances in category 3 (Fig. 5A).

In addition to an efficacy estimate, inhibitory activity can be assessed by its potency, characterized by the IC_{50}. In this study, the IC_{50} values of the reference item 6PTU were used to estimate whether a test substance inhibited DIO1 similarly to 6PTU (Fig. 5B). Again, the mean +/−3 times SD approach was used to estimate the upper range results, resulting in a threshold of 7.1 μM.

Test substances of category 1 that had an IC_{50} lower than the defined threshold of 7.1 μM were at least as potent as 6PTU and are subcategorized into category 1A. Test substances with a higher IC_{50} are less potent than 6PTU and are categorized into category 1B. As such, three test substances could be categorized as category 1A (full inhibition, "6PTU like," high potency) and four test substances as category 1B (full inhibition, lower potency than 6PTU) (Fig. 5B). Substances of category 2 were not subdivided by potency because derived IC_{50} values tend to be unreliable or could not be derived.

TABLE 6. SUMMARY OF THE DISTRIBUTION OF THE IODIDE RELEASE ACTIVITY VALUES OF CONTROL DATA GENERATED IN PART 2 OF THE DIO1-SK ASSAY

Control	*Mean*	*SD*	*Range of mean +/−3*SD*
IRA of solvent control, %	100.0	6.9	79.2; (120.8)
IRA of negative control, %	99.9	9.1	72.6; (127.2)
IRA of reference item, %	0.00	3.1	(−9.4); 9.4
IRA of positive control, %	1.0	4.1	(−11.2); 13.2

Ranges that were not used for threshold derivation are shown in brackets.

IRA, iodide release activity; SD, standard deviation.

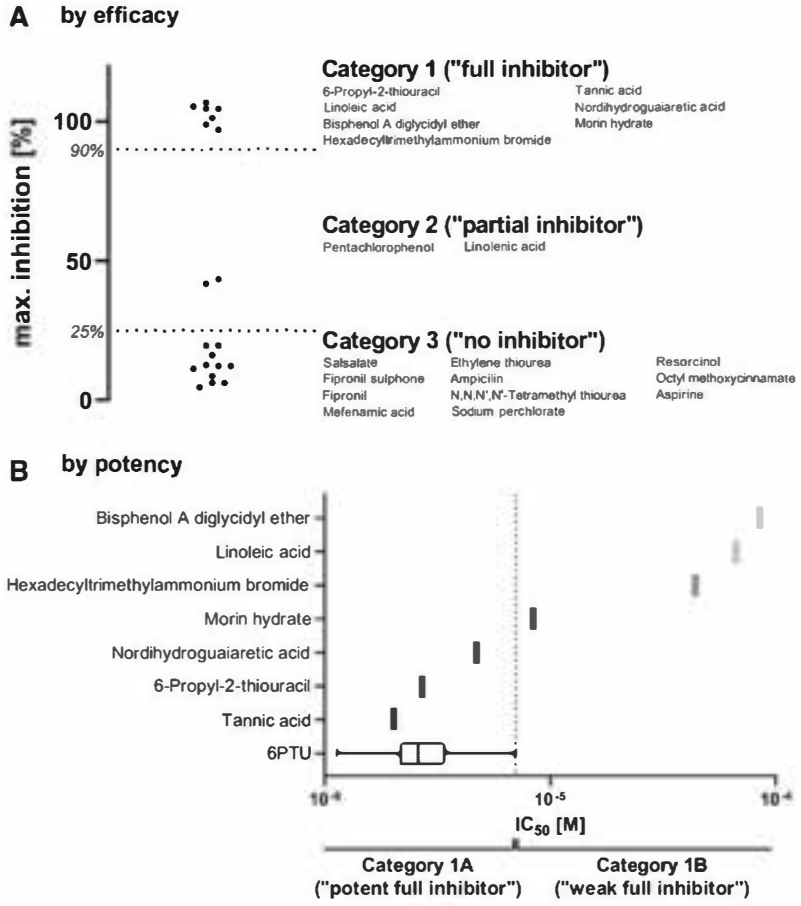

FIG. 5. Test substance inhibition data were filtered by inhibition efficacy **(A)** and inhibition potency **(B)**. The 20 applicable test substances were clustered into categories based on 2 defined thresholds (90% and 25%) of maximum DIO1 inhibition **(A)**. The seven test substances categorized as category 1 were subclassified into category 1A and 1B based on the defined threshold visualized as dotted line (reference item 6PTU, mean IC_{50} + 3*SD; 7.1 μM). In addition, the variation of the reference 6PTU of all assay runs is shown as a box plot visualization showing 1–99 percentile as whiskers (lower whisker: 1.1 μM, lower quartile: 2.2 μM, median: 2.6 μM, upper quartile: 3.5 μM, upper whisker: 7.0 μM) **(B)**.

In vivo data regarding effects on DIO of the test substances are rare. Therefore, the correlation of the *in vitro* DIO1 inhibition data generated here with *in vivo* data was not applicable to estimate the *in vivo* predictivity of the DIO1-SK assay. The results of this study can, however, be compared to previously published data. The results of this study are summarized in Table 7 along with previously published data from *in vitro* screenings and *in silico* methods.

Discussion

DIO1 inhibition and specificity testing

In this study, a set of 22 test substances was examined in the DIO1-SK assay, as well as additionally checked for nonspecific interactions with the test system. The performance of control items was comparable to part 1.[9] The IC_{50} of the reference item, 6PTU, ranged from 1.1 to 5.4 μM with a mean of 3 μM. This was a slightly lower IC_{50} compared to part 1 (4 μM). Usage of the robust z'-factor versus the z'-factor was discussed based on some runs not meeting the set acceptance criterion of a z'-factor above 0.5. If outlier exclusion, for example, by box plot outlier testing is used, the z'-factor can be safely used. Assay runs of the same test substance were generally consistent and produced similar results confirming the reproducibility of part 1.[9]

Data of some test items had high variability, which, however, did not affect the final result (classification according to the DIP, see Table 8): linolenic acid (classified as category 2 by the mean inhibition of three runs) inhibited DIO1 by 81% in the first assay run, but markedly less in following runs, possibly due to degradation of the test substance. Nonetheless, inhibition in the first assay run was not sufficient to classify linolenic acid into category 1. Inhibition–concentration curves of BADGE and hexadecyltrimethylammonium bromide were steep so that the tested concentrations resulted in either full or no inhibition. Consequently, the IC_{50} values varied across the three assay runs. Applying the DIP to the individual assay runs or the mean of all runs assigns BADGE and Hexadecyltrimethylammonium bromide to category 1B.

Testing of substances without microsomes during incubation has proven to be a valuable strategy of identifying potential interferences with the method. Possible causes may be inherent activity of the substances in the SK reaction, through the presence of either iodide or other atoms that drive the SK reaction. Using this approach, false classifications for DIO1 inhibition can be reduced and the confidence in the method can be increased. As such, 1 of the 22 test substances, Silichristin, was found false positive in the DIO1-SK assay with a seeming maximum inhibition of 62%. Silichristin is a natural substance of the fruit of milk thistle and is a

TABLE 7. SUMMARIZED INFORMATION ABOUT THE 22 DEBLINDED TEST SUBSTANCES, INCLUDING DISCLOSED INFORMATION, DIO1 INHIBITION AND SPECIFICITY DATA, AS WELL AS CATEGORIZATION OF THIS STUDY, AND RELEVANT *IN VITRO* AND *IN SILICO* METHODS

Disclosed information		*DIO1-SK assay*						*U.S. EPA Deiodinase Screening (Olker et al., 2018)*[3]			*QSAR thyroid models (Garcia de Lomana et al., 2020)*[19]		*U.S. EPA Iodotyrosine deiodinase Screening (Olker et al., 2020)*[20]		
Identifier	*CAS number*	*Max. tested concentration, M*	*Max. inhibition, %*	*IC_{50}, µM*	*Activity without microsomes?*[a]	*Active in ALP activity testing?*[b]	*Category*	*Max. tested concentration, M*	*Max. DIO1 inhibition, %*	*IC_{50}, µM*	*QSAR DIO1*	*No. of active QSAR models (9 in total)*	*Max. tested concentration, M*	*Max. IYD inhibition, %*	*IC_{50}, µM*
6PTU	51-52-5	1.00E-03	107.1	2.7	No	No	Category 1A	2.00E-04	101	3.8	Active	2	2.00E-04	0	—
Tannic acid	1401-55-4	1.00E-03	105.4	2.0	No	—[c]	Category 1A	6.00E-05	99	17.4	Active	9	7.00E-05	101	7.9
Linoleic acid	60-33-3	1.00E-03	104.9	66.6	No	No	Category 1B	2.00E-04	88	20.0	Active	3	2.00E-04	77	119.2
Nordihydroguaiaretic acid	500-38-9	3.16E-04	104.3	4.7	No	—[c]	Category 1A	2.00E-04	75	11.0	Active	5	2.00E-04	13	—
Bisphenol A diglycidyl ether	1675-54-3	1.00E-03	101.3	85.4	No	—[c]	Category 1B	2.00E-04	103	1.3	Active	3	2.00E-04	9	—
Morin hydrate xH_2O	654055-01-3	1.00E-04	99.0	8.4	No	—[c]	Category 1B	2.00E-04	96	7.4	Active	6	2.00E-04	70	127.6
Hexadecyltrimethylammonium bromide	57-09-0	1.00E-04	97.2	44.0	No	No	Category 1B	2.00E-04	98	12.9	Active	6	2.00E-04	104	24.7
Silichristin[d]	33889-69-9	1.00E-03	(55.1)	—	Yes	Yes	Not applicable	—	—	—	Active	9	—	—	—
Pentachlorophenol	87-86-5	1.00E-05	43.5	—	No	No	Category 2	1.00E-04	46.9	—	Active	5	1.02E-04	8	—
Linolenic acid	463-40-1	3.16E-05	41.7	—	No	No	Category 2	2.00E-04	92	20.0	Active	3	2.00E-04	8	—
Ketoconazole[d]	65277-42-1	1.00E-03	(28.0)	—	No	Yes	Not applicable	2.00E-04	—2.8	—	Inconclusive	1	2.00E-04	21	—
Salsalate	552-94-3	3.16E-04	19.6	—	No	No	Category 3	—	—	—	Inactive	1	—	—	—
Ethylene thiourea	96-45-7	1.00E-03	19.4	—	No	No	Category 3	2.00E-04	4	—	Inactive	1	2.00E-04	4	—
Resorcinol	108-46-3	1.00E-03	16.2	—	No	No	Category 3	2.00E-04	—4.4	—	Inactive	0	2.00E-04	0	—
Fipronil sulfone	120068-36-2	3.16E-04	12.8	—	No	No	Category 3	—	—	—	Active	8	—	—	—
Ampicillin	69-53-4	1.00E-04	12.3	—	No	No	Category 3	—	—	—	Inconclusive	0	—	—	—
Octyl methoxycinnamate/ 2-ethylhexyl 4-methoxycinnamate	5466-77-3	3.16E-04	11.9	—	No	—[c]	Category 3	2.00E-04[e]	7[e]	—	Inactive	0	2.00E-04[e]	5[e]	—
Fipronil	120068-37-3	1.00E-06	11.2	—	No	No	Category 3	2.00E-04	57.8	—	Active	7	2.01E-04	14	—
N,N,N′,N′-tetramethyl thiourea	2782-91-4	1.00E-03	8.7	—	No	No	Category 3	2.00E-04[f]	4.4[f]	—	Inactive	1	2.00E-04[f]	−2[f]	—
Aspirin	50-78-2	1.00E-03	6.2	—	No	No	Category 3	2.00E-04	—5	—	Inactive	0	2.00E-04	—8	—
Mefenamic acid	61-68-7	1.00E-06	6.2	—	No	No	Category 3	—	—	—	Inactive	2	—	—	—
Sodium perchlorate	7601-89-0	1.00E-03	4.4	—	No	No	Category 3	—	—	—	Inactive	1	—	—	—

[a]A threshold of 20% IRA increase in the samples without microsomes was used to classify test substances as nonapplicable in the DIO1-SK assay.

[b]A threshold of 20% inhibition of ALP activity after 60 minutes of incubation time was used to classify test substances as active.

[c]These test substances interfered with the ALP activity test system.

[d]Silichristin and ketoconazole were determined as not applicable in the DIO1-SK assay based on specificity testing and max. inhibition values were put in brackets.

[e]2-Ethylhexyl trans-4-methoxycinnamate (CAS: 83834-59-7) was tested here.

[f]The structural analog *N,N′*-diethylthiourea (CAS: 105-55-5) was tested here.

ALP, alkaline phosphatase; IYD, iodotyrosine deiodinase.

described inhibitor of the TH transporter monocarboxylate transporter 8.[25] Silichristin most likely interferes with the SK reaction, by driving the iodide release from substrate or the SK reaction itself. Hence, the DIO1-SK assay results must be disregarded.

In addition to DIO1 inhibition, ketoconazole inhibited ALP activity by 41%. Obviously, any DIO1 inhibition that is observed, in addition to the ALP inhibition, is not strictly specific to DIO1. This is either due to a certain promiscuity of the test substance, making it an inhibitor of two unrelated enzymes, or the test substances interfering with the microsomes, and thus disturbing any activity of its membrane-bound enzymes, for example, by perturbing the lipid membrane or unspecific protein binding. It can, however, never be ruled out that the test substance specifically inhibits both DIO1 and ALP. Ketoconazole is a known inhibitor of CYP3A[26] and produces direct hepatocellular toxicity in humans.[27] Decreased activity of a second liver enzyme that is present in human liver microsomes, the ALP, might hint toward unspecific interaction of the test substance with the microsomal protein. As such, DIO1 inhibition data from ketoconazole was also disregarded.

Comparison to literature data

In vitro data from an U.S. EPA DIO screening[2] is available for 16 of the 22 test substances. Generally, inhibition data are comparable to our study. The maximum concentrations tested are, however, different. While the U.S. EPA largely used fixed upper concentrations of 200 μM in a primary screening run, the upper concentration in our study is determined by solubility pretests and dilutions were tested in all cases. Testing up to higher concentrations might result in higher IC_{50} values and maximum inhibition, as observed for linolenic acid.

Interestingly, the IC_{50} of BADGE was 70 times higher in our study compared to the study by the U.S. EPA. The U.S. EPA study used recombinant enzyme as DIO1 protein source, which lacks metabolic competence compared to liver microsomes. Metabolism of BADGE to DIO1-inactive metabolites by microsomal hydrolysis in the DIO1-SK assay is a likely cause for the different IC_{50}s since BADGE is known to be hydrolyzed *in vitro* by epoxide hydrolases in human, rat, and mouse liver, as well as lung fraction.[28]

The DIO1-SK assay results were compared to another screening by the U.S. EPA, which investigated IYD inhibition.[20] IYDs are different from DIO enzymes: they lack the selenocysteine residue in the catalytic center, belong to the NADH oxidase/flavin reductase superfamily, use iodinated single tyrosine residues as substrate, and are inhibited by different substance classes. However, the method as originally developed[21] and used for the screening is very similar to the DIO1-SK methods of this study and the U.S. EPA DIO assay[2]: all three use a buffer system, DTT (or β-mercaptoethanol) as a reductive agent, and ion-exchange separation using resin and the SK reaction for iodide quantification. Interestingly, four test substances (tannic acid, linoleic acid, hexadecyltrimethyl ammonium bromide, and morin hydrate) are active in IYD and DIO assays.

Apart from morin hydrate, the IC_{50} values are similar in the IYD and the two DIO assays. This inhibitory activity may be due to a common nonspecific effect in all three test systems or concomitant specific effects on DIO and IYD. Whether they interact with DTT, seal the ion-exchange resin, or interfere by another test system-specific effect cannot be clarified. To investigate whether released iodide is prevented from passing through potentially sealed ion-exchange resin, preparations with the test substance and a defined, added iodide concentration that resembles the assay situation could be tested.

The DIO1-SK results of our study were compared to results from computational models by Garcia de Lomana et al.[19] All the test substances that produced more than 40% inhibition in this study were classified as "active" in the DIO1 *in silico* model. The computational model used the ToxCast database and other scientific literature as training sets. Many of the 22 test substances of this study were tested by Olker et al. and are thus part of the ToxCast database, and hence most likely part of the training set of the computational DIO1 model. There is likely a strong agreement between the experimental and computational datasets because there is little independent comparative data.

Fipronil and the structural analog fipronil sulfone were both classified as "active" in the computational model, but did not produce inhibition in our study. This could possibly be attributed to the tested concentrations in Olker et al. (200 μM). This high concentration resulted in precipitations in our study and therefore lower concentrations were tested. Obviously precipitated test substance is contributing to the inhibitory effect, potentially by continuous dissolution from the solid phase. Then again, the nominal concentration, including the precipitate, is neither the actual effective concentration of the test substance nor is it of relevance *in vivo*.

There are only few specific *in vivo* DIO inhibition data available. For 6PTU—one of the substances that was assigned into category 1A by DIO1-SK assay data—effects on DIO1 and TPO are well described *in vitro* as well as *in vivo*.[5,6,29,30] Two unsaturated fatty acids, linoleic and linolenic acid, were categorized as category 1B and category 2, respectively, by DIO1-SK assay data. Data from rats show increased biliary excretion of thyroid metabolites with iodinated outer rings after treatment with ethanol and linoleic acid, suggesting inhibition of outer ring deiodination by unsaturated fatty acids[31]; DIO1 is catalyzing the ring deiodination.

Conversion of T4 to T3 was reduced after treatment of rat liver homogenate with fatty acids like linoleic and linolenic acid.[32] There is evidence that linoleic and linolenic acid also effect other modes of action in the THS, as they are able to displace T4 from serum proteins in human serum.[33] The flavonoid morin was categorized into category 1B by DIO-SK assay data. Several flavonoids, including morin, inhibited the DIO1-catalyzed degradation of rT3 in murine thyroid microsomes.[34]

Pentachlorophenol (PCP) inhibited DIO1 by 43.5% in the DIO1-SK assay and was classified into category 2. Treatment with up to 30 mg/kg b.w. PCP decreased total and free T4 and T3 levels, as well as thyroid-stimulating hormone levels and T4:T3 ratio in Wistar rats, suggesting interference at pituitary or hypothalamic level.[35] PCP downregulated DIO1 transcription in rat pituitary GH3 cells and—in zebrafish embryos—upregulated the expression of genes along the THS, including DIO1 and DIO2.[36] In summary, there are few data on DIO1 inhibition *in vivo* and hence classical reference data linking *in vitro* inhibition directly to *in vivo* inhibition are sparse.

Disturbance of the THS *in vivo*, on the other hand, can hardly be traced back to DIO1 inhibition only. Single methods addressing THS disturbance, like many other effects on complex systems, are neither addressed by a single *in vitro*

assay nor validated one-on-one. Rather a battery of assays addressing different steps are employed and are validated by their physiological or mechanistic relevance.[37,38] Moreover, if *in vitro* data are used, one should keep in mind that the DIO1-SK assay is a cell-free test system. *In vitro* to *in vivo* extrapolation is needed to extrapolate *in vitro* DIO1 inhibition data to external exposure to humans.[39,40]

Data interpretation procedure

For chemical safety assessment, results of the DIO1-SK assay and their relevance toward potential adverse effects on human health need to be clarified. A correlation of generated *in vitro* DIO1 inhibition data to *in vivo* data is difficult because (i) information on DIO1 inhibition *in vivo* is rare, (ii) described *in vivo* DIO1 inhibitors often target multiple thyroid mode of actions, and (iii) the biological relevance of DIO1 inhibition is unclear. In this study, we propose a DIP to assess DIO1 *in vitro* inhibition data in relation to the well-characterized reference item 6PTU and used control items (Table 8). A DIP uses a fixed algorithm (e.g., rules or decision criteria) in the context of a defined approach to interpret data from defined methods and is regularly used in the interpretation of data from NAMs,[41] for example, OECD guideline number 497.[42]

The proposed DIP expands the DIO1-SK assay and allows the testing of unknown test substances for DIO1 inhibition with subsequent categorization (Fig. 6). This categorization potentially informs follow-up activities, for example, prioritization for further testing.

Assessment criteria were derived to assess the DIO1 inhibition qualitatively and quantitatively. 6PTU is known as a complete and potent DIO1 inhibitor. 6PTU is used as the reference item in this method to control its performance. It is also used as a reference to classify the results of a test substance regarding its efficacy (complete vs. incomplete inhibition) and potency (inhibition at low, up to 7.1 μM, and high substance concentrations). Likewise, negative and vehicle controls are used to set thresholds to distinguish inhibitors from noninhibitory substances (efficacy only without additional potency classification for partial inhibitors). These thresholds are based on the variability of the control data from this study, which was performed in one laboratory by different technicians. DIO1-SK assays performed in another laboratory may show a different variability, potentially requiring different thresholds. Therefore, each laboratory should derive historical control data.

It might be beneficial for each laboratory to derive their own historical database and derive thresholds for inhibition assessment. Until this historical database is built, a daily derived IC_{50} value for 6PTU could be derived to estimate DIO1 inhibition. The threshold here could be based on the SD of the derived IC_{50} value (e.g., mean IC_{50} +/−3*SD). This means that the threshold values for the respective laboratory conditions are valid, and a lengthy buildup of laboratory data is prevented. The use of the same controls and performance standards across different laboratories helps to adjust for interlaboratory differences.

The proposed DIP is based on data derived from the DIO1-SK assay. Thus, it may not be directly applicable to data generated by other assays. Hence, we are using the DIO1-SK assay results of four test substances previously published and categorizing them according to our DIP[9]: 5-Propyl-2-thiouracil, an isomer of 6PTU, would be classified in 1A. 2-Chloro-*N*-phenylacetamide fully inhibited DIO1 with an IC_{50} of 7.4 μM and would hence be classified in category 1B. Tetrabromobisphenol A and genistein were poorly soluble and the maximum tested concentration only partially inhibited DIO1 by 51% and 41%, respectively. Accordingly, both substances would be classified in category 2. Other studies tested both substances at higher, potentially not fully soluble, concentrations, leading to complete DIO1 inhibition.[3,5]

Obviously, undissolved fraction of the test substances can contribute to the inhibitory effect *in vitro*, which is probably of no relevance to the *in vivo* situation. This demonstrates the importance of carefully examining the solubility of test substances and setting relevant upper concentrations. We have also tested gold-containing substances, including organic substances like the positive control ATG, sodium aurothiomalate or auranofin, and inorganic salts [gold(I) chloride, gold(III) chloride] in the DIO1-SK assay.[43] All tested organic and inorganic gold substances fully inhibited DIO1. The least potent gold substance was gold(I) chloride with an IC_{50} of 0.95 μM. Thus, all tested gold-containing substances would be classified in category 1A.

Conclusion

The DIO1-SK had previously been demonstrated to be reproducible.[9] In this study, the predictivity of the assay has been demonstrated: The results were obtained by testing 22

TABLE 8. DATA INTERPRETATION PROCEDURE OF DIO1 *IN VITRO* INHIBITION IN THE DIO1-SK ASSAY

Category (by efficacy)	*Subcategory (by potency)*	*Inhibition activity*	*Threshold*
Category 1: full inhibitor	A: Potent full inhibitor	Fully inhibits DIO1 activity at a concentration comparable to the reference item 6PTU	Max. inhibition greater 90% and IC_{50} at or below upper range IC_{50} of 6PTU (7.1 μM in this study)
	B: Weak full inhibitor	Fully inhibits DIO1 at higher concentrations than the reference item 6PTU	Max. inhibition greater 90% and IC_{50} above upper range IC_{50} of 6PTU (7.1 μM in this study)
Category 2: partial inhibitor	—	No full inhibition, but greater than negative and solvent control	Max. inhibition between 25% and 90%
Category 3: not inhibitor	—	Does not inhibit DIO1 (same as negative and solvent control)	Max. inhibition below 25%

The model uses the generated qualitative maximum inhibition data of control data to define categories ("by efficacy") and the quantitative IC_{50} values of the reference item 6PTU to subcategorize category 1 test substances ("by potency").

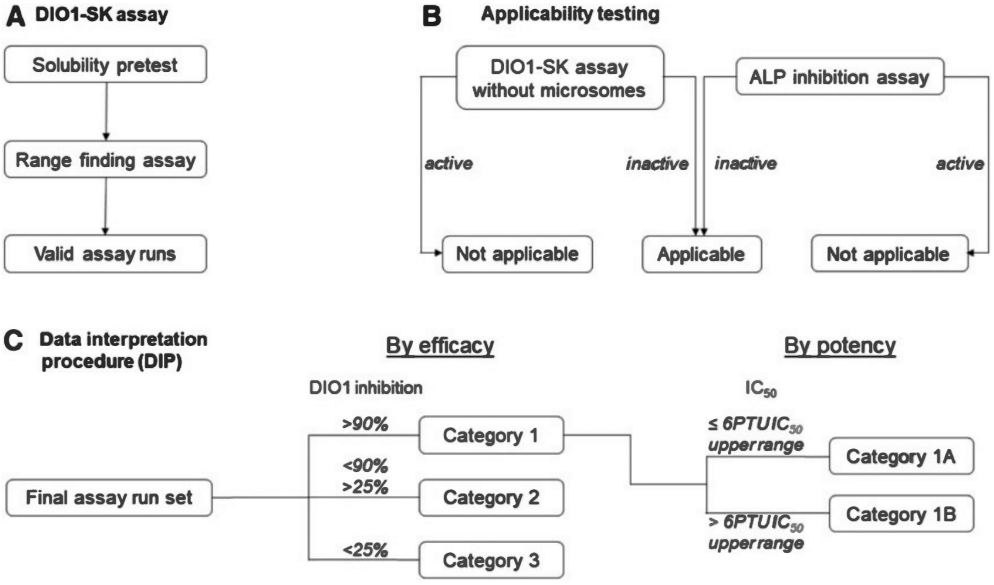

FIG. 6. Testing strategy for unknown test substances in the DIO1-SK assay. The highest assay concentration is defined in a solubility pretest and used for testing in an initial range-finding assay. If DIO1 inhibition was observed, concentrations were changed for the following assay runs. In any case, at least three valid assay runs are performed. **(A)** If DIO1 inhibition was greater than 20% in the range-finding assay, the specificity of observed DIO1 inhibition is checked using the DIO1-SK assay without microsomes and the ALP inhibition assay. If the test substance is active in either of the two specificity assays, the test substance is not applicable in the DIO1-SK assay **(B)**. Test substances are categorized using a DIP by efficacy using the maximum inhibition and by potency using the IC_{50} of the test substance **(C)**. DIP, data interpretation procedure.

blinded test substances and, after deblinding, were compared to already published *in vitro* and *in silico* data. As the results of the DIO1-SK assay are intended to be used for regulatory decision making, we propose to use inhibitor categories rather than IC_{50} values. A DIP using maximum inhibition (efficacy) and IC_{50} (potency) was used to classify test substances into "potent full inhibitors" (category 1A), "weak full inhibitors" (category 1B), "partial inhibitors" (category 2), and "no inhibitors" (category 3).

Inhibition categories as determined by the DIO1-SK assay were in accordance with previously published data. To avoid mistaking unspecific activities of a test substance for specific DIO1 inhibition, two control assays were used (DIO1-SK without microsomes and ALP assay). Based on these, 2 of the 22 test substances were excluded from the assessment of their DIO1 inhibition activity as being nonspecific.

With this, we developed a reproducible DIO1 inhibition assay, including controls for unspecific effects and a DIP to transfer assay results into inhibition categories, which are pertinent to regulatory decision making. Based on previously available *in vitro* and *in silico* data, the results of this DIO1-SK assay with its DIP are predictive. *In vivo* data as reference are, however, not available, and hence the *in vivo* relevance of *in vitro* assay results is not yet explored. Obviously, DIO1 inhibition is but one of many events, which could disturb the THS leading to adverse effects. Ultimately, the DIO1-SK assay must be combined with other *in vitro* and *in silico* assays addressing other disturbances of the THS. DIO1 inhibition along with other key events of THS disturbance need linking to THS AOP networks[44] to allow valid conclusions on the hazard of a test substance and risk arising from the exposure to this substance.

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Author Disclosure Statement

The authors A.G.W., B.B., V.G., S.S., D.F.-W., and R.L. are employees of BASF SE, a chemical company, which may use the DIO1 assay to develop and register commercial products in the future. S.H., S.C., and K.R. declare no conflict of interests.

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Supplementary Material

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4. CONCLUSION

4.1 Identifying and addressing pitfalls of the DIO1-SK assay

There is increasing concern about adverse effects based on the exposure to endocrine active substances, both in public and scientific communities. In addition, the EU wants to switch from animal testing (*in vivo*) to NAMs (*in vitro*, *in silico, in chemico* and *ex vivo*) for testing of substances. Therefore, thyroid NAMs are needed that can predict endocrine adverse effects in a similar way. Currently, there is NAM-based OECD guideline for the detection of thyroid effects available. The development of new *in vitro* methods is challenging for several reasons. GIVIMP can help to reduce uncertainties of *in vitro* methods by applying scientific, technical and quality practices and gives advice for potential pitfalls and to address occurring challenges (144). Pitfalls and challenges in the context of the DIO1-SK and GIVIMP are illustrated below, marked in *italic*.

Test system

In vitro methods can use many types of test systems. They can range from simple enzyme assays to demanding cell culturing methods. Enzyme assays are often cheaper and faster to perform but are unable to offer the level of information as cell culturing systems. Enzyme assays are usually only able to test one enzyme as an inhibition target. Cell culturing systems involve whole living cells, allowing more physiological testing. In addition, human cells can be used. For cell cultures and human *ex vivo* material, the absence of known human pathogens must therefore be proven.

The DIO1-SK assay uses human liver microsomes as a source for DIO1 enzyme. Other methods investigated substance-induced DIO1 inhibition by using recombinant enzyme (106, 110). This opens testing of all three isoenzymes of the deiodinases. *Ex vivo* material is only available for DIO1 in larger amounts. DIO2 and DIO3 are primarily expressed in tissues where obtaining sufficient cell material is challenging as well as having ethical issues (see Table 1). The source of recombinant enzyme is often of academic nature and there are no commercial suppliers, as in the case of recombinant DIO enzyme. This complicates the global use of the test system in a method. Human liver microsomes can be purchased in sufficient quantities from several international suppliers. As such, they are superior to recombinant enzyme for an aspiring, globally used OECD method. This might change if well characterized, recombinant DIO enzyme is commercially available.

The use of microsomes has several advantages and disadvantages compared to using primary cells like human hepatocytes as enzyme source. Primary hepatocytes can differ in

differentiation state, are usually limited in quantity and number of donors, and require more expertise in culturing (188), making them more expensive. As a living, functional cell however, they possess more enzymes and thus greater metabolization competence compared to microsomes. Testing for DIO1 inhibition in primary human hepatocytes might help to confirm results from the DIO1-SK assay.

Test substances and control items

The performance of an *in vitro* method can change over time. Substances are needed to control reproducible results that specifically inhibit the target or that demonstrably have no effect. These substances are called control items. GIVIMP differentiates between reference items, and positive, negative as well as solvent controls (or vehicle controls) (Table 5).

For the DIO1-SK assay, the control items were defined early in the development of the assay (Table 5). 6PTU was defined as the reference item because the mechanism of DIO1 inhibition is well understood (87, 189) and there is sufficient *in vitro* as well as *in vivo* data available. Based on the use as a pharmaceutical against hyperthyroidism, there are also human data available (190, 191).

Aurothioglucose (ATG) was defined as the positive control in the method. The gold moiety inhibits DIO1 competitively based on high affinity of the gold towards the selenocysteine of DIO1 enzyme (104, 105). ATG fully inhibits DIO1 activity in vitro (106, 107). Finding a suitable negative control for the assay is more challenging since most substances should have no effect on DIO1. A substance with a similar chemical structure as the positive control ATG without effect on DIO1 was chosen. 1-Thio-β-D-glucose sodium salt lacks the gold ligand, therefore having no effect on DIO1 (104, 107), and was defined as the negative control.

Dimethyl sulfoxide (DMSO) was defined as a suitable solvent for the assay since most organic solvents are soluble. For cell culture methods, DMSO might not be the solvent of choice since DMSO can show high cytotoxicity in many cell types as well as affecting differentiation (192, 193). In the DIO1-SK assay, 1% DMSO had no effect on deiodination activity (data not shown).

Many *in vitro* methods have a limited applicability domain, potentially excluding testing of poorly soluble substances or certain groups of substances such as liquids or nanoparticles (144). During the performed studies, a variety of test items were tested in the DIO1-SK assay including liquids, solids, powders, inorganic salts as well as nanoparticles. An even distribution of the test item can be guaranteed since the incubation with the microsomes takes place under constant shaking.

Table 5: Definition of control items according to GIVIMP (144) and their adaption in the context of the DIO1-SK assay, marked in italic.

Control item	Function
Reference item	Quantitative control to grade the response of the test system to the test item. The response of the reference item shall be well described as it is used as a basis for comparison with the test item. *Used in this method: 6-Propyl-2-thiouracil (6PTU) at a maximum assay concentration of 10^{-3} M tested on each assay plate. Concentration-response curves of 6PTU ranged from 10^{-3} to 10^{-8} M.*
Positive control	Qualitative control that has a consistent and predictive effect on the test system. *Used in this method: Aurothioglucose (ATG) at a maximum assay concentration of 10^{-4} M.*
Negative control	Qualitative control that does not lead to a response in the test system. *Used in this method: 1-Thio-β-D-glucose sodium salt at a maximum assay concentration of 10^{-4} M on each assay plate.*
Solvent control	Blank control. The solvent control ensures that the response does not originate from the applied solvent and shows no response in the method. *Used in this method: Dimethyl sulfoxide (DMSO) at a concentration of 1%.*

Test concentrations and solubility

The highest concentrations tested can have major impact on the predictivity of a test system. The maximum solubility is often used as the upper limit of testing. If concentrations above the solubility are tested, precipitations occur which can physically affect the test system. In addition, the nominal concentrations applied do not correspond to the bioavailable, dissolved concentrations. This might influence the reproducibility as well as significance of generated results. Precipitations could also represent a reservoir of steady supply of bioavailable molecules that might enhance an observed effect in the test system. However, the relevance to the *in vivo* situation is questionable since undissolved substances are unlikely to be absorbed systemically.

In the DIO1-SK assay, the highest assay concentration was defined as 1 mM. This is in line with the OECD accepted guideline 493: “Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) In Vitro Assays to Detect Chemicals with ER Binding Affinity” which defined that final concentrations tested in the assay should not exceed 1 mM (194). Serial dilutions were used for testing in an initial range finding experiment. If effects in the test system were observed,

Substances are exclusively tested in dissolved concentrations. The solubility was guaranteed using a detailed testing scheme. All substance dilutions prepared in the assay are checked beforehand by microscopical analysis. Some substances cannot be categorized as soluble or insoluble at certain concentrations. They are microscopically visible as small bubbles or tiny dots, also called homogenous solutions. Homogenous solutions of substances might also be accepted for testing.

Testing for specificity of observed interaction and increasing predictivity

The predictivity of an *in vitro* method depends on its ability to exclude unspecific interference by the test substance. These are often unspecific interactions with the test system itself or the endpoint/measurement method. Cell viability assay often use whole cell number counting, metabolic activity of catalytic enzymes or membrane integrity as an indicator to determine the proportion of living cells, testing for interference with the test system. Depending on the used endpoint, cell viability assays can provide information about early substance-induced toxic effects, like initial changes in cell metabolism. Other endpoints display later steps of toxicity mechanisms, cell death either by apoptosis or necrosis. They rely on the leakage of intracellular metabolites in surrounding media, or the entrance of dyes to visualize cell viability. Testing for cell viability is complex and can be influenced by culture conditions, cell number, used material and reagents, and by the substance itself.

Testing for interference with the measurement method is highly dependent on the endpoint. In luciferase-based methods, some substances can inhibit the endpoint-specific luciferase itself and have no effect on the endpoint of interest (195, 196). Fluorescence-based methods need to rule out substances with fluorescent capabilities e.g., by fluorescent impurities (196). Creative and adequate methods to ensure specificity are needed.

For enzyme assays, there are also possibilities to differentiate between non-specific and target-mediated toxicity. The used endpoint in the DIO1-SK assay is the SK reaction, specifically the catalytic property of iodide on the SK reaction. Many ions are known to drive the SK reaction itself, even without iodide present (175, 197, 198). Therefore, an additional test was added to the method, which examines the effect of the test substance on the SK reaction. This test uses a regular assay, but without the addition of the test system, the human

liver microsomes. This way, no iodide is formed, and an ongoing SK reaction indicates interference by the test substance. This test would also detect substances that can cleave or exchange iodide from the used substrate, rT3. It is important to perform this specificity test in the whole assay and not just add the test item to the SK reaction, as the impact of the entire method on the test substance and vice versa is preserved.

There are readouts available to test for DIO activity that measure the forming TH metabolites. This readout is obviously a more specific approach as no additional chemical reaction as the assays readout is involved. Multiple studies used liquid chromatography-tandem mass spectrometry (LC-MS/MS) as quantification method to detect substance-induced inhibition of DIO activity (126, 173). The method, however, is demanding in cost and expertise, both in laboratory handling and data interpretation, and testing of many substances is not feasible. Still, testing of substances that were positive in the DIO1-SK assay in a more specific test system, such as LC-MS/MS, might further improve the significance and verify the derived DIO1 inhibition assessment of the substance.

To test for interference of the test substance with the test system, a similar approach to cytotoxic effects in cell culture systems was chosen. If a secondary enzyme, in addition to DIO1, is inhibited, this may indicate effects on the microsomes, rather than both enzymes. Nonetheless, specific inhibition of both enzymes cannot be ruled out if the test substance is active in both assays. For the DIO1-SK assay, a second enzyme (Alkaline Phosphatase) that occurs in human liver microsomes was tested for its activity. If both DIO1 and the alkaline phosphatase are inhibited by a test item, this might indicate non-specific protein inhibition.

Whether both specificity tests can prevent false positive test substances must be investigated in future runs including more test items. During the testing of a blinded substance set in the context of the EURL ECVAM validation, two substances could be excluded from further analysis as possibly interfering. To rule out that cytotoxic concentrations are tested in the DIO1-SK assay, primary or immortalized hepatocytes could be cultivated, treated with the substance and cell viability can be analysed using a cell viability readout. The transfer of generated results to the DIO1-SK assay is still challenging and the differing biokinetics in the assays would have to be translated by modelling.

Reproducibility and predictivity

In addition to increasing confidence in the specificity of the observed *in vitro* effect, the predictivity of an *in vitro* method also depends on its ability to emulate the toxicokinetics of living organisms. Many test systems are unable to capture *in vivo* effects based on missing xenobiotic metabolization. Some substances exert their toxic effect only after metabolization in phase I or phase II, generating the toxicological active form. Also, metabolism can lead to

increased elimination of an active toxicant, thus preventing the accumulation of the substance and potential toxic effects. *In vitro* assays that lack a functional metabolization comparable to the *in vivo* situation, can either under- or overestimate the toxic effect of a substance, depending on whether the toxic metabolite is formed, or the toxic form is not eliminated. Integration of a metabolization step is therefore crucial for the interpretation of generated in *vitro* results and can help to fill the gap between data discrepancies of *in vivo* and *in vitro* data (199). Cellular test systems already contain some toxicokinetic properties such as cellular uptake or different metabolizing enzymes. Organ-on-a-chip methods can be amended by organ systems such as the liver to achieve metabolic competence (200). In the DIO1-SK assay, the used enzyme source, human liver microsomes, possess many metabolizing enzymes. In the current form of the assay, no additional cofactors that are required for functional enzymatic metabolization are added. As such only the cofactor-independent enzymes like epoxide hydrolases or esterases are presumably active in the assay, potentially explaining observed differences between data generated in our study and literature data (183, 187) Using functional enzyme assays for typical phase I and phase II metabolic enzymes, it should be investigated whether the addition of different cofactors to human liver microsome preparations can lead to a functional metabolic competence comparable to liver cells. Otherwise, a metabolic competence can possibly be achieved by adding enzyme-rich liver fractions such as S9 mix, a liver homogenate mix.

Many published *in vitro* methods lack information on their reproducibility. They are often only carried out in one laboratory by a few people. Published information on methodology is often poor, significantly increasing the difficulty of data reproduction. Robust assessment criteria are needed to ensure consistent performance of the method. Historic control databases need to be created and can help to monitor and recognize changes in method performance. The generated data of the studies with the DIO1-SK assay were conducted by three different people. No influence on method performance was determined. Detailed SOPs including precise information on all work steps were made publicly available. In addition, advice for the first steps of establishing the method in laboratories was published (183). Historical data from all performed assays was created and monitored closely. Using the historical control data of the control items, multiple assessment criteria to assess assay performance were established. These must be met to classify an assay run as valid and to be evaluated. Set acceptance criteria were mostly met. Monitoring of assessment criteria helped to identify potential issues with assay performance, especially after changes in assay material and batch variation of assay constituents or microsomes (183).

The generation of *in vitro* data is only the first step in the evaluation of an endpoint. To estimate the degree of an observed effect, a prediction model for categorization is needed. This is usually achieved through assay-specific thresholds and dichotomous classification that

separates effects into active and inactive. We established a DIP that uses the assays variability of used control items to categorize test items based on maximum inhibition (“efficacy”) and their IC_{50}s (“potency”). Compared to available *in vitro* and *in silico* DIO1 inhibition data, the DIO1-SK assay data that was categorized using the DIP was comparable (187).

Each test system has natural uncertainties and variability, resulting from the sum of all experimental steps performed. As such, there can be effect ranges that can be classified neither as active nor inactive. These ranges are called borderline ranges. They are an extension to classic prediction models by addition of the method-internal data variability (201). This methodology can also be applied in the DIO1-SK assay. This will be of special importance, once data on the variability between different laboratories is available.

4.2 Extrapolation of *in vitro* results

Comparison of generated data to *in vivo* DIO1 inhibitors is usually done to show the predictivity of the predictivity of an *in vitro* assay. However, *in vivo* substance data on DIO1 inhibition are scarce. And even if available, the *in vivo* data are mostly specific to adverse effects and the affected KE was often not examined. The KE of DIO1 inhibition is only one of many KEs in the THS and AOP networks (see Figure 2). NAMs might be required for each of these endpoints. Exemplary, inhibitors for the uptake of iodide via the NIS or the synthesis of TH via TPO are well described, and observed effects can be connected to adverse effects in humans; test systems to investigate substance-induced inhibition are available. For other KEs such as the modulation of the TSH receptor, there are only a few known, mostly synthetically produced substances available (202, 203). Whether these are a potential threat or no frequent target of substance-induced inhibition is unclear. For the case of DIO1 as an affected KE, the comparison to substances that have effects on thyroid parameters *in vivo* would be inaccurate since DIO1 inhibition is not directly associated with changes in classic thyroid parameters such as T3/T4 serum levels or histopathological changes (95). Further basic research on *in vivo* DIO1 inhibitors is needed to allow a predictivity estimation for DIO1 using *in vivo* data.

Once established, *in vitro* and *in silico* assays targeting individual KEs in the THS must be integrated in an integrated approach to testing and assessment (IATA), covering all relevant KEs and integrating multiple methods of the THS. An IATA has been developed for skin sensitization, integrating *in chemico*, *in vitro* and *in vivo* methods (204). A comparison of a defined approach using DIO1 inhibition as well as information from methods targeting other thyroid KEs to thyroid *in vitro* data would be more accurate and will likely result in a higher predictivity. We developed a DIP to categorize generated data in the DIO1-SK assay into categories to aid data interpretation, also at a regulatory level. At the time of this work, neither the development and validation of the other THS covering *in vitro* methods of the EURL

ECVAM validation project nor data on substance-inhibition had been published. As such, the development of an IATA was not scope of this work.

The integration of toxicokinetic modelling including IVIVE and PBPK will be needed to extrapolate *in vitro* data to the *in vivo* situation of humans (162). The resulting battery of test methods must be extensively validated by using different chemical classes to check its applicability and predictability, and to prevent over- or under sensitive assessments.

5. OUTLOOK

Using standardization and optimization measures, a robust and reproducible assay could be created that can investigate *in vitro* DIO1 inhibition by test substances. A data interpretation procedure (DIP) that uses the variability of the DIO1-SK data of these studies was generated to interpret the results. So far, these data have only been generated in one laboratory by different laboratory staff, showing intralaboratory reproducibility. Comparable data must be generated in other laboratories to demonstrate the transferability of the method as well as interlaboratory reproducibility. Likewise, the DIP must be examined for its suitability. Data generation in NAMs like the DIO1-SK assay can be performed much faster compared to animal studies. As such, testing of complex mixtures or nanoparticles can be performed cost-effective in medium-throughput *in vitro* assays.

The data of this method must be compared to other thyroid data to be able to estimate the predictivity of the method. Comparison to *in vivo* data is not the appropriate choice due to (i) lack of *in vivo* data for most substances, (ii) inhibition of multiple KE for many described *in vivo* inhibitors and (iii) the uncertainty of the biological relevance of DIO1 inhibition on assessed thyroid-related adverse effects in animal studies. Comparison to other more specific methods that address the KE of DIO1 inhibition like LC-MS/MS based methods or cell-based methods (organ-on-a-chip, thyroid cell models) is a more appropriate method to show the methods predictivity. Since this data is not available at this time for the used substances, this data must be generated. In addition, the KE of DIO1 inhibition needs to be further investigated and possible associated adversities need to be shown.

In silico modelling of receptor binding is available for multiple endocrine endpoints (205, 206). Regarding the DIOs, a computer-aided drug design approach was published which modelled DIO3 and used binding site analysis to identify selective ligands that specifically bind to DIO3 (207). Modelling of DIO1 and comparing the binding site information of potential inhibitors that were identified in the DIO1-SK assay could provide further evidence on the specificity of observed inhibition. An assessment on the human situation using the generated *in vitro* DIO1 inhibition data will only be possible in combination with computational models such as PBPK or IVIVE to extrapolate to an organismal level.

An IATA integrating all methods covering the different KEs in the THS (either as individual methods for each KE or using holistic models) must be generated. It must be noted that validated assays, positive substances, and prediction models are missing for many KEs. Also, assays need to be optimized and standardized to increase their reproducibility. High predictability can only be shown in extensive validation studies. The connected costs with these studies are high. Very few publications deal with the reproducibility of a method and rather try to develop new, technologically complex methods. Research funds are needed to

encourage researchers and companies to demonstrate the reproducibility and predictivity of *in vitro* methods. Only this way, methods can gain scientific and regulatory acceptance and will be fit for regular use. In addition, the current regulatory process from method development to an accepted OECD guideline takes many years. With the current processes, it may be several decades before subchronic animal experiments might be replaced (208).

6. SUMMARY (ENGLISH)

The human body can be targeted by many man-made and natural-occurring chemicals, potentially leading to adverse effects. This includes the endocrine system, essentially consisting of the EATS modalities (estrogen, androgen, thyroid, and steroidogenesis). Substances that interfere with the endocrine systems are called endocrine active substances and can finally be assessed as endocrine disruptors. Endocrine disruptors cannot be registered in the EU, unless exposure to the environment is negligible.

The thyroid hormone system (THS) is important in regulating various physiological processes, including metabolism, energy regulation, and organ function. The THS controls gene expression and pathways that impact metabolism, thermoregulation, cardiovascular function, and neural connectivity. Perturbations in the THS can lead to increased or decreased thyroid hormone (TH) signalling as well as thyroid-related diseases including cancer. The developing foetus is dependent on maternal TH supplementation, and very susceptible towards alterations in TH supply. Resulting developmental changes are also called developmental neurotoxicity.

Testing for THS parameters is routinely done in animal experiments. The EU wants to accelerate the switch from animal to non-animal methods (NAMs). Currently, there is no OECD validated NAM for the THS available. Newly developed NAMs should be standardized and reproducible. They should adhere to the Guidance Document on Good In Vitro Method Practices (GIVIMP) that provides guidance on critical aspects for the development of reliable and reproducible *in vitro* methods.

The THS is a complex signalling pathway that includes production, secretion, transport, uptake, signalling and metabolization of TH as well as a negative feedback loop to control TH release. The iodothyronine deiodinases (DIO) are one important key event (KE) in the THS and is linked to adverse neurodevelopmental outcomes in mammals. DIOs regulate TH signalling by metabolizing TH to more or less active TH metabolites.

Historically, testing for DIO1 inhibition was done in animals. A novel *in vitro* method using mouse liver enzyme as a source for DIO1 and the Sandell-Kolthoff (SK) reaction to quantify released iodide from a substrate was published in 2014. This method is part of a validation study led by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) with the goal to develop NAMs that cover different endpoints in the THS. The validation consists of two parts where the reproducibility of the method is demonstrated (part 1) and the method is checked for its relevance and reliability (part 2).

In this work, the DIO1-SK assay was optimized and standardized. Mouse liver microsomes were substituted by human liver microsomes to adhere to the human situation. Variability of generated results was reduced by implementing measures to control batch-specific microsome

activity, inherent activity of assay constituents in the SK reaction and optimal enzymatic iodide release during microsome incubation. Linear reaction kinetics in the SK reaction was ensured by optimizing incubation time and variable SK reagent concentrations. The reproducibility of the DIO1-SK assay was demonstrated by testing of six described DIO1 inhibitors in at least five independent assay runs (part 1). Guidance for establishing the method at other laboratories was provided; an initial Standard Operation Procedure (SOP) as well as acceptance criteria to assess the validity of future runs were defined.

Using the derived SOP, the DIO1-SK assay was tested for its predictivity using a described class of DIO inhibitors, gold-containing substances. As such, organic, inorganic gold substances as well as gold nanoparticles were tested. A strategic approach using the mentioned gold substances as well as substances lacking the gold moiety was chosen to verify that the observed DIO1-inhibition was mediated by the gold. All tested organic and inorganic gold substances produced DIO1 inhibition at concentrations comparable to the literature. Structural analogues lacking the gold moiety did not produce DIO1 inhibition. Interestingly, small gold nanoparticles (5 nm) caused DIO1 inhibition, while bigger nanoparticles (>30 nm) did not. It was verified that the observed inhibition was not caused by dissolved gold in the incubation media. This study verified the potential of the DIO1-SK assay to predict DIO1 inhibition and has shown the applicability of the method towards nanoparticles.

For the part 2 of the validation study of EURL ECVAM, the predictivity of the assay was assessed. A blinded set of substances, provided by the EU, was tested in the method. This included thorough solubility testing as well as two newly introduced methods to assess the specificity of observed DIO1 inhibition. Upon experimental completion, the data was deblinded and compared to available literature data. A data interpretation procedure (DIP) using the variability of the assay was used to categorize the results. Multiple substances produced high inhibition. The introduced specificity methods identified two substances as method-interfering leading to exclusion for further analysis. Inhibition data was consistent with available *in vitro* and *in silico* literature data showing the methods relevance. A DIP was derived using three categories classifying substance into full, partial or no inhibitor based on efficacy. Additionally, full inhibitors were subcategorized into potent and weak full inhibitors using the substances IC_{50} (“potency”). An updated SOP was derived and published to allow other laboratories to use the method.

In this thesis, an *in vitro* method was developed that predicts the KE of DIO1 inhibition optimized, standardized, and reproducibly. It includes methods to exclude undissolved substance, observed effects that are not specific to inhibition of DIO1 and a method-specific DIP that classifies the generated data based on assay-inherent variability. The work presented is the basis for the acceptance of the method at OECD level and is a forerunner in the validation

study coordinated by EURL ECVAM. Once methods covering other KEs in the THS are validated, they must be combined with the DIO1-SK assay to form an *in vitro* test battery. This includes computational methods that can predict different KEs and modelling that extrapolates enzymatic data to an organismal level. The current acceptance process for methods on an OECD level takes multiple years. Acceptance processes for NAMs at the OECD must be accelerated to make validated methods available more quickly and further drive the switch from animal to non-animal testing.

7. SUMMARY (GERMAN)

Viele menschengemachte und natürlich vorkommende Chemikalien können auf den menschlichen Körper einwirken und möglicherweise schädliche Effekte haben. Dazu gehört das endokrine System, das im Wesentlichen aus den EATS-Modalitäten (Östrogen, Androgen, Schilddrüse und Steroide) besteht. Stoffe, die in das endokrine System eingreifen, werden als endokrin aktive Stoffe bezeichnet und können letztlich als endokrine Disruptoren eingestuft werden. Endokrine Disruptoren können in der EU nicht registriert werden, es sei denn, die Exposition gegenüber der Umwelt ist vernachlässigbar.

Das Schilddrüsenhormonsystem (THS) ist wichtig für die Regulierung verschiedener physiologischer Prozesse, einschließlich Stoffwechsel, Energieregulierung und Organfunktion. Das THS steuert die Genexpression und Signalwege, die den Stoffwechsel, die Thermoregulation, die Herz-Kreislauf-Funktion und die neuronale Konnektivität beeinflussen. Störungen im THS können zu einer erhöhten oder verminderten Schilddrüsenhormon (TH)-Signalübertragung sowie zu Schilddrüsenerkrankungen, einschließlich Krebs, führen. Der sich entwickelnde Fötus ist besonders auf die mütterliche TH-Ergänzung angewiesen und daher anfällig für Veränderungen in der TH-Versorgung. Daraus resultierende Entwicklungsveränderungen werden auch als Entwicklungsneurotoxizität bezeichnet.

Die Prüfung der THS-Parameter erfolgt routinemäßig in Tierversuchen. Die EU will die Umstellung von tierischen zu tierversuchsfreien Methoden(NAMs) genannt, beschleunigen. Derzeit ist keine von der OECD validiertes NAM für das THS verfügbar. Neu entwickelte NAMs sollten standardisiert und reproduzierbar sein. Sie sollten sich an dem *Guidance Document on Good In Vitro Method Practices* (GIVIMP) orientieren, das Leitlinien zu kritischen Aspekten der Entwicklung zuverlässiger und reproduzierbarer *in-vitro* Methoden bietet.

Das THS ist ein komplexer Signalweg, der Produktion, Sekretion, Transport, Aufnahme, Signalübertragung und Metabolisierung des TH sowie eine negative Rückkopplungsschleife zur Steuerung der TH-Freisetzung umfasst. Die Iodothyronin-Deiodasen (DIO) sind ein wichtiges Schlüsselereignis (KE) im THS und werden mit negativen Folgen für die neurologische Entwicklung bei Säugetieren in Verbindung gebracht. DIOs regulieren die TH-Signalübertragung, indem sie TH zu mehr oder weniger aktiven TH-Metaboliten metabolisieren.

In der Vergangenheit wurden Tests zur DIO1-Inhibition an Tieren durchgeführt. Im Jahr 2014 wurde eine neuartige *in-vitro* Methode veröffentlicht, die Mausleberenzym als Quelle für DIO1 und die Sandell-Kolthoff (SK)-Reaktion nutzt, um freigesetztes Iodid aus einem Substrat zu quantifizieren. Diese Methode ist Teil einer Validierungsstudie unter der Leitung des Referenzlabors der Europäischen Union für Alternativen zu Tierversuchen (EURL ECVAM) mit

dem Ziel NAMs zu entwickeln, die verschiedenen Endpunkte im THS abdecken. Die Validierung besteht aus zwei Teilen, in denen die Reproduzierbarkeit der Methode nachgewiesen wird (Teil 1) und die Methode auf ihre Relevanz und Zuverlässigkeit überprüft wird (Teil 2).

In dieser Arbeit wurde der DIO1-SK-Assay optimiert und standardisiert. Mauslebermikrosomen wurden durch menschliche Lebermikrosomen ersetzt, um der menschlichen Situation zu entsprechen. Die Variabilität der generierten Ergebnisse wurde durch die Implementierung von Maßnahmen zur Kontrolle der chargenspezifischen Mikrosomenaktivität, der inhärenten Aktivität der Testbestandteile in der SK-Reaktion und der optimalen enzymatischen Iodidfreisetzung während der Mikrosomeninkubation verringert. Die lineare Reaktionskinetik der SK-Reaktion wurde durch die Optimierung der Inkubationszeit und variabler SK-Reagenzienkonzentrationen sichergestellt. Die Reproduzierbarkeit des DIO1-SK-Assays wurde durch das Testen von sechs beschriebenen DIO1-Inhibitoren in mindestens fünf unabhängigen Wiederholungen nachgewiesen (Teil 1). Es wurden Informationen zur Etablierung der Methode in anderen Laboratorien bereitgestellt. Eine initiale Standardarbeitsanweisung (SOP) sowie Akzeptanzkriterien zur Beurteilung der Gültigkeit zukünftiger Läufe definiert.

Unter Verwendung der abgeleiteten SOP wurde der DIO1-SK-Assay auf seine Vorhersagekraft unter Verwendung einer beschriebenen Klasse von DIO-Inhibitoren, goldhaltigen Substanzen, getestet. Dabei wurden sowohl organische, anorganische Goldsubstanzen als auch Goldnanopartikel getestet. Um zu verifizieren, dass die beobachtete DIO1-Hemmung durch das Gold vermittelt wurde, wurde ein strategischer Ansatz gewählt, bei dem die genannten Goldsubstanzen sowie Substanzen ohne Goldanteil verwendet wurden. Alle getesteten organischen und anorganischen Goldsubstanzen führten verglichen zu Literaturdaten zur kompletten DIO1-Inhibition bei vergleichbaren Konzentrationen. Strukturanaloge ohne Goldanteil führten nicht zu einer DIO1-Hemmung. Interessanterweise verursachten kleine Goldnanopartikel (5 nm) zu einer DIO1-Hemmung, größere Nanopartikel (>30 nm) hingegen nicht. Es wurde sichergestellt, dass die beobachtete Hemmung nicht durch gelöstes Gold in den Inkubationsmedien verursacht wurde. Diese Studie bestätigte das Potenzial des DIO1-SK-Assays zur Vorhersage der DIO1-Inhibition und zeigte die Anwendbarkeit der Methode auch auf Nanopartikel.

Für Teil 2 der Validierungsstudie von EURL ECVAM wurde die Vorhersagekraft des Assays bewertet. In der Methode wurden von der EU bereitgestellte, verblindete Substanzen getestet. Dazu gehörten gründliche Löslichkeitstests sowie zwei neu eingeführte Methoden zur Beurteilung der Spezifität der beobachteten DIO1-Inhibition. Nach Abschluss des Experiments wurden die Daten entblindet und mit verfügbaren Literaturdaten verglichen. Zur

Kategorisierung der Ergebnisse wurde ein Dateninterpretationsverfahren (DIP) verwendet, das die Variabilität des Assays nutzte. Mehrere Substanzen führten zu einer starken Hemmung von DIO1. Die eingeführten Spezifitätsmethoden identifizierten zwei Substanzen als interferierend, was zum Ausschluss für die weitere Analyse führte. Die Inhibitionsdaten stimmten mit den verfügbaren *in-vitro* und *in-silico* Literaturdaten überein und zeigen die Relevanz der Methode. Ein DIP wurde mithilfe von drei Kategorien abgeleitet, die die Substanz basierend auf ihrer Wirksamkeit in vollständige, teilweise oder Nicht-Inhibitoren einteilten. Zusätzlich wurden vollständige Inhibitoren anhand der IC_{50} der Substanz („Potenz“) in potente und schwache, vollständige Inhibitoren unterteilt. Eine aktualisierte SOP wurde erstellt und veröffentlicht, um anderen Laboren die Nutzung der Methode zu ermöglichen.

In dieser Arbeit wurde eine *in-vitro* Methode entwickelt, die das KE der DIO1-Hemmung optimiert, standardisiert und reproduzierbar vorhersagen kann. Es umfasst Methoden zum Ausschluss des Testens von ungelösten Substanzen, beobachtete Effekte, die nicht spezifisch für die Hemmung von DIO1 sind, und einen methodenspezifischen DIP, der die generierten Daten basierend auf der testinhärenten Variabilität klassifiziert. Die vorgestellte Arbeit bildet die Grundlage für die Akzeptanz der Methode auf OECD-Ebene und ist Vorreiter der von EURL ECVAM koordinierten Validierungsstudie. Sobald Methoden validiert sind, die andere KEs im THS abdecken, müssen sie mit dem DIO1-SK-Assay kombiniert werden, um eine *in-vitro* Testbatterie zu bilden. Dazu gehören computerbasierte Methoden, die verschiedene KEs vorhersagen können, und Modellierungen, die die enzymatischen Daten auf die Ebene des Organismus extrapolieren können. Der aktuelle Akzeptanzprozess für Methoden auf OECD-Ebene ist langwierig und dauert mehrere Jahre. Die Akzeptanzprozesse für NAMs bei der OECD müssen beschleunigt werden, um validierte Methoden schneller verfügbar zu machen und die Umstellung der Toxizitätstestung mit Tierversuchen auf tierversuchsfreie Tests weiter voranzutreiben.

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