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Do new generations of active pharmaceuticals for human use require an adaption of the environmental risk assessment? Part II: Case studies

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Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Schmallenberg

Coors Anja, Weil Mirco
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Umweltbundesamt
Wörlitzer Platz 1
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Tel: +49 340-2103-0
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buergerservice@uba.de
Internet: www.umweltbundesamt.de

Report performed by:
Fraunhofer Institute for Molecular Biology and Applied Ecology
Auf dem Aberg 1
57392 Schmallenberg
Germany

ECT Oekotoxikologie GmbH
Böttgerstraße 2
65439 Flörsheim am Main
Germany

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Edited by:
Section IV 2.2, Pharmaceuticals
Jasmin Brückner (Fachbegleitung), Ute Kühnen (Lemna), Kathi Westphal-Settele

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Abstract: Do new generations of active pharmaceuticals for human use require an adaption of the environmental risk assessment?, Part II: Case studies

In 2006, the European Medicines Agency (EMA) adopted a guideline on the environmental risk assessment (ERA) of medicinal products for human use. Since then, a large number of active pharmaceutical ingredients (APIs) with specific Mode-of-Actions (MoAs) that are potentially effective in the aquatic environment in low concentrations have been approved. This raised concern that the guideline does not allow a sufficiently protective environmental risk assessment for all of these new and very specific APIs and adaptations might be necessary.

A test strategy proposed after a previously conducted literature research (FKZ 3718654201 - Part I) should enable identification of effects specifically related to the MoA of the API and/or effects which occur at concentrations lower than the endpoints derived in the current standard long-term toxicity test set on fish, daphnia and algae. During the literature research, a number of test systems with potentially high sensitivity to these new APIs were identified. These tests included the *Lemna sp.* Growth Inhibition Test (OECD 221), the Danio rerio Fish Embryo Test (OECD 236) amended with sublethal endpoints, and the comet assay with environmentally relevant cell types derived from *Daphnia magna* and *D. rerio*.

Within the project Part II, 18 substances of the group oncologicals or cardiologicals and statins were assayed in these tests. The data generated according to the proposed new test strategy was compared to data from European public assessment reports (EPARs) to evaluate the level of protectiveness of the standard risk assessment approach.

The fish embryo toxicity test amended with sub-lethal endpoints were in none of the examined cases more sensitive than the currently employed apical endpoints from chronic aquatic toxicity tests (OECD 201, 211, 210) and can at best provide additional information.

The test with *Lemna sp*. was more sensitive than the standard endpoints (available data) for two substances with a pharmacological MoA relating to the mevalonate pathway (atorvastatin, rosuvastatin), one kinase inhibitors (dabrafenib) and two dihydrofolate reductase inhibitor (pemetrexed, methotrexate). Thus, the *Lemna* growth inhibition test might be a relevant additional test for the ERA of at least some APIs, e.g. the statins or the dihydrofolate reductase inhibitors. However, given that the difference to the NOECs provided in the EPARs is often less than 10-fold, the higher sensitivity likely reflects normal variation in species-specific sensitivity.

Lemna sp. displays specific sensitivity to the statins (HMG-CoA reductase inhibitors) and to methotrexat and pemetrexed (dihydrofolate reductase inhibitors). However, for some of the substances no EPARs but other information (e.g. FASS data or material safety data sheets) on effect levels were available. Thus, it could not be concluded if the sensitivity in Lemna sp. is substantially lower compared to the standard endpoints assessed during ERA. The concern that APIs from newer generations would show higher ecotoxicity than those from older generations was thus not confirmed, but also not clearly refuted. The performance of additional standard studies according to OECD TG 201 and 211 (210 exempted due to animal welfare reasons) with the above-mentioned substances would add additional value on interpretation of the results obtained in this study.

Kurzbeschreibung: „Erfordern neue Wirkstoffgenerationen bei Humanarzneimitteln eine Anpassung der Umweltbewertung?“, Teil II: Fallbeispiele

Im Jahr 2006 hat die Europäische Arzneimittel-Agentur (EMA) einen Leitfaden zur Umweltrisikobewertung von Humanarzneimitteln verabschiedet. Seitdem wurden zahlreiche pharmazeutische Wirkstoffe (APIs) mit spezifischen Wirkmechanismen (MoA) zugelassen, die in der aquatischen Umwelt in niedrigen Konzentrationen potenziell wirksam sind. Dies gab Anlass zur Besorgnis, dass die Leitlinie keine ausreichend schützende Umweltrisikobewertung für neue und sehr spezifische Wirkstoffe ermöglicht und Anpassungen erforderlich sein könnten.

Eine Teststrategie, basierend auf einer zuvor durchgeführten Literaturrecherche (FKZ 3718654201 - Teil I), sollte die Identifizierung von Wirkungen ermöglichen, die spezifisch mit dem MoA des Wirkstoffs zusammenhängen und/oder Wirkungen, die bei Konzentrationen auftreten, die niedriger sind als die Endpunkte, die in den derzeitigen Standardtests zur Langzeittoxizität an Fischen, Daphnien und Algen abgeleitet werden. Bei der Literaturrecherche wurde eine Reihe von Testsystemen mit potenziell hoher Empfindlichkeit gegenüber diesen neuen Wirkstoffen ermittelt. Zu diesen Tests gehörten der *Lemna sp. Growth Inhibition Test* (OECD 221), der *Danio rerio Fish Embryo Test* (OECD 236), der mit subletalen Endpunkten ergänzt wurde, und der Comet Assay mit umweltrelevanten Zelltypen von *Daphnia magna* und *D. rerio*.

Im Rahmen des Projekts Teil II wurden 18 Substanzen aus der Gruppe der Onkologika oder Kardiologika und Statine in diesen Tests untersucht. Die nach der vorgeschlagenen neuen Teststrategie gewonnenen Daten wurden mit Daten aus europäischen öffentlichen Bewertungsberichten (EPARs) verglichen, um das Schutzniveau des Standardrisikobewertungsansatzes zu bewerten.

Der mit subletalen Endpunkten ergänzte Fischembryo-Toxizitätstest war in keinem der untersuchten Fälle empfindlicher als die derzeit verwendeten apikalen Endpunkte aus chronischen aquatischen Toxizitätstests (OECD 201, 211, 210) und können bestenfalls zusätzliche Informationen liefern.

Der Test mit *Lemna sp*. war empfindlicher als die Standardendpunkte (verfügbare Daten) für zwei Substanzen mit einem pharmakologischen MoA in Bezug auf den Mevalonat-Stoffwechselweg (Atorvastatin, Rosuvastatin), ein Kinase-Inhibitoren (Dabrafenib,) und zwei Dihydrofolat-Reduktase-Inhibitor (Pemetrexed, Methotrexat). Somit könnte der Lemna-Wachstumshemmungstest ein relevanter zusätzlicher Test für das ERA zumindest einiger Wirkstoffe sein, z. B. der Statine oder der Dihydrofolat-Reduktase-Hemmer. Da der Unterschied zu den in den EPARs angegebenen NOECs jedoch oft weniger als das Zehnfache beträgt, spiegelt die höhere Empfindlichkeit eventuell die normale Variation der artspezifischen Empfindlichkeit wider.

Lemna sp. zeigt eine spezifische Empfindlichkeit gegenüber den Statinen (HMG-CoA-Reduktase-Hemmer) sowie gegenüber Methotrexat und Pemetrexed (Dihydrofolat-Reduktase-Hemmer). Für die meisten Stoffe lagen jedoch keine EPARs, sondern andere Informationen (z. B. FASS-Daten oder Sicherheitsdatenblätter) über die Höhe der Wirkung vor. Daher konnte nicht festgestellt werden, ob die Empfindlichkeit von *Lemna sp*. im Vergleich zu den während der ERA bewerteten Standardendpunkten wesentlich geringer ist. Die Befürchtung, dass Wirkstoffe neuerer Generationen eine höhere Ökotoxizität aufweisen würden als Wirkstoffe älterer Generationen, wurde somit nicht bestätigt, aber auch nicht eindeutig widerlegt. Die Durchführung zusätzlicher Standardstudien gemäß OECD TG 201 und 211 (210 aus Gründen des Tierschutzes ausgenommen) mit den oben genannten Stoffen würde die Interpretation der in dieser Studie erzielten Ergebnisse zusätzlich aufwerten.

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List of abbreviations

AAP	Algal Assay Procedure
AP	apurinic/apyrimidinic
API	Active pharmaceutical ingredient
5-FU	5-Flourouracil
ATCC	American Type Culture Collection
CML	Chronic myelogenous leukemia
DIN	Deutsches Institut für Normung
DMEM	Eagle's Minimal Essential Medium
DNA	Deoxyribonucleic acid
EC	Effective concentration
EGFR	Estimated Glomerular Filtration Rate
EMA	European Medicines Agency
EPAR	European public assessment report
ERA	Environmental risk assessment
EU	European Union
FASS	Pharmaceutical Specialities in Sweden
FBS	Fetal Bovine Serum
FELS	Fish Early Life Stage
FET	Fish Embryo Test
FKZ	Forschungskennzahl
GIST	Gastrointestinal Stromal Tumor
GLP	Good Laboratory Practice
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hpf	Hours post fertilization
HPLC-MS/MS	High Performance Liquid chromatography with tandem mass spectrometry
LMP	Low melting point
LOEC	Lowest observed effect concentration
LOQ	Limit of quantification
MoA	Mode of action
NILU	Norwegian Institute for Air Research
OECD	Organisation for Economic Co-operation and Development
OTM	Olive tail moment
PBS	Phosphate buffered saline
PEC	Predicted Environmantel Concentration
PNEC	Predicted No Effect Concentration

PPP	Plant protection product
PVDF	Polyvinylidenfluorid
ROS	Reactive Oxygen Species
SD	Standard deviation
SOP	Standard Operating Procedure
TG	Test guideline
TKI	Tyrosine kinase inhibitor
ZF-L	Zebrafish liver cell line

Summary

Since the 1990s, it has been known that pharmaceuticals pose risks to the environment. In 2006, the European Medicines Agency (EMA) adopted a guideline on the environmental risk assessment of medicinal products for human use (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2) that allows an assessment of the hazards and risks of human pharmaceuticals based on the data available on pharmaceutically active substances (European Medicines Agency 2006). The guideline describes a tiered approach for the assessment of pharmaceuticals. In Phase I, the assumed environmental concentrations in surface waters (PEC = Predicted Environmental Concentration) are estimated, while in Phase II the environmental behaviour and the effects on different trophic levels are determined to derive Predicted No Effect Concentrations (PNEC). In the risk assessment, PEC and PNEC are then compared with each other. This system has strengths and weaknesses, but has been successfully applied in recent years.

Since 2006, a large number of pharmaceuticals that are already very effective at their pharmacological target have been newly approved. The high pharmacological specificity and potency raised concern that these new-generation pharmaceuticals are more potent and specific than first generation pharmaceuticals and that they therefore also affect non-target organisms in the environment at very low concentrations. Furthermore, pharmaceuticals with new modes of action (MoA) or with innovative molecular mechanisms (RNA-technology) were marketed in recent years. These new-generation pharmaceuticals are possibly not adequately evaluated following the current guideline, as the active substances may have been not yet as potent when the guideline was developed.

The tests currently required for Phase II risk assessment for the aquatic compartment include the determination and evaluation of chronic toxicity using the growth inhibition test in algae (OECD Guideline 201) (OECD 2011), the reproduction test in Daphnia sp. (OECD Guideline 211) (OECD 2012) and the Fish Early Life Stage toxicity test (OECD Guideline 210) (OECD 2013). The apical endpoints (survival, growth and reproduction) covered in these studies may not cover all effects, especially those caused by very specific modes of action (MoAs) or only occurring in organisms that have the appropriate receptors. The available draft version of the EMA guideline, which is currently under revision and has not yet been published, will also only take to a limited extent into account the sensitivity to specific MoAs and for individual substance categories.

Literature review

The literature search preceding this project (FKZ 3718 65 420 1 - Project Part I, Kohler et al. (2019)), which was carried out at the Fraunhofer IME under the same leadership, revealed that 448 new pharmaceuticals were approved in the period from 2006 to 2018 (extracted from 2006 – 2018th Nature Reviews Drug Discovery). Most of the newly approved pharmaceuticals belonged to the category's "oncology" (83), "neurology" (50) and "infections" (79). However, in the assessment strategies within the above-mentioned literature study, the category "infections" was not considered, as this category mainly included antibiotics, that are already represented by tests with cyanobacteria and which represent the most sensitive species for this substance class. Another category into which many of the pharmaceuticals were classified was "cardiology" (28). Based on these data, the categories of oncology, neurology and cardiology were selected for the individual development of tailored assessment strategies, or to propose test systems that are potentially more sensitive than those listed in the EMA guideline. Three additional test systems were identified that could potentially be sensitive to one or more of the three major categories. These systems included the Lemna sp. growth inhibition test according to OECD Guideline 221 (OECD 2006), the zebrafish embryo toxicity test (FET) according to OECD Guideline 236 (OECD

2013) and *in vitro* assays such as the comet assay (following OECD guideline 489 (OECD 2016)) to be performed with environmentally relevant organisms or cell types.

The aim of the project was to test the theoretically developed adapted ecotoxicological test strategies for new generation pharmaceuticals identified in the preceding literature study, which might not be adequately assessed by the existing guideline. The focus was on pharmaceuticals that were either administered in large quantities or in a large number of different preparations, or whose specificity indicated a particular sensitivity of individual organisms.

The three above-mentioned systems were proposed, the suitability of which was to be examined as an alternative or supplement to the study types proposed in the EMA guideline. These test systems were to be evaluated on the basis of suitable new generation human pharmaceuticals for their usefulness in the development of a tailored evaluation strategy.

In total, 18 different substances were identified (see Table 1) for investigation using the three test systems. These test substances were selected from the drug classes oncology and cardiology, as the test systems to be investigated were determined to be sensitive for these categories.

Applied test strategy

As a result, GLP-approved test reports were provided for the studies carried out according to the OECD guideline (Lemna sp., FET), which can be used for the evaluation of the test substances and which can be directly compared with the available studies carried out within the framework of the prescribed risk assessment. Furthermore, the most sensitive endpoints of a substance were compared with those of the other tested substances of the same substance class in order to determine a MoA-specific sensitivity. In order to be able to make valid statements, not only the substances of the defined drug categories were examined, but also adequate controls (e.g. negative controls: other MoA; positive controls: model substances). These substances were treated like new-generation pharmaceuticals and were counted in the number of studies to be conducted. Thus, similarities and differences in the most sensitive endpoint could be identified in comparison to the controls.

Table 1: List of test substances, the mode of action, and an assignment to the aquatic toxicity tests.

Substance name	CAS number	MoA category	MoA	Lemna	FET	Comet
Edoxaban tosylate hydrate	1229194-11-9	Cardiology	Faktor Xa inhibitor; anti-coagulant	y	y	y
Rivaroxaban	366789-02-8	Cardiology	Faktor Xa inhibitor; anti-coagulant	y	y	y
Atorvastatin calcium	134523-03-8	Cardiology	HMG-CoA reductase inhibitor	y	n	n
Pitavastatin calcium	147526-32-7	Cardiology	HMG-CoA reductase inhibitor	y	n	y

Substance name	CAS number	MoA category	MoA	Lemna	FET	Comet
Rosuvastatin calcium	147098-20-2	Cardiology	HMG-CoA reductase inhibitor	y	y	y
Propranolol hydrochloride	318-98-9	Cardiology	ß-blocker	n	y	n
Dabrafenib mesylate	1195768-06-9	Oncology; cytostatics	BRAF-Serin-Threonin kinase inhibitor	y	n	y
Abemaciclib	1231929-97-7	Oncology; cytostatics	CDK4/CDK6; kinase inhibitor	y	n	y
Palbociclib	571190-30-2	Oncology; cytostatics	CDK4/CDK6; kinase inhibitor	y	y	y
Ribociclib succinate	1374639-75-4	Oncology; cytostatics	CDK4/CDK6; kinase inhibitor	y	n	y
Methotrexat	59-05-2	Oncology; cytostatics	Dihydrofolate reductase inhibitior	y	n	n
Pemetrexed disodium heptahydrate	357166-29-1	Oncology; cytostatics	Dihydrofolate reductase inhibitior Thymidylat synthase inhibitor	y	n	n
Cabazitaxel	183133-96-2	Oncology; cytostatics	Mikrotubule inhibitor (Beta-Tubulin)	y	n	n
Paclitaxel	33069-62-4	Oncology; cytostatics	Mikrotubule inhibitor (Beta-Tubulin)	y	n	n
Imatinib mesylate	220127-57-1	Oncology; cytostatics	Thyrosin-kinase inhibitor	y	y	y
Afatinib dimaleate	850140-73-7	Oncology; cytostatics	TKI erbB-2	y	y	n
Neratinib maleate	915942-22-2	Oncology; cytostatics	TKI	y	n	n
Cyclo-phosphamide monohydrate	6055-19-2	Oncology; cytotoxics	Alkylating antineoplastic	n	y	y

Results

Lemna sp. Growth inhibition test (OECD TG 221)

In general, both cardiologically active substances and oncologically active substances tested in the Lemna sp. growth inhibition test in accordance to OECD 221 affected the frond area stronger than the frond number, which is the definitive parameter in this test. The cardiologically active substances of the substance class of statins strongly affected the growth of either *Lemna minor* and *Lemna gibba*. The effect of atorvastatin and rosuvastatin on both species with different test media varying in their individual pH (Steinberg pH 5.5 vs. 20x AAP pH 7.5) were tested, with comparable or slightly stronger effects using *Lemna gibba* with 20x AAP medium. The E_rC_{10} values for frond area, the most sensitive endpoint, varied between 0.006 and 0.083 mg a.s./L for the three statins atorvastatin, rosuvastatin and pitavastatin. Here, rosuvastatin was the most toxic test substance with an E_rC_{10} of 0.006 mg a.s./L, however, it has to be considered that for the two other statins a semi-static test design was chosen due to a fast degradation of the test substance over time, while rosuvastatin appeared to be stable over 7 days exposure. Therefore, the exposure was probably longer and the evaluation was based on nominal concentrations, while it was based on the time weighted average (TWA) for atorvastatin and pitavastatin.

The Lemna sp. growth inhibition test (OECD 221) was chosen in this project because the literature review in the 1st part of the project had shown that the mode of action of cardiologically active substances might lead to effects on the growth of aquatic plants. The strong inhibitions of growth of *Lemna minor* and *Lemna gibba* at already very low concentrations indicate that this assumption could be confirmed.

Published data from European public assessment reports (EPAR) were available for each of the oncologically active substances. In most cases these were NOEC data, for ribociclib the EC_{10} was given. Thus, a comparison might be hampered. However, it was assumed that the data evaluation was performed based on measured concentrations as it was done for the actual Lemna tests as well. Methotrexate was the most toxic oncologically active test substance, resulting in an E_rC_{10} of 0.0008 mg a.s./L and an E_rC_{50} of 0.002 mg a.s./L (TWA). In the respective EPAR for Methotrexat an E_rC_{50} of 10 mg/L was stated for the algal growth inhibition test (OECD 201), which is 5000x higher than in the Lemna study. This clearly indicates that Lemna is a sensitive test species to dihydrofolate reductase inhibitors. Also, pemetrexed was quite toxic (E_rC_{10} = 0.159 µg a.s./L) to aquatic plants. Here, available data from the Swedish FASS database indicated a NOEC value of 4 mg/L for algae (OECD 201) and 1.2 mg/L for daphnia (OECD 211). Therefore, *Lemna minor* appeared to be more sensitive than the other organisms by a factor of around 7.5. In general, the NOEC/EC_{10} values for ribociclib, cabazitaxel and imatinib in the Lemna test were comparable to the values from the algal test. The values differ by less than a factor of 2 and are thus also within the range of biological variability. For the substance abemaciclib the effect in the Lemna test by a factor of 9.5 was weaker than in the algae test as reference test for the aquatic primary producers. For palbociclib Lemna was more sensitive than algae (factor ~4.1), however, the results of a Fish Early Life Stage (FELS) test were more sensitive with a NOEC value of 0.13 mg/L. In the case of afatinib and dabrafenib, the NOEC values in the Lemna test were lower than in the algae test and showed that *Lemna gibba* was more sensitive to the active substances than the green algae. Here, the differences were approximately a factor of 3.5 (NOEC for imatinib was <0.334) and 27.5 (dabrafenib). However, for afatinib considering all data fish was the most sensitive organism with a NOEC of 0.032 mg/L (factor 6) from a FELS test.

Comet assay with Daphnia magna and zebrafish cells

The assay selected to assess the genotoxicity of the chosen pharmaceuticals was the comet assay, or single cell gel electrophoresis. It is capable of visualizing the frequency of strand breaks and apurinic/apyrimidinic (AP) sites in the nuclear DNA of eukaryotic cells. Multiple endpoints were established and used for the comet assay, which are all based on the size of the so-called comet tail, usually in proportion to the head. This can be explained with the fact that strand breaks in the DNA cause it to be connected to the nucleus more loosely and thus let a larger fraction of the DNA get pulled out by the electric current and form a comet tail. The OECD Test No. 489 “In Vivo Mammalian Alkaline Comet Assay” (OECD 2016) as well as a majority of literature of the comet assay (e.g. Pellegri et al. (2014)) uses the TI% (% tail integrity) as endpoint. TI% was also the endpoint agreed on by the international Workshop on Genotoxicity Test Procedures in 2005 (Burlinson et al. 2007). For this reason, the TI% was used as endpoint for the comet assay in this study.

The comet assay was performed to figure out if this test system could provide more sensitive data for testing active substances, that might exert effects on DNA integrity. Therefore, we investigated the antineoplastic substance cyclophosphamide as well as H_2O_2 as positive control, and several TKIs. with *D. magna* and *D. rerio cells*.

Correct functioning of the test system was demonstrated, as strand breaks were observed for the positive control H_2O_2 for the comet assays with both cell types and for cyclophosphamide with the ZF-L cell line. It was observed that the comet assay was less sensitive for the investigated pharmaceuticals compared to test systems already included in ERA of pharmaceuticals, andmost of the test substances did not result in any effect in the assay. This also is probably due to the circumstances that the pharmaceuticals tested in this approach do not possess a genotoxic MoA.

However, the comet assays performed in the course of this project were not sufficient to allow any assumptions with respect to the validity of the test system. This was was caused by the restricted flexibility in substance selection, as the selection only included a limited number of MoAs excluding substances with an explicitly genotoxic MoA.

Zebrafish embryo toxicity test

The fish embryo toxicity (FET) tests with the eight selected substances (afatinib, cyclophosphamid, edoxaban, imatinib, palbociclib, propranolol, rivaroxaban, and rosuvastatin) were designed to comply with the OECD Guideline for Testing of Chemicals, No. 236 "Fish Embryo Acute Toxicity (FET) Test" (OECD 2013), and complemented with additional sublethal endpoints. An additional test with the standard reference substance 3,4-dichloroaniline was conducted to provide supporting information on the specificity of these endpoints. All nine tests fulfilled the validity criteria described in the test guideline.

Significant lethal effects were observed only for 3,4-dichloroanline and cyclophosphamide with 70 and 100 % mortality in the highest investigated substance concentrations. In all tests with the seven remaining substances, mortality was ≤ 10 % of introduced embryos. No larvae hatched in the first 48 ± 3 h of exposure in any of the tests. Significantly delayed hatching was determined in the test with propranolol (excluding the tests with occurrence of significant mortality). The number of somites counted after 24 ± 3 h of exposure were similar in all tests across all test substances and concentration levels. Hence, none of the test items influenced the number of somites during embryonic development of *D. rerio*. Effects on the pigmentation of the body or the eyes were observed occasionally during exposure to rosuvastatin, propranolol, edoxaban, cyclophosphamide and 3,4-dichloroaniline. In all cases, reduced body pigmentation

was observed in less than five embryos per treatment at test end and without relation to increasing concentrations.

Heart beat rate was a sensitive endpoint for three of the nine selected test substances, namely propranolol, 3,4-dichloroanline and cyclophosphamide. For the latter two substances, lethal effects were observed at test end in similar concentration ranges influencing the heart beat rate at 48 h exposure, indicating a general stress response. For propranolol, however, all introduced embryos survived until test end in the highest test concentration of 50 mg/L. Heart beat rate was significantly reduced in all four propranolol treatments with concentrations above 0.5 mg/L, indicating a specific mode of action.

Body length of hatched larvae was negatively influenced by four test substances (3,4-dichloroanline, cyclophosphamide, propranolol and rosuvastatin). For propranolol and rosuvastatin the difference determined for larvae from the control and the respective highest test concentration was below 10 %, restricting the calculation of valid ECx values and allowing the determination of a NOEC only. Oedema in the area of the yolk were observed occasionally, but no significant effect was determined. However, presence of oedema in the heart was significantly increased by four of the nine substance tests (3,4-dichloroaniline, cyclophosphamide, rosuvastatin and propranolol). Malformation of otoliths was observed in the test with cyclophosphamide, malformations of the yolk of the embryos were observed in the tests with 3,4-dichloronaline and cyclophosphamide. Malformation of spine were observed in embryos exposed to 3,4-dichloronaline, cyclophosphamide and rosuvastatin.

Additional effects were noted: a significant number of embryos showed no spontaneous movement after 24 h of exposure to 3,4-dichloroanline and cyclophosphamide. Propranolol and cyclophosphamide caused loss of the swim ability of surviving larvae. Cyclophosphamide caused spasmic movement surviving embryos. Exposure to 3,4-dichloroanline delayed the development of the eyes, and propranolol caused a dark coloured yolk sac (nearly lacking any transparency). Palbociclib caused a defective development of the head, notably by the reduced size of the head. This observation was, however, assessed qualitatively and no measurement of the head dimensions was performed.

Discussion

The first hypothesis of the present project stated that the positive model substances and the test substances from the specific MoA group would induce specific effects in the respective selected test systems. This was confirmed in the FET for both positive model substances: propranolol influenced heart beat rate and cyclophosphamide induced teratogenic effects. The hypothesis was not confirmed for the test substances since those from the group ‘cardiology’ did not all influence heart beat rate and those from the group ‘oncology’ did not all induce teratogenic effects. For Lemna sp., the three selected statins (atorvastatin, pitavastatin, rosuvastatin) were suspected to result in strong effects and in the experimental phase these expectations were confirmed. Whether this finding indeed relate to the MoA as HMG-CoA reductase inhibitor, i.e., indicates specific effects related to the pharmacological MoA, was confirmed by a recent study, which investigated the MoA of atorvastatin in transcriptome analyses (Loll et al. 2022). No EPAR data was available for the three statins, but data from a material safety data sheet could be used for atorvastatin and data from the literature for rosuvastatin.

The second hypothesis (effects at lower concentrations than in standard test systems required in the ERA) was clearly refuted for the FET. For none of the test substances, any endpoint in the FET was lower than the standard endpoint given in the EPAR of the respective API. For 6 out of 10 APIs (atorvastatin, rosuvastatin, dabrafenib, palbociclib, pemetrexet, imatinib) for which this

comparison was possible, at least one endpoint derived with Lemna was lower than the endpoints provided in the EPARs of the respective API.

Lemna sp. is specifically sensitive to the dihydrofolate reductase inhibitors pemetrexet and methotrexat, which were both no APIs approved after 2006, but were proven to result in strong adverse effects in the above-mentioned test system. Methotrexat, the first-in-class API, was however much more potent compared to pemetrexet (factor >300). As both substances display only minimal differences in their structure, it was suspected that the sensitivity was equal between these APIs. As even small differences in the structure could however result in different potencies, i.e. with respect to binding potential to a receptor or to enzyme inhibition, the effect could be more pronounced for one substance compared to a similar substance. Comparable observations were also made for other pairs of APIs with similar structure, e.g. imatinib and afatinib, however only with a factor of 2 (afatinib more effective than the first-in-class API imatinib). This is a common problem in ecotoxicology, as the effect size of substances with similar MoA in different species is difficult to predict, and also QSARs could only give hints on hazards rather on risks. Based on the EPAR data for methotrexat it can be concluded that an assessment following the EMA guideline would not be as protective as an assessment that includes the Lemna endpoint derived in the present study.

The concern that APIs from newer generations would show higher ecotoxicity than those from older generations (third hypothesis) was not confirmed, but also not clearly refuted. The newer generation antineoplastics did show higher toxicity in the FET than the first-in-class API imatinib. The same was true for the Lemna test system and the Comet assay.

Recently approved first-in-class pharmaceuticals with specific MoAs were for example the Xa-inhibitor edoxaban and the microtubule inhibitor paclitaxel, which were both marketed after 2006. For both substances, second substances were chosen in order to proof the sensitivity of the test systems to specific MoAs, which were rivaroxaban as an API of newer generation compared to edoxaban, and cabazitaxel compared to paclitaxel. Furthermore, as in both cases the first-in-class pharmaceutical was included, it was possible to verify or falsify the third hypothesis.

As the factor Xa inhibitors edoxaban and rivaroxaban were chosen as negative controls for Lemna studies, as the MoA was suspected to be not evident in plants, the hypothesis could not be tested in this case. These substances were initially chosen based on a suspected effect in the FET, which was however not observed.

The microtubule inhibitors paclitaxel and cabazitaxel were suspected to have effects on actively dividing organisms, i.e. Lemna sp. ec. An effect was indeed observed for cabazitaxel, while no effect was observed for paclitaxel, the first-in-class. Even though the Lemna studies were not explicitly sensitive to this MoA, in this case the API of the newer generation was at least more effective than the first-in-class.

For the statins, no EPARs are available but for atorvastatin data from a material safety data sheet was available indicating that Lemna sp was more sensitive to the statin than the standard test organisms (here: Daphnia magna NOEC: 0.2 mg/L) varying by a factor 3 to 15 in dependence of the species used in the test Lemna minor or Lemna gibba respectively. The differences are also related to the pH of the test medium which is much higher in the test with Lemna minor (Steinberg medium, pH 7.5) than in the test with Lemna gibba (20 x AAP, pH 5.5). Thus, the comparison of the data obtained with the standard testing results is difficult. For rosuvastatin, literature data for algae are available, which indicate a comparably low sensitivity of algae (330 mg/L-350 mg/L; studies performed in accordance to the FDA Technical Assistance Document 4.01). It was however shown that rosuvastatin resulted in relatively high chronic toxicity to

Daphnia magna, with a NOEC after 21 days of 18 µg/L (study performed according to FDA Technical Assistance Document 4.09). In Lemna sp., the most sensitive endpoint (front area) resulted in a NOEC of 6 µg/L for rosuvastatin in the non-GLP study with L. gibba and a NOEC of 17 µg/L (frond area) and 5 µg/L (frond number) in the GLP study with L. minor. Thus, the test system seems to be very sensitive to the substance class of statins, with a difference of around factor 4 related to the most sensitive endpoint of standard test organisms (here daphnia) and a much higher sensitivity compared to the standard aquatic primary producer (algae).

It was furthermore suspected that Lemna sp. as actively dividing organisms react sensitive to oncologically active substances. Indeed, the results confirm this hypothesis in most cases. However, Lemna sp. was not consistently more sensitive than algae. Thus, the sensitivity might be additionally dependent on the time of exposure, which for example was postulated as a potential reason for the increased sensitivity of Lemna sp. to methotrexat (Białk-Bielińska et al. 2017). Other oncologically active substances with different MoAs, which showed lower toxicity to algae compared to Lemna sp. were afatinib, imatinib and dabrafenib, while cabazitaxel, abemaciclib, and palbociclib were less toxic in Lemna sp. than to green algae.

The comet assay was performed to figure out if this test system might be applied for assessment of genotoxicity of an active pharmaceutical ingredient in the environmentally relevant cell types. Therefore, we investigated the antineoplastic substance cyclophosphamide as positive control, and several TKIs as presumptive substances which might result in DNA strand breaks. As negative controls, the cardiologically active substances edoxaban and pitavastatin were tested.

Correct functioning of the test system was confirmed, as strand breaks were observed for the positive control H_2O_2 and for cyclophosphamide. It was observed that the comet assay was less sensitive compared to test systems already included in ERA of pharmaceuticals, and most of the test substances did not result in any effect in the assay. Interestingly, exposure of daphnids to pitavastatin resulted in effects in the corresponding comet assay. This was likely due to the suspected increased sensitivity of this species to statins based on the MoA of HMG-CoA reductase inhibition, which is also known to be a sensitive pathway in daphnids, resulting in effects on moulting and reproduction (Miyakawa et al. 2018, Miura 2019), endpoints which likely results in effects in Daphnia reproduction studies.

Finally, it was investigated whether the assessment of sublethal endpoints in the FET results in lower sensitivities than those determined during Fish Early Life Stage toxicity tests according to OECD TG 210 (OECD 2013), which are performed during the pharmaceutical ERA.

Only some of the tested substances caused in effects in the FET. None of the selected test substances resulted in effective concentrations lower than those provided in the EPARs for FELS tests. Hence, the FET amended with sublethal endpoints appears not as a more sensitive method than the standard Fish Early Life Stage test required in the EMA guideline.

To conclude, from the three test systems applied during the current project, the Lemna sp. growth inhibition test according to OECD TG 221 (OECD 2006) is a test system with a potential to be integrated as additional aquatic effect test into the ERA of specific classes of human pharmaceuticals. For example, it is sensitive to substances which act on MoAs which are conserved during evolution, like the HMG-CoA pathway. Furthermore, substances affecting actively dividing organisms result in low effective concentrations, and the most sensitive species can be missed if the guideline only considers those species already included. Also, by comparing the determined NOEC to all endpoints provided in the EPARs, studies according to OECD TG 221 (OECD 2006) in most of the test substances resulted in the effects which were either equally sensitive to or more sensitive to the most sensitive endpoint presented in the EPAR (if

available). An exception to this was, for example, cabazitaxel for which the lowest EPAR endpoint was 17-fold lower than the NOEC derived with Lemna sp..

Growth inhibition in *Lemna sp*. was a more sensitive endpoint than the standard endpoints in the EPARs for some of the substances. Given that the difference to the NOECs provided in the EPARs is often less than 10-fold, the higher sensitivity likely reflects normal variation in species-specific sensitivity. Independently of the underlying reasons, the Lemna growth inhibition test provides relevant additional endpoints for the ERA of at least some APIs as it represents a so far not considered species group (aquatic macrophytes).

Outlook

The project raises several questions, which could be addressed in a follow-up project. Of special interest would be the following:

Lemna sp. displays specific sensitivity for two substances with a pharmacological MoA relating to the mevalonate pathway (atorvastatin, rosuvastatin), one kinase inhibitor (dabrafenib) and two dihydrofolate reductase inhibitors (pemetrexed, methotrexat). However, for some of the substances no EPARs or other information (e.g. FASS data or material safety data sheets) on effect levels (except for rosuvastatin) were available. Thus, it could not be concluded if the sensitivity in Lemna sp. is substantially lower compared to the standard endpoints assessed during ERA. The performance of additional standard studies according to OECD TG 201 and 211 (210 exempted due to animal welfare reasons) with the above-mentioned substances would add additional value on interpretation of the results obtained in this study. Furthermore, in order to verify that the effects are based on the specific MoA of the substances, transcriptomic analyses as performed in Loll et al., 2022 for atorvastatin in *Lemna minor* would add information on the specific genomic responses in the test organisms.

The *Lemna sp*. growth inhibition test is thus a relevant additional test for the ERA of at least some APIs – dihydrofolate reductase inhibitors, statins and BRAF serine threonine kinase inhibitors, like Dabrafenib.

Zusammenfassung

Seit den 1990er Jahren ist bekannt, dass Arzneimittel Risiken für die Umwelt bergen. Im Jahr 2006 verabschiedete die Europäische Arzneimittel-Agentur (EMA) einen Leitfaden zur Umweltrisikobewertung von Humanarzneimitteln (Dok. Ref. EMEA/CHMP/SWP/4447/00 corr 2), der eine Bewertung der Gefahren und Risiken von Humanarzneimitteln auf der Grundlage der für pharmazeutische Wirkstoffe verfügbaren Daten ermöglicht (European Medicines Agency 2006). Der Leitfaden beschreibt einen stufenweisen Ansatz für die Bewertung von Arzneimitteln. In Phase I werden die angenommenen Umweltkonzentrationen in Oberflächengewässern (PEC = *Predicted Environmental Concentration*) abgeschätzt, während in Phase II das Umweltverhalten und die Auswirkungen auf verschiedene trophische Ebenen bestimmt werden, um *Predicted No Effect Concentrations* (PNEC) abzuleiten. Bei der Risikobewertung werden dann PEC und PNEC miteinander verglichen. Dieses System hat Stärken und Schwächen, wurde aber in den letzten Jahren erfolgreich angewandt.

Seit 2006 wurde eine große Zahl von Arzneimitteln neu zugelassen, die an ihrem pharmakologischen Ziel bereits sehr wirksam sind. Die hohe pharmakologische Spezifität und Potenz gab Anlass zur Sorge, dass diese Arzneimittel der neuen Generation stärker und spezifischer sind als die Arzneimittel der ersten Generation und dass sie daher auch Nichtzielorganismen in der Umwelt in sehr niedrigen Konzentrationen beeinträchtigen. Außerdem wurden in den letzten Jahren Arzneimittel mit neuen Wirkmechanismen (MoA) oder mit innovativen molekularen Mechanismen (RNA-Technologie) auf den Markt gebracht. Diese Arzneimittel der neuen Generation werden nach der aktuellen Richtlinie möglicherweise nicht ausreichend bewertet, da die Wirkstoffe zum Zeitpunkt der Entwicklung der Richtlinie möglicherweise noch nicht so potent waren.

Die derzeit für die Phase II der Risikobewertung für das aquatische Kompartiment vorgeschriebenen Tests umfassen die Bestimmung und Bewertung der chronischen Toxizität anhand des Wachstumshemmungstests bei Algen (OECD-Richtlinie 201) (OECD 2011), des Reproduktionstests bei Daphnia sp. (OECD-Richtlinie 211) (OECD 2012) und des Toxizitätstests für Fische im frühen Lebensstadium (FELS; OECD-Richtlinie 210) (OECD 2013). Die in diesen Studien abgedeckten apikalen Endpunkte (Überleben, Wachstum und Fortpflanzung) decken möglicherweise nicht alle Wirkungen ab, insbesondere solche, die durch sehr spezifische MoA verursacht werden oder nur bei Organismen auftreten, die die entsprechenden Rezeptoren haben. Auch der vorliegende neue Entwurf des EMA-Leitfadens, der derzeit überarbeitet wird und noch nicht veröffentlicht ist, wird die Empfindlichkeit gegenüber bestimmten MoA und für einzelne Stoffkategorien nur bedingt berücksichtigen.

Literaturrecherche

Die diesem Projekt vorausgehende Literaturrecherche (FKZ 3718 65 420 1 - Projektteil I, Kohler et al. (2019)), die am Fraunhofer IME unter gleicher Leitung durchgeführt wurde, ergab, dass im Zeitraum von 2006 bis 2018 448 neue Arzneimittel zugelassen wurden (entnommen aus 2006 - 2018 Nature Reviews Drug Discovery). Die meisten der neu zugelassenen Arzneimittel gehörten zu den Kategorien "Onkologie" (83), "Neurologie" (50) und "Infektionen" (79). Bei den Bewertungsstrategien im Rahmen der oben genannten Literaturstudie wurde die Kategorie "Infektionen" jedoch nicht berücksichtigt, da diese Kategorie hauptsächlich Antibiotika umfasst, für die bereits spezielle Tests mit Cyanobakterien, der für diese Substanzklasse empfindlichsten Spezies, durchzuführen sind. Eine weitere Kategorie, in die viele der Arzneimittel eingeordnet wurden, war "Kardiologie" (28). Auf der Grundlage dieser Daten wurden die Kategorien Onkologie, Neurologie und Kardiologie für die individuelle Entwicklung maßgeschneiderter Bewertungsstrategien ausgewählt, oder um Testsysteme vorzuschlagen, die potenziell

empfindlicher sind als die in der EMA-Richtlinie aufgeführten. Es wurden drei zusätzliche Testsysteme ermittelt, die potenziell für eine oder mehrere der drei Hauptkategorien empfindlich sein könnten. Zu diesen Systemen gehörten der Lemna sp. Wachstumshemmungstest nach OECD-Richtlinie 221 (OECD 2006), der Zebrafisch-Embryo-Toxizitätstest (FET) nach OECD-Richtlinie 236 (OECD 2013) und ein In-vitro-Test, nämlich der Comet-Assay (nach OECD-Richtlinie 489 (OECD 2016)), der mit umweltrelevanten Organismen oder Zelltypen durchgeführt werden sollte.

Ziel des Projekts war es, die theoretisch entwickelten, angepassten ökotoxikologischen Teststrategien für solche in der vorangegangenen Literaturstudie identifizierten Arzneimittel der neuen Generation zu testen, die durch die bestehende Richtlinie möglicherweise nicht angemessen bewertet werden. Der Schwerpunkt lag dabei auf Arzneimitteln, die entweder in großen Mengen oder in einer großen Anzahl verschiedener Anwendungen verabreicht werden oder deren Spezifität auf eine besondere Empfindlichkeit einzelner Organismen hinweist.

Vorgeschlagen wurden die drei oben genannten Systeme, deren Eignung als Alternative oder Ergänzung zu den in der EMA-Leitlinie vorgeschlagenen Studientypen geprüft werden sollten. Diese Testsysteme sollten anhand von geeigneten Humanarzneimitteln der neuen Generation auf ihre Nützlichkeit für die Entwicklung einer maßgeschneiderten Bewertungsstrategie hin überprüft werden.

Insgesamt wurden 18 verschiedene Substanzen identifiziert (siehe Tabelle 1), die in bis zu drei Testsystemen untersucht werden sollten. Diese Testsubstanzen wurden aus den Arzneimittelklassen Onkologie und Kardiologie ausgewählt, da die zu untersuchenden Testsysteme für diese Kategorien als empfindlich eingestuft wurden.

Angewandte Prüfstrategie

Auf dieser Basis wurden für die nach der OECD-Richtlinie durchgeführten Studien (Lemna sp., FET) GLP-genehmigte Prüfberichte erstellt, die für die Bewertung der Prüfsubstanzen herangezogen werden können und die direkt mit den vorliegenden Studien, die im Rahmen der vorgeschriebenen Risikobewertung durchgeführt wurden, verglichen werden können. Darüber hinaus wurden die empfindlichsten Endpunkte einer Substanz mit denen der anderen getesteten Substanzen der gleichen Substanzklasse verglichen, um eine MoA-spezifische Empfindlichkeit zu ermitteln. Um valide Aussagen machen zu können, wurden nicht nur die Substanzen der definierten Wirkstoffklassen untersucht, sondern auch adäquate Kontrollen (z.B. Negativkontrollen: andere MoA; Positivkontrollen: Modellsubstanzen). Diese Substanzen wurden wie Arzneimittel der neuen Generation behandelt und bei der Anzahl der durchzuführenden Studien mitgezählt. So konnten Gemeinsamkeiten und Unterschiede im empfindlichsten Endpunkt im Vergleich zu den Kontrollen festgestellt werden.

Tabelle 1: Liste der Prüfsubstanzen, deren Wirkweise und eine Zuordnung zu den Tests der aquatischen Toxizität.

Substanzname	CAS Nummer	MoA Kategorie	MoA	Lemna	FET	Comet
Edoxaban tosylate hydrate	1229194-11-9	Kardiologie	Faktor Xa Inhibitor; Anti-Koagulant	ja	ja	ja
Rivaroxaban	366789-02-8	Kardiologie	Faktor Xa Inhibitor; Anti-Koagulant	ja	ja	ja
Atorvastatin calcium	134523-03-8	Kardiologie	HMG-CoA Reduktase Inhibitor	ja	nein	nein
Pitavastatin calcium	147526-32-7	Kardiologie	HMG-CoA Reduktase Inhibitor	ja	nein	ja
Rosuvastatin calcium	147098-20-2	Kardiologie	HMG-CoA Reduktase Inhibitor	ja	ja	ja
Propranolol hydrochloride	318-98-9	Kardiologie	ß-Blocker	nein	ja	nein
Dabrafenib mesylate	1195768-06-9	Onkologie; Zytostatikum	BRAF-Serin-Threonin Kinase Inhibitor	ja	nein	ja
Abemaciclib	1231929-97-7	Onkologie; Zytostatikum	CDK4/CDK6; Kinase Inhibitor	ja	nein	ja
Palbociclib	571190-30-2	Onkologie; Zytostatikum	CDK4/CDK6; Kinase Inhibitor	ja	ja	ja
Ribociclib succinate	1374639-75-4	Onkologie; Zytostatikum	CDK4/CDK6; Kinase Inhibitor	ja	nein	ja
Methotrexat	59-05-2	Onkologie; Zytostatikum	Dihydrofolate Reduktase Inhibitor	ja	nein	nein
Pemetrexed disodium heptahydrate	357166-29-1	Onkologie; Zytostatikum	Dihydrofolate Reduktase Inhibitor Thymidylat Synthase Inhibitor	ja	nein	nein
Cabazitaxel	183133-96-2	Onkologie; Zytostatikum	Mikrotubule	ja	nein	nein

Substanzname	CAS Nummer	MoA Kategorie	MoA	Lemna	FET	Comet
			inhibitor (Beta-Tubulin)			
Paclitaxel	33069-62-4	Onkologie; Zytostatikum	Mikrotubuli Inhibitor (Beta-Tubulin)	ja	nein	nein
Imatinib mesylate	220127-57-1	Onkologie; Zytostatikum	Thyrosin- Kinase Inhibitor	ja	ja	ja
Afatinib dimaleate	850140-73-7	Onkologie; Zytostatikum	TKI erbB-2	ja	ja	nein
Neratinib maleate	915942-22-2	Onkologie; Zytostatikum	TKI	ja	nein	nein
Cyclo-phosphamide monohydrate	6055-19-2	Onkologie; Zytotoxikum	Alkylierendes Antineoplastikum	nein	ja	ja

Ergebnisse

Lemna sp. Wachstumshemmungstest (OECD TG 221)

Generell beeinflussten sowohl die kardiologisch wirksamen Substanzen als auch die onkologisch wirksamen Substanzen, die im Wachstumshemmungstest von Lemna sp. nach OECD 221 getestet wurden, die Blattfläche stärker als die Blattanzahl, die in diesem Test der maßgebliche Parameter ist. Die kardiologisch wirksamen Substanzen aus der Substanzklasse der Statine beeinflussten das Wachstum sowohl von *Lemna minor* als auch von *Lemna gibba* stark. Getestet wurde die Wirkung von Atorvastatin und Rosuvastatin auf beide Arten mit unterschiedlichen Testmedien, die sich in ihrem individuellen pH-Wert unterscheiden (Steinberg pH 5.5 vs. 20x AAP pH 7.5), mit vergleichbaren oder etwas stärkeren Effekten bei *Lemna gibba* mit 20x AAP-Medium. Die ErC_{10}-Werte für die Blattfläche, den empfindlichsten Endpunkt, variierten zwischen 0.006 und 0.083 mg a.s./L für die drei Statine Atorvastatin, Rosuvastatin und Pitavastatin. Dabei war Rosuvastatin mit einem ErC_{10} von 0.006 mg a.s./L die toxischste Prüfsubstanz, wobei jedoch zu berücksichtigen ist, dass für die beiden anderen Statine ein halbstatisches Testdesign gewählt wurde, da die Prüfsubstanz im Laufe der Zeit schnell abgebaut wird, während Rosuvastatin über eine 7-tägige Exposition stabil zu sein scheint. Daher war die Exposition wahrscheinlich länger, und die Bewertung basierte auf nominalen Konzentrationen, während sie für Atorvastatin und Pitavastatin auf dem zeitgewichteten Durchschnitt (TWA) basierte.

Der Lemna sp. Wachstumshemmungstest (OECD 221) wurde in diesem Projekt gewählt, da die Literaturrecherche im ersten Teil des Projekts gezeigt hatte, dass die Wirkungsweise kardiologisch aktiver Substanzen Auswirkungen auf das Wachstum von Wasserpflanzen haben könnte. Die starken Wachstumshemmungen von *Lemna minor* und *Lemna gibba* bei bereits sehr geringen Konzentrationen deuten darauf hin, dass sich diese Annahme bestätigen konnte.

Für jeden der onkologisch wirksamen Stoffe lagen veröffentlichte Daten aus europäischen öffentlichen Bewertungsberichten (EPAR) vor. In den meisten Fällen handelte es sich um NOEC-Werte, für Ribociclib wurde der EC_{10}-Wert angegeben. Dies könnte einen Vergleich erschweren. Methotrexat war die toxischste onkologisch wirksame Prüfsubstanz, was zu einem ErC_{10} von

0.0008 mg a.s./L und einem E_rC_{50} von 0.002 mg a.s./L (TWA) führte. Im entsprechenden EPAR für Methotrexat wurde für den Algenwachstumshemmungstest (OECD 201) eine E_rC_{50} von 10 mg/L angegeben, was 5000-mal höher ist als in der Lemna-Studie. Dies zeigt eindeutig, dass Lemna eine empfindliche Spezies für Dihydrofolat-Reduktase-Hemmer ist. Außerdem war Pemetrexed für Wasserpflanzen vergleichsweise toxisch (ErC_{10} = 0.159 µg a.s./L). Die verfügbaren Daten aus der schwedischen FASS-Datenbank ergaben hier einen NOEC-Wert von 4 mg/L für Algen (OECD 201) und 1.2 mg/L für Daphnien (OECD 211). *Lemna minor* scheint also um einen Faktor von etwa 7.5 empfindlicher zu sein als die anderen Organismen. Im Allgemeinen waren die NOEC/EC_{10}-Werte für Ribociclib, Carbazitaxel und Imatinib im Lemna-Test mit den Werten aus dem Algentest vergleichbar. Die Werte unterscheiden sich um weniger als einen Faktor 2 und liegen damit ebenfalls im Bereich der biologischen Variabilität. Für die Substanz Abemaciclib war die Wirkung im Lemna-Test um den Faktor 9,5 schwächer als im Algentest als Referenztest für die aquatischen Primärproduzenten. Für Palbociclib war Lemna empfindlicher als Algen (Faktor ~4,1), jedoch zeigten die Ergebnisse des FELS-Tests mit einem NOEC-Wert von 0,13 mg/L, dass Fische den empfindlichsten Organismus für diese Substanz darstellen. Im Falle von Afatinib und Dabrafenib waren die NOEC-Werte im Lemna-Test niedriger als im Algentest und zeigten, dass *Lemna gibba* empfindlicher auf die Wirkstoffe reagierte als die Grünalgen. Hier betrugen die Unterschiede etwa einen Faktor von 3.5 (NOEC für Imatinib war <0.334) und 27.5 (Dabrafenib). Bei Afatinib war jedoch der Fisch unter Berücksichtigung aller Daten der empfindlichste Organismus mit einer NOEC von 0.032 mg/L (Faktor 6) aus einem FELS-Test.

Comet-Test mit *Daphnia magna* und Zebrafischzellen

Als Test zur Bewertung der Gentoxizität der ausgewählten Arzneimittel wurde der Comet-Assay oder die Einzelzellgelelektrophorese gewählt. Mit ihm lässt sich die Häufigkeit von Strangbrüchen in der Kern-DNA eukaryontischer Zellen sichtbar machen. Für den Comet-Assay wurden mehrere Endpunkte festgelegt und verwendet, die alle auf der Größe des so genannten Kometenschweifs basieren, der in der Regel im Verhältnis zum Kopf dargestellt wird. Dies lässt sich damit erklären, dass Strangbrüche in der DNA dazu führen, dass diese lockerer mit dem Zellkern verbunden ist und somit ein größerer Teil der DNA durch den elektrischen Strom herausgezogen wird und einen Kometenschweif bildet. Der OECD-Test Nr. 489 "In Vivo Mammalian Alkaline Comet Assay" (OECD 2016) sowie ein Großteil der Literatur zum Comet-Assay verwendet den TI% (% tail integrity) als Endpunkt. Der TI% war auch der Endpunkt, auf den sich der internationale Workshop über Genotoxizitätstestverfahren im Jahr 2005 geeinigt hatte. Aus diesem Grund wurde in dieser Studie der TI% als Endpunkt für den Comet-Assay verwendet.

Der Comet-Assay wurde durchgeführt, um herauszufinden, ob dieses Testsystem Daten für aktive Substanzen liefern könnte, die eine Wirkung auf die DNA-Integrität haben könnten. Daher untersuchten wir die antineoplastische Substanz Cyclophosphamid sowie H_2O_2 als Positivkontrolle und verschiedene TKIs *D.-magna*- und D. rerio Zellen Kometen zeigte, so dass die Hypothese, dass Kardiologika keine DNA-Schäden hervorrufen, nicht bestätigt werden konnte.

Die Funktionalität des Testsystems wurde bestätigt, da Strangbrüche bei der Positivkontrolle H_2O_2 in beiden Zelltypen und für Cyclophosphamid mit der ZF-L-Zelllinie nachgewiesen wurden. Es wurde festgestellt, dass der Comet Assay für die untersuchten Arzneimittel weniger empfindlich war als die Testsysteme, die bereits in der ERA von Arzneimitteln enthalten sind, und die meisten Testsubstanzen keine Wirkung im Comet-Assay zeigten. Dies war dadurch bedingt, dass die bei diesem Ansatz getesteten Arzneimittel keine genotoxische Wirkung besitzen.

Die im Rahmen dieses Projekts durchgeführten Comet-Assays reichten also nicht aus, um Aussagen über die über die Validität des Testsystems im Rahmen der Umweltrisikobewertung von von Humanarzneimitteln zu treffen. Dies lag vor allem an den eingeschränkten Möglichkeiten hinsichtlich der Substanzauswahl, die nur eine begrenzte Anzahl von MoAs umfasste.

Zebrafisch-Embryo-Toxizitätstest

Die Tests zur Fischembryo-Toxizität (FET) mit den acht ausgewählten Substanzen (Afatinib, Cyclophosphamid, Edoxaban, Imatinib, Palbociclib, Propranolol, Rivaroxaban und Rosuvastatin) wurden in Übereinstimmung mit der OECD-Richtlinie für die Prüfung von Chemikalien Nr. 236 "Fish Embryo Acute Toxicity (FET) Test" (OECD 2013) konzipiert und durch zusätzliche subletale Endpunkte ergänzt. Ein zusätzlicher Test mit der Standardreferenzsubstanz 3,4-Dichloranilin wurde durchgeführt, um unterstützende Informationen über die Spezifität dieser Endpunkte zu liefern. Alle neun Tests erfüllten die in der Prüfrichtlinie beschriebenen Validitätskriterien.

Signifikante letale Wirkungen wurden nur für 3,4-Dichloranilin und Cyclophosphamid mit 70 und 100 % Mortalität bei den höchsten untersuchten Substanzkonzentrationen beobachtet. Bei allen Tests mit den sieben übrigen Substanzen lag die Sterblichkeit bei ≤ 10 % der eingesetzten Embryonen. In den ersten 48 ± 3 Stunden der Exposition schlüpfte in keinem der Tests eine Larve. Ein signifikant verzögerter Schlupf wurde im Test mit Propranolol festgestellt (mit Ausnahme der Tests mit signifikanter Mortalität). Die Anzahl der Somiten, die nach 24 ± 3 Stunden Exposition gezählt wurden, war in allen Tests bei allen Testsubstanzen und Konzentrationsstufen ähnlich. Folglich beeinflusste keiner der Prüfgegenstände die Anzahl der Somiten während der Embryonalentwicklung von *D. rerio*. Auswirkungen auf die Pigmentierung des Körpers oder der Augen wurden gelegentlich während der Exposition mit Rosuvastatin, Propranolol, Edoxaban, Cyclophosphamid und 3,4-Dichloranilin beobachtet. In allen Fällen wurde eine verminderte Körperpigmentierung bei weniger als fünf Embryonen pro Behandlung am Ende des Tests und ohne Zusammenhang mit steigenden Konzentrationen beobachtet.

Die Herzschlagrate war ein empfindlicher Endpunkt für drei der neun ausgewählten Prüfsubstanzen, nämlich Propranolol, 3,4-Dichloranilin und Cyclophosphamid. Bei den beiden letztgenannten Substanzen wurden am Testende tödliche Wirkungen in ähnlichen Konzentrationsbereichen beobachtet, die die Herzschlagrate bei 48-stündiger Exposition beeinflussen, was auf eine allgemeine Stressreaktion hinweist. Bei Propranolol hingegen überlebten alle eingeführten Embryonen bis zum Testende in der höchsten Testkonzentration von 50 mg/L. Die Herzschlagrate war bei allen vier Propranolol-Behandlungen mit Konzentrationen über 0.5 mg/L signifikant reduziert, was auf eine spezifische Wirkungsweise hinweist.

Die Körperlänge der geschlüpften Larven wurde durch vier Prüfsubstanzen (3,4-Dichloranlin, Cyclophosphamid, Propranolol und Rosuvastatin) negativ beeinflusst. Bei Propranolol und Rosuvastatin lag die für Larven aus der Kontrolle und der jeweils höchsten Testkonzentration ermittelte Differenz unter 10 %, was die Berechnung gültiger EC_x-Werte einschränkte und nur die Bestimmung einer NOEC ermöglichte. Ödeme im Bereich des Dotters wurden gelegentlich beobachtet, es wurde jedoch keine signifikante Wirkung festgestellt. Das Auftreten von Ödemen im Herzbereich wurde jedoch durch vier der neun getesteten Substanzen (3,4-Dichloranilin, Cyclophosphamid, Rosuvastatin und Propranolol) signifikant erhöht. Missbildungen der Otolithen wurden bei dem Test mit Cyclophosphamid beobachtet, Missbildungen des Dotters der Embryonen wurden bei den Tests mit 3,4-Dichloronalin und Cyclophosphamid beobachtet.

Missbildungen der Wirbelsäule wurden bei Embryonen beobachtet, die 3,4-Dichloronalin, Cyclophosphamid und Rosuvastatin ausgesetzt waren.

Es wurden zusätzliche Wirkungen festgestellt: Eine signifikante Anzahl von Embryonen zeigte nach 24-stündiger Exposition gegenüber 3,4-Dichloranilin und Cyclophosphamid keine spontanen Bewegungen. Propranolol und Cyclophosphamid verursachten den Verlust der Schwimmfähigkeit der überlebenden Larven. Cyclophosphamid verursachte krampfartige Bewegungen überlebender Embryonen. Die Exposition gegenüber 3,4-Dichloranilin verzögerte die Entwicklung der Augen, und Propranolol verursachte einen dunkel gefärbten Dottersack (fast ohne jegliche Transparenz). Palbociclib verursachte eine mangelhafte Entwicklung des Kopfes, insbesondere durch die reduzierte Größe des Kopfes. Diese Beobachtung wurde jedoch qualitativ bewertet, und es wurde keine Messung der Kopfgröße durchgeführt.

Diskussion

Die erste Hypothese des vorliegenden Projekts besagte, dass die positiven Modellsubstanzen und die Testsubstanzen aus der spezifischen MoA-Gruppe spezifische Wirkungen in den jeweils ausgewählten Testsystemen hervorrufen würden. Dies wurde im FET für beide positiven Modellsubstanzen bestätigt: Propranolol beeinflusste die Herzschlagrate und Cyclophosphamid induzierte teratogene Effekte. Für die Testsubstanzen konnte die Hypothese nicht bestätigt werden, da die Substanzen aus der Gruppe "Kardiologie" nicht alle die Herzschlagrate beeinflussten und die Substanzen aus der Gruppe "Onkologie", nicht alle teratogene Wirkungen hervorriefen. Für *Lemna sp*. wurden bei den drei ausgewählten Statinen (Atorvastatin, Pitavastatin, Rosuvastatin) starke Wirkungen vermutet und in der Versuchsphase wurden diese Erwartungen bestätigt. Ob dieser Befund tatsächlich mit dem MoA als HMG-CoA-Reduktase-Hemmer zusammenhängt, also auf spezifische, mit dem pharmakologischen MoA zusammenhängende Effekte hinweist, wurde durch eine aktuelle Studie bestätigt, die den MoA von Atorvastatin in Transkriptomanalysen untersuchte (Loll et al. 2022). Für nur eines der drei Statine lagen EPAR-Daten zum Vergleich vor. Für die drei Statine lagen keine EPAR-Daten vor, aber für Atorvastatin konnten Daten aus einem Sicherheitsdatenblatt und für Rosuvastatin Daten aus der Literatur verwendet werden,

Die zweite Hypothese (Wirkungen bei niedrigeren Konzentrationen als in den in der ERA geforderten Standardtestsystemen) wurde für den FET eindeutig widerlegt. Bei keiner der Prüfsubstanzen war irgendein Endpunkt im FET niedriger als der im EPAR des jeweiligen Wirkstoffs angegebene Standardendpunkt. Für 6 von 10 Wirkstoffen (Atorvastatin, Rosuvastatin, Dabrafenib, Palbociclib, Pemetrexet, Imatinib), für die dieser Vergleich möglich war, war mindestens ein mit Lemna abgeleiteter Endpunkt niedriger als die in den EPARs des jeweiligen Wirkstoffs angegebenen Endpunkte.

Lemna sp. ist besonders empfindlich gegenüber den Dihydrofolatreduktase-Hemmern Pemetrexet und Methotrexat, die beide nicht zur Kategorie der nach 2006 als Wirkstoffe gehören, aber in dem oben genannten Testsystem nachweislich starke unerwünschte Wirkungen hatten. Methotrexat, der erste Wirkstoff in der Klasse, war jedoch im Vergleich zu Pemetrexet wesentlich stärker (Faktor >300). Da beide Substanzen nur minimale Unterschiede in ihrer Struktur aufweisen, wurde initial vermutet, dass die Empfindlichkeit zwischen diesen Wirkstoffen gleich ist. Da jedoch selbst kleine Unterschiede in der Struktur zu unterschiedlichen Potenzen führen können, z. B. in Bezug auf das Bindungspotenzial an einen Rezeptor oder die Enzymhemmung, könnte die Wirkung bei einer Substanz im Vergleich zu einer ähnlichen Substanz ausgeprägter sein. Vergleichbare Beobachtungen wurden auch bei anderen Wirkstoffpaaren mit ähnlicher Struktur gemacht, z. B. Imatinib und Afatinib, allerdings nur mit dem Faktor 2 (Afatinib ist wirksamer als der erste Wirkstoff der Klasse, Imatinib). Dies ist ein

häufiges Problem in der Ökotoxikologie, da die Größe der Wirkung von Stoffen mit ähnlichem MoA in verschiedenen Spezies schwer vorherzusagen ist und auch QSARs nur Hinweise auf Gefahren und nicht auf Risiken geben können. Basierend auf den EPAR-Daten für Methotrexat zeigt sich, dass eine Bewertung gemäß der EMA-Richtlinie weniger protektiv wäre wie eine Bewertung basierend auf den abgeleiteten Lemna-Endpunkten.

Die Befürchtung, dass die Wirkstoffe der neueren Generationen eine höhere Ökotoxizität aufweisen würden als die der älteren Generationen (dritte Hypothese), konnte nicht bestätigt, aber auch nicht eindeutig widerlegt werden. Die Antineoplastika der neueren Generation wiesen im FET eine höhere Toxizität auf als der erste Wirkstoff der Klasse, Imatinib. Das Gleiche galt für das Lemna-Testsystem und den Comet-Assay.

Kürzlich zugelassene *First-in-Class*-Arzneimittel mit spezifischen MoA waren zum Beispiel der Xa-Hemmer Edoxaban und der Mikrotubuli-Inhibitor Paclitaxel, die beide nach 2006 auf den Markt kamen. Für beide Substanzen wurden zweite Substanzen ausgewählt, um die Empfindlichkeit der Testsysteme gegenüber spezifischen MoA zu belegen, nämlich Rivaroxaban als Wirkstoff der neueren Generation im Vergleich zu Edoxaban und Cabazitaxel im Vergleich zu Paclitaxel. Da in beiden Fällen der erste Wirkstoff der Klasse einbezogen wurde, bot sich die Möglichkeit, die dritte Hypothese zu verifizieren oder zu falsifizieren.

Da die Faktor-Xa-Inhibitoren Edoxaban und Rivaroxaban als Negativkontrollen für die Lemna-Studien gewählt wurden, weil man vermutete, dass der MoA in Pflanzen nicht nachweisbar ist, konnte die Hypothese in diesem Fall nicht geprüft werden. Diese Substanzen wurden ursprünglich aufgrund einer vermuteten Wirkung in der FET ausgewählt, die jedoch nicht beobachtet wurde.

Die Mikrotubuli-Inhibitoren Paclitaxel und Cabazitaxel standen im Verdacht, Auswirkungen auf sich aktiv teilende Organismen zu haben, d. h. z.B. *Lemna sp.*. Bei Cabazitaxel wurde in der Tat eine Wirkung beobachtet, während bei Paclitaxel, dem ersten Vertreter seiner Klasse, keine Wirkung festgestellt wurde. Auch wenn die Lemna-Studien nicht ausdrücklich auf diesen MoA empfindlich waren, war in diesem Fall der Wirkstoff der neueren Generation zumindest wirksamer als der der ersten Klasse.

Für die Statine liegen keine EPARs vor, aber für Atorvastatin waren Daten aus einem Sicherheitsdatenblatt verfügbar, aus denen hervorging, dass *Lemna sp.* empfindlicher auf das Statin reagierte als die Standard-Testorganismen (hier: *Daphnia magna* NOEC: 0.2 mg/L), wobei die Unterschiede je nach der im Test verwendeten Art *Lemna minor* bzw. *Lemna gibba* um den Faktor 3 bis 15 variierten. Die Unterschiede hängen auch mit dem pH-Wert des Testmediums zusammen, der bei dem Test mit *Lemna minor* (Steinberg-Medium, pH 7.5) viel höher ist als bei dem Test mit *Lemna gibba* (20 x AAP, pH 5.5). Daher ist der Vergleich der erhaltenen Daten mit den Ergebnissen der Standardtests schwierig. Für Rosuvastatin liegen Literaturdaten für Algen vor, die auf eine vergleichsweise geringe Empfindlichkeit der Algen hinweisen (330 mg/L-350 mg/L; Untersuchungen gemäß FDA *Technical Assistance Document* 4.01). Es wurde jedoch gezeigt, dass Rosuvastatin zu einer relativ hohen chronischen Toxizität für *Daphnia magna* führt, mit einer NOEC nach 21 Tagen von 18 µg/L (Studie durchgeführt gemäß FDA *Technical Assistance Document* 4.09). Bei *Lemna sp.* ergab der empfindlichste Endpunkt (Blattfläche) eine NOEC von 6 µg/L für Rosuvastatin in der Nicht-GLP-Studie mit *L. gibba* und eine NOEC von 17 µg/L (Blattfläche) und 5 µg/L (Blattanzahl) in der GLP-Studie mit *L. minor*. Das Testsystem scheint also sehr empfindlich auf die Substanzklasse der Statine zu reagieren, mit einem Unterschied von etwa Faktor 4 in Bezug auf den empfindlichsten Endpunkt der Standardtestorganismen (hier Daphnien) und einer wesentlich höheren Empfindlichkeit im Vergleich zum Standard-Primärproduzenten im Wasser (Algen).

Es wurde außerdem vermutet, dass *Lemna sp*. als sich aktiv teilende Organismen empfindlich auf onkologisch aktive Substanzen reagieren. In der Tat bestätigen die Ergebnisse diese Hypothese in den meisten Fällen. Allerdings war *Lemna sp*. nicht durchgängig empfindlicher als Algen. So könnte die Empfindlichkeit zusätzlich vom Zeitpunkt der Exposition abhängen, was zum Beispiel als möglicher Grund für die erhöhte Empfindlichkeit von *Lemna sp*. gegenüber Methotrexat postuliert wurde. Andere onkologisch aktive Substanzen mit unterschiedlichen MoA, die eine geringere Toxizität für Algen im Vergleich zu *Lemna sp.* zeigten, waren Afatinib, Imatinib und Dabrafenib, während Cabazitaxel, Abemaciclib und Palbociclib für *Lemna sp.* weniger toxisch waren als für Grünalgen.

Der Comet-Assay wurde durchgeführt, um herauszufinden, ob dieses Testsystem empfindlichere Daten für die Prüfung onkologisch aktiver Substanzen liefern könnte, da diese eine Wirkung auf die DNA-Integrität haben könnten. Daher untersuchten wir die antineoplastische Substanz Cyclophosphamid als Positivkontrolle und mehrere TKIs als mutmaßliche Substanzen, die zu DNA-Strangbrüchen führen könnten. Als Negativkontrollen wurden die kardiologisch aktiven Substanzen Edoxaban und Pitavastatin getestet.

Das korrekte Funktionieren des Testsystems wurde validiert, da Strangbrüche für die Positivkontrolle H_2O_2 und für Cyclophosphamid beobachtet wurden. Es wurde festgestellt, dass der Comet-Assay im Vergleich zu den bereits in der ERA für Arzneimittel enthaltenen Testsystemen weniger empfindlich war und die meisten Testsubstanzen im Assay keine Wirkung zeigten. Interessanterweise führte die Exposition von Daphnien gegenüber Pitavastatin zu Auswirkungen im entsprechenden Comet-Assay. Dies war wahrscheinlich auf die vermutete erhöhte Empfindlichkeit dieser Spezies gegenüber Statinen zurückzuführen, die auf der Hemmung der HMG-CoA-Reduktase beruht, die bekanntermaßen auch ein empfindlicher Signalweg bei Daphnien ist und zu Effekten auf die Häutung und die Fortpflanzung führt; Endpunkte, die wahrscheinlich zu Auswirkungen in Daphnien-Reproduktionsstudien führen.

Da unsere Studien jedoch nicht zu den vermuteten Effekten führten, ist die Methode wahrscheinlich nicht für die Umweltrisikobewertung von Humanarzneimitteln geeignet, da sie nicht die genetischen Schäden bestätigt, die die Substanz bei einem umweltrelevanten Modellorganismus voraussichtlich verursachen würde.

Schließlich wurde untersucht, ob die Bewertung subletaler Endpunkte im FET zu niedrigeren Empfindlichkeiten führt als die, die bei den Toxizitätstests für Fische im frühen Lebensstadium gemäß OECD TG 210 (OECD 2013) ermittelt werden, wie sie im Rahmen der Umweltrisikobewertung durchgeführt werden.

Nur einige der getesteten Substanzen verursachten Effekte im FET. Keine der ausgewählten Prüfsubstanzen führte zu wirksamen Konzentrationen, die unter den in den EPARs für FELS-Tests angegebenen lagen. Daher scheint der mit subletalen Endpunkten ergänzte FET keine empfindlichere Methode zu sein als der in der EMA-Richtlinie geforderte Standardtest für Fische im frühen Lebensstadium.

Zusammenfassend lässt sich sagen, dass von den drei im Rahmen dieses Projekts angewandten Testsystemen der Wachstumshemmungstest für *Lemna sp.* gemäß OECD TG 221 (OECD 2006) ein Testsystem ist, das als zusätzlicher Test für aquatische Wirkungen in die Umweltrisikobewertung bestimmter Klassen von Humanarzneimitteln integriert werden könnte. Es ist zum Beispiel empfindlich gegenüber Substanzen, die auf MoA wirken, die während der Evolution konserviert wurden, wie der HMG-CoA-Weg. Darüber hinaus führen Substanzen, die auf sich aktiv teilende Organismen einwirken, zu niedrigen effektiven Konzentrationen, und die empfindlichsten Arten können übersehen werden, wenn der Leitfaden nur die bereits einbezogenen Arten berücksichtigt. Auch beim Vergleich der ermittelten NOEC mit allen in den

EPARs angegebenen Endpunkten ergaben Studien gemäß OECD TG 221 (OECD 2006) bei den meisten Prüfsubstanzen Wirkungen, die entweder gleich empfindlich oder empfindlicher als der empfindlichste im EPAR angegebene Endpunkt (sofern verfügbar) waren. Eine Ausnahme bildete z. B. Cabazitaxel, für das der niedrigste EPAR-Endpunkt 17-mal niedriger war als die mit *Lemna sp.* abgeleitete NOEC.

Die Wachstumshemmung bei *Lemna sp.* war ein empfindlicherer Endpunkt als die Standardendpunkte in den EPARs für mehrere Substanzen. Da der Unterschied zu den in den EPARs angegebenen NOECs oft weniger als das Zehnfache beträgt, spiegelt die höhere Empfindlichkeit in manchen Fällen wahrscheinlich die normale Variation der artspezifischen Empfindlichkeit wider. Unabhängig von den zugrundeliegenden Gründen liefert der Lemna-Wachstumshemmungstest relevante zusätzliche Endpunkte für das ERA von zumindest einigen Wirkstoffen, da er eine bisher nicht berücksichtigte Artengruppe (aquatische Makrophyten) repräsentiert.

Ausblick

Das Projekt wirft mehrere Fragen auf, die in einem Folgeprojekt behandelt werden könnten. Von besonderem Interesse wären die folgenden:

Lemna sp. zeigt eine spezifische Empfindlichkeit für zwei Substanzen mit einem pharmakologischen MoA, der sich auf den Mevalonatweg bezieht (Atorvastatin, Rosuvastatin), einen Kinaseinhibitor (Dabrafenib) und zwei Dihydrofolat-Reduktase-Inhibitoren (Pemetrexed, Methotrexat). Für einige dieser Stoffe lagen jedoch keine EPARs oder andere Informationen (z. B. FASS-Daten oder Sicherheitsdatenblätter) zu den Effektlevel vor (außer für Rosuvastatin). Daher konnte nicht festgestellt werden, ob die Empfindlichkeit von *Lemna sp.* im Vergleich zu den während der ERA bewerteten Standardendpunkten wesentlich geringer ist. Die Durchführung zusätzlicher Standardstudien gemäß OECD TG 201 und 211 (210 aus Gründen des Tierschutzes ausgenommen) mit den oben genannten Substanzen würde die Interpretation der in dieser Studie erzielten Ergebnisse zusätzlich aufwerten. Um zu verifizieren, dass die Wirkungen auf dem spezifischen MoA der Substanzen beruhen, würden außerdem transkriptomische Analysen, wie sie in Loll et al. 2022 für Atorvastatin in *Lemna minor* durchgeführt wurden, Informationen über die spezifischen genomischen Reaktionen in den Testorganismen liefern.

Der Wachstumshemmungstest bei *Lemna sp.* Ist somit ein relevanter zusätzlicher Test für die ERA zumindest einiger Wirkstoffe - Dihydrofolatreduktase-Hemmer, Statine und BRAF-Serin-Threonin-Kinase-Hemmer, wie Dabrafenib.

1 Introduction

Since the 1990s, it has been known that pharmaceuticals pose risks to the environment. In 2006, the European Medicines Agency (EMA) adopted a guideline on the environmental risk assessment of medicinal products for human use (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2) that allows an assessment of the hazards and risks of human pharmaceuticals based on the data available on pharmaceutically active substances (European Medicines Agency 2006). The guideline describes a tiered approach for the assessment of pharmaceuticals. In Phase I, the assumed environmental concentrations in surface waters (PEC = Predicted Environmental Concentration) are estimated, while in Phase II the environmental behaviour and the effects on different trophic levels are determined to derive Predicted No Effect Concentrations (PNEC). In the risk assessment, PEC and PNEC are then compared with each other. This system has strengths and weaknesses, but has been successfully applied in recent years.

Since 2006, a large number of pharmaceuticals that are already very effective at their pharmacological target have been newly approved. The high pharmacological specificity and potency raised concern that these new-generation pharmaceuticals are more potent and specific than first generation pharmaceuticals and that they therefore also affect non-target organisms in the environment at very low concentrations. Furthermore, pharmaceuticals with new modes of action (MoA) or with innovative molecular mechanisms (RNA-technology) were marketed in recent years. These new-generation pharmaceuticals are possibly not adequately evaluated following the actual Directive, as the active substances may have been not yet as potent when the Directive was developed.

The tests currently required for Phase II risk assessment for the aquatic compartment include the determination and evaluation of chronic toxicity using the growth inhibition test in algae (OECD Guideline 201) (OECD 2011), the reproduction test in Daphnia sp. (OECD Guideline 211) (OECD 2012) and the Fish Early Life Stage toxicity test (OECD Guideline 210) (OECD 2013). The apical endpoints (survival, growth and reproduction) covered in these studies may not cover all effects, especially those caused by very specific modes of action (MoAs) or only occurring in organisms that have the appropriate receptors. The draft EMA guideline, which is currently under revision and has not yet been published, will also only take to a limited extent into account the sensitivity to specific MoAs and for individual substance categories.

The literature search preceding this project (FKZ 3718 65 420 1 - Project Part I, Kohler et al. (2019)), which was carried out at the Fraunhofer IME under the same leadership, revealed that 448 new pharmaceuticals were approved in the period from 2006 to 2018 (extracted from 2006 – 2018th Nature Reviews Drug Discovery).

Most of the newly approved pharmaceuticals belonged to the category's "oncology" (83), "neurology" (50) and "infections" (79). However, in the assessment strategies within the above-mentioned literature study, the category "infections" was not considered, as this category mainly included antibiotics, that are already represented by tests with cyanobacteria and which represent the most sensitive species for this substance class. Another category into which many of the pharmaceuticals were classified was "cardiology" (28). Based on these data, the categories of oncology, neurology and cardiology were selected for the individual development of tailored assessment strategies, or to propose test systems that are potentially more sensitive than those listed in the EMA guideline.

The pharmaceuticals belonging to these three categories were classified into subgroups based on e.g. the respective target structures such as receptors, transporters, ion channels or enzymes, or the DNA itself.

During the literature search in project part I, it was found that no studies with alternative test organisms or test systems were available for the new generation pharmaceuticals. For some substances, the data for the environmental risk assessment are publicly available in the European Public Assessment Report (EPAR) for the test systems algae (OECD guideline 201) (OECD 2011), daphnia (OECD guideline 211) (OECD 2012) and fish (OECD guideline 210) (OECD 2013), however, further public studies that would have enabled the creation of a tailored assessment strategy based on the new pharmaceuticals themselves were not accessible. Thus, a literature search was conducted on model substances with similar mechanism of action, or the same categorisation (e.g. cytostatics, cytotoxics, ion channel, receptors, enzymes), in order to identify sensitive test systems for specific substance groups, which included a large number of the new generation pharmaceuticals.

With this in mind, three additional test systems were identified that could potentially be sensitive to one or more of the three major categories. These systems included the *Lemna sp*. growth inhibition test according to OECD Guideline 221 (OECD 2006), the zebrafish embryo toxicity test (FET) according to OECD Guideline 236 (OECD 2013) and *in vitro* assays such as the comet assay (following OECD guideline 489 (OECD 2016)) to be performed with environmentally relevant organisms or cell types.

The aim of the project was to test the theoretically developed adapted ecotoxicological test strategies for new generation pharmaceuticals identified in the preceding literature study, which might not be adequately assessed by the existing guideline. The focus was on pharmaceuticals that were either administered in large quantities or in a large number of different preparations, or whose specificity indicated a particular sensitivity of individual organisms.

In the literature study, test guidelines and systems were identified that were not considered in the current EMA guideline, but which could react sensitively to substances of the identified MoAs. Both standardised studies according to OECD guidelines and *in vitro* assays to support the weight-of-evidence approach were considered.

The three above-mentioned systems were proposed, the suitability of which was to be examined as an alternative or supplement to the study types proposed in the EMA guideline. These test systems were to be evaluated on the basis of suitable new generation human pharmaceuticals for their usefulness in the development of a tailored evaluation strategy.

In total, 18 different substances were identified for investigation using the three test systems. These test substances were selected from the drug classes oncology and cardiology, as the test systems to be investigated were determined to be sensitive for these categories.

As a result, GLP-approved test reports are provided for the studies carried out according to the OECD guideline (Lemna sp., FET), which can be used for the evaluation of the test substances and which can be directly compared with the available studies carried out within the framework of the prescribed risk assessment. Furthermore, the most sensitive endpoints of a substance should be compared with those of the other tested sub-substances of the active substance class in order to determine a MoA-specific sensitivity. In order to be able to make valid statements, not only the substances of the defined drug categories were examined, but also test substances as adequate controls (e.g. negative controls: other MoA; positive controls: model substances). These substances were treated like new-generation pharmaceuticals and were counted in the number of studies to be conducted. Thus, similarities and differences in the most sensitive endpoint could be identified in comparison to the controls.

2 Goal of the project

The following hypotheses were investigated in the project:

- Standard endpoints and approaches of the current environmental risk assessment (ERA) are not sufficiently sensitive and not specific for assessing the potential effects of new-generation active pharmaceutical ingredients (APIs).
- Due to their increased target specificity and/or efficacy, new-generation APIs may induce stronger effects or effects at lower concentrations in non-standard approaches than first-generation APIs.

The test strategies proposed in the literature research (FKZ 3718 65 420 1, Kohler et al. (2019)) should enable identification of effects specifically related to the mode of action of the API and/or effects which occur at concentrations lower than the endpoints derived in the current standard tests according to the guideline on the environmental risk assessment of medicinal products for human use (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2) (European Medicines Agency 2006).

During the above-mentioned literature research, a number of test systems which might be sensitive to these new APIs were identified. These tests included the Growth Inhibition Tests with *Lemna sp.* (OECD TG 221) (OECD 2006) as well as Fish Embryo Tests (FET) with zebrafish (OECD TG 236) (OECD 2013). These test systems were of special interest due to the following reasons:

The current EMA guidance does not include any studies on macrophytes. The test species *Lemna sp*. is ideally suited to fill this gap as it is rapidly dividing, possesses a short generation time and is known to be sensitive to at least some of the proposed modes of action (MoA).

The zebrafish (*Danio rerio*) embryo is well suited to be included into the test strategy as it is a versatile and well-known organism, which was already used for a number of studies investigating different MoAs. Besides the lethal endpoints that should be assessed according to the OECD TG 236 (OECD 2013) (coagulation, no heartbeat at 48 hours post fertilization (hpf), no detachment of tail, no somite formation), a number of sublethal endpoints can be assessed additionally, e.g. the heartbeat rate, malformations of tail, head, or spine, or others. Furthermore, the fish embryo is not considered as animal test, while the inner organs are already functioning to a large extent, and it is thus sensitive to a high number of MoAs.

Additionally, to the two test systems according to OECD TGs, Comet assays with environmentally relevant cell types were performed. The cells were obtained from the water flea species *Daphnia magna*. Furthermore, the applicability of fish cell lines was investigated.

In total, 18 different test substances were tested. These substances belonged to the categories of oncologically or cardiologically active substances. The substances selection took also into account that:

- European public assessment reports (EPARs) should ideally be available for the test systems algae (OECD TG 201) (OECD 2011), *Daphnia sp*. (OECD TG 211) (OECD 2012), and fish (OECD TG 210) (OECD 2013).
- Literature data for the specific MoA of the API, either of the substance itself or of a model substance with a similar MoA were available.
- The substance was commercially available and in the budget of the project.

Additionally, to the substances of interest, i.e. substances of the new generation, appropriate positive and negative controls were chosen, which were either known to elicit a specific effect on the test system (positive substance), or which were supposed to have no effects on the test system (negative substance).

3 Substance selection

3.1 Substance selection for *Lemna sp*. studies (OECD TG 221)

In order to adopt the general approach for the identification of appropriate test substances as described above, appropriate substances for the test system *Lemna sp*. (OECD TG 221) (OECD 2006) were identified. These included substances which were likely to act on *Lemna sp*., as well as substances which should not induce any effect in this species. These substances should also belong to the same category, in order to demonstrate the specificity of the system. Positive APIs likely reacted on targets that are also present in the test species, like the mevalonic acid pathway or microtobules.

3.1.1 Cardiologically active substances

3.1.1.1 Selection of positive substances

For cardiologically active substances, the substance class of statins was of special interest for studies with *Lemna sp*.. Statins are among the cardiovascular medications that can be divided into the group of statins, which lower cholesterol, and the group of fibrates, which are known to maintain fatty acids and triglycerides. The statins in particular are competitive inhibitors of HMG-CoA reductase. In plants, these compounds inhibit HMG-CoA reductase, which regulates cytosolic isoprenoid biosynthesis in the mevalonic acid pathway (Brain et al. 2004)). In crustaceans, the mevalonate pathway is upstream of the synthesis of methyl-farnesoate, a juvenile hormone that plays essential roles in crustacean moulting, sex determination and reproduction (Santos et al. 2016)

The statins atorvastatin and lovastatin were found to be phytotoxic for *Lemna gibba* after exposure for 7 days. Brain et al. (2004) found decreased levels of both stigmasterin and β-sitosterol, which are critical components of plant membranes and regulate morphogenesis and development, and which are the end products of the mevalonic acid pathway. The EC_{10} values for atorvastatin and lovastatin were 26.1 µg/L and 32.8 µg/L, respectively.

There is also evidence that the HMG-CoA is involved in plant root growth (Liao et al. 2020), thus, this endpoint might also be affected by statins.

Based on these observations, the group of statins was chosen as substance class with a potential effect on *Lemna sp*.. During the literature research, only one statin approved after 2006 was identified (pitavastatin), while the others were approved before the EMA guideline came into effect. However, in order to prove the susceptibility of the test system to this substance class, it was decided to combine more than one test substance of the new generation, together with one positive model substance (atorvastatin), into these evaluations. During the kick off meeting, the UBA suggested for example the substances atorvastatin (for which already data for *Lemna sp*. exist; compare Brain et al. (2004)), or rosuvastatin for inclusion into the case studies.

The test substances finally chosen for the class of statins were as follows:

- Pitavastatin (C10AA08)
- Atorvastatin (C10AA05)
- Rosuvastatin (C10AA07)

3.1.1.2 Selection of another substance class

Additionally, to the group of statins, the literature research identified a new substance class with three potential test candidates. These candidates belonged to the factor Xa inhibitors, which were anti-coagulants. During the literature research, no data for any of the substances or to a potential model substance were identified. These might be due to the fact that rivaroxaban was the first orally active factor Xa inhibitor (www.drugbank.com). The mode-of-action of this substance class is the competitive inhibition of free and clot bound factor Xa, which is needed to activate prothrombin to thrombin. Thrombin is a serine protease that is required to activate fibrinogen to fibrin, which is the loose meshwork that completes the clotting process. Since one molecule of factor Xa can generate more than 1000 molecules of thrombin, selective inhibitors of factor Xa are profoundly useful in terminating the amplification of thrombin generation.

As this pathway is not present in plants in general, our hypothesis was that these substance class might not result in as pronounced effects as suspected for the statins, and thus might represent appropriate negative controls. This comparison of different MoAs was considered to be of interest in order to demonstrate that this test system acted more sensitive to APIs which act on pathways present in *Lemna sp.,* than to APIs which act on pathways absent in *Lemna sp.*. Thus, the following anticoagulant substances were tested as negative control in the Lemna studies:

- Edoxaban (B01AF03)
- Rivaroxaban (B01AF01)

3.1.2 Oncologically active substances

3.1.2.1 Selection of positive substances

The test system *Lemna sp.* (OECD 2006) was indicated to be sensitive to oncologically active substances due to its short generation time. It was chosen based on a study of Białk-Bielińska et al. (2017) that tested six oncological agents and one metabolite in a test battery to determine the biological effects of the substances. They used six of the most commonly used cancer drugs, i. e. cyclophosphamide, ifosfamide, 5-fluorouracil (5-FU), imatinib, tamoxifen and methotrexate. The test organisms included the bacterium *Vibrio fischeri* (DIN 38412-L34), the standard alga *Raphidocelis subcapitata* (OECD TG 201) (OECD 2011), the water flea *Daphnia magna* (OECD TG 202) (OECD 2004) and the duckweed *Lemna minor* (OECD TG 221) (OECD 2006). They found that *Lemna minor* was the most sensitive organism. The most toxic substances in this study were 5-FU (highly toxic for algae, EC_{50} = 0.075 mg/L) and methotrexat (depending on the test conditions, EC_{50} = 0.08-0.16 mg/L (*Lemna minor*)). It was assumed that the increased sensitivity to aquatic primary producers (algae, water plants) can be attributed to the extended exposure time compared to bacteria, since the specific MoA (inhibition of DNA replication) of the two substances could thereby produce a specific effect. This led to the conclusion that actively dividing cells are susceptible to substances that affect DNA replication. After the kick off meeting, the UBA suggested to test the substances methotrexat and pemetrexed, which are both known inhibitors of DNA replication. However, these substances do not belong to the new APIs approved after 2006. Thus, these substances were chosen to be tested:

- Methotrexate (L01BA01)
- Pemetrexed (L01BA04)

An additionally performed literature research revealed that some herbicides (e.g. pendimethalin) specifically act on microtubule formation (Cedergreen and Streibig 2005), which is also a MoA of one substance class of oncologically active substances. Cedergreen and Streibig

(2005) determined an EC_{10} for pendimethalin for the test species *Lemna minor* of 90 µg/L. Thus, it was assumed that oncologically active substances with a similar MoA may have similar results on this test system. We thus suggested cabazitaxel and paclitaxel to be tested in as part of these case studies.

- Cabazitaxel (L01CD04)
- Paclitaxel (L01CD01)

A third potential substance class for oncologically active substances was the class of kinase inhibitors, which belonged to the cytostatics. For this class, information on the thyrosin kinase inhibitors erlotinib and imatinib were obtained during the literature research. Białk-Bielińska et al. (2017) determined an EC_{50} of 61.05 mg/L for *Lemna minor* for imatinib. Thus, we suggested to use this substance as model substance, which resulted in an effect in the test species. To the class of kinase inhibitors, substance like abemaciclib, palbociclib, or ribociclib (CDK4/CDK6 kinase inhibitors), neratinib or afatinib (TKI growth factor), and dabrafenib or vemurafenib (BRAF-Serin-Threonin kinase inhibitors) can be attributed to.

Finally, the following test substances were chosen as cytostatics:

- Dabrafenib (L01XE23)
- Abemaciclib (L01XE50)
- Palbociclib (L01XE33)
- Ribociclib (L01XE42)
- Neratinib (L01XE45)
- Afatinib (L01EB03)
- Imatinib (L01XE01)

3.1.2.2 Selection of a negative substance

As suggested above, the class of anticoagulants (apixaban, edoxaban, rivaroxaban) was tested as negative controls for the *Lemna* studies.

3.2 Substance selection for comet assays (non-GLP)

In general, this test system was chosen as it was suspected to be sensitive for substances with act on DNA, i.e. oncologically active substances, rather than other substances. The comet assay is usually performed for assessement of genotoxicity using mammalian cell lines. The test is validated as OECD test guideline 489 (OECD 2016) and applied in toxicological risk assessment in order to identify DNA damage. In order to increase environmental relevance, it was decided to apply this assay to cells from daphnids and zebrafish.

The literature research performed in the previous studyfocussed on the comet assay with cells either from *Daphnia magna* or *Ceriodaphnia dubia* and its sensitivity to APIs. In the current study, the obtained data were again evaluated for the general sensitivity of daphnids to the identified substances. Due to the availability and better comparability of later results with available EPARs on Daphnia acute studies, which mainly utilized *Daphnia magna* as test species, it was decided to focus on this species in the comet assay.

Additionally, to the cells from *Daphnia magna*, an appropriate fish cell line was chosen to be tested. The choice considered a) the origin of the cells, which should be preferably zebrafish (*Danio rerio*) as well as b) their availability. We obtained the cell line ZF-L, which is a zebrafish liver cell line also used in comet assays already described in the literature.

The comet assay was primarily focussed on Daphnia cells and was performed secondly with the ZF-L cell line. Thus, in total 9 substances were tested with Daphnia cells and 7 substances were tested with the cell line. The rational for substance selection is described below.

The choice of test substances was focussed on those substances which were also used for the other test systems in order to allow a comparison of the effect concentrations. Furthermore, for the Comet assays with cells from Daphnia magna and ZF-L cells, similar test substances were chosen. Furthermore, for most of the substances there was no evidence if the comet assay has effects on cells from *Daphnia magna*, the choice of test items focussed furthermore on substances with known effects in Daphnia acute and chronic studies. In this case, it was considered that a potential effect on the %TI could be due to cytotoxic effects due to high test item concentrations resulting in acute toxitiy.

3.2.1 Cardiologically active substances

In general, as described above, the comet assay is designed to detect substances which cause DNA damage. This DNA damage could be induced potentially by specific classes of oncologicals, i.e. those that result in genotoxicity or which induce oxidative stress. Thus, testing of cardiologically active substances was to verify the specificity of the test system and effects were not expected. However, available EPARs for statins revealed that daphnids were the most sensitive species. Thus, we suggested to choose this substance class to be tested in the comet assay. The following substances were chosen for the comet assay with Daphnia cells:

- Pitavastatin (C10AA08)
- Rosuvastatin (C10AA07)

As it was not evident if these substances indeed induced an effect in the comet assay or were rather negative controls, it was decided to test another cardiologically active substance from the class of factor Xa inhibitors, which definitely should not result in any effect:

- Edoxaban (B01AF03)

For the comet assays with the cell line, no effects were suspected for both substance classes. Thus, it was decided to only test one substance of each class, which were the following:

- Pitavastatin (C10AA08)
- Edoxaban (B01AF03)

3.2.2 Oncologically active substances

The literature search showed that the comet assay was sensitive to cytotoxic and cytostatic substances. *Ceriodaphnia dubia* was chosen as the test system in the comet assay in one study due to its short life span, high reproductive capacity and genetic uniformity (Russo et al. 2018). They investigated the pyrimidine analogue 5-fluorouracil (5-FU), Cis-pT, etoposide and ifosfamide.

Other studies also used *Daphnia magna* as a test system (Pellegri et al. 2014) and proved its suitability with known genotoxicants ($CdCl_2$ and H_2O_2). Parrella et al. (2015) tested 5-

fluorouracil (5-FU) cisplatin (CisPt), doxorubicin (DOX), etoposide (ET), imatinib (IM), and capecitabine (CAP) in acute and chronic daphnia studies. They identified acute toxicity to CisPt and DOX, and chronic toxicity of daphnids to 5-FU, at concentration of 26.4 µg/L for *Daphnia magna* (21 d reproduction) and 3.35 µg/L for *Ceriodaphnia dubia* (7 d reproduction). This indicated that daphnids are generally susceptible to this class of oncologically active substances, in this case cytotoxic substances. Furthermore, Parella et al. (2015) identified positive results in the Comet assay with *Daphnia magna* cells for the same substances in a mg/L range.

Novak et al. (2017) investigated the cytotoxicity of anticancer drugs in ZF-L cells and found a LOEC of 37.5 mg/L for cyclophosphamide. Further substances that were tested in this study included CisPt, imatinib and etoposide. Thus, cyclophosphamide was found to result in effects in both test systems, daphnid cells and the cell line.

The final choice of substances from the pool of oncologic substance was based on available information from the literature (compare above) and known general sensitivity of *Daphnia magna* to the test item. It was furthermore considered that effects on %TI might be a result of systemic toxicity. Thus, only concentrations not resulting in acute toxitiy were considered for evaluation.

The EPARs revealed *Daphnia magna* as most sensitive species for example for the BRAF-Serin-Threonin kinase inhibitors vemerufanib and dabrafenib, and for the microtubule inhibitor cabazitaxel. Thus, these APIs, together with the substances of similar MoA were identified to be best suited to be tested in the comet assay with cells from *Daphnia magna*. Based on the above presented information, it was finally agreed to test the following substances in *Daphnia magna* cell comet assay, considering the information on sensitivity as well as availability and price:

- Cyclophosphamide (L01AA01)
- Abemaciclib (L01XE50)
- Dabrafenib (L01XE23)
- Palbociclib (L01XE33)
- Ribociclib (L01XE42)
- Imatinib (L01XE01)

Similar substances should be also tested for the cell line. However, as initially only 15 comet assays should be performed in total, it was decided not to perform a test with the substance ribociclib, as other substances with similar MoA were included.

3.2.3 Positive control

Additionally, to the substances of interest, the comet assay was performed with H_2O_2 as positive control.

3.3 Substance selection for 8 Fish Embryo Tests (OECD TG 236)

The hypotheses of the proposed test strategies involving fish embryo toxicity (FET) (OECD 2013) tests that shall be tested in the present project are:

1. The model substances induce specific effects in the FET (effect on heart beat rate for cardiological substances and teratogenic effects for antineoplastics).

2. These specific effects in the FET occur at concentrations below available standard endpoints for a given API.
3. APIs of newer generations show higher toxicity related to these specific endpoints than APIs from first generations.

3.3.1 Selection of test substances for the FET

It was decided to test specific substances based on the following criteria:

- Positive model (control) substances that should confirm the test strategy and in particular hypotheses 1 and 2, e.g. substances based on which the test strategy had been developed.
- One positive model substance per strategy, i.e. one cardiological and one oncological.
- Negative model (control) substances that should confirm the specificity of the test strategy, i.e. that should not show the effects stated in hypotheses 1 and 2.

Since heart beat rate is the specific endpoint for cardiological substances and teratogenic effects the specific endpoint for oncological substances, the selected positive model substances served as negative model substance for the respective other test strategy. This reduced the testing of control substances, and allowed the investigation of 6 actual test substances in the FET.

Hence, for each test strategy one positive (control) substance and three test substances needed to be selected. Of the test substances, one should ideally be characterized as first-generation and the other two as a newer generation in order to test hypothesis 3.

3.3.2 Cardiologically active substances

3.3.2.1 Selection of positive model substance

Statins and factor Xa inhibitors have been proposed as test substances for the cardiological test strategy in the report of the first project. Yet, no positive model substance for the cardiological test strategy in the FET has been proposed. Given that the test strategy employed heart beat rate as endpoint, ß-blockers appeared as suitable positive model compounds since reduction of heart beat rate in the FET has been shown for this therapeutic group (Bittner et al. 2018). Based on water exposure concentrations and at neutral pH, propranolol showed the lowest EC_{50} values with regard to reduction of survival and heart beat rate as well as sublethal endpoints compared to the other three ß-blockers (Bittner et al. 2018). Similar to imatinib (see below), propranolol is considered as first-in-class, i.e. the first beta-blocker that was authorised (in 1967, www.drugbank.ca). Therefore, it was selected as positive model substance for evaluating the cardiological test strategy for the FET.

3.3.2.2 Selection of test substances

New-generation ß-blockers were identified to be ideal to test in comparison to propranolol. However, even the newest third-generation ß-blockers such as carvedilol and nebivolol have been invented well before 2006. No EPARs with an ERA appear to be available for them. Hence, other new-generation APIs used to treat hypertension or, in general, symptoms related to the cardiological system were proposed as test substances. Lomitapid as new cholesterol-lowering substance (Coudert and Daulhac-Terrail 2020) has been suggested by UBA.

However, the three test substances selected for the cardiological test strategy in the FET were simply chosen among the APIs selected for the Lemna testing in order to keep the analytical method development within the limits of the project. Thus, the following test substances were selected:

- Propranolol (C07AA05)
- Edoxaban (B01AF03)
- Rivaroxaban (B01AF01)
- Rosuvastatin (C10AA07)

3.3.3 Oncologically active substances

3.3.3.1 Selection of positive model substance

Imatinib and erlotinib were identified in the report of the first project as positive model substances; both are cytostatic thyrosin-kinase inhibitors (TKIs) (Table 2).

5-Fluorouracil is the only API for which the evaluation in the first project identified literature data that indicated a higher susceptibility of sublethal endpoints in the FET than standard endpoints in fish (see literature data below); it is cytotoxic.

Imatinib was first authorised in the EU in 2001. It is recognized as first in class of a new group of agents, i.e. tyrosine kinase inhibitors (TKIs), which have a highly specific mode of action (drugbank.ca, accessed 22 Sept 20; Ravichandran et al. (2015)). Imatinib is a kinase inhibitor with specificity for the tyrosine kinase ABL, which is mutated (gene fusion of ABL gene to a fragment of the BCR gene, resulting in the expression of a fusion protein with increased kinase activity) in patients with chronic myeloid leukemia (CML). Imatinib effectively inhibits also other tyrosin kinase enzymes. It became and still is the standard treatment for CML, gastrointestinal stromal tumors (GISTs) and some other forms of cancer, except in many developing countries where the access of patients to TKIs is limited (Gómez-Almaguer et al. 2016). There is an EPAR available for imatinib as well as literature data on effects on a range of non-standard endpoints.

Erlotinib was first authorised in the EU in 2005. It is a TKI that specifically targets an epidermal growth factor receptor gene (EGFR) and nuclear receptor genes (NR1I2) and is used in the treatment of non-small cell lung cancer, pancreatic cancer and several other types of cancer (drugbank.ca, accessed 24 Sept 2020).

5-Fluorouracil is an antineoplastic antimetabolite that interferes with synthesis of DNA blocking the thymidylate synthetase (pyrimindine analogue). It is not clear whether it is currently marketed in the EU: it is not contained in market authorisations listed at the EMA webpage, but a DDD for Germany is available. It is marketed in the US and Canada. It was introduced well before 2006 (all: drugbank.ca, accessed 24 Sept 2020).

For all three substances, a full data set of standard endpoints according to EMA is available, either as EPAR or in the literature.

Table 2: Overview on standard endpoints for potential positive model substances for the FET test strategy with teratogenic compounds

API	Date of market authorization in EU	DDD (mg)*	Aquatic toxicity – chronic standardendpoints (mg/L) A / D / F **	Source of toxicity data
Imatinib	2001	500	**0.96** / 5.6 / 10	EPAR
Erlotinib	2005	125	1.39 / 0.6 / **0.52** (erlotinib HCl)	Vestel et al. (2016)
5-Fluorouracil	unclear	150	**0.002** / 0.0028 / 32	Vestel et al. (2016)

* From: Amtliche Fassung des ATC-Index mit DDD-Angaben für Deutschland im Jahre 2020 (DIMDI 2020); ** Algae / Daphnia / Fish, lowest endpoint in bold

3.3.3.2 Additional possibly relevant literature data

For imatinib, Kovács et al. (2016) report an LC_{50} of 70.8 mg/L for acute fish toxicity measured according to OECD 203 (OECD 2019). In the same publication, an LC_{50} of 65.9 mg/L is reported for a FET with zebrafish. The authors state that „Imatinib-treated zebrafish embryos were characterized with a downward curvature and thinning of the tail as well as deformation and retarded development of the fin folds at concentrations of 100 mg/L or higher. In several cases, deformed yolk sacs and disturbances in pigmentation were observed. From a 33-day early life stage test with zebrafish, Kovács et al. (2016) derived a NOEC of 1 mg/L based on survival (no significant effects on weight and length were observed). However, the chronic study suffered from high control mortality (about 25%) and none of the studies in Kovács et al. (2016) included analytical verification of test concentrations. Due to these shortcomings, the data must be seen as not reliable in a regulatory context. However, they were considered relevant and hence used to develop the propose test strategy.

For erlotinib, Besse et al. (2012) summarized ecotoxicity data particularly from the online data base fass.se. Yet, these data appeared to be erroneous (e.g. 'Daphnia reproduction 48 h'), and are likely a mistake in either fass.se or Besse et al. (2012). Since data in fass.se are usually those supplied by pharmaceutical companies, Vestel et al. (2016) was identified to be the more reliable reference (see Table 1). There are no other ecotoxicity data for erlotinib cited in the first literature study and none were found in a Web of Science search in September 2020.

For 5-fluorouracil (5-FU), Kovács et al. (2016) reported an LC_{50} of >100 mg/L for acute fish toxicity measured according to OECD 203 (OECD 2019) and an LC_{50} of 2222 mg/L for a FET with zebrafish. The EC50 for embryonic deformities in the FET was determined as 1723 mg/L at 120 hpf. This lower EC50 for sublethal effects (i.e. deformities indicating teratogenic effects) compared to acutely toxic effects was at the basis of the test strategy for oncological substances developed in the first project. However, the concentration-response curve for deformities was very steep and actual deformities were recorded at about 2000 mg/L, i.e. at about the LC_{50} for mortality in the FET. Hence, deformities are not necessarily more sensitive than mortality, but may have been linked to each other. The NOEC for a 33-day early life stage study with zebrafish was determined as 0.1 mg/L (length) and 1 mg/L (survival) in Kovács et al. (2016). Control mortality is not reported, but may have been impaired as for imatinib in the same publication. In a different study conducted by the same group (Kovacs et al. 2015), zebrafish was exposed semi-statically over 2 generations (adult F0 at exposure start) to 5-FU concentrations of 0.01, 1.0 and 100 µg/l, verified at two occasions during all of the test (about 100% of nominal in freshly prepared, but quickly disappearing within 48 h). There was high control mortality during the whole test duration (35.8% in control in FO at test end and 63% in control in F1), which renders

this study not reliable. However, while there was no impact of 5-FU on survival, length, weight, and fecundity of the F1 was observed, 5-FU related genotoxicity, DNA damage in tissues (measured by comet assay), and changes in transcriptomics were reported.

Ng et al. (2020) exposed zebrafish larvae for 8 days to low (ng/l) 5-FU concentrations and measured an increase in body length but no effects on any other parameter. However, this study suffered from a lack of analytical verification of test concentrations and high control mortality (30%), and must therefore been seen as not reliable.

3.3.3.3 Conclusion

Imatinib appears as first in class API with a relatively unspecific inhibition of tyrosin kinases as perfectly suited test substance and could be compared to two new-generation APIs. However, there is little evidence from the literature whether sublethal endpoints in the FET will be lower than standard endpoints or whether teratogenic effects will be observed at all. Erlotinib has a very limited data basis, i.e. no evidence from the literature that it could serve as a good positive model substance.

5-Fluorouracil is a cytotoxic substance that interacts with DNA synthesis. Hence, teratogenic effects can be expected and tend to have been documented in the literature. However, it is questionable whether these effects indeed occur below standard endpoints (particularly considering the sensitivity of *Daphnia* and algae).

It was therefore proposed to select none of these three APIs as positive model substance. Instead, cyclophosphamide was selected for this purpose. Imatinib however was chosen as test substance in order to provide potential comparison to the other kinase inhibitors of the new generation, afatinib and palbociclib.

Cyclophosphamide is an alkylating antineoplastic that has been on the market since 1959 (drugbank.ca). Cyclophosphamide has been used as positive model substance for teratogenic effects in tests with frog tadpoles (Turani et al. 2019), lizard embryos (Schaumburg et al. 2016), and fish embryos (Busquet et al. 2008). Cyclophosphamide needs metabolic activation and may therefore have to be tested in combination with a metabolic activation system as described by Busquet et al. (2008). Cyclophosphamide has been pointed out to be the antineoplastic most frequently measured in aquatic systems globally (Wormington et al. 2020), which renders it not only a suitable positive model substance, but indicates potential environmental relevance on its own.

Finally, the following substances were chosen for testing:

- Cyclophosphamide (L01AA01)
- Imatinib (L01XE01)
- Afatinib (L01EB03)
- Palbociclib (L01XE33)

3.3.4 Selection of test substances

The following 12 substances were chosen to be tested in the Lemna test: palbociclib, afatinib, imatinib, dabrafenib, ribociclib, abemaciclib, cabazitaxel, atorvastatin, rosuvastatin, pitavastatin, pemetrexed and methotrexate. While paclitaxel, neratinib, rivaroxaban and edoxaban were only tested in a range finder, for atorvastatin and rosuvastatin, it was decided to perform tests with *Lemna gibba* and *Lemna minor*.

The following 8 substances were chosen to be tested in the FET: afatinib, cyclophosphamid, edoxaban, imatinib, palbociclib, propranolol, rivaroxaban and rosuvastatin.

The following 9 substances were chosen to be tested in the comet Assay with cells of *Daphnia magna* and a fish cell line: abamaciclib, cyclophosphamid, dabrafenib, edoxaban, imatinib, palbociclib, pitavastatin, ribociclib and rosuvastatin (ribociclib and rosuvastatin only tested in Daphnia cells).

The final list of substances is presented in Table 26 in the Appendix A.1.

4 Analytical method development and validation

The analytical methods for the determination of each active substance in the corresponding ecotoxicological tests were developed and validated in three separate GLP validation studies. For each of the media used in the tests (Steinberg medium, 20xAAP medium, modified reconstituted water), methods were developed and were successfully validated according to the SANTE/2020/12830 guideline (European Commission 2021) (guidance document on pesticide analytical methods for risk assessment and post-approval control and monitoring purposes). All limits of quantification (LOQs) were on a level of 0.1 or 1.0 µg a.s./L, respectively.

The detailed methods and results of the validation studies were reported in the corresponding GLP study reports. All three reports were shared with all project partners as official GLP report files (scans with signatures) and as unofficial working documents (pdf files without signatures). Table 3 gives an overview of the analytical methods used for each medium and each active substance as well as their corresponding LOQs.

Table 3: Overview of the analytical methods used for each medium and each active substance and their corresponding limit of quantification (LOQ)

Medium	Active substance	Study code (Method)	LOQ
Steinberg medium	Pitavastatin	IME-021/6-22	0.1 or 1.0 µg a.s./L
	Rosuvastatin		
	Atorvastatin		
	Methotrexate		
	Pemetrexed		
20xAAP medium	Neratinib	IME-022/6-22 (Method 1)	0.1 µg a.s./L
	Afatinib		
	Dabrafenib		
	Imatinib		
	Paclitaxel		
	Cabazitaxel		
	Rivaroxaban		
	Edoxaban	IME-022/6-22 (Method 2)	1.0 µg a.s./L
	Ribociclib	IME-022/6-22 (Method 3)	1.0 µg a.s./L
	Abemaciclib		
	Palbociclib		
Modified reconstituted water	Propranolol	IME-023/6-22 (Method 1)	0.1 µg a.s./L
	Edoxaban		
	Afatinib		
	Cyclophosphamide		

Medium	Active substance	Study code (Method)	LOQ
	Rosuvastatin		
	Imatinib		
	Rivaroxaban		
	Palbociclib	IME-023/6-22 (Method 2)	1.0 µg a.s./L

The analytical methods were successfully applied in the corresponding ecotoxicological GLP studies (Lemna growth inhibition and fish embryo tests).

In addition to the initially planned studies, it was agreed to conduct additional growth inhibition tests on *Lemna gibba* with atorvastatin and rosuvastatin in 20xAAP medium. As the analytical determination of atorvastatin and rosuvastatin in this medium was not validated in the above mentioned GLP studies, these tests were performed in separate non-GLP studies under GLP-like conditions. Therefore, the analytical method that was previously validated for Steinberg medium was used for the 20xAAP medium without further validation. Corresponding matrix calibrations as well as quality control samples (both prepared in 20xAAP medium) showed the applicability of the analytical method as well as its transferability from one medium to another.

5 Lemna sp. Growth inhibition test (OECD 221)

5.1 Test principle

The objective of each study was to assess the intrinsic ecotoxicity of the test item to the freshwater floating macrophyte of the genus Lemna (duck-weed, *Lemna minor* or *Lemna gibba*) in a sediment-free system and defined standard medium (modified Steinberg medium or 20x AAP medium).

The tests were conducted according to OECD Guideline 221 (OECD 2006). The plants were exposed to five to six test concentrations arranged in a geometric series in static test system without media renewal or in a semi-static system with one or two media renewals after e.g. 2 and 5 days, respectively.

The primary parameter recorded was frond number. In addition, total frond area and/or dry weight were measured, depending on the results of a pre-test. Measurement of frond number and total frond area or dry weight were performed at the beginning of the test and at test termination after 7 days. Additionally, frond number and frond area (if necessary) were recorded on two occasions during the 7-days test period, e.g. day 3 and 5 of the test. Based on the range finder results the test concentrations and measurement parameters for the GLP main were chosen. Regarding the three parameters frond number, frond area and dry weight, in all tests either frond area or frond number were the most sensitive parameters. The OECD 221 (OECD 2006) states that additionally to the parameter frond number only one further parameter has to be observed in the main test. The test concentrations and the measurement parameters for the GLP main tests are summarized in Table 27.

To quantify substance-related effects, the inhibition of growth in relation to control cultures was determined. Growth was expressed as logarithmic increase in measurement variable (average specific growth rate) during the exposure period. As additional response variable, yield of the measured parameters was determined.

The concentration causing a 10, 20 and 50% inhibition of growth was determined and expressed as the EC_{10}, EC_{20}, and EC_{50} value. Additionally, NOEC and LOEC values were determined.

The concentration of the test item in the water phase was assessed by chemical analysis (UHPLC-MS/MS) in all test media and controls at test initiation, at each media renewal (fresh and aged), if occurred and at test termination.

5.2 Test substances

16 substances were identified to be tested in a range finding test to determine the effect on the growth of the duckweed *Lemna gibba* or *Lemna minor*. In addition, it was decided to perform further range finding tests on the effect of atorvastatin and rosuvastatin on *L. gibba* in addition to the studies with *L. minor*.

The results revealed that for the two negative controls rivaroxaban and edoxaban, there was no inhibition up to the highest test concentration of 100 mg/L. Accordingly, the test system was validated and a GLP main test was not performed with the two substances.

An analytical pre-test on the solubility of paclitaxel and neratinib, which showed no effect in the range finding tests, was performed using the same approach for the test media preparation as in the range finding tests. Both substances had only a low solubility even after 48 hours stirring (Table 28). Due to this and the nonexistent effects, it was decided to exclude paclitaxel and neratinib from the further testing.

The analytical data of the first GLP main test with Atorvastatin (IME-025/4-11/I, static) revealed a fast decrease of the test concentration with values below the LOQ (1 µg a.s./L) in the two lowest test concentrations. In addition, an analytical issue (occurrence of metabolites and no separation of phase transition) was identified, and measured concentrations in the three higher treatments might be overestimated. For rosuvastatin, this issue did not occur. However, it was decided to briefly investigate the fate of pitavastatin, pemetrexed and methotrexate before performing the GLP main tests. The highest test concentration was prepared as done in the range finding test and as proposed for the main test and test concentrations were quantified at test initiation, after 3 days and after 7 days. The measured values revealed that there was also a strong decrease of the test concentrations for pitavastatin and methotrexate, while pemetrexed remained relatively stable. The data are presented in Table 29. It was decided to perform the test with atorvastatin with two media renewals and the tests with pitavastatin and methotrexat with one media renewal. For all test substances the test concentrations were adapted.

The test concentrations, test media used and the results of all range finding tests are summarized in Table 30. In all tests the three parameters frond number, frond area and dry weight were determined.

5.3 Validity criteria

The following conditions for the validity of a standard growth inhibition test are listed in the guideline OECD 221 (OECD 2006):

The doubling time of frond number in the continuously growing control cultures (7-day growth period) (T_d = ln 2/average specific growth rate) must be less than 2.5 days (60 hours), corresponding to an average specific growth rate for frond number of 0.275 d^{-1} (approximately a seven-fold increase in seven days).

5.4 Materials and methods

5.4.1 Test organism

The genus *Lemna gibba* or *Lemna minor* (Lemnaceae, Monocotyledonous, Spermatophyta) was chosen by OECD-experts (OECD 2006) as a test organism representing freshwater aquatic plants. They were purchased from the Friedrich-Schiller-Universität Jena (Institut für Allgemeine Botanik und Pflanzenphysiologie Dornburger Str. 159, 07743 Jena, Germany). Stock cultures were maintained fulfilling the criteria of the OECD guideline (OECD 2006). Around 7 days before testing a pre-culture was established in test medium to obtain exponentially growing plants for the test. The cultures were maintained under the conditions of the test.

5.4.2 Reference substance

The sensitivity of the test organism was routinely checked using 3,5-dichlorophenol as primary standard following internal SOPs in a non-GLP test. The E_rC_{50} value for the inhibition on frond number was always in good agreement with the E_rC_{50} results of an international ring test (Sims et al. 1999).

5.4.3 Growth media

As growth medium the either synthetic 20x AAP growth medium (*Lemna gibba,* Table 3) or Steinberg medium (*Lemna minor,* Table 4) according to OECD 221 (OECD 2006) was used. All stock solutions and the medium were prepared with purified water processed using an ELGA

„PURELAB Ultra“. For 20x AAP growth medium, the pH of the medium should be 7.5 ± 0.1, while the pH of the Steinberg medium should be at 5.5 ± 0.2. The pH was adjusted by addition of a small volume of HCl (5% acid) or NaOH.

Table 4: Composition of 20x AAP growth medium (as presented in the OECD 221).

20x AAP growth medium	
Nutrients - Macroelements	**Final concentration in medium [mg/L]**
$NaNO_3$	510
$MgCl_2$ x 6 H_2O	240
$CaCl_2$ x 2 H_2O	90
$MgSO_4$ x 7 H_2O	290
K_2HPO_4 x 3 H_2O	30
$NaHCO_3$	300
Nutrients - Microelements	**Final concentration in medium [µg/L]**
H_3BO_3	3700
$MnCl_2$ x 4 H_2O	8300
$FeCl_3$ x 6 H_2O	3200
Na_2EDTA x 2 H_2O	6000
$ZnCl_2$	66
$CoCl_2$ x 2 H_2O	29
Na_2MoO_4 x 2 H_2O	145
$CuCl_2$ x 2 H_2O	0.24

Note: If individual elements were not available but those were present with different amounts of water, this element could also be used as an alternative by adjusting the initial weight.

Table 5: Composition of Steinberg growth medium (as presented in the OECD 221).

Steinberg growth medium	
Nutrients - Macroelements	**Final concentration in medium [mg/L]**
KNO_3	350.0
$Ca(NO_3)_2$ x 4 H_2O	295.0
KH_2PO_4	90.0

Steinberg growth medium	
K_2HPO_4	12.6
$MgSO_4$ x 7 H_2O	100.0
Nutrients - Microelements	**Final concentration in medium [µg/L]**
H_3BO_3	120.0
$ZnSO_4$ x 7 H_2O	180.0
Na_2MoO4 x 2 H_2O	44.0
$MnCl_2$ x 4 H_2O	180.0
$FeCl_3$ x 6 H_2O	760.0
Na_2EDTA x 2 H_2O	1500

Note: If individual elements were not available but those were present with different amounts of water, this element could also be used as an alternative by adjusting the initial weight.

5.4.4 Test concentrations

Based on the results of range finding tests and after discussion with the UBA, five to six nominal test concentrations were arranged in a geometric series and a separation factor between test concentrations of approx. 2 to 3.16. The results of the various range finding tests are summarized in Table 30, while in Table 27 the final test concentrations of the definitive test are summarized.

5.4.5 Experimental design and introduction of Lemna plants

5.4.5.1 Initiation of the toxicity test

For the growth test, eight replicates were prepared for the control plants and four replicates for each treatment. The culture vessels were 400 mL glass beakers (low form) filled with 150 mL test solution and covered with glass plates.

Colonies consisting of 2 to 4 visible fronds each were transferred from the inoculum culture (pre-culture, see 5.4.1) and randomly assigned to the test vessels. Each vessel contained in total 12 fronds. The number of fronds and colonies was the same in each test vessel.

5.4.5.2 Test media preparation

Mainly the highest test concentration was prepared by addition of an appropriate of the test item into a glass bottle. An acetone washed, sterilized star shaped stirring bar was added to the bottle followed by 1 L of the growth medium, either 20x AAP or Steinberg growth medium. The test solution was stirred vigorously for 2 to up to 48 hours at room temperature (about 20°C) with sonication steps for 15 minutes after 2 hours stirring and every 24 hours, if necessary. If after 48 hours still undissolved test item remained, the solution was filtered through a 0.45 µm PVDF filters. The (filtered) test solution was used as highest test concentration. The other test concentrations were prepared by serial dilution. Growth medium without test item was used for the controls.

For each test substance the used test media preparation is described separately in the prepared GLP study reports.

5.4.5.3 Observations and measurements

Exemplarily, the test set-up and assessment schedule for the growth inhibition test in a static test design is shown in Table 6 (static design) and Table 7 (semi-static design).

Table 6: Scheme for test set-up and assessment schedule for the static growth inhibition test.

Time [d]		Control	Per treatment
	Number of test vessels, replicates	8	4
0 - 7	Additional replicate without plant: temperature	1	-
0	Sampling for chemical analysis of fresh test media prior to the distribution into the test vessels. Determination of pH of fresh media.	+	+
0	Start of growth test: frond number and area, photography. Dry weight of representative plants. Light measurements, visual inspections, temperature measurement (1)	+	+
2 or 3	Frond number and area, photography. Temperature measurement (1)	+	+
4 or 5	Frond number and area, photography. Temperature measurement (1)	+	+
7	Frond number and area, photography. Dry weight determination. Light, pH and temperature measurements (1), visual inspections.	+	+
7	Sampling for chemical analysis of aged test media (2).	+	+

(1) Temperature was recorded with a thermometer in a separate beaker.

(2) Chemical analysis of the test item in the test media (representative replicate(s) per treatment)

Table 7: Scheme for test set-up and assessment schedule for the semi-static growth inhibition test with two media renewals.

Time [d]		Control	Per treatment
	Number of test vessels, replicates	8	4
0 - 7	Additional replicate without plant: temperature	1	-
0	Sampling for chemical analysis of fresh test media prior to the distribution into the test vessels (1). Determination of pH of fresh media.	+	+

Time [d]		Control	Per treatment
0	Start of growth test: frond number and area, photography. Light measurements, temperature measurement [2], visual inspections.	+	+
3	Frond number and area, photography. Temperature measurement [2].	+	+
2	1st test media renewal Sampling for chemical analysis of fresh test media prior to the distribution into the test vessels [1]. Determination of pH pf fresh media.	+	+
3	Sampling for chemical analysis of aged test media [1]. Determination of pH of aged media.	+	+
5	Frond number and area, photography. Temperature measurement [2]	+	+
5	2nd test media renewal Sampling for chemical analysis of fresh test media prior to the distribution into the test vessels [1]. Determination of pH of fresh media.	+	+
5	Sampling for chemical analysis of aged test media [1]. Determination of pH of aged media.	+	+
7	Frond number and area, photography. Light, pH and temperature measurements, visual inspections.	+	+
7	Sampling for chemical analysis of aged test media [1].	+	+

[1] Temperature was recorded with a thermometer in a separate beaker.

[2] Chemical analysis of the test item in the test media (representative replicate(s) per treatment)

5.4.5.4 Test conditions and physico-chemical parameters

Light intensity

The light intensity was measured at the water surface at the start of the test and after the 7-days test period.

Temperature

The temperature of the test media was recorded at least at each observation date using a thermometer placed in an additional test vessel without plants (prepared for this purpose).

pH value

The pH value of the test medium was checked at test initiation and after the 7-days test period in a static test system. If the test was performed under semi-static conditions, the pH was measured at test initiation (fresh), at each media renewal (fresh, aged) and at test termination.

Determination of measurement variables

According the OECD 221 (OECD 2006), at least one other measurement variable (dry weight or frond area) should be measured in addition to the primary measurement variable frond number. In the various experiments, different decisions were made in consultation with the UBA. The

measurement parameters chosen in each of the studies for the various test substances are listed in Table 27.

The measurement variables frond number and frond area were determined using the medeaLAB device for Lemna tests with an integrated Basler Gigabit Ethernet camera. Both measurement variables were determined by image analysis using the medeaLAB Count & Classify 6.8 (medea AV Multimedia und Software GmbH, Am Weichselgarten 23, 91058 Erlangen, Germany). The silhouettes of the plants were captured using a camera and the resulting image was digitised. The fronds were recognised automatically by the software and the identified fronds were highlighted. Afterwards every image was checked and if necessary manually retouched by going over the silhouettes of the fronds in order to include those fronds that were incompletely recognised by the image analyser.

In addition to the determinations of frond number, effects of the test item on a second measurement variable, frond area, was assessed according to OECD 221 (OECD 2006). The frond number and frond area were determined at the start of the test (day 0 growth test). Number of fronds appearing normal or abnormal and frond area were also determined at day 2 or 3, day 4 or 5 and at test end (day 7), depending on the chosen test system (static or semi-static). At the semi-static study with Atorvastatin (GLP study number: IME-025/4-11/I) and the non-GLP study with rosuvastatin, the medeaLab device could not be used. Here, pictures were prepared from each replicate and the software ImageJ was used to determine frond area.

Dry weight was determined at the test initiation from a sample of the inoculum culture representative of what was used for the test, and at test termination with the plant material from each biological treatment vessel and the control. All colonies were collected from each of the test vessels. They were blotted to remove excess water and then dried at 60 °C. Root fragments were included.

Remarkable morphological changes in plant development (e.g. frond size, deformations, appearance, indications of necrosis, chlorosis or gibbosity, discolorations, colony break-up or loss of buoyancy, and in root length and appearance) were documented.

5.4.5.5 Sampling and chemical analysis of the test solutions

The concentrations of the test substance in the water phase was assessed by chemical analysis of the active substance using UHPLC-MS/MS at test initiation and test termination in a static test system and additional at each media renewal (fresh, aged) in semi-static tests.

Samples (mainly 2 x about 5 mL) were taken from the test and control media preparations at the start of the exposure period (day 0, fresh media) and from representative replicates per treatment level and control at the end of the exposure period (day 7, aged media) or at each media renewal. Prior to the sampling, 1 mL of methanol was added into each vial to stabilize the samples.

If chemical analysis was not possible at the same day, the samples were stored frozen at ≤-18°C until analysis. The analytical method was validated following SANTE/2020/12830, rev. 1 (European Commission 2021).

5.4.5.6 Data evaluation and statistics

The evaluation of the test was based on either measured initial concentration, the geometric mean measured concentrations or the time weighted average (TWA) of the active substance, depending on the recoveries of the measured concentrations over time.

The mean value of the measurement variable for each concentration plot was used for plotting growth curves. For each measurement variable, frond number, frond area and dry weight, mean average growth rates and yield were calculated according to OECD 221 (OECD 2006).

Calculation of the percent inhibition compared to controls of growth rate [r] and yield [y] of the measurement variables were performed according to the OECD 221 (OECD 2006). The percent inhibition values of growth rate and yield of the measurement variables were plotted as a function of the test item concentration. All statistical evaluations were based on the control.

To determine the doubling time (T_d) of frond number, the following formula was used with data obtained from the control vessels: $T_d = \ln 2 / \mu$, where μ is the average specific growth rate determined.

According to the OECD 221 (OECD 2006), both average specific growth rate and yield of the measurement variables of untreated and treated plants were determined. Statistical calculations were made on the results obtained for individual vessels.

For yield the increase was calculated per plant or treatment according to the following formula:

Increase = $N_t - N_0$, where N_t is the measurement variable in the test or control vessel at time t and N_0 is the measurement variable in the test or control vessel at test termination.

The average specific growth rate for a specific period was calculated as the logarithmic increase in the growth variables using the formula below for each replicate of control and treatments:

$\mu_{i-j} = (\ln (N_j) - \ln(N_i))/t$,

where: μ_{i-j}: average specific growth rate from time I to j, N_i: measurement variable in the test or control vessel at time I, N_j: measurement variable in the test or control vessel at time j and t: time period from I to j.

Percent inhibition of growth rate (I_r) was calculated for each test concentration according to the following formula:

$\%I_r = ((\mu_r - \mu_T)/ \mu_C) \times 100$,

where: $\%I_r$: percent inhibition in average specific growth rate, μ_C: mean value for μ in the control and μ_T: mean value for μ in the treatment group.

The data was statistically analyzed to determine an EC_{50}, EC_{20} and EC_{10} value together with 95% confidence intervals using non-linear regression procedures as outlined in OECD 221 (OECD 2006). However, due to a lack of fit and/or a low r^2 (below 0.7) for the non-linear regression models for some endpoints a linear regression procedure (Probit) was used. More information on the statistical approaches can be found in OECD 54 “OECD current approaches in the statistical analysis of ecotoxicity data: a guidance to application” (OECD Series On Testing and Assessment 2006).

The NOEC values were determined using the appropriate statistical procedures as e.g. the Williams t-test (Williams 1971, Williams 1972), following the guidance outlined in OECD 54 (OECD Series On Testing and Assessment 2006).

The computer program ToxRat® 3.3.0 (ToxRat® Solutions GmbH 2022) was used for all statistical evaluations.

5.5 Results

In general, both cardiologically active substances and oncologically active substances tested in the Lemna sp. Growth inhibition test in accordance to OECD 221 affected the frond area stronger than the frond number, which is the definitive parameter in this test, and dry weight (if determined). This was already indicated in the range finding tests (Table 30) and could be confirmed with the main tests (Table 8).

The results of the range finding tests are presented in Annex A.2 in Table 30, while an overview on the chosen main test concentrations and the measurement parameters is presented in Table 27.

5.5.1 Cardiologically active substances

The cardiologically active substances tested were very sensitive and strongly affected the growth of either *Lemna minor* and *Lemna gibba*. The effect of atorvastatin and rosuvastatin on both species with different test media varying in their individual pH (Steinberg 5.5 vs. 20x AAP 7.5) were tested, with comparable or slightly stronger effects using *Lemna gibba* with 20x AAP medium. The E_rC_{10} values for frond area, the most sensitive endpoint, varied between 0.006 and 0.083 mg a.s./L for the three statins atorvastatin, rosuvastatin and pitavastatin. Here, Rosuvastatin was the most toxic test substance with an E_rC_{10} of 0.006 mg a.s./L, however, it has to be considered that for the two other statins a semi-static test design was chosen due to a fast degradation of the test substance over time, while rosuvastatin appeared to be stable over 7 days exposure and therefore the exposure was probably longer and the evaluation was based on nominal concentrations while it was based on the time weighted average for atorvastatin and pitavastatin.

The Lemna sp. growth inhibition test (OECD 221) was chosen in this project because the literature review in the 1st part of the project had shown that the mode of action of cardiologically active substances might lead to effects on the growth of aquatic plants. The strong inhibitions of growth of *Lemna minor* and *Lemna gibba* at already very low concentrations indicate that this assumption could be confirmed.

5.5.2 Oncologically active substances

EPAR data were available for each of the oncologically active substances. In most cases these were NOEC data, for ribociclib the EC_{10} was given. Thus, a comparison might be hampered. However, it was assumed that the data evaluation was performed based on measured concentrations as it was done for the actual Lemna tests as well.

Methotrexate was the most toxic oncologically active test substances, resulting in an E_rC_{10} of 0.0008 mg a.s./L (TWA) and an E_rC_{50} of 0.002 mg a.s./L (TWA). In the respective EPAR for Methotrexat an E_rC_{50} of 10 mg/L was stated for the algal growth inhibition test (OECD 201), which is 5000x higher than in the Lemna study. Also, pemetrexed was quite toxic (E_rC_{10} = 0.159 µg a.s./L) to aquatic plants. Here, FASS data indicated a NOEC value of 4 mg/L for algae (OECD 201) and 1.2 mg/L for daphnia (OECD 211). Therefore, *Lemna minor* appeared to be more sensitive than the other organisms by a factor of around 7.5.

In general, the NOEC/EC_{10} values for ribociclib, cabazitaxel and imatinib in the Lemna test were comparable to the values from the algal test. The values differ by less than a factor of 2 and are thus also within the range of biological variability.

For the substance abemaciclib the effect in the Lemna test by a factor of 9.5 was weaker than in the algae test as reference test for the aquatic primary producers. For palbociclib Lemna was more sensitive than algae (factor ~4.1), however, the results of a FELS test were more sensitive with a NOEC value of 0.13 mg/L.

In the case of afatinib and dabrafenib, the NOEC values in the Lemna test were lower than in the algae test and showed that *Lemna gibba* was more sensitive to the active substances than the green algae. Here, the differences were approximately a factor of 3.5 (NOEC for imatinib was <0.334) and 27.5 (dabrafenib). However, for afatinib considering all data fish was the most sensitive organism with a NOEC of 0.032 mg/L (factor 6) from a FELS test.

Table 8: Summary the GLP and Non-GLP main tests performed with *Lemna minor*/*Lemna gibba*.

Substance name	Study number and details	Recoveries and concentrations used for evaluation	Frond number [mg a.s./L]			Frond area [mg a.s./L]			Dry weight [mg a.s./L]		
			ErC_{50}	ErC_{10}	NOEC	ErC_{50}	ErC_{10}	NOEC	ErC_{50}	ErC_{10}	NOEC
Atorvastatin	IME-025/4-11/I/a semi-static, two media renewals L. minor	D0 (Fresh): 84.2 – 102% of nominal D2 (aged): 2.4 – 13.0% of nominal and 2.6 – 14.9% of initial D2 (fresh): 70.9 – 91.0% of nominal D5 (aged): <LOQ – 4.1% of nominal and <LOQ and 4.7% of initial D5 (fresh): 79.1 – 96.4% of nominal D7 (aged): 4.5 – 10.3% of nominal and 4.7 – 11.3% of initial **Time weighted average:** **0.674, 2.18, 6.14, 21.1, 71.3 and 331 µg a.s./L**	0.305	0.088	0.071	**0.272**	**0.083**	**0.071**	n.d.	n.d.	n.d.
Atorvastatin	Non-GLPc semi-static, two media renewals L. gibba	D0 (Fresh): 88.5 – 109% of nominal D2 (aged): 11.6 – 35.8% of nominal and 11.0 – 37.5% of initial D2 (fresh): 93.1 – 109% of nominal D5 (aged): 6.9 – 30.8% of nominal and 7.0 - 29.7% of initial D5 (fresh): 97.4 – 112% of nominal D7 (aged): 26.4 – 68.9% of nominal and 27.1 – 65.3% of initial **Time weighted average:** **1.74, 4.47, 12.9, 41.9, 107 and 435 µg a.s./L**	0.122	**0.039**	**0.013**	**0.119**	**0.043**	**0.013**	n.d.	n.d.	n.d.
Pitavastatin	IME-026/4-11/I semi-static, one media renewal L. minor	D0 (Fresh): 78.6 – 81.0% of nominal D3 (aged): 12.6 – 17.4% of nominal and 15.7 – 21.7% of initial D3 (fresh): 91.8 – 93.4% of nominal D7 (aged): 18.9– 29.1% of nominal	0.115	0.050	0.070	**0.093**	**0.048**	**0.034**	n.d.	n.d.	n.d.

Substance name	Study number and details	Recoveries and concentrations used for evaluation	Frond number [mg a.s./L]			Frond area [mg a.s./L]			Dry weight [mg a.s./L]		
		and 20.2 – 31.7% of initial **Time weighted average:** **14.5, 33.7, 69.8, 155 and 357 µg a.s./L**									
Rosuvastatin	IME-027/4-11/I static, L. minor	D0: 88.5 – 90.7% of nominal D7: 76.2 – 82.6% of nominal and 84.0 – 93.0% of initial Measured initial: 0.005, 0.017, 0.050, 0.149, 0.444 mg a.s./L	0.076	0.042	**0.005**	**0.072**	**0.036**	**0.017**	n.d.	n.d.	n.d.
Rosuvastatin	Non-GLPc static, L. gibba	D0: 98.7 – 102% of nominal D7: 91.9 – 112% of nominal and 93.0 – 113% of initial Nominal concentration: 0.006, 0.019, 0.056, 0.167, 0.500 mg a.s./L	0.036	0.012	0.006	**0.062**	**0.006**	**0.006**	n.d.	n.d.	n.d.
Pemetrexed	IME-028/4-11/I/a semi-static, two media renewals L. minor	D0 (Fresh): 88.2 – 92.5% of nominal D3 (aged): <LOQ – 0.11% of nominal and <LOQ – 0.12% of initial D3 (fresh): 100 – 107% of nominal D5 (aged): <LOQ – 3.9% of nominal and <LOQ and 3.8% of initial D5 (fresh): 92.2 – 95.7% of nominal D7 (aged): <LOQ – 0.29% of nominal and <LOQ – 0.03% of initial **Time weighted average:** **9.88, 20.6, 54.0, 159, 475 and 1604 µg a.s./L**	1.49	0.245	0.159	**0.744**	**0.159**	**0.159**	n.d.	n.d.	n.d.
Methotrexat	IME-029/4-11/I semi-static, one media renewal L. minor	D0 (Fresh): 103 – 107% of nominal D3 (aged): 72.9 – 89.7% of nominal and 70.0 – 87.0% of initial D3 (fresh): 98.8 – 103% of nominal D7 (aged): <LOQ – 8.4% of nominal and <LOQ – 8.4% of initial	0.006	0.002	0.001	**0.002**	**0.0008**	**<0.0005**	n.d.	n.d.	n.d.

Substance name	Study number and details	Recoveries and concentrations used for evaluation	Frond number [mg a.s./L]			Frond area [mg a.s./L]			Dry weight [mg a.s./L]		
		Time weighted average: 0.466, 1.42, 4.71, 14.8 and 47.3 µg a.s./L									
Cabazitaxel	IME-030/4-11/J static, L. gibba	D0: 37.6 – 50.2% of nominal D7: 16.3 – 22.2% of nominal and 37.0 – 47.8% of initial **Geometric mean measured: 0.046, 0.153, 0.455, 1.21 and 3.78 mg a.s./L**	2.58	0.930	0.455a	**2.15**	**0.588**	**0.153**	>3.78	0.558	0.455a
Abemaciclib	IME-034/4-11/J static, L. gibba	D0: 9.93 – 20.5% of nominal D7: 2.4 – 6.7% of nominal and 14.5 – 65.2% of initial **Geometric mean measured: 0.020, 0.056, 0.146, 0.459 and 2.08 mg a.s./L**	>2.08 (2.39)b	0.089	0.056	**0.802**	**0.065**	**0.056**	2.08	0.082	**0.020**
Palbociclib	IME-035/4-11/J static, L. gibba	D0: 6.1 – 7.1% of nominal D7: 0.4 – 4.3% of nominal and 6.3 – 61.0% of initial **Geometric mean measured: 0.017, 0.065, 0.220, 1.11 and 5.49 mg a.s./L**	>5.49 (5.82)b	0.313	0.220	**2.08**	**0.236**	**0.220**	3.42	0.280	**0.065**
Ribociclib	IME-036/4-11/J static, L. gibba	D0: 106 – 113% of nominal D7: 33.2 – 88.7% of nominal and 31.0 – 83.5% of initial **Geometric mean measured: 0.298, 1.11, 4.58, 15.3 and 47.8 mg a.s./L**	5.09	**1.02**	**1.11**	**4.27**	**1.31**	**1.11**	43.3	1.93	1.11
Afatinib	IME-037/4-11/J static, L. gibba	D0: 75.5 – 97.1% of nominal D7: 15.2 – 78.4% of nominal and 18.2 – 81.0% of initial **Geometric mean measured: 0.334, 0.911, 2.88, 6.53 and 12.8 mg a.s./L**	3.25	0.267	<0.334	**1.65**	**0.195**	**<0.334**	n.d.	n.d.	n.d.
Imatinib	IME-038/4-11/J static,	D0: 59.9 – 66.8% of nominal	21.4	1.56	<0.794	**10.2**	**1.64**	**0.794**	24.9	1.59	<0.794

Substance name	Study number and details	Recoveries and concentrations used for evaluation	Frond number [mg a.s./L]			Frond area [mg a.s./L]			Dry weight [mg a.s./L]		
	L. gibba	D7: 17.5 – 47.2% of nominal and 29.2 – 78.6% of initial **Geometric mean measured:** **0.794, 2.67, 9.61, 28.7 and 52.5 mg a.s./L**									
Dabrafenib	IME-040/4-11/J static, L. gibba	D0: 5.0 – 5.3% of nominal D7: 2.4 – 3.1% of nominal and 49.0 – 60.8% of initial **Geometric mean measured:** **0.008, 0.021, 0.064, 0.213 and 0.645 mg a.s./L**	0.083	0.006	0.008	**0.080**	**0.006**	**0.008**	>0.645	0.021	0.008

n.d.: The parameter dry weight was not determined for the test substance. According to the OECD 221, frond number is the primary measurement variable. At least one other measurement variable (total frond area or dry weight) is also measured, since some substances may affect other measurement variables much more than frond numbers. Based on the results of the range finding tests the respective measurement variables were chosen.

Bold: Most sensitive endpoint determined in the study.

[a]: The NOEC was determined to be at 0.046 mg a.s./L. However, effects at 0.046 and 0.153 mg a.s./L were below 10% compared to the control treatment and considered to be not environmentally relevant. Therefore, the NOEC was adjusted by expert judgement.

[b]: The determined value is above the highest test concentration and therefore extrapolated. Nevertheless, the value is only slightly above the highest test concentration and is therefore also presented.

[c]: In addition to the GLP studies on the effect of atorvastatin and rosuvastatin on *Lemna minor* using modified Steinberg medium (pH 5.5), the effect of the two substances on *Lemna gibba* using 20x AAP medium (pH 7.5) was also observed to determine potential effects based on different pH values of the test media. However, while the studies were performed as outlined in the study plans IME-025/4-11/I/a and IME-027/4-11/I, it was decided that for the two studies no separate GLP studies have to be performed.

Table 9: Comparison of the most sensitive endpoints of the OECD 221 with the available data.

Substance name	Study number and details	Most sensitive endpoint Frond number or area [mg a.s./L]			EPAR data [mg a.s./L]	Sensitivity compared to standard
		ErC_{50}	ErC_{10}	NOEC	EC_{10}/NOEC	
Atorvastatin	IME-025/4-11/I/a semi-static, two media renewals *L. minor*	0.272	0.083	0.071	Safety Data Sheet for Atorvastatin: NOEC algae (OECD 201): 14 mg/L NOEC fish (OECD 210): 0.49 mg/L NOEC daphnia (OECD 211): 0.2 mg/L	>
Atorvastatin	Non-GLP semi-static, two media renewals *L. gibba*	0.119	0.043	0.013	Safety Data Sheet for Atorvastatin: NOEC algae (OECD 201): 14 mg/L NOEC fish (OECD 210): 0.49 mg/L NOEC daphnia (OECD 211): 0.2 mg/L	>
Pitavastatin	IME-026/4-11/I semi-static, one media renewal *L. minor*	0.093	0.048	0.034	Not available	
Rosuvastatin	IME-027/4-11/I static, *L. minor*	0.072	0.036	0.017 0.005 (frond number)	Gunnarsson et al. (2019): 0.018 mg/L (OECD TG 211); 330 mg/L (OECD 201, algae)	>
Rosuvastatin	Non-GLP static, *L. gibba*	0.062	0.006	0.006	Gunnarsson et al. (2019): 0.018 mg/L (OECD TG 211); 330 mg/L (OECD 201, algae)	>
Pemetrexed	IME-028/4-11/I/a semi-static, two media renewals *L. minor*	0.744	0.159	0.159	FASS data for pemetrexed: NOEC algae (OECD 201): 4 mg/L NOEC fish (OECD 210): 13 mg/L NOEC daphnia (OECD 211): 1.2 mg/L	>
Methotrexat	IME-029/4-11/I semi-static,	0.002	0.0008	<0.0005	EC50 algae (OECD 201): 10 mg/L (Methotrexate EPAR EMA/78284/2017 2017)	>

Substance name	Study number and details	Most sensitive endpoint Frond number or area [mg a.s./L]			EPAR data [mg a.s./L]	Sensitivity compared to standard
	one media renewal *L. minor*					
Cabazitaxel	IME-030/4-11/J static, *L. gibba*	2.15	0.588	0.153	Gunnarsson et al. (2019): 0.064 mg/L (OECD 201, algae)	<
Abemaciclib	IME-034/4-11/J static, *L. gibba*	0.802	0.065	FA: 0.056 DW: 0.020[c]	NOEC algae (OECD 201): 0.0059 mg/L NOEC fish (OECD 210): 0.075 mg/L NOEC daphnia (OECD 211): 0.020 mg/L (Abemaciclib EPAR EMA/551438/2018 2018)	<
Palbociclib	IME-035/4-11/J static, *L. gibba*	2.08	0.236	FA: 0.220 DW: 0.065[c]	NOEC algae (OECD 201): 0.9 mg/L (growth rate); 0.091 mg/L (yield) Note: Yield is stated as additional information due to the large discrepancy between NOEC for yield and growth rate of factor 10. NOEC fish (OECD 210): 0.13 mg/L NOEC daphnia (OECD 211): 0.27 mg/L (Palbociclib EPAR EMA/652627/2016 2016)	<
Ribociclib	IME-036/4-11/J static, *L. gibba*	4.27	1.31	1.11	EC_{10} algae (OECD 201): 0.71 mg/L NOEC fish (OECD 210): 1.0 mg/L NOEC daphnia (OECD 211): 1.4 mg/L (Ribociclib EPAR EMA/CHMP/506968/2017 2017)	=
Afatinib	IME-037/4-11/J static, *L. gibba*	1.65	0.195	<0.334	NOEC algae (OECD 201: 1.2 mg/L NOEC fish (OECD 210): 0.032 mg/L NOEC daphnia (OECD 211): 2.7 mg/L (Afatinib EPAR EMA/491185/2013 2013)	<
Imatinib	IME-038/4-11/J static, *L. gibba*	10.2	1.64	0.794	NOEC algae (OECD 201): 0.96 mg/L NOEC fish (OECD 210): 10 mg/L NOEC daphnia (OECD 211): 5.6 mg/L	>

Substance name	Study number and details	Most sensitive endpoint Frond number or area [mg a.s./L]			EPAR data [mg a.s./L]	Sensitivity compared to standard
					(Imatinib EPAR EMA/CHMP/161314/2013 2013)	
Dabrafenib	IME-040/4-11/J static, *L. gibba*	0.080	0.006	0.008	NOEC algae (OECD 201): 0.22 mg/L NOEC fish (OECD 210): 1.47 mg/L NOEC daphnia (OECD 211): 0.0583 mg/L (Dabrafenib EPAR EMA/550929/2018 2018)	>

n.d.: The parameter dry weight was not determined for the test substance. According to the OECD 221, frond number is the primary measurement variable. At least one other measurement variable (total frond area or dry weight) is also measured, since some substances may affect other measurement variables much more than frond numbers. Based on the results of the range finding tests the respective measurement variables were chosen.

Bold: Most sensitive endpoint determined in the study.

[a]: The NOEC was determined to be at 0.046 mg a.s./L. However, effects at 0.046 and 0.153 mg a.s./L were below 10% compared to the control treatment and considered to be not environmentally relevant. Therefore, the NOEC was adjusted by expert judgement.

[b]: The determined value is above the highest test concentration and therefore extrapolated. Nevertheless, the value is only slightly above the highest test concentration and is therefore also presented.

[c]: In most studies, frond area or number were the most sensitive endpoints. Only in the studies with palbociclib and abemaciclib NOEC values for dry weight were lower than those for frond number or area. In general, however, the dry weight parameter is considered to be less reliable and the comparison of sensitivities was accordingly based on the data for frond number and area.

6 Comet assay with *Daphnia magna* and zebrafish cells

6.1 Test principle

The assay selected to assess the genotoxicity of the chosen pharmaceuticals was the comet assay, or single cell gel electrophoresis. It is capable of visualizing the frequency of strand breaks in the nuclear DNA of eukaryotic cells. The method, which was first described by Ostling and Johanson (1984) and later modified by Singh et al. (1988) was used to assess genetic damage in a variety of fields, including (eco)toxicology or human biomonitoring (Ostling and Johanson 1984, Singh et al. 1988, Anderson et al. 2013). One advantage of the method is its versatility, since it can be used on all eukaryotic cells which present nuclei, and be applied *in vivo*, *in vitro* as well as *ex vivo*. Additionally, the method is relatively fast and does not require biological processes, as the sister chromatid exchange assay or the *in vitro* micronucleus assay.

The general principle of the comet assay involves embedding eukaryotic cells in an agarose gel, lysating the cell membranes, and exposing the gels to an electric current. The gels are then treated with fluorescent DNA dye and examined via fluorescence microscopy, in order to visualize nuclei. As with conventional gel electrophoresis, the electric current causes DNA to migrate towards the anode due to its negative charge. However, as the nuclei are too large to move through the agarose gel, only loose DNA loops migrate out of the nuclei, resembling the tail of a comet, with the nucleus being the head (Ostling and Johanson 1984, Singh et al. 1988).

Multiple endpoints were established and used for the comet assay, which are all based on the size of the comet tail, usually in proportion to the head. This can be explained with the fact that strand breaks in the DNA cause it to be connected to the nucleus more loosely and thus let a larger fraction of the DNA get pulled out by the electric current and form a comet tail. Originally, visual scoring was used, and comets were visually classified with grades from 0 to 4, with class 0 cells being undamaged and resembling a circle, and class 4 cells being highly damaged, so called “hedgehog cells”. The term hedgehog refers to the optical similarity of highly damaged cells to the bird’s eye view of a hedgehog (Collins et al. 1995).

Of the (semi-)automated measurements, both Olive tail moment (OTM) (according to Olive et al. (1990)) and Tail Intensity percentage (TI%) were found to be the most reliable comet measurements (Kumaravel and Jha 2006). However, results on OTM seem to raise difficulties regarding interlaboratory comparison (Kumaravel et al. 2009). The OECD Test No. 489 “In Vivo Mammalian Alkaline Comet Assay” (OECD 2016) as well as a majority of literature of the comet assay (e.g. Pellegri et al. (2014)) uses the TI% as endpoint. TI% was also the endpoint agreed on by the international Workshop on Genotoxicity Test Procedures in 2005 (Burlinson et al. 2007). For this reason, the TI% was used as endpoint for the comet assay in this study.

6.1.1 Method development

Literature research was conducted in order to compare multiple methods for both isolation of single daphnia cells or nuclei, and the comet Assay. From the results of the literature research, as well as the SOP provided by the Norwegian institute for Air Research (NILU), which, however, was not specific to daphnia, protocols were created, for the isolation of *Daphnia magna* cells or nuclei, as well as the performance of the comet Assay itself. The final comet assays were performed according to an optimised protocol. A description of the final method can be found in chapter 6.3.

Chemicals and further assay specific materials were ordered, including two comet Assay test kits, namely the comet Assay Kit (3-well slides) (Catalog No.: ab238544) by Abcam, and the R&D

Systems™ comet assay™ Single Cell Gel Electrophoresis Assay (Catalog No.: 4250050K). Solutions for Comet Assay and extraction of daphnid nuclei were also prepared.

6.1.1.1 Cell isolation

In preliminary experiments, the comet assay was tested and two methods for the isolation of Daphnia cells described in the literature were compared:

1. Pellegri et al. (2014), where a homogenizer was used in the presence of glass microspheres, and
2. David et al. (2011), where Daphnia were mechanically dissociated by serial pipetting in cold, hypotonic citrate buffer.

For the homogenizer method 1, around 15 juvenile daphnids were placed into 200 µL of either citrate extraction buffer or phosphate buffer and approximately 100 µL of 0.5 to 0.75 mm glass beads. The samples were homogenized in a FastPrep24 homogenizer (MP biomedicals Germany GmbH). For the serial pipetting method 2, around 20 juvenile daphnids were placed in 900 µL of citrate extraction buffer and the solution was repeatedly pipetted up and down using an aspiration pipette. After both cell isolation methods, the samples were filtered through a 50 µm PET centrifugation filter (PluriSelect, Germany) by loading the sample onto the filter, which was placed onto a 50 mL Falcon Tube, and briefly centrifuging, in order to remove larger fragments as well as the glass beads.

The methods were compared with each other afterwards to determine the most appropriate method to perform the tests with the selected test substances. The microscopy slide containing the agarose gels was washed in water. As shown in Figure 1, multiple nuclei were visible using a Leica Microsystems fluorescence microscope DMI6000 B (Leica microsystems, Germany), thus the method for cell separation in order to run a comet assay was successful. The results were similar for both isolation methods, and for all project related experiments the method according to Pellegri et al. (2014) was used, namely the homogenization with glass microspheres.

6.1.1.2 Embedding of cells in gels

In the method suggested by the NILU, 40 µL of isolated cells in a concentration of approximately 250,000 cells/mL were mixed with 130 µL of 0.8% low melting point (LMP) agarose at 37°C. Up to 12 gels of 10 µL were pipetted onto a cold microscopy slide previously pre-coated with normal melting point agarose. Pre-coating was done by dipping a clean microscopy slide into liquid normal melting point agarose (0.5%) at 55°C, and letting the slide rest overnight. Per slide, two gels per cell isolation approach plus two blank gels of pure LMP agarose (0.8%) were pipetted. For both test kits, the two-well-plates contained in the R&D Systems Test kit or the three-well-plates contained in the Abcam test kit were used instead of pre-coated microscopy plates. A mixing of cell solution with LMP agarose in a 1:10 ratio was performed, as suggested in both kits, as well as in the NILU protocol, at 37°C. For the Abcam test kit, 75 µL of the mixture of cells and agarose were pipetted onto a base layer of 75 µL LMP agarose, while the R&D Systems Test kit suggested pipetting 50 µL of the mixture directly into the well. For all methods, the slides were let cool down for around 10 minutes at 4°C, in order to let the agarose, solidify.

6.1.1.3 Lysis

For method implementation, the isolated daphnid cells were exposed to either 1 or 10 µM hydrogen peroxide (H_2O_2) in order to induce DNA damage prior to lysis. This was done by holding the slides into the solution for 10 seconds, and washing the slide in phosphate buffered saline (PBS) afterward. Lysis buffer was either contained in the test kit, or prepared as described in the NILU protocol. The duration of lysis was either given as 1 hour or overnight, as both test

kits suggested a duration of 30 to 60 minutes and the NILU a duration of at least 1 hour, with both the NILU as well as the R&D Systems test kit recommending lysis overnight for increased sensitivity.

6.1.1.4 Alkaline treatment and gel electrophoresis

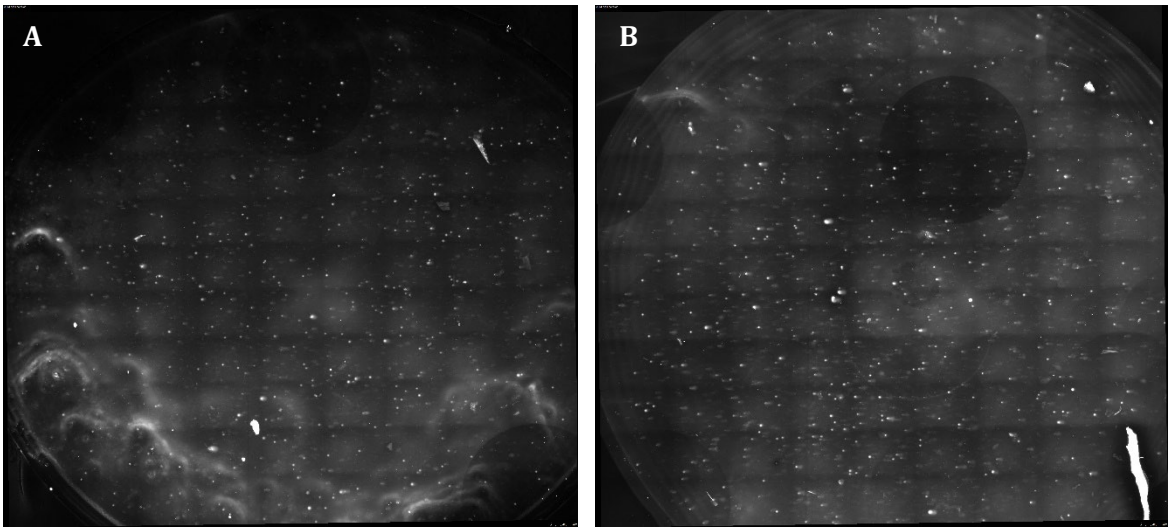
After lysis, slides were washed by briefly immersing them in double-distilled water. Alkaline treatment and gel electrophoresis were done in alkaline gel electrophoresis solution, which consisted of 0.3 M NaOH and 0.001 M Na_2EDTA in double distilled water. Unwinding was done for 20 min according to the R&D systems test kit and the NILU protocol, and for 30 min according to the Abcam test kit. Gel electrophoresis was performed with a duration of 30 min for the test kits, and 20 min for the NILU protocol. The voltage used was always 1 V/cm distance between the electrodes of the electrophoresis chamber, or 15 V for the chamber used. Subsequently, slides were washed, twice in double distilled water, then once in 70% ethanol, for 5 minutes each time. Slides were then dried overnight.

6.1.1.5 Straining and scoring

Comet assay slides were stained using either peqGREEN or Vista Green as DNA dyes, and visualized using the Leica Microsystems fluorescence microscope DMI6000 B (Leica Microsystems, Germany). Staining was either done by bathing the slides in a 1x solution of the dyes for 30 minutes, and then washing them in double distilled water for the same time, or by evenly spreading 50 µL of the 1x dye over the slide using a pipette. After staining, slides were dried at room temperature for approximately 90 minutes, or until no droplets of staining solution were visible on the plate. The I3 filter cube of the Leica EL6000 120W Fluorescence light source (Leica microsystems, Germany) was used, as it has an excitation range of 450 to 490 nm and a dichromatic mirror blocking wavelengths up to 510 nm, matching the excitation maxima (488 nm for Vista Green, 490 nm for peqGREEN) and emission maxima (520 for Vista Green, 530 for peqGREEN) of both DNA stains.

Pictures of the nuclei in the gels were saved and later digitally analyzed with a selection of software, including Comet Assay IV (perceptive systems, United Kingdom), Open Comet (OpenComet, USA) and CaspLab (CaspLab, Poland). Overlapping comets, or ones with visible contamination were not scored (Figure 1).

Figure 1. Fluorescence microscopy image of *D. magna* nuclei stained with VistaGreen after H_2O_2 treatment and Comet Assay. Nuclei were isolated using the FastPrep24 homogenizer; (A) control treatment and (B) treatment with 10 µM H_2O_2 after overnight lysis



Source: Own illustration, Fraunhofer IME

6.1.1.6 Result of the method development

Cell isolation using the serial pipetting approach yielded in a low quantity of nuclei of around 10 nuclei per gel.

Both isolation methods utilizing the homogenizer provided sufficient density of cells, however the method using the phosphate isolation buffer showed comets with slightly clearer outlines.

There was no noticeable effect of the usage of test kits, except the gels being bigger in diameter and thus containing more nuclei. Running gel electrophoresis for 20 minutes produced comets with well recognizable tails, which were not excessively long, as longer tails are more likely to overlap with other comets and thus be unscorable. For staining, both bathing slides in staining solution, as well as dropping 50 µL of staining solution on each gel stained DNA sufficient for comets to be evaluated. When recording pictures of comets from fluorescence microscopy, noticeable photobleaching took place, as the fluorescence decreased with time. This could be prevented by working quickly and setting the fluorescence source to the lowest level. From the three Software options, OpenComet, CaspLab and Comet Assay IV, both CaspLab and Comet Assay IV showed satisfactory capability to correctly recognize the comets and analyze them. The automatic image recognition with OpenComet was often problematic, as comets were either not correctly recognized, or objects other than comets were falsely analyzed as comets. As CaspLab is freely accessible, it was selected to be used for the evaluation of future experiments.

6.2 Validity criteria

There are no general validity criteria for the comet assay. The validity criteria of the OECD 202 (OECD 2004) were applied for the exposure of *D. magna* with the chosen pharmaceuticals. Such criteria were not available for the tests with ZF-L.

As the Comet assay was only recently established at the Fraunhofer IME in the scope of this project, the validity of the method still has to be discussed. After all, the TI% value is not only dependent on DNA damage, but can also vary depending on the experimental conditions (Collins et al. 2014)

6.3 Materials and methods

6.3.1 Preparation of test solutions

The test solutions for the comet assay were created by weighing in the respective substance (normalized for active substance in case the substance is present as a salt) in either Daphnia medium or the growth medium for the ZF-L cells. The solutions were treated with ultrasonification for 15 minutes and stirred for 24 hours. Afterwards, the solutions were filtered through a 0.45 µm Polivindildifluorid (PVDF) bottle top filter (Merck Millipore, USA), and lower test concentrations were obtained via serial dilution with the respective medium.

For the *in vitro* experiments, the test solution was not prepared under sterile conditions, but filtered into a sterile bottle in order to remove potential contaminants. For this purpose, serum-free medium was used, and serum, as well as insulin and EGF were added to the test solutions after filtration.

6.3.2 Culturing of *Daphnia magna*

D. magna were kept in 2 L beakers at 20°C filled with Daphnia medium. An artificial day-and-night cycle with a light intensity of below 1000 lux for 16 hours interrupted by dark periods of 8 hours was applied. In order to exchange medium and clean the beakers, daphnids were removed from the medium by pouring them over a set of sieves with mesh sizes of 180, 560 and 900 µm, separating them by size. After cleaning the beakers, approx. 5 mL of a suspension of the green algae *Desmodesmus subspicatus* mixed with approximately five drops of ArtemioFluid (JBL, Germany) were added into the beaker and approximately 85% of the former Daphnia medium was placed back into the beaker. The beaker was then filled to the top with fresh Daphnia medium, and the Daphnia were placed back into the medium.

Juvenile daphnids aged below 24 hours, which were in the size group retained by the finest sieve (180 µm), were not released into the same beaker, but either discarded or placed into a fresh beaker, in order to keep populations of daphnids homogenous in age.

In accordance to the OECD guideline 202 (OECD 2004) juvenile *D. magna* of an age below 24 hours were required for tests. Therefore, the beakers containing the Daphnia culture were sieved 24 hours before the experiment in order to remove all juvenile Daphnia. Due to this, the next day it was ensured that test animals were of an age of below 24 hours.

Daphnia medium consisted of dechlorinated, purified tap water, which was hardened with calcium chloride to reach a Ca^{2+}-hardness of above 140 mg/L as recommended in OECD guideline 211 (OECD 2012).

6.3.3 Culturing of *Danio rerio* liver cell line ZF-L

D. rerio liver cells were cultured according to the protocol given by the American Type Culture Collection (ATCC). The cells were grown in culture flasks with a treated surface fore adhesive cells, which were stored in an incubator at 28°C with free gas exchange with atmospheric air. The growth medium consisted of 50 % L-15 medium, 35 % DMEM high glucose medium, and 15 % Ham's F12 medium, which was supplemented with 0.15 g/L sodium bicarbonate, 15 mM HEPES, 10 mg/L bovine insulin, 50 µg/L mouse EGF, 5% heat-inactivated fetal bovine serum (FBS) and 0.5% trout serum.

Cells were passaged twice a week. Passaging was done by washing the cells with PBS, adding 28°C warm Trypsin-EDTA solution (0.25%) for approx. 5 minutes, deactivating the Trypsin with medium containing 10 % FBS, and centrifuging the suspension for 10 minutes at 400*g. The pellet was then resuspended in growth medium and transferred into culture flasks. The sub cultivation ratio was 3:1.

6.3.4 Treatment of D. magna for comet assay

The daphnids were exposed to the pharmaceuticals following the OECD guideline 202 (OECD 2004). Five concentration groups were tested, as well as a control of Daphnia medium. For each treatment group, eight replicates were tested, each one consisting of a 50 mL beaker containing 50 mL of either test solution or Daphnia medium, as well as five juvenile *D. magna*.

Test concentrations were chosen based on the NOEC values as stated in the EPARs (Table 10) to achieve acute effects only at the highest concentration, but no effects at the lower concentrations to observe sublethal effects. For cyclophosphamide and pitavastatin, no EPAR or any other data was available. Therefore, for both test items a range finding test with concentrations of 100, 10, 1.0 and 0.1 mg a.s./L was performed and the test concentrations for the comet assay were chosen based on the data afterwards.

Table 10: Test concentrations for the comet assay on *D. magna* and the corresponding EPAR data (NOEC) used to choose test concentrations.

Substance name	Test concentrations [mg a.s./L]	EPAR data
Abamaciclib	0.1/0.32/1.00/3.16/10.0	OECD 211: NOEC: 0.02 mg/L OECD 202: NOEC: 5.6 mg/L (Abemaciclib EPAR EMA/551438/2018 2018)
Cyclophosphamid	10/31.6/100	Not available
Dabrafenib	0.5/1.58/5.00/15.8/50.0	OECD 211: NOEC: 0.0583 mg/L (Dabrafenib EPAR EMA/550929/2018 2018)
Edoxaban	0.5/1.58/5.00/15.8/50.0	OECD 211: NOEC: 5.57 mg/L (Edoxaban EPAR EMA/321083/2015 2015)
Imatinib	0.5/1.58/5.00/15.8/50.0	OECD 211: NOEC: 5.6 mg/L (Imatinib EPAR EMA/CHMP/161314/2013 2013)
Palbociclib	0.5/1.58/5.00/15.8/50.0	OECD 211: NOEC: 0.27 mg/L (Palbociclib EPAR EMA/652627/2016 2016)
Pitavastatin	0.5/5.0/50.0	Not available
Ribociclib	0.4/1.00/10.0/20.0/40.0	OECD 211: NOEC: 1.4 mg/L (Ribociclib EPAR EMA/CHMP/506968/2017 2017)
Rosuvastatin	0.05/0.16/0.5/1.58/5.00	OECD 211: NOEC: 0.018 mg/L (Gunnarsson et al. 2019)

The test conditions were the same as the culturing conditions. An artificial day-and-night cycle was maintained by illuminating them with electrical light for 16 hours a day, with a light intensity up to 1000 lux, and a temperature of 20 ± 1°C. Exposure time was set to 48 hours for the pharmaceuticals, and after this time immobility was evaluated by observing daphnids, and gently agitating those not immediately moving with a Pasteur pipette. Immobile daphnids were discarded, the rest was used for the comet assay. The pH of the different test solutions was measured after the test.

The majority of experiments were performed on a regular laboratory work surface. For some experiments, non-concentration-dependent immobility occurred. In order to investigate, if this immobilization occurred due to the conditions in the laboratory, the second calibration with H_2O_2 (the one with the 24-hour lysis duration) was performed in an incubator instead of the laboratory. A control of daphnids (8 replicates of 5 daphnids each) was also incubated in the laboratory, in order to compare immobilization.

6.3.5 Exposition of ZF-L cells for comet assay

The comet assay was performed as described by Novak et al. (2017). Cells were washed and trypsinated and the trypsin was deactivated by adding medium containing 10 % FBS. After

centrifugation (10 minutes, 400*g), the supernatant was discarded and the cells were resuspended in growth medium. The cells were then seeded into a sterile 24-well-plate at a density of 100 000 cells/well and left to attach for 24 hours at 28°C.

After attachment, the growth medium was discarded and replaced by the test solution. With the exception of the validation experiment, which compared how the test system reacted to a 3, 24 and 48-hour exposition, the exposition time was set to 48 hours. Per control and test concentration, three replicates were used.

After 48 hours, exposition was stopped by removing the test solutions from the cells, washing them, and detaching the cells from the wells via Trypsin-EDTA solution (0.25%). 30 µL of this solution were mixed with 70 µL of LMPA for the comet assay.

Test concentrations for the comet assay were selected based on effect concentrations from comparable test systems, such as the chronic Fish Early Life Stage test with *D. rerio* for the ZF-L cell line. The test concentrations are presented in Table 11.

Table 11: Test concentrations chosen for the comet assay with ZF-L.

Substance	Use	ZF-L Test concentration [mg a.s./L]
Abemaciclib	Chemotherapy	0.1, 1.0, 10
Cyclophosphamid	Chemotherapy	6.25, 12.5, 25, 50, 100
Dabrafenib	Chemotherapy	0.15, 1.5, 15
Edoxaban	Cardiology	0.25, 2.5, 25
Imatinib	Chemotherapy	1, 10, 100
Palbociclib	Chemotherapy	0.1, 1.0, 10
Pitavastatin	Cardiology	0.5, 5.0, 50

6.3.6 Comet assay

Per replicate, six 10µL drops of the LMPA-cell suspension were pipetted onto a microscopy slide pre-coated with agarose (normal melting point). After letting the agarose solidify at 4°C for 10 minutes, the slides were incubated in lysis solution for 60 minutes. The slides were then washed by briefly placing them into ddH_2O (4°C) and incubated in alkaline gel electrophoresis solution 4°C for 20 minutes. Gel electrophoresis was run for 20 minutes at 1 V/cm chamber length, or 24 V for the gel electrophoresis chamber used. After gel electrophoresis, the slides were washed by first placing them in ddH_2O for 5 minutes, and then in in 70% EtOH for 5 minutes. The slides were dried overnight and recorded under the microscope the next day to observe comet formation.

6.3.7 Fluorescence microscopy

In order to make DNA visible, it was stained with the fluorescent DNA dye Vista Green (Abcam, United Kingdom) by placing a drop onto each gel with a pipette and letting the slide dry at room temperature for approx. 2 hours. The dye has an excitation maximum of 488 nm and an emission maximum of 520 nm. After incubation, the gels were observed using the Leica DMI6000 B Fluorescence Microscope (Leica Microsystems, Germany). For fluorescence microscopy, the I3 filter cube of the Leica EL6000 120W Fluorescence light source was used, as it has an excitation range of 450 to 490 nm as well as the dichromatic mirror's wavelength of above 510 nm, and thus works with the Vista Green DNA dye. The gels were scanned using the area scan function, and later scored using the CaspLab (CaspLab, Poland) software. Overlapping comets, hedgehog cells, or comets overlapping with contamination, such as fragments of Daphnia exoskeletons or air bubbles in the gel, were not scored in order to avoid error.

Each experiment was performed in two (cells from *D. magna*) and three (ZF-L) replicates, respectively, consisting of 3 microscopy slides per replicate. In total, approximately 150 to 400 cells per replicate and concentration were evaluated.

6.3.8 Statistical analysis

For statistical anlyses, the median TI% value per replicate was calculated (as suggest in the OECD TG 489). The software ToxRat 3.3.0 (ToxRat Solutions GmbH) was used to perform statistical analysis. For the comet assay, Levene's test was used to determine variance homogeneity. If variance homogeneity was detected, the Step-down Jonckheere-Terpstra Test was used in order to determine whether or not a statistically significant effect occurred, and for variance heterogeneity, the Welch t-test was used.

6.4 Viability testing

Next to genotoxic effects, viability was assessed in order to determine acute effects caused by the substances. For *D. magna*, immobility was investigated by observing the test animals after the 48 hours of exposition. Daphnia which did not move, even after gently agitating them with a pipette, were counted as immobile.

For the ZF-L cell line, viability was assessed by staining cells with trypan-blue. The cells were detached from the multi-well plate via trypsin, which was deactivated with 10% FBS medium, and removed by centrifugation. The pellet was resuspended in a 1:1 mixture of serum free medium and trypan blue. Since trypan blue is not absorbed by viable cells, viable cells could be distinguished from non-viable cells during microscopy.

6.4.1 Method approval

In order to approve the functionality of the method, both with ZF-L cells and with *D. magna*, two reference substances with known genotoxicity were tested: H_2O_2, which acts as a reactive oxygen species (ROS) and cyclophosphamide, which acts as a DNA alkylating agent.

For *Daphnia magna*, H_2O_2 was tested in concentrations of 8.24, 14.7 and 26.5 µM (or 280, 500 and 900 µg/L) and cyclophosphamide was tested in concentrations of 10.0, 31.6 and 100 mg/L.

For ZF-L, H_2O_2 was tested in concentrations of 6.25, 12.5, 25, 50 and 100 µM and cyclophosphamide was tested in concentrations of 6.25, 12.5, 25, 50 and 100 mg/L. In order to

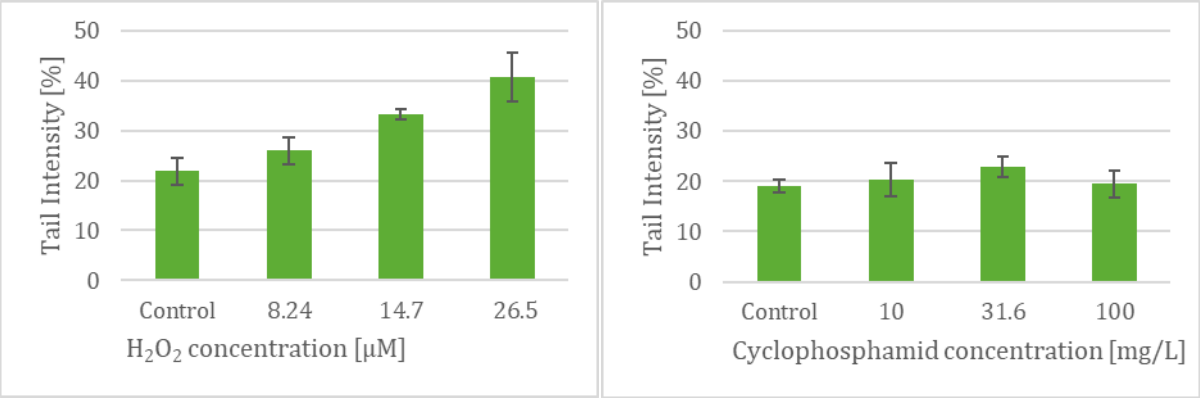
determine, which exposition duration would result in the most sensitive test system, exposure durations of 3 and 48 hours were compared.

6.5 Results

6.5.1 Method validation

The comet assay with reference substances on *D. magna* showed a concentration-dependent increase in TI% for hydrogen peroxide, however not with cyclophosphamide. For H_2O_2, the TI% values the two treatment groups with the highest concentrations (14.7 µM and 26.5 µM) showed a statistically significant difference towards the control (Figure 3)

Figure 2: Results of the comet assay on the genotoxic effect of H_2O_2 and cyclophosphamide on *D. magna*.



Source: Own illustration, Fraunhofer IME

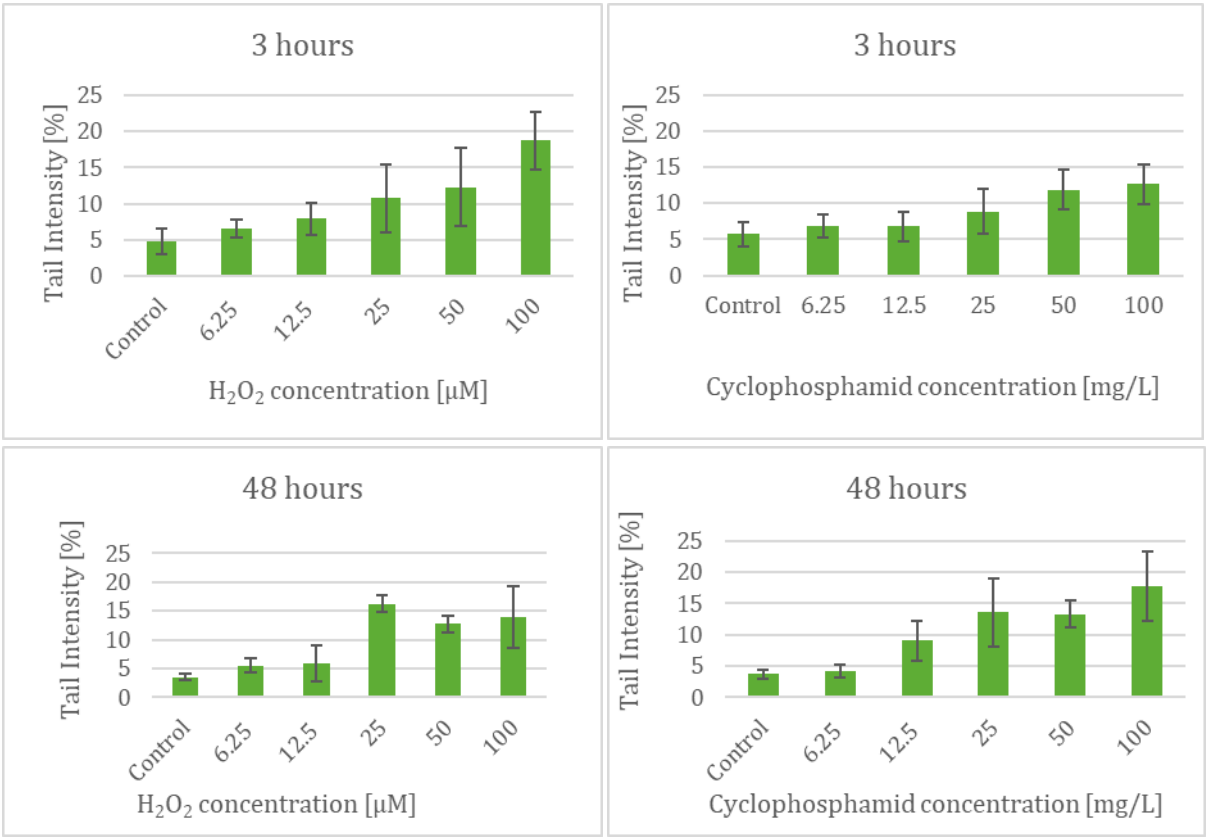
Remark: Results from 2 independent replicates per concentration, with 3 slides/replicate. The number of counted cells was between 150 and 400 cells per concentration.

The validation experiment with the ZF-L cells on the other hand showed a statistically significant effect in the two highest exposure concentrations for both reference substances with an exposure duration of 3 hours (Figure 3). For the 48-hour exposure duration, a statistically significant effect could be observed in the three highest exposure concentrations for H_2O_2 (25, 50 and 100 µM) and for the four highest exposure concentrations for Cyclophosphamide (12.5, 25, 50 and 100 µM).

Figure 3: Results of the comet assay validation experiment with ZF-L cells.

Source: Own illustration, Fraunhofer IME
Remark: Results from 3 independent replicates per concentration, with 3 slides/replicate. The number of counted cells was between 150 and 400 cells per concentration.

Thus, the *in vitro* comet assay with the ZF-L cell line was shown to be capable to detect genetic damage caused by both known genotoxic agents. The sensitivity of the method was slightly increased by prolonging exposure form 3 hours to 48 hours, thus for the remainder of the experiments, an exposure duration of 48 hours was used. This further allowed better comparability to the studies with cells from *D. magna*.



The comet assay with *D. magna* was also shown to be capable to detect genetic damage caused by H_2O_2. The experiment with cyclophosphamide could not show a concentration-dependent increase in TI% for the cells from *D. magna*.

6.5.2 Genotoxicity testing of human pharmaceuticals

For the human pharmaceuticals, the comet assay was performed, and the lowest concentration showing a statistically significant difference to the control was determined. The statistically significant values determined by these experiments are presented in Table 12. If no statistically significant effect occurred, the effect was indicated to be above the highest test concentration.

Table 12: Statistically significant concentrations of the test substances determined in the comet assay with *D. magna* and ZF-L.

Substance name	Use	Significant effect concentrations [mg/L] *D. magna* comet assay	Significant effect concentrations [mg/L] ZF-L in vitro comet assay	EPAR data NOEC/EC_{10} [mg a.s./L]
Abemaciclib	Chemotherapy	> 10	> 10	NOEC algae (OECD 201): 0.0059 mg/L NOEC fish (OECD 210): 0.075 mg/L NOEC daphnia (OECD 211): 0.020 mg/L (Abemaciclib EPAR EMA/551438/2018 2018)
Cyclophosphamid	Chemotherapy	> 100	**12.5**	**No data available**
Dabrafenib	Chemotherapy	> 50	> 15	NOEC algae (OECD 201): 0.22 mg/L NOEC fish (OECD 210): 1.47 mg/L NOEC daphnia (OECD 211): 0.0583 mg/L (Dabrafenib EPAR EMA/550929/2018 2018)
Edoxaban	Cardiology	> 50	> 25	NOEC algae (OECD 201): Not available NOEC fish (OECD 210): 2.32 mg/L NOEC daphnia (OECD 211): 5.57 mg/L (Edoxaban EPAR EMA/321083/2015 2015)
Imatinib	Chemotherapy	> 50	**100**	NOEC algae (OECD 201): 0.96 mg/L **NOEC fish (OECD 210): 10 mg/L** NOEC daphnia (OECD 211): 5.6 mg/L **(Imatinib EPAR EMA/CHMP/161314/2013 2013)**
Palbociclib	Chemotherapy	> 10	> 10	NOEC algae (OECD 201): 0.0091 mg/L NOEC fish (OECD 210): 0.13 mg/L NOEC daphnia (OECD 211):

Substance name	Use	Significant effect concentrations [mg/L] *D. magna* comet assay	Significant effect concentrations [mg/L] ZF-L in vitro comet assay	EPAR data NOEC/EC_{10} [mg a.s./L]
				0.27 mg/L (Palbociclib EPAR EMA/652627/2016 2016)
Pitavastatin	Cardiology	**50.0**	> 50	No data available
Ribociclib	Chemotherapy	> 40	Not determined	EC10 algae (OECD 201): 0.71 mg/L NOEC fish (OECD 210): 1.0 mg/L NOEC daphnia (OECD 211): 1.4 mg/L (Ribociclib EPAR EMA/CHMP/506968/2017 2017)
Rosuvastatin	Cardiology	> 5	Not determined	No data available

Bold: Bold values indicate tests with a statistically significant difference to the control treatment.
Remark: Results from two (cells from D. magna) and three (ZF-L) independent replicates per concentration, with 3 slides/replicate. The number of counted cells was between 150 and 400 cells per concentration.

In general, the results show that the comet assay with *D. magna* or ZF-L appeared to be less sensitive or equally sensitive compared to the data received from a Fish Early Life Stage (FELS, OECD 210) test or the *D. magna* reproduction test (OECD 211) as shown in Table 12.

There were statistically significant effects compared to the control treatment in the test with *Daphnia magna* only for pitavastatin. Here, unfortunately no data from a FELS test (OECD 210) or a *D. magna* reproduction test (OECD 211) was available for comparison. Therefore, it is difficult to consider the comparative sensitivity of the test system.

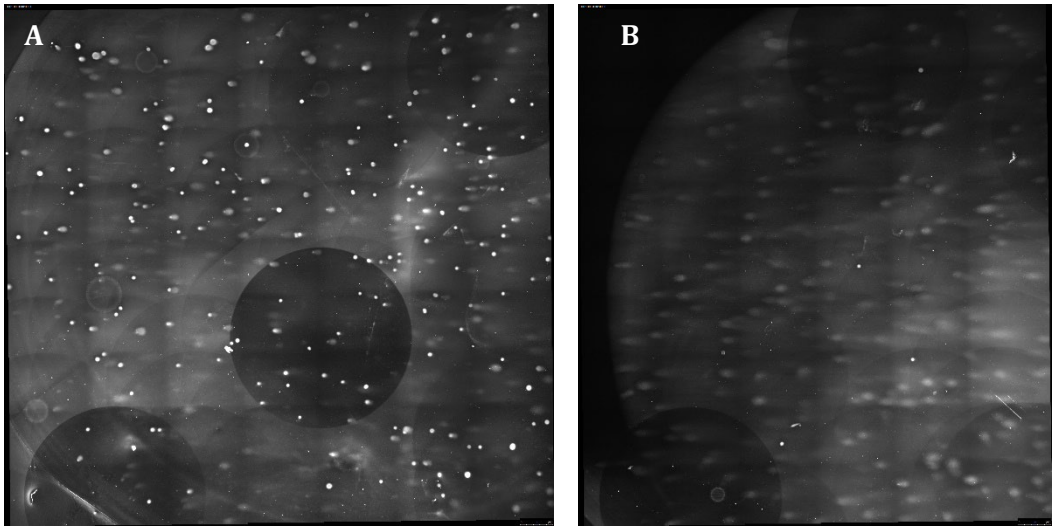
Pitavastatin was the only test substance which induced a stronger effect in the test with *Daphnia magna* than in the test with ZF-L cells.

For the comet assay with ZF-L cells, initially only controls and the highest tested concentration were evaluated. In the case of statistically significant results, also the other test concentrations were evaluated. Thus, for the test items Abameciclib, Dabrafenib, Edoxaban, Imatinib, Palbociclib and Pitavastatin, only the results of the highest test concentration are available.

It was observed, that less nuclei were found in the comet assay with substances that have a cytostatic effect. Namely, these substances were abemaciclib, cyclophosphamid, dabrafenib and imatinib. For imatinib, it has to be noted, that the effect recorded in the 100 mg/L concentration was not an increase in TI%, rather than the comets appearing shapeless. While an effect was observable, due to the appearance of the comets, it was not possible to determine the TI%, as no clear comet tail and head were distinguishable (Figure 4).

All generated %TI values are presented in Table 13.

Figure 4: Nuclei of ZF-L cells after exposition to (A) control conditions and (B) 100 mg/L Imatinib.



Source: Own illustration, Fraunhofer IME

%TI values for all pharmaceuticals and cell sytems

Substance name	Test system	Control [%TI ± SD]	Conc. 1 [%TI ± SD]	Conc. 2 [%TI ± SD]	Conc. 3 [%TI ± SD]	Conc. 4 [%TI ± SD]	Conc. 5 [%TI ± SD]
Abemaciclib	*D. magna* cells						
	Concentration [mg/L]	Ctrl	0.1	0.316	1	3.16	10
	%TI	8.21	7.60	8.44	9.65	9.93	-
	SD	1.59	0.38	1.72	0.53	0.21	-
	ZF-L cells						
	Concentration [mg/L]	Ctrl	100	-	-	-	-
	%TI	9.45	12.73	-	-	-	-
	SD	1.26	7.57	-	-	-	-
Cyclophosphamid	*D. magna* cells						
	Concentration [mg/L]	Ctrl	10	31.6	100	-	-
	%TI	19.00	20.31	22.86	19.53	-	-
	SD	1.24	3.37	1.99	2.69	-	-
	ZF-L cells						
	Concentration [mg/L]	Ctrl	6.25	12.5	25	50	100
	%TI	3.68	4.19	9.04	13.57	13.34	17.75
	SD	0.78	1.08	3.20	5.38	2.24	5.62

Substance name	Test system	Control [%TI ± SD]	Conc. 1 [%TI ± SD]	Conc. 2 [%TI ± SD]	Conc. 3 [%TI ± SD]	Conc. 4 [%TI ± SD]	Conc. 5 [%TI ± SD]
Dabrafenib	*D. magna* cells						
	Concentration [mg/L]	Ctrl	0.5	1.58	5	15.8	50
	%TI	15.60	20.55	19.54	16.75	19.77	16.34
	SD	0.75	0.77	0.97	0.23	2.56	0.92
	ZF-L cells						
	Concentration [mg/L]	Ctrl	15	-	-	-	-
	%TI	1.94	1.60	-	-	-	-
	SD	0.85	0.49	-	-	-	-
Edoxaban	*D. magna* cells						
	Concentration [mg/L]	Ctrl	0.5	1.58	5	15.8	50
	%TI	14.21	9.69	11.14	13.49	15.37	16.52
	SD	2.39	2.47	1.54	3.17	2.63	1.85
	ZF-L cells						
	Concentration [mg/L]	Ctrl	25	-	-	-	-
	%TI	5.93	3.16	-	-	-	-
	SD	1.38	0.28	-	-	-	-
Imatinib	*D. magna* cells						
	Concentration [mg/L]	Ctrl	5.0	16	50	-	-
	%TI	19.18	19.78	17.16	18.34	-	-
	SD	2.13	3.49	5.78	1.29	-	-
	ZF-L cells						
	Concentration [mg/L]	Ctrl	31.6	100	-	-	-
	%TI	9.45	1.47	-	-	-	-
	SD	1.26	0.67	-	-	-	-
Palbociclib	*D. magna* cells						
	Concentration [mg/L]	Ctrl	0.4	2.0	10	-	-
	%TI	19.55	17.16	13.44	14.34	-	-

Substance name	Test system	Control [%TI ± SD]	Conc. 1 [%TI ± SD]	Conc. 2 [%TI ± SD]	Conc. 3 [%TI ± SD]	Conc. 4 [%TI ± SD]	Conc. 5 [%TI ± SD]
	SD	4.49	1.71	2.23	1.89	-	-
	ZF-L cells						
	Concentration [mg/L]	Ctrl	10	-	-	-	-
	%TI	0.91	0.34	-	-	-	-
	SD	0.48	0.33	-	-	-	-
Pitavastatin	*D. magna* cells						
	Concentration [mg/L]	Ctrl	0.5	5.0	50	-	-
	%TI	12.00	12.48	14.59	17.74	-	-
	SD	1.64	1.60	1.10	1.00	-	-
	ZF-L cells						
	Concentration [mg/L]	Ctrl	50	-	-	-	-
	%TI	6.94	1.23	-	-	-	-
	SD	1.18	0.41	-	-	-	-
Ribociclib	*D. magna* cells						
	Concentration [mg/L]	Ctrl	0.4	1	10	2	40
	%TI	40.94	36.74	22.60	23.85	38.94	20.16
	SD	1.14	6.33	3.12	2.45	9.73	2.55
Rosuvastatin	*D. magna* cells						
	Concentration [mg/L]	Ctrl	0.05	0.16	0.50	1.58	5.00
	%TI	12.76	20.37	15.15	13.59	14.17	13.61
	SD	0.59	4.55	1.64	1.09	2.38	0.41

6.5.3 Viability

For *D. magna*, no acute effect on immobilization was observed after exposing juveniles to palbociclib, pitavastatin, cyclophosphamide and edoxaban. The LOEC for *Daphnia* immobilization of imatinib and dabrafenib was at 50 mg a.s./L. For abemaciclib a LOEC of 10 mg a.s./L, and for ribociclib a LOEC of 40 mg a.s./L was determined. The experiment with rosuvastatin showed non-concentration dependent immobilization, also in the control, thus no statement can be made on the acute effect of the substance. For the ZF-L cell line, no notable loss in viability occurred for any of the substances tested.

6.6 Discussion

6.6.1 Method development

The method for the comet assay was developed by applying it to both *Daphnia magna* and ZF-L cells, previously exposed to H_2O_2, a ROS, and cyclophosphamide, a DNA alkylating agent, two substances with known genotoxic potential (Benhusein et al. 2010, Abdella 2012).

6.6.2 *D. magna in vivo* comet Assay

For *D. magna*, this confirmed, that the method was capable of detecting genetic damage caused by H_2O_2 with statistically significant differences at the two highest test concentrations in terms of an increase in TI% compared to the control. However, for cyclophosphamide, no such effect was observed. Other ecotoxicological studies for example from Grzesiuk et al. (2019) confirmed *D. magna* to be sensitive toward cyclophosphamide. An effect to life history parameters was detected at concentrations as low as 10 ng/L. Thus, it is unlikely that *D. magna* is insenstive to the effects of the DNA alkylating agent, and a lower sensitivity of the method compared to other endpoints is the more plausible explanation.

Comparing the results of this study with those of Pellegri et al. (2014), who established a similar method for the Comet assay on *D. magna*, it can be observed, that the calibration with H_2O_2 showed statistically significant effects at lower concentrations. Namely, while this study detected a statistically significant increase in the 14.7 µM treatment group and upwards, Pellegri et al. (2014) detected a statistically significant effect for test concentrations as low as 5 µM. The main difference between the two methods is the cell extraction. Both methods did use mechanical disruption with glass microbeads, however while here we used a mechanical homogenizer, Pellegri et al. (2014) used an amalgamator device. As the TI% in the control groups for *D. magna* were higher in our study than those in comparable studies (Pellegri et al. 2014, Parrella et al. 2015), it is likely that the homogenization caused the TI% to increase by damaging the DNA. DNA damage from mechanical disruption is a known factor capable of influencing the outcome of the comet assay (Møller et al. 2020).

6.6.3 Genotoxic effects caused by human pharmaceutical

For most of the other human pharmaceuticals, no genotoxic effects were detected, which is consistent with the genotoxicity results published in the respective European Public Assessment Reports for the substances. Pre-clinical testing revealed no genotoxicity or mutagenicity for most of the substances, and their MoA also did not imply genotoxic activity, as none of the substances interacts with the genetic material. A summary of the MoA and the genotoxicity tests performed during pre-clinical tests can be found in the following table (Table 31).

The only substance that caused a statistically significant genotoxic effect for *D. magna*, was pitavastatin. While no EPAR is available for the substance, the Australian Public Assessment Report states that the substance did not have a genotoxic effect in the AMES test, as well as several *in vivo* tests, including the comet assay with mammalian animal models. While the test concentrations were not stated, the Australian Public Assessment Report states that “adequate exposure” for clinical use was tested. As the daily dose for the substance ranges from 1 and 4 mg for an adult human, it is possible, that the genotoxic effect described in this study can be explained by the fact that the test concentrations being higher by several orders of magnitude.

However, it is also possible, that the effect did only occur due to biological variation, as the comet assay with the ZF-L cell line showed a higher sensitivity.

For the comet assay with the ZF-L cell line, the only substance, which showed a genotoxic effect, was imatinib. However, while an effect on the nuclei was visible, the cells appeared blurry, that scanning them in order to determine TI% was not possible. While an effect was clearly visible, it was not quantifiable, due to the appearance of the comets. This indicates that the oberved effect could rather be assigned to systemic toxicity than to DNA damage.

7 Fish Embryo Acute Toxicity Test

The fish embryo toxicity (FET) tests with the eight selected substances (afatinib, cyclophosphamid, edoxaban, imatinib, palbociclib, propranolol, rivaroxaban, and rosuvastatin) were designed to comply with the OECD Guideline for Testing of Chemicals, No. 236 "Fish Embryo Acute Toxicity (FET) Test" (OECD 2013), and complemented with selected sublethal endpoints. An additional test with the standard reference substance 3,4-dichloroaniline was conducted to provide supplemental information on the specificity of the sublethal endpoints. All nine tests were formally conducted under the conditions of Good Laboratory Praxis (GLP) at ECT Oekotoxikolgie GmbH with analytical support under GLP by Fraunhofer-IME. Hence, all critical phases of each study were inspected and quality-checked by an experienced quality assurance unit under GLP, and the study records are archived under GLP at the test facilities.

Range-finding tests (results are provided in Annex A4, Figure 6-8) were conducted to identify the optimal test concentrations for each test item. Subsequently, definitive studies were performed either as concentration-response (5 test concentrations), extended limit (three test concentrations) or limit test (one test concentration). Newly fertilized zebrafish (*Danio rerio*) eggs were exposed to aqueous solutions of the respective test item and in parallel to control treatments for 96 h without renewal of test solutions (static exposure design). Lethal and sublethal effects were assessed at several time points during the exposure period. The data obtained were statistically analyzed to estimate a No-Observed Effect Concentration (NOEC) and to calculate concentrations that cause the respective effect in 10 % and 50 % of the embryos (EC_{10} and EC_{50}), if the data supported these calculations. The performance and the results of each individual test is described in detail in the respective study reports. In the following, summaries of the methods, general performance and results are provided.

7.1 Material and Methods

7.1.1 Preparation of and exposure to test solutions

Modified reconstituted water (detailed composition is provided in Annex A4) was used as test medium to prepare stock and diluted test solutions. In cases of limited water solubility of a test item, a saturated stock solution was prepared, filtered (0.45 µm polyvinylidenfluorid (PVDF) filter), and the filtrate further diluted to generate the test solutions. Modified reconstituted water (i.e., test medium) was used also for the negative control (no addition of test item) and the positive control (4.0 mg/L 3,4-dichloroaniniline) in each test.

On the day of test start, eggs were collected from an in-house culture of adult zebrafish (*Danio rerio*) within 30 min of fertilization. Eggs were collected in one glass vessel with temperature-adapted test medium (26 ± 2 °C) and fertilization rate was immediately confirmed to be ≥ 80 %. Before the eggs reached the 32-cell stage, they were distributed to temperature-adapted pre-exposure vessels with the respective test solutions, which marks the start of exposure. Subsequently, fertilized eggs were transferred individually from the pre-exposure vessels to one well of a 24-well polystyrene plate filled with the 2.5 mL of corresponding test or control solution. The plates were subsequently incubated at 26 ± 1 °C for 96 hours. The solutions were not aerated during incubation and no food was added. The light conditions were 16/8 hours light/dark cycle, and light intensity was 547 to 936 lx across all tests.

There were 20 replicates (i.e., individual wells with one egg) on one 24-well plate for each test item concentration level and the positive control. The remaining four wells on each plate served as internal plate negative control, in addition to the one 24-well plate that was prepared as negative control (n=24 replicates). The 24-well plates were conditioned for approx. three days

prior to the start of the exposure period with the corresponding test solutions. Test solutions from the conditioning phase were exchanged on the day of exposure start with freshly prepared test and control solutions.

7.1.2 Water quality measurements

The following physico-chemical parameters were measured and recorded at least in the controls and the highest test concentration at start and at the end of exposure:

- temperature,
- pH,
- dissolved oxygen content,
- total water hardness,
- conductivity.

For measurement of physico-chemical parameters in test solutions at the end of the exposure, samples from the individual wells were pooled to achieve sufficient sample volume. At the start of exposure, the parameters were measured in aliquots of the freshly prepared test and control solutions.

The temperature of the test solutions was measured manually before start of the exposure and at test end. During the exposure, online measurement with logging intervals ≤ 30 minutes was used to monitor the temperature in the test chamber. When assessing effects under the stereomicroscope, the room temperature was monitored manually and kept at 26 ± 1 °C.

7.1.3 Sampling for analytical verification

In all tests except that with the 3,4 dichloroaniline, all solutions from all test concentration levels and the negative control were sampled from freshly prepared solutions immediately before filling the 24-well plates and at the end of the exposure to determine the actual active ingredient concentrations in comparison to the nominally applied concentrations. Analytical samples were stabilized by addition of methanol and stored in amber glass bottles at ≤-18 °C. Samples were transferred to the test site for chemical analysis on dry ice and temperature was recorded during shipping with a data logger.

7.1.4 Observations of developing embryos during the test

The OECD test guideline 236 (OECD 2013) describes the determination of acute toxicity of chemicals on embryonic stages of *D. rerio*. Hence, only observations relating to lethality are included as standard during the 96-hour exposure. In the present project, approaches to include sublethal endpoints in the FET were explored. These additional sublethal observations were first conducted in the range-finding tests to check their feasibility and then integrated in the GLP study plans for the definitive tests.

Depending on the age of the embryo, observations for various lethal and sublethal effects were performed at defined timepoints during the exposure phase in test organisms in the negative controls and the treatments (Table 13). For embryos in the internal plate negative controls on each 24-well plate and the positive controls, only lethal effects were assessed at 24, 48, 72 and 96 h after start of the exposure.

Table 13: Observations performed in the FET at defined time points during the 96 hours of exposure (x: observation performed; -: observation not performed)

Observation	24 ± 3 hours after start of exposure	48 ± 3 hours after start of exposure	72 ± 3 hours after start of exposure	96 ± 3 hours after start of exposure
Coagulation (presence/absence)	x	x	x	x
Somite formation (presence/absence)	x	x	x	x
Detachment of the tail (yes/no)	x	x	x	x
Heart beat (presence/absence)	–	x	x	x
Hatching (yes/no)	–	x	x	x
Spontaneous movement (yes/no)	x	–	–	–
Pigmentation of body	–	x	–	x
Development of eyes	–	x	–	x
Pigmentation of eyes	–	x	–	x
Malformation of otoliths	–	x	–	x
Occurrence of oedema	–	–	–	x
Deformations of spinal cord	–	x	–	x
Yolk deformation	–	x	–	x
Any other malformations	–	x	–	x
Number of somites	x [1]	–	–	–
Heart beat rate (beats per minute)	–	x [1]	–	–
Body length	–	–	–	x

(1) observation performed in 12 embryos per treatment.

7.1.4.1 Lethal effects

An egg or embryo was considered dead if at least one of the following criteria was fulfilled:

- Coagulation: indicated by a marked loss of translucency and a change in coloration, leading to an opaque appearance.

- Lack of somite formation: indicated if no somites were observed (in a normally developing zebrafish embryo about 20 somites should have developed at 24 hours post fertilization (hpf).
- Non-detachment of the tail: indicated if the tail was not detached from the yolk after posterior elongation of the embryonic body.
- Lack of heartbeat: indicated if no heartbeat was visible. Normally, heartbeat is visible at 48 hpf and older embryos. To record this criterion, embryos were observed under a minimum magnification of 80x for up to one minute. If no heartbeat was observed during this time, lack of heartbeat was recorded.

For embryos considered dead no further observations were conducted.

7.1.4.2 Additional effects

Hatch of the embryos was recorded starting 48 hours post fertilization (hpf). Embryos were considered as hatched if they had left the chorion completely.

7.1.4.3 Sublethal effects

Development of embryos was recorded as delayed or accelerated compared to the embryos of the negative control.

Presence or absence was documented for spontaneous movement (present if an embryo moved within 1 minute of observation), malformation of otoliths (appearing in the head region of an embryos) and any other malformations, e.g. on head, heart, tail (in comparison to the negative control).

For the following selected sublethal effects, the severity was documented using a rating scheme (none, minor, medium and strong differences compared to the negative control):

- Oedema, often visible e.g. in the regions of the heart and the yolk sac.
- Deformations of the spinal cord (e.g. scoliosis): In dorsal view, the spinal cord usually forms a straight line. Scoliosis describes an ‘S’ or ‘C’-shaped spine.
- Yolk deformation: depending on the developmental stage, the yolk should be of circular shape, similar to a ‘9’ or further elongated.
- Pigmentation of embryo body usually develops in approximately 36 hpf old embryos.
- Development of eyes: Eyes develop during the first 24 hpf. In embryos aged 48 hpf and older, iridophore pigmentation should be visible in the eyes.

Three parameters were recorded as continuous metric measures:

- Number of somites: approx. 20 somites should develop in embryos aged 24 hpf. The number of somites of 12 embryos per treatment (or less if fewer embryos survived) were counted. To avoid a bias due to the proceeding development of the embryos, counting was performed in a sequential manner over all test concentrations, i.e., the first four replicates of all treatments were assessed, followed by the observation of number of somites in the next four replicates of all other treatments etc.
- Heartbeat rate: the heartbeat of 12 embryos per treatment (or less if fewer embryos survived) was counted during a suitable time span of 30 seconds. Similar to assessment of

smite numbers, the first four replicates of all treatments were assessed, followed by the observation of heartbeat in the next four replicates of all other treatments etc.

- Additionally, length of all surviving hatched fish (except for the plate internal controls and the positive control) was individually measured and recorded at test end.

Heartbeat rate or number of somites were counted after all other relevant lethal and sublethal parameters had been assessed. Counting of heartbeat and of somites was performed successively for the first 4 replicates of all controls and treatments and repeated until the heartbeat of 12 embryos for the negative control and the test concentrations was assessed.

After the documentation of observations was concluded, the test vessels were relocated within the test chamber in a randomized manner.

7.1.5 Procedures at the end of the test

Observations of lethal effects were recorded first at the end of the test. For each test concentration and the negative control, 1.5 mL of the respective test solution in each replicate well were removed using a 5 mL piston-stroke pipette and pooled in a glass vessel. From these pooled test solutions, samples for chemical analysis were taken. After sampling of analytical samples, the physico-chemical parameters were measured in these pooled samples.

Each egg/larva in the 24-well plates was then killed by adding 40 µL benzocaine (25 g/L) to each well. Observations of sublethal effects were recorded for each egg and developing embryo. The body length of individual fish was measured from the tip of the snout to the tip of the tail fin using a stereomicroscope and subsequent photographic image processing. Subsequently, each egg/embryo was frozen in the test units in the freezer to confirm death.

7.1.6 Data Assessment and Statistical Evaluation

The statistical software package ToxRat Professional 3.3.0 (ToxRat Solutions GmbH, Naheweg 15, D-52477 Alsdorf) (ToxRat® Solutions GmbH 2022) was used for these calculations.

All statistical evaluations were based on the geometric mean of the measured concentrations of the active ingredient in the respective test item. For each observation time during the 96-hour exposure, the number of dead embryos (i.e. embryos with at least one lethal effect) per treatment were calculated. Numbers of hatched embryos per treatment were determined and used for statistical analysis of hatch after 48, 72 and 96 h of exposure. For each sublethal effect, the sums of embryos per treatment with the respective sublethal effect were calculated. Effect concentrations were based on the number of embryos showing the respective effect compared to the number of surviving embryos at the specific time. For the observation of effects specifically on larvae, i.e. the effect „larvae laying on the lateral side“, numbers of larvae showing this effect were compared with the number of hatched larvae. Number of somites and heartbeat rate were assessed in 12 embryos per treatment 24 and 48 hours after start of the exposure, respectively. Body length of each surviving larvae was measured at test end, 96 hours after start of the exposure. For these data, mean values per treatment were calculated and used for further statistical calculations.

Normal-distribution of errors was tested with the Shapiro-Wilk's test (alpha = 0.01) and variance homogeneity was checked with Levene's test (p = 0.01). The respective data for each active ingredient concentration were then statistically compared to the data from the negative control with post-hoc tests to determine the highest active ingredient concentration that was significantly different from the control (Lowest Observed Effect Concentration, LOEC) and deduce from that the next lower concentration level that was not significantly different (at α =

0.05) from the control (No Observed Effect Concentration, NOEC). Details on selection of these post-hoc tests are described in the following for the respective endpoints. If the slope of the concentration-response curve was significant, EC_x values were calculated additionally.

As post-hoc tests for evaluation of data on lethal and sublethal effects and on hatch, the Step-down Cochran-Armitage test was performed on data showing a linear trend. If no linear trend could be observed, the Chi^2-2 x 2 Test with Bonferroni Correction was applied. If the number of embryos with the respective effect was too low to fulfil the requirements of the Chi^2-test, the multiple sequentially-rejective Fisher Test after Bonferroni-Holm correction was used.

Determination of NOEC values in post-hoc tests for data on number of somites after 24 h of exposure, heartbeat rate after 48 h of exposure and length of hatched larvae after 96 h of exposure was performed with the William's multiple sequential t-test if assumptions on normal distribution and homogenous variances were fulfilled. If assumptions were not fulfilled, the multiple sequentially-rejective Welsh-t-test after Bonferroni-Holm or the step-down Jonckheere-Terpstra test, or the multiple sequentially-rejective Median (2x2-table) test after Bonferroni-Holm was chosen. All post-hoc tests were performed at alpha = 0.05.

The calculation of EC_x values for hatch, lethal and sublethal effects was performed with a 2-parametric probit analysis using linear maximum likelihood regression. For data continuous (number of somites, heartbeat rate and length), EC_x values were calculated with a 3-parametric non-linear cumulative distribution regression with Downhill-Simplex optimization.

7.2 Results

7.2.1 Validity of conducted FET test

In all nine tests, the validity criteria defined in the OECD test guideline (OECD 2013) were met (Table 14). The light intensity in the test chamber was between 582 and 936 lux. Excluding the test with cyclophosphamide, at the start and end of the exposure, hardness was 161-196 mg $CaCO_3$/L, conductivity was 773-904 µS/cm, and measured pH values were between 7.2 and 7.7 in all test solutions.

In the test with cyclophosphamide high substance concentrations were used which influenced the physico-chemical parameters in the test solutions. Hardness, conductivity and pH at test start were within the limits mentioned above and the values measured in the negative control and the lowest three test concentrations at test end similarly were in this range. However, at test end hardness, conductivity and pH in the two highest test concentrations were 177 mg $CaCO_3$/L, 1333 µS/cm, and 3.2, respectively.

Table 14: Validity criteria for the FET as prescribed in the OECD guideline (2013) and the values observed in each of the nine tests.

Test N°	Active Ingredient	Fertility (%)	Survival negative control (%)	Tempera-ture (°C)	Oxygen (% saturation)	Hatch (%)	Mortality positive control (%)
-	**Validity criteria**	**≥ 70**	**≥ 90**	**26 ± 1**	**≥ 80**	**≥ 80**	**≥ 30**
1	3,4-dichloroanline	70	100	25.3–26.7	≥ 87	100	70
2	Propranolol	85	92	25.5–26.7	≥ 98	92	80
3	Afatinib	90	100	25.4–26.9	≥ 99	100	80
4	Rosuvastatin	85	100	25.5–26.2	≥ 101	100	55
5	Edoxaban	90	100	25.4–26.2	≥ 101	100	80
6	Rivaroxaban	90	100	25.5–26.4	≥ 100	100	80
7	Cyclophosphamide	90	100	25.8–26.2	≥ 99	96	75
8	Imatinib	90	96	25.0–26.7	≥ 102	83	80
9	Palbociclib	90	96	25.0–26.7	≥ 101	83	80

Fertility: Fertilization rate of the respective batch of eggs used for the tests in % of all collected eggs; Survival: survival of embryos in the negative control at test end in % of introduced eggs; Temperature: temperature in the test vessels during the exposure; Oxygen: dissolved oxygen levels measured at least in the negative control and the highest test concentration in % of air saturation; Hatch: hatching rate in the negative controls at test end in % of introduced eggs; Mortality positive control: mortality in the positive control with 4.0 mg/L 3,4-dichloroaniline in % of introduced eggs.

7.2.2 Analytically verified exposure concentrations

Measured test concentrations are summarized in Table 15. The measured concentrations of propranolol, rosuvastatin, cyclophosphamide and imatinib deviated by less 20% from the respective nominal concentrations, i.e., they were in good agreement.

Stock solutions of rivaroxaban, imatinib and palbociclib had been filtered before the preparation of test solutions. In the case of rivaroxaban and palbociclib, the low recovery in relation to nominal concentration indicates therefore incompletely dissolved test item in the stock solution (i.e., exceedance of water solubility). For imatinib with recoveries ≥ 100% of the nominal concentration, the filtration step did not remove significant amounts of the test substance. In test solutions with afatinib and edoxaban, measured concentrations were between 60 and 83 % of the nominal concentrations. Reasons for the low recovery could not be identified.

Chemical analysis did not reveal significant loss of the respective active ingredient during the 96-hour exposure of the embryos, indicating stability under the exposure conditions. Thus, the geometric mean of the measured test concentrations at test start and end were used to represent exposure concentrations at each concentration level, even for those test substances with less than 20% deviation between nominal and measured concentrations.

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Table 15: Summary of analytically verified exposure concentrations and relation to nominal test concentrations

Active Ingredient	Nominal test concentrations [mg a.s./L]	Measured in relation to nominal concentration (%) at test start	Measured in relation to nominal concentration (%) at test end	Geometric mean of test concentrations measured at test start and end [mg a.s./L]
3,4-dichloroanline	0.04/0.086/0.186/0.4/0.862/1.86/4	-	-	not measured
Propranolol	0.5/1.58/5/15.8/50	100–104	101–108	0.531/1.62/5.03/16.5/51.2
Afatinib	0.696/1.50/3.23/6.96/15.0	60–84	63–83	0.428/1.02/2.36/5.48/12.5
Rosuvastatin	2.15/4.6/410.0	81–83	82–84	1.77/3.80/8.24
Edoxaban	10.0	68	59	6.33
Rivaroxaban	5.00	60	64	3.10
Cyclophosphamide	371/800/1723/3713/8000	94–96	92–94	346/749/1640/3516/7555
Imatinib	100	116	100	107
Palbociclib	10.0/17.8/31.6/56.2/100	4.3–5.1	3.2–5.2	0.372/0.730/1.42/2.78/5.13

7.2.3 Biological results

7.2.3.1 Lethal effects

Significant lethal effects were observed only for 3,4-dichloroanline and cyclophosphamide with 70 and 100 % mortality in the highest investigated substance concentrations (Table 16). According to the Validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test (OECD 2013) the LC_{50} (96 h)-values for 3,4-dichloroaniline should range between 1.80 mg/L and 3.60 mg/L. Hence, the results of the reference test are acceptable, the test conditions are reliable and the sensitivity of the test system was demonstrated. In all investigated test concentrations in the tests with the seven remaining substances, mortality was ≤ 10 % of introduced embryos.

Table 16: Summary of lethal effects in the FET tests

Category of test substance	Active ingredient	LC_{50} (mg/L) with 95% confidence intervals	Mortality at highest test concentration (% of introduced)
Reference substance	3,4-dichloroaniline	3.56 (1.75-2.25)	70
Cardiologically active, positive model substance, first-in-class	Propranolol	> 51.2	0
Cardiologically active	Edoxaban	> 6.33	0
Cardiologically active, first-in-class	Rivaroxaban	> 3.10	0
Cardiologically active	Rosuvastatin	> 8.24	0
Oncologically active, positive model substance	Cyclophosphamide	2,820 (1,351-5,887)	100
Oncologically active, first-in-class	Imatinib	> 107	0
Oncologically active, new generation	Afatinib	> 12.5	5
Oncologically active, new generation	Palbociclib	> 5.13	5

7.2.3.2 Hatching rate

Hatch of larvae was observed at the earliest at 72 ± 3 hours of exposure, i.e., larvae hatched in none of the tests after 48 h. Not including tests with those substances causing significant mortality (3,4-dichloroanline and cyclophosphamide), 35 to 95 % (mean ± sd = 66.1 ± 14.3 %) of the introduced embryos hatched after 72 ± 3 hours of exposure. For this observation time point, significantly delayed hatching was determined in the test with propranolol (see Table 19). After 96 hours of exposure, 83.3 to 100% (mean ± sd = 95.2 ± 4.85 %) of the introduced embryos hatched (Table 17) and no influence of the respectively investigated substances on hatching rate was discernible.

Table 17: Summary of hatching rates observed in the FET tests after 96 hours of exposure.

Category of test substance	Active ingredient	LC_{50} (mg/L) with 95% confidence intervals	Hatching rate at highest test concentration, 96 hpf (% of introduced)
Reference substance	3,4-dichloroaniline	3.56 (2.25-1.75)	0 (70% mortality)
Cardiologically active, positive model substance, first in class	Propranolol	> 51.2	90
Cardiologically active	Edoxaban	> 6.33	100
Cardiologically active	Rivaroxaban	> 3.10	95
Cardiologically active	Rosuvastatin	> 8.24	100
Oncologically active, positive model substance	Cyclophosphamide	2820 (1351-5887)	0 (100% mortality)
Oncologically active, first-in-class	Imatinib	> 107	100
Oncologically active, new generation	Afatinib	> 12.5	95
Oncologically active, new generation	Palbociclib	> 5.13	95

7.2.3.3 Sublethal effects

Observed sublethal effects are summarized in the following in Table 18 for the reference substance 3,4-dichloroaniline, in Table 19 for the cardiologically active substances and in Table 20 for the oncologically active substances.

The number of somites at 24 h of exposure were similar in all tests across all test substances and concentration levels, ranging from 16 to 32 somites (mean± sd = 23.5 ± 2.47 somites) across all tests performed. Hence, none of the test items influenced the number of somites during embryonic development of *D. rerio*. The results obtained by the assessment of the number of somites as sublethal endpoint is therefore not included in the following tables.

A difference between the pigmentation of the body or the eyes between the negative control and any test item was observed only occasionally under exposure to rosuvastatin, propranolol, edoxaban, cyclophosphamide and 3,4-dichloroaniline. In all cases, reduced body pigmentation was observed in less than five embryos per treatment at test end and without relation to increasing concentrations. Pigmentation of eyes was reduced in 2, 1 and 2 embryos in the lowest, a median and the highest test concentration at the end of the test with 3,4-dichloroanline, similarly lacking a concentration-dependence. Pigmentation of body or eyes is therefore also not included in the following tables (Table 18, Table 19, and Table 20), which summarize the observed sublethal effects.

Heart beat rate was a sensitive endpoint for three of the nine selected test substances, namely propranolol, 3,4-dichloroanline and cyclophosphamide. For the latter two substances, lethal effects were observed at test end in the concentration range where the heart beat rate was influenced at 48 h exposure (3,4-dichloroaniline: LC50 = 3.56 mg/L, EC50(heart beat rate) =

3.62 mg/L; cyclophosphamide: LC50 = 2820 mg/L, EC50(heart beat rate) = 3195 mg/L), indicating a general stress response. For propranolol, however, all introduced embryos survived until test end in the highest test concentration of 50 mg/L. Heart beat rate was significantly reduced in all four treatments with concentrations above 0.5 mg/L.

Body length was negatively influenced by four test substances (3,4-dichloroanline, cyclophosphamide, propranolol and rosuvastatin). For propranolol and rosuvastatin the difference determined for larvae from the control and the respective highest test concentration was below 10 %, restricting the calculation of valid ECx values and allowing the determination of a NOEC only.

Oedema in the area of the yolk were observed at test end in 2 embryos in the negative control in the test with cyclophosphamide, in 3 embryos in the highest rosuvastatin concentration and in 3 embryos in the two highest 3,4-dichloroaniline concentrations, but no significant effect was determined. However, presence of oedema in the heart was significantly increased by four of the nine substance tests (3,4-dichloroaniline, cyclophosphamide, rosuvastatin and propranolol).

Malformation of otoliths was observed in the test with cyclophosphamide, malformations of the yolk of the embryos were observed in the tests with 3,4-dichloronaline and cyclophosphamide. Malformation of spine were observed in embryos exposed to 3,4-dichloronaline, cyclophosphamide and rosuvastatin.

The assessed effects were not an exhaustive selection of all possible physiological changes in zebrafish embryos. Any additional potential effect noted in a single embryo at test end was evaluated for all embryos. Among these additional observations, a significant number of embryos showed no spontaneous movement after 24 h of exposure to respective highest test concentrations of 3,4-dichloroanline (40 % of surviving embryos) and cyclophosphamide (54 % of surviving embryos).

Propranolol and cyclophosphamide caused 90 % and 38 % of the surviving larvae to lose their ability to swim upright in the water phase, laying laterally on the bottom of the test vessels instead. Other observations were relevant only for one of the selected substances: 40 % of the surviving embryos showed spasmic movement in the treatment with 1640 mg cyclophosphamide/L, 3,4-dichloroanline delayed the development of the eyes of 29 % and 83 % of the surviving embryos in the two highest test concentrations, and propranolol delayed the hatching of exposed embryos at 72 ± 3 h and at test end, and caused a dark coloured yolk sac (nearly lacking any transparency that is common for zebrafish embryos at this developmental stage) in a significant number of embryos (30 % of the surviving embryos).

Palbociclib in concentrations between 1.06 and 5.07 mg/L caused the defective development of the head in 26 to 79 % of the surviving embryos, notably by the reduced size of the head. This observation was, however, assessed qualitatively and no measurement of the head dimensions was performed.

Table 18: Summary of sublethal endpoints (mg a.i./L) with the reference substance 3,4-dichloroaniline in the FET test

Reduced heart beat rate at 48 h	Reduced body length at 96 h	Occurrence of oedema at 96 h	Presence of malformations at 96 h	Other significant sublethal effects
EC_{10} = 2.04 (1.64-2.53) EC_{50} = 3.62 (3.24-4.17) NOEC = 0.862	EC_{10} = 0.989 (0.807-1.213) EC_{50} > 4.00 NOEC = 0.186	Heart oedema: EC_{10} = 0.166 (0.0187-1.47) EC_{50} = 0.552 (0.129-2.36) NOEC = 0.400	Spinal malformation: EC_{10} = 1.23 (0.047-32.3) EC_{50} > 4.0 NOEC = 1.86 Yolk malformation: EC_{10} = 2.07 (0.210-20.3) EC_{50} > 4.00 NOEC = 1.86	Lack of spontaneous movement at 24 h: EC_{10} = 1.34 (0.104-17.3) EC_{50} > 4.0 NOEC = 1.86 Delayed eye development at 96 h: EC_{10} = 0.600 (0.0592-6.07) EC_{50} > 4.0 NOEC = 0.862

Table 19: Summary of sublethal endpoints (mg a.i./L) in the FET tests with cardiologically active test substances

Active ingredient	Reduced heart beat rate at 48 h	Reduced body length at 96 h	Occurrence of oedema at 96 h	Presence of malformations at 96 h	Other significant sublethal effects
Propranolol	EC_{10} = 4.01 (1.72-8.22) EC_{50} > 51.2 NOEC = 0.531	EC_{10} > 51.2 NOEC < 0.531	Heart oedema: EC_{10} = 17.2 (0.429-688) NOEC = 16.4	none	Hatch (72 h): NOEC = 16.4 Larvae laying on side (96 h): NOEC = 16.4 Yolk sac dark coloured (96 h): NOEC =16.4
Edoxaban	EC_{10} > 6.33 NOEC ≥ 6.33	EC_{10} > 6.33 NOEC ≥ 6.33	none	none	none
Rivaroxaban	EC_{10} > 3.10 NOEC ≥ 3.10	EC_{10} > 3.10 NOEC ≥ 3.10	none	none	none
Rosuvastatin	EC_{10} > 8.24 NOEC ≥ 8.24	EC_{10} > 8.24 NOEC < 1.77	Heart oedema: EC_{10} = 2.45 (1.56-3.09) EC_{50} = 4.33 (3.52-5.36) NOEC = 1.77	Spinal malformation: EC_{10} > 8.24 NOEC = 3.80	none

Table 20: Summary of sublethal endpoints (mg a.i./L) in the FET tests with oncologically active test substances

Active ingredient	Reduced heart beat rate at 48 h	Body length at 96 h	Occurrence of oedema at 96 h	Malformations	Other significant sublethal effects
Cyclophosphamide	EC_{10} = 1.978 (1.775-2.430) EC_{50} = 3.195 (3.051-2.276) NOEC = 749	EC_{10} = 1.627 (n.a.) EC_{50} = 1.708 (n.a.) NOEC = 749	Heart oedema: EC_{10} = 1.120 (798-1.572) EC_{50} = 2.171 (1.600-2.947) NOEC = 749	Otolith malformation: EC_{10} = 1.522 (249-9.295) EC_{50} = 3.471 (201-59.901) NOEC = 1.640 Yolk malformation: EC_{10} = 1.522 (249-9.295) EC_{50} = 3.471 (201-59.901) NOEC = 1.640 Spinal malformation: EC_{10} = 1.135 (556-2.318) EC_{50} > 7.555 NOEC ≥ 3.516	Lack of spontaneous movement at 24 h: EC_{10} = 6.012 (n.a.) EC_{50} = 7.435 (n.a.) NOEC = 3.516 Spasm: EC_{10} = 1.000 (75.2-13.299) EC_{50} = 3.889 (41.5-364.437) NOEC = 749 Larvae laying on side: EC_{10} = 1.360 (n.a.) EC_{50} =1.734 (n.a.) NOEC = 749
Imatinib	EC_{10} > 107 NOEC ≥ 107	EC_{10} > 107 NOEC < 107	none	none	none
Afatinib	EC_{10} > 12.5 NOEC ≥ 12.5	EC_{10} > 12.5 NOEC ≥ 12.5	none	none	none
Palbociclib	EC_{10} > 5.13 NOEC ≥ 5.135	EC_{10} > 5.13 NOEC ≥ 5.13	none	none	Small head: EC_{10} = 0.485 (0.224-0.732) EC_{50} = 0.763 (0.437-1.07) NOEC = 0.372

na: not available due to mathematical reasons

7.3 Discussion

7.3.1 Specificity of sublethal endpoints in the FET

One of the hypotheses in the present project postulated that heart beat rate would be a specific endpoint for cardiologically active substances. Indeed, a significant reduction of heart beat rate in *D. rerio* embryos was observed for the selected positive model substance propranolol, a first-in-class beta-blocker. This finding is consistent with Bittner et al. (2018), who reported reduction of heart beat rate 96 hpf at the lowest tested propranolol concentration of 0.01 mM (equivalent to 2.59 mg/L). However, the reference substance 3,4-dichloroaniline and the oncologically active substance cyclophosphamide also caused a significant reduction of heart beat rate although with slightly (3,4-dichloroaniline) or substantially (cyclophosphamide) higher NOECs. Hence, beta-blockers such as propranolol were confirmed in the present study to specifically influence heart beat rate in *D. rerio* embryos, which is in accordance with their pharmacological mode of action. However, heart beat rate was revealed as an endpoint that lacks specificity to such a mode of action, because other chemicals with a different pharmacological mode of action (cyclophosphamide) or unspecific baseline toxicity (3,4-dichloroaniline) can also influence it. This is in agreement with a recent study of Schweizer et al. (Schweizer et al. 2022) who report a high correlation between mortality and heart beat rate measured in the FET for ten ionizable substances.

In addition, the other three cardiologically active substances did not influence heart beat rate at concentrations up to their limit of water solubility. Yet, the specificity of heart beat rate for the effects of the beta-blocker propranolol appears apparent when considering NOEC values for heart beat rate in relation to LC50 values for lethal effects: there is an almost 100-fold deviation between the two values for propranolol, while NOEC and LC50 differ less than 5-fold for 3,4-dichloroaniline and cyclophosphamide. In the study of Schweizer et al. (Schweizer et al. 2022), propranolol's LC50 in the FET and its EC20 for heart beat rate differ less than 5-fold across a range of pH values in the test medium (EC_{10} and NOEC have not been determined in that study).

The hypothesis for the oncologically active substances postulated that antineoplastics would cause teratogenic effects. Cyclophosphamide was selected to serve as positive model substance based on previous such suggestions (Busquet et al. 2008, Schaumburg et al. 2016, Turani et al. 2019). Indeed, cyclophosphamide caused malformations of otoliths, the yolk and the spinal cord in developing *D. rerio* embryos, which indicates teratogenic activity. Yet, these effects were observed at high concentrations (generally greater than 1 g/L), which are not environmentally relevant. The three selected antineoplastic test substances showed generally no impact on sublethal endpoints at concentrations up to their limit of water solubility. The only exception was palbociclib that caused a peculiar form of a small head in the embryos, which indicates teratogenic activity. No other test substance caused such a phenotype nor was it observed in any of the controls in the nine tests. Hence, this sublethal effect appears to be specific for palbociclib. Whether it is specifically linked to the pharmacological mechanisms of action of palbociclib, cyclin-dependent kinase inhibition, cannot be concluded, because the other tested antineoplastics target different receptors (tyrosine kinases). It remains also unclear, whether malformations and oedema are specifically pointing at teratogenic activity since 3,4-dichloroanline as well as the lipid-lowering drug rosuvastatin both caused spine and yolk malformations and heart oedema, and heart oedema were observed under propranolol exposure, all at concentrations above 1 mg/L. However, this finding could in principle also

indicate teratogenic potential of rosuvastatin or even statins in general. Indeed, there has been discussion in the medicinal literature about teratogenic activity of statins, particularly in view of their usage during pregnancy. Meta-studies and critical reviews are for example Karalis et al. (2016), Gheorghe et al. (2020) and Vahedian-Azimi et al. (2021). Yet, there is apparently no clear consensus yet, apart from continuation of considering pregnancy a strong contra-indication for statin treatment.

7.3.2 Sensitivity of sublethal endpoints in the FET

A key hypothesis driving the present study was that the apical standard endpoints according to the current guideline on environmental risk assessment of medicinal products for human use may not be sufficiently sensitive to ensure a protective risk assessment. The FET test had been proposed based on a previous project to provide sublethal endpoints that could be more sensitive, i.e., deliver lower NOEC values. The values for the lethal (LC50) and sublethal endpoints (lowest NOEC among determined sublethal NOECs) in the FET derived in the present study and apical standard endpoints for fish are summarized in Table 21. This comparison demonstrates that in fact the apical standard endpoints (most of them originating from regulatory accepted risk assessments) are in all cases lower. In two cases, the sublethal endpoint for body length delivered the lowest non-standard sublethal NOEC and was determined as smaller-than value, but not necessarily as smaller than the standard apical endpoint. Hence, this comparison clearly demonstrates that the standard apical endpoint derived in a Fish Early Life Stage study with fish is a more protective endpoint than any of the sublethal or lethal endpoints evaluated in the FET in the present study.

Table 21: Comparison of lethal and sublethal endpoints in the FET and apical endpoints in adult fish

Active ingredient	LC50 (mg/L) in present study	Lowest NOEC (mg/L) for the most sensitive sublethal endpoint in the present study	Apical endpoint (NOEC) in early life stage test with fish
3,4-dichloroaniline	3.56	0.166	not available
Propranolol	> 51.2	< 0.531	0.110 mg/L Egg production and hatchability Gunnarsson et al. (2019) referring to Giltrow et al 2009)
Edoxaban	> 6.33	≥ 6.33	2.32 mg/L (Edoxaban EPAR EMA/321083/2015 2015)
Rivaroxaban	> 3.10	≥ 3.10	0.086 mg/L (Rivaroxaban EPAR EMA/CHMP/301607/2011 2011)
Rosuvastatin	> 8.24	< 1.77	1.0 mg/L (Gunnarsson et al. 2019)
Cyclophosphamide	2,820	749	not available

Active ingredient	LC50 (mg/L) in present study	Lowest NOEC (mg/L) for the most sensitive sublethal endpoint in the present study	Apical endpoint (NOEC) in early life stage test with fish
Imatinib	> 107	< 107	10 mg/L (Imatinib EPAR EMA/CHMP/161314/2013 2013)
Afatinib	> 12.5	≥ 12.5	0.032 mg/L (Afatinib EPAR EMA/491185/2013 2013)
Palbociclib	> 5.13	0.372	0.13 mg/L (Palbociclib EPAR EMA/652627/2016 2016)

7.3.3 Suitability of the selected sublethal endpoints

In the nine tests performed, four and three substances induced significant heart oedema and malformations in the zebrafish embryos, respectively. A significant concentration-dependence of the endpoints heart beat rate and body length was observed in three and four of the tests, respectively.

Presence of oedema in heart and yolk, the pigmentation of eyes and body, the development of eyes and malformations of yolk and spine were ranked on a scale of severity from 0 (no difference to control), 1 (minor severity of the respective effect) to 4 (strongest possible severity of the respective effect). Though observations on the developing embryos in the present project were performed by experienced technicians, the classification of severity for these effects was often a matter of discussion and a certain degree of subjectivity remained.

Among the selected test substances, 3,4-dichloroanline and cyclophosphamide influenced the highest number of different sublethal effects after 96 hours of exposure (see Table 18 and Table 20). To illustrate two examples for the severity rating for sublethal endpoints, data for the most sensitive sublethal effect for 3,4-dichloroaninline (heart oedema, Table 22 and cyclophosphamide (heart oedema, Table 23) are summarized. Obviously, in both studies increasing substance concentrations led to more pronounced oedema in the exposed embryos, indicated by rating into higher severity categories. However, consideration of the severity of the observed oedema did not lead to any difference in the determined NOEC values. Thus, sensitivity of the test could not be improved by determination of severity of the sublethal effects.

Table 22: Data on severity of heart oedema determined in embryos exposed to 3,4-dichloroaniline for 96 h. The NOEC when performing statistics on presence/absence of the respective effect is 0.400 mg/L (bold).

Test conc. [mg/L]	Surviving embryos (n)	Embryos with heart oedema (n)	Embryos without heart oedema (n)	Embryos with severity 1 (n)	Embryos with severity 2 (n)	Embryos with severity 3 (n)	Embryos with severity 4 (n)
0.00	24	1	23	0	1	0	0
0.04	20	1	19	0	1	0	0
0.086	20	0	20	0	0	0	0

Test conc. [mg/L]	Surviving embryos (n)	Embryos with heart oedema (n)	Embryos without heart oedema (n)	Embryos with severity 1 (n)	Embryos with severity 2 (n)	Embryos with severity 3 (n)	Embryos with severity 4 (n)
0.186	19	0	19	0	0	0	0
0.400	18	3	15	3	0	0	0
0.862	19	19	0	12	7	0	0
1.86	17	17	0	0	15	2	0
4.00	6	4	2	0	4	0	0

Table 23: Data on severity of heart oedema determined in embryos exposed to cyclophosphamide for 96 h. The NOEC for presence of oedema is indicated in bold.

Test conc. [mg/L]	Surviving embryos (n)	Embryos with heart oedema (n)	Embryos without heart oedema (n)	Embryos with severity 1 (n)	Embryos with severity 2 (n)	Embryos with severity 3 (n)	Embryos with severity 4 (n)
0.00	24	1	23	0	1	0	0
346	20	0	20	0	0	0	0
749	**19**	**1**	**18**	0	0	1	0
1640	20	4	16	4	0	0	0
3516	5	5	0	0	3	2	0
7555	0	-	-	-	-	-	-

NOEC values for the endpoint length were the most sensitive sublethal endpoint in the FET for four of the five test substances (3,4-dichloroaniline, cyclophosphamide, propranolol, and rosuvastatin) that caused significant effects in the embryos in the tested concentration range.

If an embryo hatches, it successfully passed certain stages of development and will have a minimum length. In the early life stage of the embryos (96 hours post fertilization and approx. 24 – 48 hours after hatch), the larvae fed on the yolk sac and endogenous feeding was not required yet, nor was any food provided during the test. The length of a zebrafish larvae 96 hours post fertilization likely has an upper limit close the minimum length, leading to a small range of possible values.

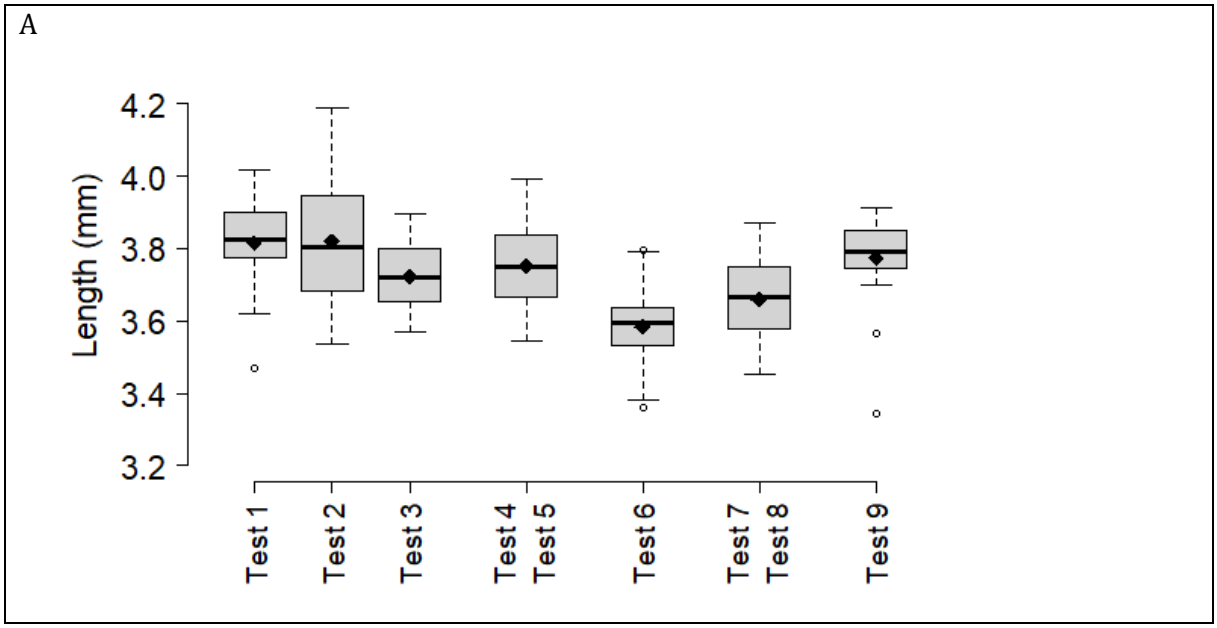
The embryos in the highest relevant test concentration (i.e. the highest test concentration with hatched larvae at test end) compared with embryos from the control were 16.6 % and 13.6 % smaller in tests with 3,4-dichloroaniline and cyclophosphamide and 8.5 % and 5.6 % smaller in the tests with propranolol and rosuvastatin. Hence, the strongest effect observed was a reduction of the mean length by approx. 17 % and the minimum mean length observed in the highest relevant test concentrations described here was 3.148 mm (1.86 mg/L 3,4-

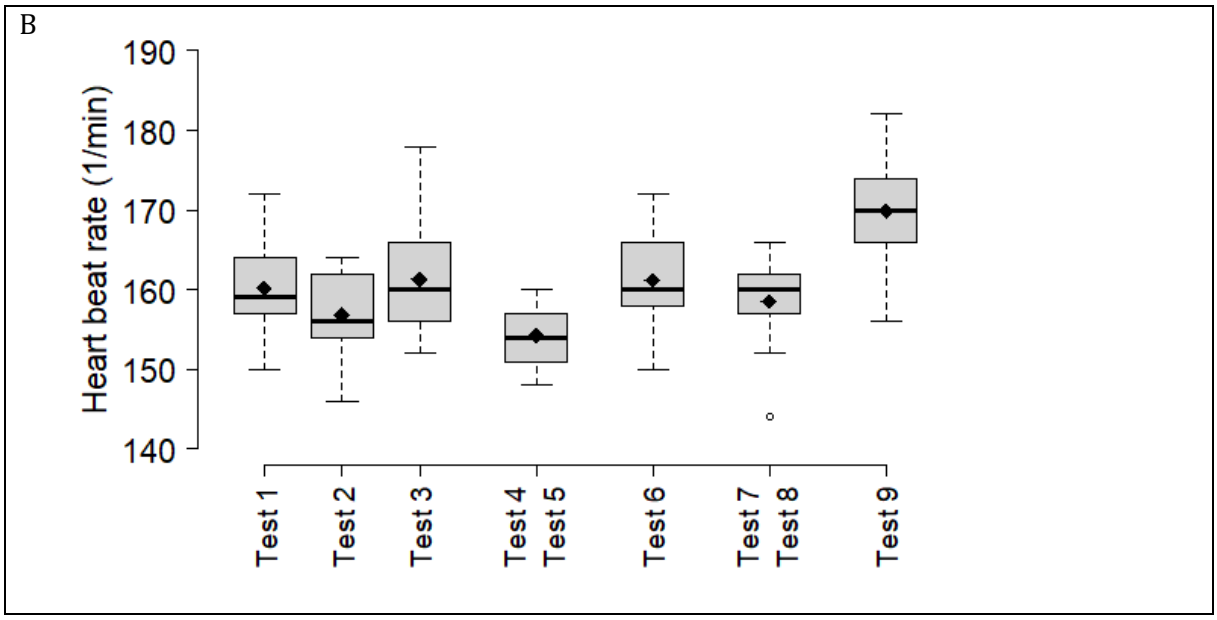
dichloroanline). The determined lowest observed effect concentrations lead to 2.5 % (3,4-dichloroanline), 12.6 % (cyclophosphamide), 3.5 % (propranolol) and 3.3 % (rosuvastatin) smaller embryos than in the negative control. With the exception of cyclophosphamide, these deviations are noticeable small.

Nevertheless, the variance in length data obtained in each of these tests is small. On the example of propranolol, the statistically minimum detectable differences (MDD) for the treatments compared to the negative control (calculated by multiple sequentially-rejective t-test after Bonferroni-Holm, one-sided smaller) were between -2.0 and -2.9 % for propranolol, meaning that an absolute difference in length of approx. 0.08 mm would be considered significant on an alpha level of 0.05. Whether such a length different is indeed biologically significant and relevant at the population level, remains open, though.

To gain insight in the usual length of zebrafish larvae 96 hours post fertilization under the test conditions of a FET, Figure 5A summarizes the measured length of embryos in the negative controls of each test. The mean values of all embryos in the negative controls of the nine tests were between 3.584 and 3.821 mm. For the tests with propranolol, rosuvastatin, cyclophosphamide and 3,4-dichloroanline the statistically significant lowest observed effect concentrations correlated to mean length values of 3.682, 3.600, 3.683 and 3.786 mm. These values are clearly within the observed variation in the controls.

Figure 5: Reproducibility and sensitivity of the sublethal endpoints body length (A) and heartbeat rate (B).





Source: Own illustration, ECT

Data shown were obtained from embryos in the negative controls of the respective substance tests. Mean values are indicated by diamonds. The boxes represent the first and third quartile of the data, the bold horizontal line is the median, whiskers extend to minimum and maximum values in the 1.5 interquartile range, outliers from this range are represented by dots. Tests were performed with propranolol (Test 1), afatinib (Test 2), rosuvastatin (Test 3), edoxaban (Test 4), rivaroxaban (Test 5), cyclophosphamide (Test 6), imatinib (Test 7), palbociclib (Test 8) and 3,4-dichloroaniline (Test 9). Note that the tests with edoxaban and rivaroxaban and the tests with imatinib and palbociclib were performed simultaneously and used the same controls.

Supplementary to length of the hatched larvae, the observations on heart beat rate in zebrafish embryos 48 hpf provide a sensitive endpoint for three of the five test substances that caused significant effects.

Heart beat rates in control embryos under the test conditions of the present studies are shown in Figure 5B for all tests performed. The mean values observed in the controls of each test range from 154.2 to 169.8 beats per minute, minimum and maximum values in the negative controls of all tests were 144 and 182 beat per minute.

The determined lowest observed effect concentrations lead to heart beat rates that were reduced by 10.6 % (3,4-dichloroanline), 6.4 % (cyclophosphamide) and 5.6 % (propranolol) compared to embryos in the negative control. Similar to the observations performed on length, these differences are small.

However, the ranges of observed mean values per test are comparable larger. Similar to length, an embryo likely has to have a certain minimum and maximum heartbeat that enables correct development and survival until the time of observation. In the studies performed, observed heart beat rates in embryos 48 hours post fertilization were 60.5 % (3,4-dichloroanline), 58.6 % (cyclophosphamide) and 25.0% (propranolol) lower in the embryos from the highest test concentrations than in the controls. The corresponding mean heart beat rates are 67.0, 66.7 and 120.2 beats per minute.

In the test performed, treatments where a mean heartbeat rate of 67.0 (4.00 mg/L 3,4-dichloroanline) and 66.7 (3516 mg/L cyclophosphamide) beats per minute was observed in embryos 48 hours post fertilization, 70.0 % and 75 % of the introduced embryos showed lethal effects at test end and none of the survivors hatched. The highest test concentration of propranolol lead to a mean heart beat rate of 120.2 beats per minute. Here, no lethal effects

were observed until test end. Hatch of embryos was significantly delayed after 72 hours of exposure but at test end, 90 % of the introduced embryos hatched.

7.3.4 Complementing the FET with sublethal endpoints

Currently, the OECD guideline 236 for the zebrafish embryo acute toxicity test (OECD 2013) requires reporting of morphological and physiological abnormalities observed in embryos exposed to the test item. Based on the results obtained in the nine tests discussed here, the following potential effects should be explicitly named and described in the guideline: Presence of oedema in the heart and yolk, and presence of malformations, e.g. of the spine and the yolk. In the current version of the guideline, these effects are described very briefly in the annex only.

Additionally, any other noticeable morphologically difference should be documented, since in the present study seven additional observations (lack of spontaneous movement, delayed eye development, delayed hatch, larvae laying laterally, yolk sac dark coloured, spasms, small head) were significantly altered by the investigated test substances. In any case, advice should be provided on the statistical evaluation of the data and the reporting of the respective endpoints. In the studies performed, the observation of these effects was facilitated by killing the embryos with an overdose of the anaesthetic benzocaine after the assessment of lethal effects at test end. Thus, ethics in animal welfare were considered and the observations were performed quickly on non-moving embryos. Provided an optimized form of documentation is prepared (e.g. score sheet for each potential effect in each embryo), these observations can be easily integrated in the standard test procedures.

Similarly, the measurement of length in fish killed after the end of the exposure does not require complex method development and comprised transfer of the organisms to an object slide with a metric scale and photographing the slide with the test organisms under a microscope with a digital camera. Length was measured later using a digital image software. Determination of length could hence be integrated in the standard procedures of the fish embryo test and would likely increase the sensitivity of the FET test. Without further investigation of this endpoint, a statement on the biological relevance of small but significant effects on length of hatched embryos, as observed here, remains unclear.

Opposed to length, the relevance of substance-induced effects on heart beat rate are more obvious and can lead to lethal effects or may have an impact on the general fitness of surviving fish. Heart beats should be counted in embryos approx. 48 hpf. After this time, the embryos begin hatching and can move in wider ranges in the test vessels. Counting heart beat for 30 seconds with a magnification of approx. factor 80 then becomes extremely laborious or impossible. Earlier assessment of heart beat rates may be possible, but visibility of the heart and its contraction movement is best in embryos aged 40 hpf and older. Provided a microscope with the appropriate magnification is available, assessment of heart beat rates can be integrated into studies on fish embryo toxicity and may lead to increased sensitivity of the test. Nevertheless, the proceeding development of the embryo and its potential effect on the heart beat rate has to be considered, requiring speedily assessment and alternating observation of embryos of all treatments to avoid a time-related bias on the results.
If the integration of heart beat rate into a standard test procedure is not desired, a modification to the existing test guideline (OECD 2013) is suggested. In the OECD test guideline, one criterion for lethal effects is the presence of heartbeat: If no heartbeat was observed during an observation time of one minute, the embryo is considered dead. This is a very pragmatic approach, since mean heartbeat rates in control zebrafish in the tests performed are in the range of approx. 160 beat per minute. Obviously, the criterion for lethality should be modified. Based on the limited data set obtained in the present project further research may be necessary.

However, it seems advisable to reduce the currently described time period of 1 minute (OECD 2013) to 10 seconds, i.e. the embryo is considered dead if no heartbeat occurs during 10 seconds of observation. In cases of strong effects of a test substance on the embryos, this could greatly reduce the time required for effect assessment.

Determination of the number of somites did not reveal significant effects of any of the investigated substances. While this is no proof of the unsuitability of this observation based on the limited number of tests performed, the counting of somites was very time intensive and laborious. Somites were counted in embryos aged 24 hpf and despite their early developmental stage, the organisms moved considerable strong in all tests (known as spontaneous movement, whose presence was assessed as a separate endpoint). This movements impeded the counting of the approx. 24 somites per embryo extremely since magnification had to be considerable high (at least factor 80) and the depth of field was too narrow to keep the respective area of the embryo in the visual focus. Thus, the embryo had to re-focused in these cases and counting needed to be started again. As mentioned for heart beat rate, the development of the embryo was proceeding while observations were made and any delay led to increasing variance between the embryos. Thus, of the sublethal effects investigated in the tests discussed here, the determination of the number of somites is the least to be recommend for integration into a standard test.

Hence, integration of sublethal effects into the FET are technically suitable based on the current results. Scoring by severity, however, is not recommended, because such a scoring is extremely difficult to standardise and did not provide any added value. As discussed above in relation to apical standard endpoints, however, the results of the present study do not provide evidence that adding sublethal endpoints in the FET would render this test more sensitive than a Fish Early Life Stage test. In fact, for most substances the sublethal endpoints in the FET were clearly less sensitive than the apical standard endpoints, which does not support usage of a FET with sublethal endpoints as a simple substitute for a Fish Early Life Stage study.

8 General discussion

The goal of the study was to determine if the EMA guideline on the environmental risk assessment of medicinal products for human use (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2) (European Medicines Agency 2006) cover the most sensitive test species and endpoints in order to not underestimate the environmental risk of possibly highly potent new-generation pharmaceuticals. The specificity of recently developed pharmaceuticals (either first-in-class, if the substance class was recently discovered, or a new generation of an older substance class) leads to higher potency of active ingredients and thus potentially to effects in non-target organisms even at very low concentrations.

The required tests for the phase II environmental risk assessment for the aquatic compartment include the determination and evaluation of chronic effects in the following studies

- Algae growth inhibition test (OECD 201) (OECD 2011)
- Reproduction test with Daphnia magna (OECD 211) (OECD 2012)
- Fish Early Life Stage toxicity test (OECD 210) (OECD 2013)

Apical endpoints may not cover all effects relevant for the respective active ingredient, e.g. if pharmaceuticals act via a specific mechanism of action or if sensitive organism groups are not covered by the studies required in the standard test strategy.

During the previously performed literature study (FKZ 3718 65 420 1), a possible testing strategy was developed, which was tested in this project. The proposed testing strategy included the determination of the MoA of the substance, a SeqAPASS analysis to determine the most sensitive species based on sequence homology of the target molecule, the performance of the comet assay with environmentally relevant organisms (*Daphnia sp.*) or fish cell lines, and studies with additional species like *Lemna sp*., (whose fast growth by division might increase their sensitivity compared to other macrophytes for example to oncologically active substances) and embryos of the zebrafish *Danio rerio* also considering sublethal endpoints.

It was agreed to use the above mentioned three test systems with substances with oncological or cardiological MoA and statins. After the performance of the studies, the applicability of the new test strategy should be either verified or falsified. In any case, a plausible explanation why such an adopted test strategy could be omitted, should be given.

The selection of the test substances was based on the MoA of the respective substance classes., The choice of suitable substances comprises those that were expected to cause effects in the test organisms as well as substances from a negative control group that were expected to cause no effects.

The first hypothesis of the present project stated that the positive model substances and the test substances from the specific MoA group would induce specific effects in the respective selected test systems. This was confirmed in the FET for both positive model substances: propranolol influenced heart beat rate and cyclophosphamide induced teratogenic effects. The hypothesis was not confirmed for the test substances since those from the group ‘cardiology’ did not all influence heart beat rate and those from the group ‘oncology’ did not all induce teratogenic effects. For *Lemna sp.,* the three selected statins (atorvastatin, pitavastatin, rosuvastatin) were suspected to result in strong effects and in the experimental phase these expectations were confirmed (Table 24). Whether this finding indeed relate to the MoA as HMG-CoA reductase inhibitor, i.e., indicates specific effects related to the pharmacological MoA, was confirmed by a recent study, which investigated the MoA of atorvastatin in transcriptome analyses (Loll et al.

2022). No EPAR data was available for the three statins, but data from a material safety data sheet could be used for atorvastatin and data from the literature for rosuvastatin.

The second hypothesis (effects at lower concentrations than in standard test systems required in the ERA) was clearly refuted for the FET. For none of the test substances, any endpoint in the FET was lower than the standard endpoint given in the EPAR of the respective API. For 6 out of 10 APIs for which this comparison was possible (atorvastatin, rosuvastatin, dabrafenib, palbociclib, pemetrexet, imatinib; compare Table 25), at least one endpoint derived with Lemna was lower than the endpoints provided in the EPARs of the respective API.

Lemna sp. is specifically sensitive to the dihydrofolate reductase inhibitors pemetrexet and methotrexat, which were both no APIs approved after 2006, but were proven to result in strong adverse effects in the above-mentioned test system. Even though data available for pemetrexet (compare www.fass.se) also demonstrated general chronic toxicity to algae, daphnids, and fish, effects in Lemna sp. showed that Lemna sp. was a factor of 25x more sensitive compared to the OECD TG 201, and a factor of 7.5x more sensitive compared to the most sensitive endpoint so far, assessed in a *Daphnia magna* reproduction assay (OECD TG 211).

Methotrexat, the first-in-class API, was furthermore much more potent compared to pemetrexet (factor >300). As both substances display only minimal differences in their structure, it was suspected that the sensitivity was equal between these APIs. As even small differences in the structure could however result in different potencies, i.e. with respect to binding potential to a receptor or to enzyme inhibition, the effect could be more pronounced for one substance compared to a similar substance. Comparable observations were also made for other pairs of APIs with similar structure, e.g. imatinib and afatinib, however only with a factor of 2 (afatinib more effective than the first-in-class API imatinib). This is a common problem in ecotoxicology, as the effect size of substances with similar MoA in different species is difficult to predict, and also QSARs could only give hints on hazards rather on risks. Given the unavailability of EPAR data for methotrexat, however, it cannot be concluded whether an assessment following the EMA guideline would be as protective as an assessment that includes the *Lemna* endpoint derived in the present study.

The concern that APIs from newer generations would show higher ecotoxicity than those from older generations (third hypothesis) was not confirmed, but also not clearly refuted. The newer generation antineoplastics did show higher toxicity in the FET than the first-in-class API imatinib. The same was true for the Lemna test system and the Comet assay.

Recently approved first-in-class pharmaceuticals with specific MoAs were for example the Xa-inhibitor edoxaban and the microtubule inhibitor Paclitaxel, which were both marketed after 2006. For both substances, second substances were chosen in order to proof the sensitivity of the test systems to specific MoAs, which were rivaroxaban as an API of newer generation compared to edoxaban, and cabazitaxel compared to paclitaxel. Furthermore, as in both cases the first-in-class pharmaceutical was included, it was possible to verify or falsify the third hypothesis.

As the factor Xa inhibitors edoxaban and rivaroxaban were chosen as negative controls for Lemna studies, as the MoA was suspected to be not evident in plants, the hypothesis could not be tested in this case. These substances were initially chosen based on a suspected effect in the FET, which was however not observed.

The microtubule inhibitors paclitaxel and cabazitaxel were suspected to have effects on actively dividing organisms, i.e. *Lemna sp.*. An effect was indeed observed for cabazitaxel, while no effect was observed for paclitaxel, the first-in-class. Even though the *Lemna* studies were not explicitly

sensitive to this MoA, in this case the API of the newer generation was at least more effective than the first-in-class.

For the statins, no EPARs are available but for atorvastatin data from a material safety data sheet was available indicating that Lemna sp was more sensitive to the statin than the standard test organisms (here: *Daphnia magna* NOEC: 0.2 mg/L) varying by a factor 3 to 15 in dependence of the species used in the test *Lemna minor* or *Lemna gibba* respectively. The differences are also related to the pH of the test medium which is much higher in the test with *Lemna minor* (Steinberg medium, pH 7.5) than in the test with *Lemna gibba* (20 x AAP, pH 5.5). For rosuvastatin, literature data for algae are available, which indicate a comparably low sensitivity of algae (330 mg/L-350 mg/L; studies performed in accordance to the FDA Technical Assistance Document 4.01). It was however shown that rosuvastatin resulted in relatively high chronic toxicity to *Daphnia magna*, with a NOEC after 21 days of 18 µg/L (study performed according to FDA Technical Assistance Document 4.09). In *Lemna sp*., the most sensitive endpoint (front area) resulted in a NOEC of 6 µg/L for rosuvastatin in the non-GLP study with *L. gibba* and a NOEC of 17 µg/L (frond area) and 5 µg/L (frond number) in the GLP study with *L. minor*. Thus, the test system seems to be very sensitive to the substance class of statins, with a difference of around factor 4 related to the most sensitive endpoint of standard test organisms (here daphnia) and a much higher sensitivity compared to the standard aquatic primary producer (algae).

It was furthermore suspected that *Lemna sp.* as actively dividing organisms react sensitive to oncologically active substances. Indeed, the results confirm this hypothesis in most cases. However, *Lemna sp*. was not consistently more sensitive than algae. Thus, the sensitivity might be additionally dependent on the time of exposure, which for example was postulated as a potential reason for the increased sensitivity of *Lemna sp*. to methotrexat (Białk-Bielińska et al. 2017). Other oncologically active substances with different MoAs, which showed lower toxicity to algae compared to *Lemna sp*. were afatinib, imatinib and dabrafenib, while cabazitaxel, abemaciclib and palbociclib were less toxic in *Lemna sp*. than to green algae.

The comet assay was performed to figure out if this test system could provide more sensitive data for testing oncologically active substances, as these might exert effect on DNA integrity. Therefore, we investigated the antineoplastic substance cyclophosphamide as positive control, and several TKIs.

Correct functioning of the test system was demonstrated, as strand breaks were observed for the positive control H_2O_2 for the comet assays with both cell types and for cyclophosphamide with the ZF-L cell line. It was observed that the comet assay was less sensitive for the investigated pharmaceuticals compared to test systems already included in ERA of pharmaceuticals, and most of the test substances did not result in any effect in the assay. This is probably due to the circumstances that the pharmaceuticals tested in this approach do not possess a genotoxic MoA. Interestingly, exposure of daphnids to pitavastatin resulted in effects in the corresponding comet assay. This was likely due to the suspected increased sensitivity of this species to statins based on the MoA of HMG-CoA reductase inhibition, which is also known to be a sensitive pathway in daphnids, resulting in effects on moulting and reproduction (Miyakawa et al. 2018, Miura 2019), endpoints which likely results in effects in Daphnia reproduction studies. As the substance has however not genotoxic potential, the observed comets might be a secondary effect.

However, the comet assays performed in the course of this project were not sufficient to allow any assumptions with respect to the validity of the test system in the context of environmental

risk assessment of human pharmaceuticals. This was caused by restricted flexibility in substance selection, as the substances selected for the other test systems included a limited number of MoAs excluding genotoxic substances, as the oncologicals were mainly genostatic. Genostatic substances are not known to induce DNA strand breaks and should thus not result in positive comet effects. As next steps, a substance choice based on available data generated by application of the OECD TG 489 should be performed, in order to define positive controls and to demonstrate the suitablility of the test system in an environmental context.

Finally, it was investigated whether the assessment of sublethal endpoints in the FET results in lower sensitivities than those determined during Fish Early Life Stage toxicity test according to OECD TG 210 (OECD 2013), which are performed during the pharmaceutical ERA.

Only some of the tested substances caused in effects in the FET. None of the selected test substances resulted in effective concentrations lower than those provided in the EPARs for FELS test. Hence, the FET amended with sublethal endpoints appears not as a more sensitive method than the standard Fish Early Life Stage test required in the EMA guideline. Even though this project did not suggest that sublethal endpoints assessed in the FET are more sensitive than chronic effects in the FELS for pharmaceuticals, it cannot be ruled out that this is also true for other chemicals tested in another regulatory context, and it might be worth investigating if the test systems applied in this study should be used for other chemicals.

To conclude, from the three test systems applied during the current project, the *Lemna sp*. growth inhibition test according to OECD TG 221 (OECD 2006) is a test system with a potential to be integrated as additional aquatic effect test into the ERA of specific classes of human pharmaceuticals. For example, it is sensitive to substances which act on MoAs which are conserved during evolution, like the HMG-CoA pathway. Furthermore, substances affecting actively dividing organisms result in low effective concentrations, and the most sensitive species can be missed if the guideline only considers those species already included. Also, by comparing the determined NOEC to all endpoints provided in the EPARs, studies according to OECD TG 221 (OECD 2006) in most of the test substances resulted in the effects which were either equally sensitive to or more sensitive to the most sensitive endpoint presented in the EPAR (if available). An exception to this was, for example, cabazitaxel for which the lowest EPAR endpoint was 17-fold lower than the NOEC derived with *Lemna sp.*.

Growth inhibition in *Lemna sp*. was a more sensitive endpoint than the standard endpoints in the EPARs for two substances (atorvastatin, rosuvastatin) with a pharmacological MoAs relating to the mevalonate pathway (which is conserved in plants) one kinase inhibitor (dabrafenib) and two dihydrofolate reductase inhibitors (pemetrexed, methotrexate). Given that the difference to the NOECs provided in the EPARs is often less than 10-fold, the higher sensitivity likely reflects normal variation in species-specific sensitivity. Independently of the underlying reasons, the Lemna growth inhibition test provides relevant additional endpoints for the ERA of at least some APIs as it represents a so far not considered species group (aquatic macrophytes).

The project raises several questions, which could be addressed in a follow-up project. Of special interest would be the following:

Lemna sp. displays specific sensitivity to the statins (HMG-CoA reductase inhibitors) and to methotrexat and pemetrexed (dihydrofolate reductase inhibitors). However, for most of the substances no EPARs but other information (e.g. FASS data or material safety data sheets) on effect levels were available. Thus, it could not be concluded if the sensitivity in *Lemna sp*. is substantially lower compared to the standard endpoints assessed during ERA. The performance of additional standard studies according to OECD TG 201 and 211 (210 exempted due to animal

welfare reasons) with the above-mentioned substances would add additional value on interpretation of the results obtained in this study.

The project so far focussed on substances approved after 2006. In order to proof the hypothesis that the sensitivity of Lemna sp. is dependent on specific MoAs, like HMG-CoA reductase inhibition or dihydrofolate reductase inhibition, also legacy pharmaceuticals approved before 2006 should be tested. This would furthermore allow predictions if the current guideline still provides appropriate environmental safety or if specific substance classes have to be re-tested with the Lemna sp. growth inhibition test.

As the project demonstrated that specific MoAs might generate the need for a tailored risk assessment, a comparison of signaling pathways in humans (addressed by specific pharmaceutical classes) to those in ecotoxicologically relevant test organisms could be performed. As shown for the similarity with respect to the HMG-CoA reductase, this could also be proven true for other MoAs, which are not yet investigated. Additionally, HMG-CoA reductase inhibition could also be important in other organisms, which are not yet subject of testing and which might act as sensitive as Lemna sp..

Furthermore, in order to verify that the effects are based on the specific MoA of the substances, transcriptomic analyses as performed in Loll et al., 2022 for atorvastatin in *Lemna minor* would add information on the specific genomic responses in the test organisms.

Taken together, the Lemna sp. growth inhibition test is a relevant additional test for the ERA of at least some APIs – dihydrofolate reductase inhibitors, statins and BRAF serine threonine kinase inhibitors, like dabrafenib.

Table 24: Sensitivity of the three test systems to the test substances based on the different MoAs.

Substance	Mode of Action	Observed effect Lemna	Lemna vs. standard endpoints	Observed effect Comet assay	Observed effect FET
Edoxaban tosylate hydrate	Faktor Xa inhibitor; anti-coagulant	-	No effect expected	-	-
Rivaroxaban	Faktor Xa inhibitor; anti-coagulant	-	No effect expected	No study performed	-
Atorvastatin calcium	HMG-CoA reductase inhibitor	+	More sensitive	No study performed	No study performed
Pitavastatin calcium	HMG-CoA reductase inhibitor	+	No data on standard endpoints	+/-	No study performed
Rosuvastatin calcium	HMG-CoA reductase inhibitor	+	More sensitive	-	+
Propranolol hydrochloride	ß-blocker	No study performed	No study performed	No study performed	+
Dabrafenib mesylate	BRAF-Serin-Threonin Kinase inhibitor	+	More sensitive	-	No study performed
Abemaciclib	CDK4/CDK6; kinase inhibitor	+	Less sensitive	-	No study performed
Palbociclib	CDK4/CDK6; kinase inhibitor	+	More sensitive	-	+
Ribociclib succinate	CDK4/CDK6; kinase inhibitor	+	Less sensitive	-	No study performed
Methotrexat	Dihydrofolate reductase inhibitor	+	No data on standard endpoints	No study performed	No study performed
Pemetrexed disodium heptahydrate	Dihydrofolate reductase inhibitor; Thymidylat	+	More sensitive	No study performed	No study performed

Substance	Mode of Action	Observed effect Lemna	Lemna vs. standard endpoints	Observed effect Comet assay	Observed effect FET
	synthase Inhibitor; Glycinamid ribonucleotide formyl transferase inhibitor				
Cabazitaxel	Mikrotubule inhibitor (Beta-Tubulin)	+	Less sensitive	No study performed	No study performed
Paclitaxel	Mikrotubule inhibitor (Beta-Tubulin)	-	Less sensitive	No study performed	No study performed
Imatinib mesylate	Thyrosin-kinase inhibitor	+	More sensitive	+/-	-
Afatinib dimaleate	Thyrosin-kinase inhibitor erbB-2	+	Less sensitive	No study performed	-
Neratinib maleate	Thyrosin-kinase inhibitor	-	Less sensitive	No study performed	No study performed
Cyclophosphamide monohydrate	Alkylating antineoplastic	No study performed	No study performed	+	+

Legend: : + = effect observed; - = no effect observed; +/- effect only in one of the two test systems

Table 25: Comparison of standard endpoints to the most sensitive endpoint assessed in the OECD TG 221.

Substance	MoA	OECD TG 221 NOEC	OECD TG 201 EC_{10}/NOEC	OECD TG 211 NOEC	OECD TG 210 NOEC
Atorvastatin calcium	HMG-CoA reductase inhibitor	**0.013 mg/L**	14 mg/L	0.2 mg/L	0.49 mg/L
Pitavastatin calcium	HMG-CoA reductase inhibitor	**0.034 mg/L**	Not available	Not available	Not available
Rosuvastatin calcium	HMG-CoA reductase inhibitor	**0.005 mg/L**	330 mg/L	0.018 mg/L	1.0 mg/L
Dabrafenib mesylate	BRAF-Serin-Threonin kinase inhibitor	**0.008 mg/L**	0.22 mg/L	0.105 mg/L	1.47 mg/L
Abemaciclib	CDK4/CDK6; kinase inhibitor	0.056 mg/L	**0.0059 mg/L**	0.02 mg/L	0.075 mg/L
Palbociclib	CDK4/CDK6; kinase inhibitor	0.220	0.90 mg/L	0.27 mg/L	0.13 mg/L
Ribociclib succinate	CDK4/CDK6; kinase inhibitor	1.11 mg/L	**0.71 mg/L**	1.4 mg/L	1.0 mg/L
Methotrexat	Dihydrofolate reductase inhibitor	**<0.0005 mg/L 0.002 mg/L (ErC_{50})**	10 mg/L (ErC_{50})	Not available	Not available
Pemetrexed disodium heptahydrate	Dihydrofolate reductase inhibitor;	**0.159 mg/L**	4 mg/L	1.2 mg/L	13 mg/L
Cabazitaxel	Mikrotubule inhibitor (Beta-Tubulin)	0.153 mg/L	0.064 mg/L	**0.0089 mg/L**	0.11 mg/L
Imatinib mesylate	Thyrosin-kinase Inhibitor (TKI)	**0.794 mg/L**	0.96 mg/L	≥ 5.6 mg/L	≥ 10 mg/L
Afatinib dimaleate	TKI erbB-2	<0.334 mg/L	1.2 mg/L	2.7 mg/L	**0.032 mg/L**

Remark: Only substances with effects in the *Lemna sp.* studies are presented.

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A Appendix

A.1 List of test substances

Table 26: List of test substances, ordered according to the mode of action, and additional information on their physico-chemical properties and the available EPAR data.

Substance name	CAS number	MoA category	MoA	Lemna	FET	Comet	Solubility	logPow	Source of information
Edoxaban tosylate hydrate	1229194-11-9	Cardiology	Faktor Xa inhibitor; anti-coagulant	y	y	y	0.0114 mg/ml	1.7 bei pH 8	EPAR
Rivaroxaban	366789-02-8	Cardiology	Faktor Xa inhibitor; anti-coagulant	y	y	y	No data	1.5	EPAR
Atorvastatin calcium	134523-03-8	Cardiology	HMG-CoA reductase inhibitor	y	n	n	No data	No data	-
Pitavastatin calcium	147526-32-7	Cardiology	HMG-CoA reductase inhibitor	y	n	y	No data	No data	-
Rosuvastatin calcium	147098-20-2	Cardiology	HMG-CoA reductase inhibitor	y	y	y	No data	<4 (pH7)	data in Vestel et al Gunnarsson

Substance name	CAS number	MoA category	MoA	Lemna	FET	Comet	Solubility	logPow	Source of information
									et al 2019
Propranolol hydrochloride	318-98-9	Cardiology	ß-blocker	n	y	n	No data	No data	data in Gunnarsson et al 2019
Dabrafenib mesylate	1195768-06-9	Oncology; cytostatics	BRAF-Serin-Threonin kinase inhibitor	y	n	y	No data	log Dow: 3.384 at pH=7	EPAR
Abemaciclib	1231929-97-7	Oncology; cytostatics	CDK4/CDK6; kinase inhibitor	y	n	y	No data	log KOW = 3.6 at pH7 (OECD123)	EPAR
Palbociclib	571190-30-2	Oncology; cytostatics	CDK4/CDK6; kinase inhibitor	y	y	y	10 mg/ml (isethionate form; PubChem)	LogD (pH7) = 1.11 (OECD 107)	EPAR

Substance name	CAS number	MoA category	MoA	Lemna	FET	Comet	Solubility	logPow	Source of information
Ribociclib succinate	1374639-75-4	Oncology; cytostatics	CDK4/CDK6; kinase inhibitor	y	n	y	No data	log D at pH 7 = 0.6 (OECD 107)	EPAR
Methotrexat	59-05-2	Oncology; cytostatics	Dihydrofolate reductase inhibitior	y	n	n	No data	No data	-
Pemetrexed disodium heptahydrate	357166-29-1	Oncology; cytostatics	Dihydrofolate reductase inhibitior Thymidylat synthase Inhibitor Glycinamid ribonucleotide formyl transferase inhibitor	y	n	n	No data	No data	-
Cabazitaxel	183133-96-2	Oncology; cytostatics	Mikrotubule inhibitor (Beta-Tubulin)	y	n	n	praktisch unlöslich	log Pow: 3,3 (25 °C)	data in Gunnarsson et al 2019
Paclitaxel	33069-62-4	Oncology; cytostatics	Mikrotubule inhibitor (Beta-Tubulin)	y	n	n	No data	No data	-

Substance name	CAS number	MoA category	MoA	Lemna	FET	Comet	Solubility	logPow	Source of information
Imatinib mesylate	220127-57-1	Oncology; cytostatics	Thyrosin-kinase inhibitor	y	y	y	No data	log Kow = 3.5 (OECD 107)	EPAR
Afatinib dimaleate	850140-73-7	Oncology; cytostatics	TKI erbB-2	y	y	n	No data	log D (pH7.4) = 3.8	EPAR
Neratinib maleate	915942-22-2	Oncology; cytostatics	TKI	y	n	n	No data	Log Pow at pH 6 = 2.98 ± 0.02; Log Pow at pH 8 = 4.41 ± 0.07 (OECD 107)	EPAR
Cyclo-phosphamide monohydrate	6055-19-2	Oncology; cytotoxics	Alkylating antineoplastic	n	y	y	40mg/ml	No data	No data

A.2 Lemna growth inhibition test

Table 27: Final test concentrations and measurement parameters for the GLP main tests.

Substance name	GLP-Code	Species	Test media	Nominal concentrations (mg a.s./L)	Measurement parameter	Comment
Paclitaxel Neratinib	-	*L. gibba*	20xAAP	-	-	Static test design. A pre-test if solubility is sufficient was performed. There was only a very low solubility of the test item. Since the solubility is low and no effects could be observed, it was decided that no further testing is necessary.
Palbociclib	IME-035/4-11/J	*L. gibba*	20xAAP	1.00, 3.17, 10.0, 31.6, 100 mg/L (Factor: 3.16)	Frond number, frond area, dry weight	Static test design.
Afatinib	IME-037/4-11/J	*L. gibba*	20xAAP	0.938, 1.88, 3.75, 7.50, 15.0 mg/L (Factor: 2)	Frond number, frond area, dry weight	Static test design.
Imatinib	IME-038/4-11/J	*L. gibba*	20xAAP	2.00, 6.00, 18.0, 54.0, 162 mg/L (Factor: 3)	Frond number, frond area, dry weight	Static test design.
Dabrafenib	IME-040/4-11/J	*L. gibba*	20xAAP	0.200, 0.600, 1.80, 5.40, 16.2 mg/L (Factor: 3)	Frond number, frond area	Static test design.
Ribociclib	IME-036/4-11/J	*L. gibba*	20xAAP	0.500, 1.58, 4.99, 15.8, 49.9 mg/L (Factor: 3.16)	Frond number, frond area, dry weight	Static test design.
Abemaciclib	IME-034/4-11/J	*L. gibba*	20xAAP	0.251, 0.792, 2.50, 7.91, 25.0 mg/L (Factor: 3.16)	Frond number, frond area, dry weight	Static test design.
Rivaroxaban Edoxaban	-	*L. gibba*	20xAAP	-	-	Static test design.

Substance name	GLP-Code	Species	Test media	Nominal concentrations (mg a.s./L)	Measurement parameter	Comment
						Negative control: No effects were observed and therefore the negative control was verified. No main test was performed. The range finder results will be reported.
Cabazitaxel	IME-030/4-11/J	*L. gibba*	20xAAP	0.15, 0.47, 1.50, 4.77, 15.0 mg/L (Factor: 3.16)	Frond number, frond area	Static test design.
Atorvastatin	2020-029 (Non-GLP)	*L. gibba*	20xAAP	0.003, 0.008, 0.025, 0.079, 0.249 mg/L (Factor 3.16)	Frond number, frond area	The range finding test was performed in a static test design. However, the analytical results of the main test with L. minor revealed a fast decrease of the test concentrations. Therefore, it was decided to perform further tests with **two media renewals**.
Rosuvastatin	2020-029 (Non-GLP)	*L. gibba*	20xAAP	0.006, 0.019, 0.056, 0.167, 0.500 mg/L (Factor 3)	Frond number, frond area	Static test design.
Atorvastatin	IME-025/4-11/I	*L. minor*	Steinberg	0.030, 0.095, 0.300, 0.974 and 2.99 mg/L (Factor 3.16)	Frond number, frond area	The 1st static main test was performed and fulfilled the validity criteria. However, the chemical analysis revealed that after 7 days most measured concentrations were below the LOQ of 1 µg a.s./L.

Substance name	GLP-Code	Species	Test media	Nominal concentrations (mg a.s./L)	Measurement parameter	Comment
						Therefore, it was concluded that the test has to be repeated in a semi-static test design (see IME-025/4-11/I/a
Atorvastatin	IME-025/4-11/I/a	*L. minor*	Steinberg	0,0025, 0,0079, 0,0251, 0,079, 0,252, 0,797 mg/L (Factor 3.16)	Frond number, frond area	Test will be performed in a semi-static test design with two times media renewal.
Pitavastatin	IME-026/4-11/I	*L. minor*	Steinberg	0.030, 0.069, 0.159, 0.365, 0.840 mg/L (Factor: 2.3)	Frond number, frond area	The range finding test was performed in a static test design. However, the analytical results of the analytical pre-test with L. minor revealed a fast decrease of the test concentrations (Table 29). Therefore, it was decided to perform further tests with **one media renewal.**
Rosuvastatin	IME-027/4-11/I	*L. minor*	Steinberg	0.006, 0.019, 0.056, 0.167, 0.500 mg/L (Factor 3)	Frond number, frond area	Static test design.
Pemetrexed	IME-028/4-11/I	*L. minor*	Steinberg	0.185, 0.556, 1.67, 5.00, 15.0 mg/L (Factor: 3)	Frond number, frond area	-
Methotrexate	IME-029/4-11/I	*L. minor*	Steinberg	0.001, 0.003, 0.010, 0.032, 0.100 mg/L (Factor 3.16)	Frond number, frond area	The range finding test was performed in a static test design. However, the analytical results of the pre-test with L. minor revealed a fast decrease of the test

Substance name	GLP-Code	Species	Test media	Nominal concentrations (mg a.s./L)	Measurement parameter	Comment
						concentrations (Table 29). Therefore, it was decided to perform further tests with **one media renewal.**

Table 28: Summary of the test on solubility for Paclitaxel and Neratinib.

Test substance	Date	Sample	Nominal conc. [µg a.s./L]	Measured conc. [µg a.s./L]	% of nominal [%]
Neratinib	Day 0	A	10000	325	3.25
		B	10000	335	3.35
	Day 7	Plant	10000	8.22	0.08
		No plant	10000	20.4	0.20
Paclitaxel	Day 0	A	10000	286	0.29
		B	10000	302	0.30
	Day 7	Plant	10000	26.8	0.03
		No plant	10000	28.4	0.03

Table 29: Summary of the test on stability of Pitavastatin, Pemetrexed and Methotrexat.

Test substance	Date	Peak area	% of nominal [%]
Pitavastatin	Day 0	13174	100
	Day 3	2606	19.8
	Day 7	1029	7.8
Pemetrexed	Day 0	272075	100
	Day 3	217705	80.0
	Day 7	182007	66.9
Methotrexat	Day 0	395876	100
	Day 3	306858	77.5
	Day 7	37904	9.6

Table 30: Summary of all range finding tests performed with *Lemna minor*/*Lemna gibba*.

Substance name	GLP-Code	Species	Test media	Nominal Concentration (mg a.s./L)	Frond number		Frond area		Dry weight	
					ErC50	ErC10	ErC50	ErC10	ErC50	ErC10
Atorvastatin	2020-029	*L. gibba*	20xAAP	0.1/1/10	0.310	0.064	0.306	0.061	44.60	0.026
Rosuvastatin	2020-029	*L. gibba*	20xAAP	0.01/0.1/1/10	0.042	0.010	0.051	0.005	n.d.	0.004
Atorvastatin[1]	IME-025/4-11/I	*L. minor*	Steinberg	1/10/100	<1	n.d.	<1	n.d.	<1	n.d.
				0.001/0.01/0.1/1	1.03	0.091	0.654	0.138	3.03	0.124
Pitavastatin	IME-026/4-11/I	*L. minor*	Steinberg	1/10/100	0.396	0.101	0.728	n.d.	160	0.025
Rosuvastatin[1]	IME-027/4-11/I	*L. minor*	Steinberg	1/10/100	<1	n.d.	<1	n.d.	<1	n.d.
				0.001/0.01/0.1/1.00	0.052	0.013	0.046	0.018	2.61	0.009
Pemetrexed	IME-028/4-11/I	*L. minor*	Steinberg	1/10/100	3.8	0.695	3.6	0.328	>100	0.489
Methotrexate[1]	IME-029/4-11/I	*L. minor*	Steinberg	1/10/100	<1	n.d.	<1	n.d.	<1	n.d.
Cabazitaxel	IME-030/4-11/J	*L. gibba*	20xAAP	0.01/0.1/1/10	6.74	1.39	5.36	1.11	80.9	1.92
Paclitaxel	IME-031/4-11/J	*L. gibba*	20xAAP	1/10/100	>100	n.d.	>100	n.d.	>100	n.d.
Edoxaban	IME-032/4-11/J	*L. gibba*	20xAAP	1/10/100	>100	n.d.	>100	n.d.	>100	n.d.
Rivaroxaban	IME-033/4-11/J	*L. gibba*	20xAAP	0.01/0.1/1/10	>10	n.d.	>10	n.d.	>10	n.d.
Abemaciclib	IME-034/4-11/J	*L. gibba*	20xAAP	0.01/0.1/1/10	21.7	0.986	8.46	0.916	20.0	0.619
Palbociclib	IME-035/4-11/J	*L. gibba*	20xAAP	0.1/1/10	30.2	4.12	22.8	3.87	31.7	3.31
Ribociclib	IME-036/4-11/J	*L. gibba*	20xAAP	0.1/1/10	55.0	1.13	22.8	2.33	46.2	1.04

Substance name	GLP-Code	Species	Test media	Nominal Concentration (mg a.s./L)	Frond number		Frond area		Dry weight	
					ErC50	ErC10	ErC50	ErC10	ErC50	ErC10
Afatinib	IME-037/4-11/J	*L. gibba*	20xAAP	0.1/1/10	6.26	1.47	5.1	1.56	5.8	1.4
Imatinib	IME-038/4-11/J	*L. gibba*	20xAAP	1/10/100	152	6.13	77.5	6.76	166	5.9
Neratinib	IME-039/4-11/J	*L. gibba*	20xAAP	0.1/1/10/100	>100	n.d.	>100	n.d.	>100	n.d.
Dabrafenib	IME-040/4-11/J	*L. gibba*	20xAAP	0.1/1/10/100	6.52	0.093	7.07	0.185	>100	1.19

n.d.: not determined due to mathematical reasons or inappropriate data.

[1]: The chosen test concentrations in the range finding tests were not appropriate. Even at the lowest test concentration strong effects on the growth of *Lemna minor* were found. Therefore, the tests were repeated with an adopted concentration range.

A.3 Comet assay

Table 31: Mechanism of action and Genotoxicity data for substances tested with comet assay (*=genotoxicity implied in some studies, **=known genotoxicant)

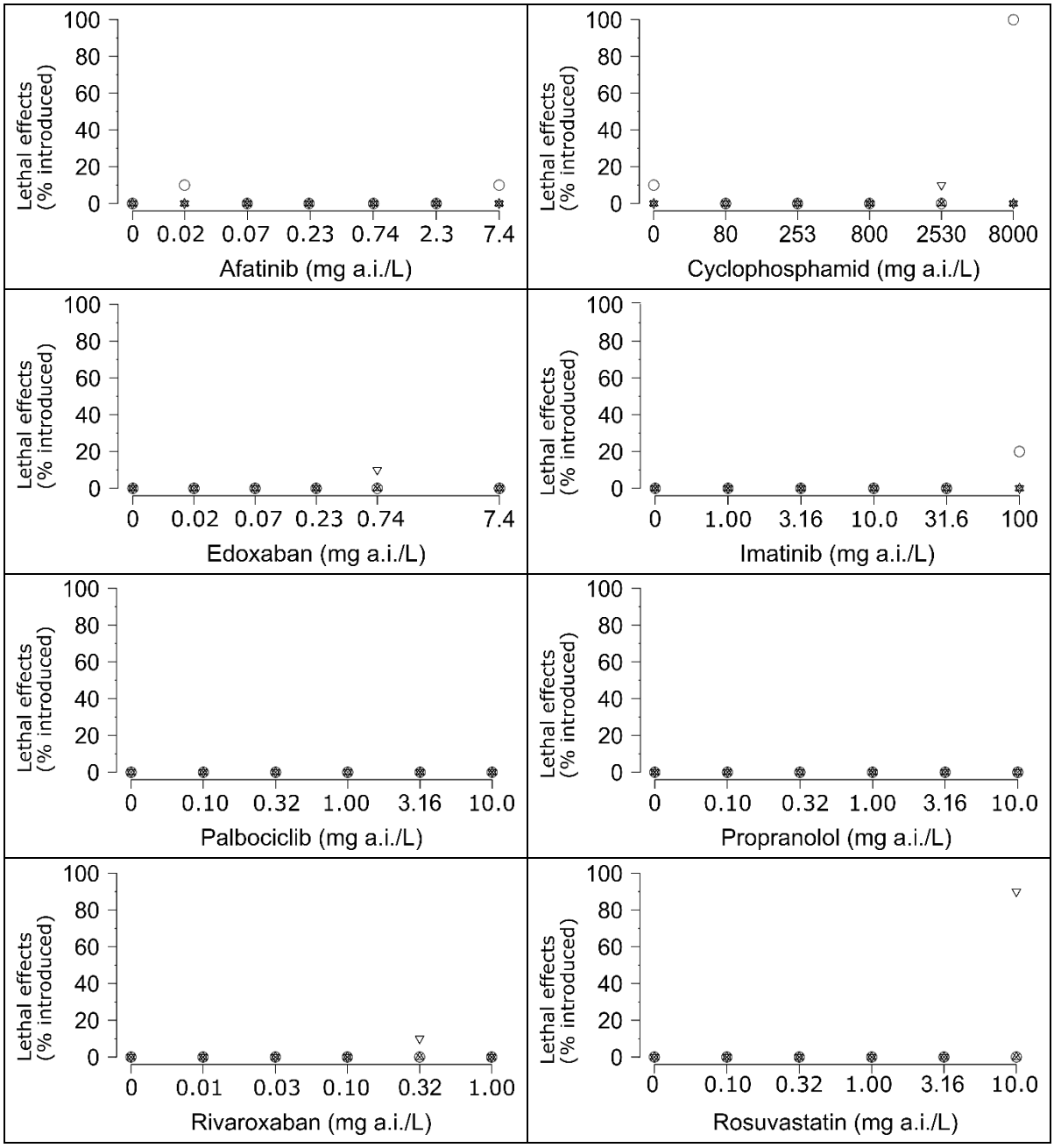
Substance name	Mode of action	Genotoxicity data from pre-clinical tests
Abamaciclib	Cytostatic, CDK4/6 inhibitor	No genotoxicity shown by Ames test, in vitro chromosomal aberration assay and in vivo micronucleus assay (European Medicines Agency 2018)
Cyclophosphamid**	Cytotoxic, alkylating antineoplastic precursor	Genotoxic and mutagen towards somatic and germ-line cells (Baxter Oncology, 2015)
Dabrafenib	Cytostatic, BRAF-Serin-Threonin kinase inhibitor	No genotoxicity shown by Ames test, mouse lymphoma assay and micronucleus assay (European Medicines Agency 2013)
Edoxaban	Anti-Coagulant, factor Xa inhibitor	No genotoxicity shown during pre-clinical testing (European Medicines Agency 2015)
Imatinib*	Cytostatic, Thyrosin-kinase inhibitor	No genotoxicity shown by Ames test, mouse lymphoma assay and in vivo micronucleus assay. Positive genotoxic effects in chromosomal aberration assay. Intermediate products contained in capsules also reacted positively to Ames test and mouse lymphoma assay. (European Medicines Agency 2009)
Palbociclib*	Cytostatic, CDK4/6 inhibitor	No genotoxicity shown by Ames test or in vitro chromosomal aberration assay. Increased micronucleus formation in in vitro and in vivo micronucleus assay in doses above 100 mg/kg/day (European Medicines Agency 2016)
Pitavastatin	Statin, HMG-CoA reductase inhibitor	No genotoxicity shown by Ames test and in vivo mammalian UDS test, micronucleus test and comet assay. Clastogenicity shown in vitro at concentrations of significant cytotoxicity (50%) (Australian Government, Department of Health, 2013)
Ribociclib	Cytostatic, CDK4/6 inhibitor	No genotoxicity shown by bacterial in vitro tests nor mammalian in vitro and in vivo tests (European Medicines Agency 2017)
Rosuvastatin	Statin, HMG-CoA reductase inhibitor	No genotoxicity shown during pre-clinical testing (European Medicines Agency 2005)

A.4 Fish Embryo Toxicity Tests

Table 32: Composition of major elements in the modified reconstituted water used in the fish embryo toxicity tests

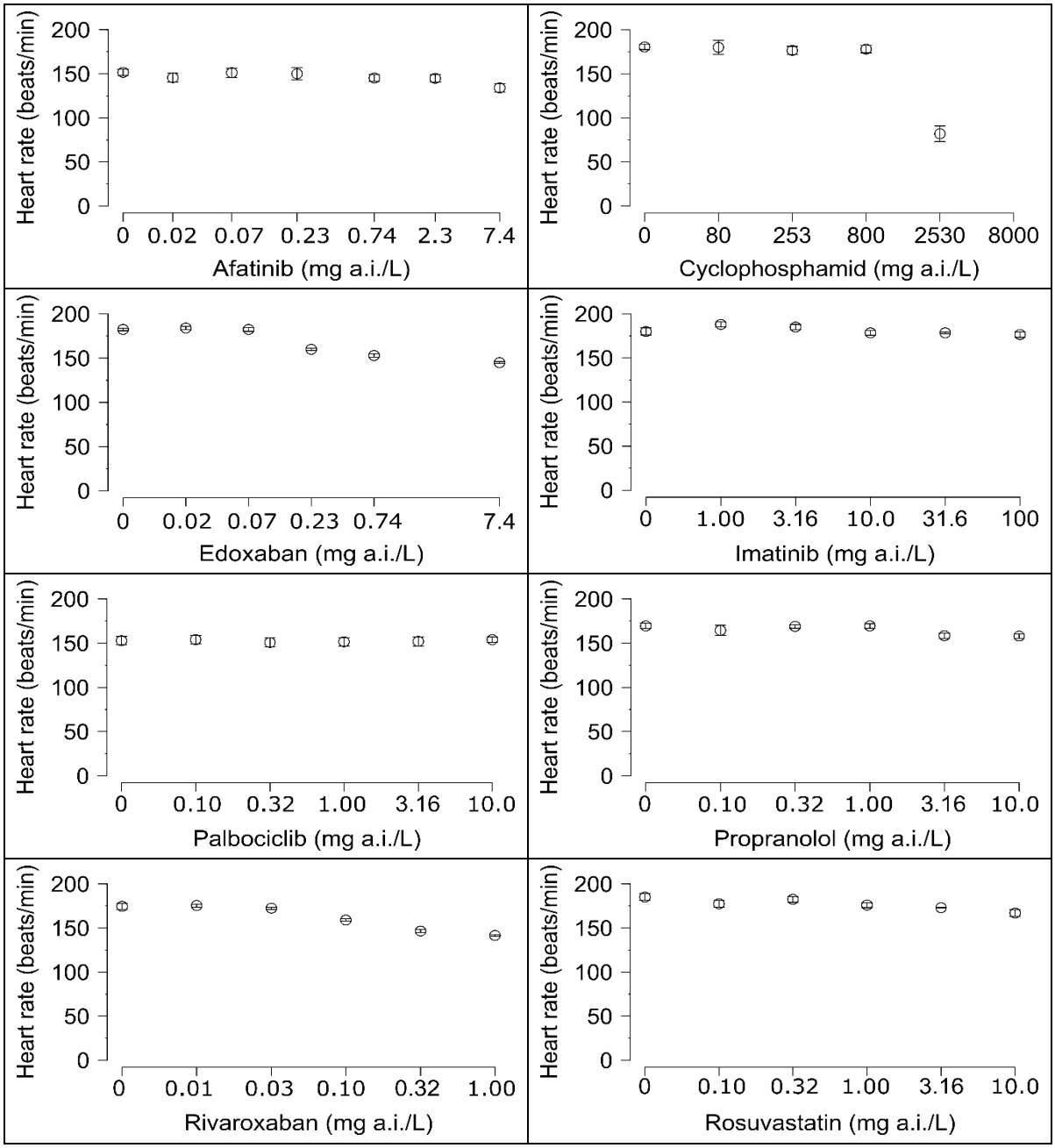
Substance	Concentration [mg/L]
Ca^{2+}	55.7
Mg^{2+}	15.0
Na^{+}	90.2
K^{+}	4.35
Sr^{2+}	0.056
Cl^{-}	189.0
SO_4^{-}	40.2
BO_3	0.169
HCO_3^{-}/CO_3^{-}	24.1

Figure 6: **Lethal effects in the range-finding experiments after 96 h exposure to the respective test items. To determine lethal effects, presence of the following characteristics was assessed: absence of heartbeat (▽), tail not detached from the yolk sac (no occurrence in present tests), coagulation (○) and no formation of somites (no occurrence in present tests).**



Source: Own illustration, ECT

Figure 7: Heartbeat rate in embryos (mean ± sd; n=4 per treatment) after 48 h exposure to the respective test items.



Source: Own illustration, ECT

Figure 8: **Sublethal effects observed in more than 20% of the introduced embryos during 96 h of exposure to the respective test items. Effects were assessed at least after 72 h (△) and 96 h (◇) of exposure.**