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Risk assessment of highly active human pharmaceuticals in the environment: Identification, development and application of a new concept for their identification

von

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16. Kurzfassung

In den letzten Jahren wurden dank verbesserter Analysemethoden eine Vielzahl von Medikamenten im Abwasser, im Oberflächenwasser und Grundwasser im Konzentrationsbereich von Nanogramm bis Mikrogramm pro Liter nachgewiesen. Mögliche Effekte dieser Stoffe auf die Umwelt sind bisher aber noch kaum bekannt.
Seit 2006 ist die neue Richtlinie der European Medicines Agency (EMEA) zur Umweltbeurteilung von neuen Arznei-Mitteln in Kraft. Danach wird ihr Umweltrisiko in einem zweistufigen Verfahren bestimmt. In der ersten Stufe wird die potentielle Umweltkonzentration eines Medikamentes abgeschätzt. Liegt diese unter 0.01 Mikrogramm pro Liter, wird wird das Medikament als unbedenklich für die Umwelt eingestuft. Wenn die Konzentration den Schwellenwert von 10 Nanogramm pro Liter überschreitet, sind experimentelle Studien zum Verbleib und zur Wirkung des Arzneimittels In der Umwelt durchzuführen. Da es jedoch Medikamente wie Ethinylestradiol und Levonorgestrel gibt, die Effekte auf aquatische Organismen unterhalb des Schwellenwertes ausüben, müssen diese identifiziert werden. Bisher gibt es Aber keine Kriterien, anhand derer solche Substanzen identifiziert werden können.
Ziel dieses Vorhaben ist es, solche hochaktiven Substanzen anhand vorhandener Daten und der wissenschaftlichen Literatur zu identifizieren. Des Weiteren wird ein neues Konzept erarbeitet, anhand dessen hochaktive Substanzen identifiziert werden können. Dabei werden Kriterien definiert. Das neue Konzept basiert auf dem Wirkmechanismus der Substanz. Zudem wird der Grad der Homologie zwischen dem Zielrezeptor beim Menschen und dem potentiellen Rezeptor bei Organismen in der Umwelt betrachtet. Ein weiteres Kriterium ist die Bedeutung des durch den beteiligten Rezeptor regulierten Signalwegs in der Zelle und des Stoffwechselwegs. Dabei werden wichtige biologische und physiologische Prozesse definiert. Zudem werden toxikologische Daten mit berücksichtigt. Das neue Konzept wird anhand von Beispielen verifiziert. Schliesslich wird das neue Konzept mit Modellen wie dem Fisch-Plasma-Modell und einem QSAR-Modell verglichen. Die Analyse zeigt, dass mit dem neuen Konzept hochaktive Substanzen erkannt werden können.

17. Schlagwörter
EMEA Guideline. Umweltrisikobeurteilung von Arzneimitteln. Hochaktive Arzneimittel in der Umwelt. Konzept zur Identifikation von hochaktiven Substanzen. Fisch-Plasma-Modell. QSAR-Modell.

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16. Abstract
Due to more sensitive analytical methods, an increasing number of pharmaceuticals are detected in wastewater, surface and ground water at concentrations from nanogram to microgram per litre in the last decade. Possible effects of these compounds in the environment are only marginally unknown. The guideline of the European Medicines Agency (EMEA) for the environmental risk assessment of new pharmaceuticals is in force since 2006. It consists of two steps. In the first step, the potential environmental concentration of the pharmaceutical is estimated. If the concentration is below 10 nanogram per litre, the pharmaceutical is classified as harmless. If the concentration is above this threshold, studies about the fate and effects of the new drug are necessary. However, drugs such as ethinylestradiol and levonorgestrel exist that exhibit effects to aquatic organisms below the threshold. So far, there are no criteria defined, nor concepts to identify such highly active compounds.
The goal of this project is to identify such highly active compound by use of existing data banks and the scientific literature. A concept for the identification of highly active compounds is developed and established. The new concept is based on the mode of action of a compound. In addition, the homology between the human drug target and the potential targets in organism in the environment is taken into account. Another criteria is the impact on the regulated cellular signalling pathways affected by the drug. Consequently, critical biological and physiological processes are defined. Furthermore, toxicological data are considered. The new concept is applied to some pharmaceuticals for verification. Finally the new concept is compared to existing models such as the fish plasma model and a QSAR model. The analysis shows that the new model, highly active compounds can be identified. Future applications will demonstrate its usefulness within the regulatory context.

17. Keywords
EMEA guideline. Environmental risk assessment of pharmaceuticals. Highly active compounds in the Environment. Concept for the identification of highly active compounds. Fish plasma model. QSAR model

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Summary

Over the last decade, traces of pharmaceuticals have been detected in many countries at concentrations in the ng/L to µg/L range in wastewater treatment plant effluents, surface water, seawater and groundwater. The effects of these compounds on the environment are not well investigated so far. Most effects are shown in acute toxicity experiments, but chronic toxicity data are missing for most of the commonly used pharmaceuticals. To estimate the potential environmental risk of human medicinal drugs, the European Medicines Agency (EMEA) issued in 2006 the "guideline on the environmental risk assessment of medicinal products for human use". According to this guideline the potential risk of pharmaceuticals to the environment is estimated by a step-wise procedure. During phase I the predicted environmental concentration (PEC) of a pharmaceutical is assessed in the surface water. If the PEC value is below 0.01 µg/L and no other environmental concerns are apparent, it is assumed that the medicinal product is unlikely to represent a risk for the environment. If the PEC value is equal or above 0.01 µg/L a phase II environmental fate and effect analysis is performed.

The threshold of 0.01 µg/L may be too high for very specific and highly potent pharmaceuticals like hormones, which can have effects on the environment at concentrations below 0.01 µg/L. Some compounds such as 17-α-ethinylestradiol and levonorgestrel display effects at aquatic organisms at concentrations below 10 ng/L. The goal of the project was the identification of such highly potent and highly selective pharmaceuticals and pharmaceuticals groups, which could exhibit a potential effect on the environment at concentrations below the threshold value of 0.01 µg/L. To identify these highly active compound ("HC"), various databases and the scientific literature were used. The ultimate aim of the present project was the development of a concept for the identifcation of such "HC". We developed a concept based on the mode of action of pharmaceuticals to identify compounds with potential ecotoxicological risk at low concentrations. This concept is based on (1) the mode of action of the pharmaceutical taking into consideration of toxicological data, (2) the degree of sequence homology between the human drug target and the potential target in aquatic organisms and (3) on the importance of the pathways affected by the drug. We evaluated the usefulness of this concept to other potential approaches such as the fish plasma model (Huggett et al., 2003) and a QSAR model (VirtualTox Lab). In the fish plasma model the fish plasma concentration of drugs is estimated and compared with the human therapeutic plasma concentration for predicting the potential risk. The QSAR (VirtualTox lab) model is based on the binding affinity of pharmaceuticals to human receptors for risk assessment. All concepts were evaluated using a set of seven pharmaceuticals. Application of the three concepts to the selected pharmaceuticals resulted in most, but not all, cases the same

categorization. The mode of action concept was most promising for identification of compounds of high ecotoxicological risks. Furthermore, the inclusion of toxicological data in the risk assessment provided useful hints in terms of identification of mode of actions of the compounds and its potential potential ecotoxicological risk at low concentrations.

1. Introduction

1.2 Pharmaceuticals in the environment

Pharmaceuticals are a class of emerging environmental contaminants that are extensively and increasingly being used in human and veterinary medicine. The consumption of pharmaceuticals is substantial. In the European Union (EU) about 3000 different substances are used in human medicine. Also a large number of pharmaceuticals are used in veterinary medicine. The main categories of human pharmaceuticals and the most commonly used products are anti-inflammatory drugs/analgesics, antibiotics, lipid regulators, beta blockers, steroids and related hormones (Daughton et al., 1999; Halling-Sorensen et al., 1998). For example, in England, Germany, the United States, Denmark and Australia, the amounts for the most frequently used drugs are in the hundreds of tons per year (Huschek et al., 2004; Jones et al., 2002).

These compounds are designed to have a specific mode of action, and many show long half-lives in the body. Pharmaceuticals usually undergo transformations in the human body, resulting in the release of significant amounts of metabolites. Pharmaceuticals and their metabolites can enter the environment mainly via excretion and disposal in wastewater. Municipal and hospital wastewaters are the most important sources of human pharmaceutical compounds, with contributions also from wastewater, manufacturers and landfill leachates, and from disposal of unused medicines into the environment. Application to fields and subsequent runoff, and direct application in aquaculture, are the main sources of veterinary pharmaceuticals in the aquatic environment (Fent et al., 2006; Johnson et al., 2006; Kay et al., 2005). In wastewater treatment, two elimination processes are generally important: adsorption to suspended solids (sewage sludge) and biodegradation. Adsorption is dependent on both hydrophobic and electrostatic interactions of the pharmaceutical with particles and microorganisms. Biodegradation can occur either in aerobic/anaerobic zones in activated sludge treatment, or anaerobically in sewage sludge digestion. Because of an incomplete elimination in wastewater treatment plants, pharmaceuticals and their metabolites are found in surface waters. The elimination efficiencies of pharmaceuticals span a large

range. Figure 1 shows the elimination rates of some non-steroid-anti-inflammatory drugs (NSAID) as an example.

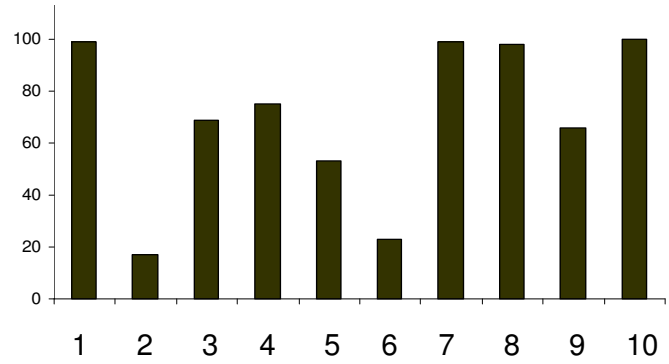

Fig.1: Removal efficiency of several NSAID (percentage) in different wastewater treatment plants. Salicylic acid (1), diclofenac (2-6), ibuprofen (7,8), naproxen (9) and paracetamol. Removal rates are variable, even for the same pharmaceutical between different treatment plants (Fent et al., 2006).

As consequence of incomplete elimination in wastewater treatment plants (and where treatment plants are lacking), pharmaceuticals and their metabolites enter the aquatic environment. The occurrence of pharmaceuticals was first reported in 1976, clofibric acid was detected in treated wastewater in the USA at concentrations from 0.8 to 2 µg/L (Fent et al., 2006). In 1981 the occurrence of pharmaceutical compounds in river waters in the UK was reported (Fent et al., 2006), and in 1986 ibuprofen and naproxen were detected in wastewaters in Canada (Fent et al., 2006). In the meantime pharmaceuticals have been detected in low concentrations in many countries in many environmental samples, for example wastewater treatment plant effluents, surface water, seawater, and groundwater (Daughton et al., 1999; Fent et al., 2006; Halling-Sorensen et al., 1998). The measured concentrations in sewage treatment plant effluents are generally in the range of ng/L to µg/L and in rivers, lakes and seawater in ng/L (Kolpin et al., 2002). In the last few years, knowledge about environmental occurrence of pharmaceuticals has increased to a large extent due to new analytical techniques able to detect polar compound at trace quantities. Since a few years, research about the fate of pharmaceuticals in the environment is increasing. Typical depletion processes in surface waters are: biodegradation in sediments, phototransformation, hydrolysis and binding to sediments (Kolpin et al., 2002, Halling-Sorensen et al., 1998, 2003; Ingerslev et al., 2001). The low volatility of pharmaceutical products indicates that distribution in the environment will occur primarily by aqueous

transport, but also by food-chain dispersal. Once in surface water, biotransformation may occur, but abiotic transformation reactions are also important. Photodegradation can play an important role at the water surface and hydrolysis may occur. There is no or very little information about the bioaccumulation potential of pharmaceuticals in biota and food chains with the exception of diclofenac, which accumulates in the prey of vultures (Oaks et al., 2004).

1.3 Ecotoxicological effects

Pharmaceuticals are designed to target specific metabolic and molecular pathways in humans and animals. When introduced into the environment they may affect the same pathways in vertebrates and invertebrates having identical/similar targets. So far, very little is known about possible counterparts of human target biomolecules of pharmaceuticals in invertebrates. In addition, for many pharmaceuticals the specific mode of action is not well characterized and often not only one but many different modes of action occur. Therefore specific toxicity analysis in lower animals is difficult to perform. Pharmaceuticals are assessed for their long-term toxicity by standard tests according to existing guidelines (for example OECD) using laboratory organisms of different trophic levels such as algae, zooplankton, other invertebrates and fish. Beyond laboratory investigations, some mathematical models were developed to estimate or predict ecotoxicological effects of chemicals. Beside the evaluation of acute effects of pharmaceuticals for aquatic animals, the characterisation of their chronic potential is very important, because many aquatic species are exposed over long periods of time or even for their entire life. But there is a general lack of chronic data and the existing ones do often not investigate the important key targets (which are based on the mode of actions) and they do not address the question in different organisms. Investigations about chronic toxicity over different life stages have only rarely been performed.

Life cycle analyses are not reported so far, except for the synthetic steroid 17α-ethinylestradiol (EE2), contained in contraceptive pills. Oviparous females of fish, reptiles, amphibians and birds secrete the steroid hormone 17β-estradiol from the thecal cells surrounding the ovaries, which in turn migrates through the bloodstream to the liver to induce vitellogenin synthesis. Activation of vitellogenin transcription occurs through transcriptional activation once the estrogenic steroid binds to the nuclear estrogen receptor. Exposure of male fish to EE2 leads to the activation of this pathway therefore vitellogenin transcription can be used as a marker for estrogen exposure. EE2 shows estrogenic effects at extremely

low and environmentally relevant concentrations. The ecotoxicological effects of EE2 were studied in a fish full life-cycle study using fathead minnows (Länge et al., 2001). EE2 had a strong impact on several key health indices, including gross development and growth, gonad development, sex determination and reproductive maturity. The effects of EE2 on development in the early life stages of fathead minnows were most obvious. After 56 days posthatch, the effect of EE2 on larval growth was severe and ovotestis was present with LOEC (lowest-observed-effect concentration) of 4 ng/L and NOEC (no-observed-effect concentration) of 1 ng/L. Fish exposed to 4 ng/L EE2 showed no male secondary sex characteristics at any age. Overall for all the endpoints monitored during the EE2 full life-cycle study, the biological derived NOEC was 1 ng/L (Lange et al., 2001). Induced transcription of vitellogenin mRNA was observed in fathead minnows exposed to concentrations as low as 2 ng/L EE2 for 24 h (Miracle et al., 2006)

1.4 Environmental risk assessment of pharmaceuticals according to the European Medicines Agency (EMEA)

According to the guideline of EMEA (2006) the assessment of the potential risks to the environment is a step-wise procedure, consisting of two phases. In the first phase the exposure of the environment to the drug substance is estimated. By application of the following mathematical formula the PEC of a pharmaceutical in the surface water is calculated during the first phase.

$$\frac{\text{Maximum daily dose consumed per inhabitant} * \text{percentage of market penetration (Fpen)}}{\text{Amount of wastewater per inhabitant per day} * \text{dilution factor}}$$

The default value for Fpen (1 %) should be used unless the Fpen can be refined based on published epidemiological data.

If the $PEC_{surfacewater}$ value is below 0.01 µg/L and no other environmental concerns are apparent, it is assumed that the medicinal product is unlikely to represent a risk for the environment. If the $PEC_{surfacewater}$ value is equal or above 0.01 µg/L, a Phase II environmental fate and effect analysis has to be performed. During this Phase II, the fate of the substance in the sewage treatment plant is investigated by performing ready biodegradability test, and analyzing the sorption behaviour of the substance to sewage sludge and soil, the distribution between octanol and water, and the transformation in water-sediment-systems. Depending on the outcome of the fate tests, further testing in Tier B might be necessary, for example on fate

and effects in soil or on bioaccumulation. The effect on sewage treatment plants is tested in a respiration inhibition test. Standard long-term toxicity tests on fish, Daphnia and algae are performed to predict the no-effect concentration ($PNEC_{water}$). The $PNEC_{water}$ is used to calculate the ratio $PEC_{surface\ water}$ versus $PNEC_{water}$. If this value is below 1, there is no further ecotoxicological testing in the aquatic environment needed. The EMEA guideline acknowledges that the threshold of 0.01 µg/L may not be applicable for very specific and highly potent pharmaceuticals like hormones, which can have effects on the environment at concentrations below 0.01µg/L. These compounds enter Phase II irrespective of the predicted environmental concentration. In Phase II, a tailored risk assessment taking into account the mode of action is required. Therefore the pharmaceutical industry has to evaluate for each product, if it is known or if it could be expected that the effect concentration is below 0.01µg/L. Pharmaceuticals that fulfil this criteria are so called compounds of high activity "HC".

1.5 Drug Categorization

To get an overview of the mostly used pharmaceuticals prescribed in Germany and of the used amount, we did an intensive database search to make a categorization of the most prescribed pharmaceuticals.

1.5.1 Consumption of pharmaceuticals in Germany

Pharmaceuticals are a class of emerging environmental contaminants that are extensively and increasingly being used in human and veterinary medicine. The consumption of pharmaceuticals is substantial. In Germany about 3000 different substances are used in human medicine (Arzneiverordnungs-Report 2007). Also a large number of pharmaceuticals are used in veterinary medicine. Figures 2 and 3 show the consumption of pharmaceuticals in Germany 2006.

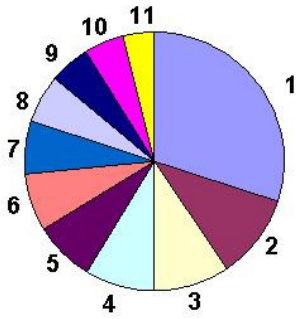

Fig. 2: Consumption of pharmaceuticals in Germany 2006 (prescriptions in millions, Arzneiverordnungs-Report 2007, Springer- Verlag Berlin-Heidelberg 2008): 1, drugs to treat heart disorders (109.2), 2, antibiotics (37.3), 3, drugs to treat inflammation and rheumatism (34.5), 4, pain killers (31.9), 5, anti diabetic drugs (26.9), 6, psycholeptics (25.9), 7, drugs to treat asthma (24.7), 8, drugs to treat ulcers (21.2), 9, stimulant drugs (18.5), 10, drugs to treat thyroid gland disorders (18.2) and 11, blood lipid lowering agents (14.1)

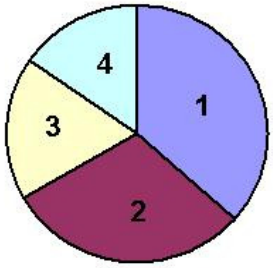

Fig. 3: Prescribed drugs to treat heart disorders in millions prescriptions 2006 in Germany (Arzneiverordnungs-Report 2007, Springer- Verlag Berlin-Heidelberg 2008): 1: angiotensin receptor inhibitors (40.0), 2: beta blockers (32.7), 3: diuretics (19.9) and 4: calcium channel inhibitors (16.6)

1.5.2 Main groups of pharmaceuticals

In order to have a general category to which the many different pharmaceuticals can be assigned to, we have followed a common scheme according to the working mechanism of the drug. To make this general categorization of pharmaceuticals, the following sources were used: Allgemeine und spezielle Pharmakologie und Toxikologie, Urban und Fischer, 9. Auflage 2004; www.meds.com; www.drugs.com; www.mayoclinic.com/health/drug-information). In the following, the different groups are briefly listed.

Antihypertensives - drugs to treat heart disorders

Antihypertensives are a class of drugs that are used in medicine and pharmacology to treat hypertension (high blood pressure). There are many classes of antihypertensives, which - by varying means - act by lowering blood pressure. A reduction of blood pressure by 5-6 mmHg can decrease the risk of stroke by 40% and of coronary heart disease by 15-20%. The likelihood of dementia, heart failure, and mortality from cardiovascular disease is also reduced.

Angiotensin-converting enzyme inhibitors

Inhibitors of angiotensin-converting enzyme (ACE inhibitors), are a group of pharmaceuticals that are used primarily in the treatment of hypertension and congestive heart failure. ACE inhibitors lower arteriolar resistance and increase venous capacity. Beside of these effects, ACE inhibitors increase cardiac output and cardiac index, stroke work and volume. Other important functions of this group of drugs are the lowering of renovascular resistance and the increasing of the excretion of sodium in the urine.

Angiotensin II receptor antagonists

Their main use is to treat hypertension (high blood pressure), diabetic nephropathy (kidney damage due to diabetes) and congestive heart failure. Angiotensin II receptor antagonists are primarily used for the treatment of hypertension, where the patient is intolerant of ACE inhibitor therapy.

Aldosterone antagonists

Aldosterone is a steroid hormone produced by the outer-section of the adrenal cortex in the adrenal gland, and acts on the distal tubules and collecting ducts of the kidney to cause the conservation of sodium, secretion of potassium, increased water retention, and increased blood pressure. The overall effect of aldosterone is to increase reabsorption of ions and water in the kidney. Aldosterone antagonist refers to drugs which antagonise the action of aldosterone. This group of drugs is often used as adjunctive therapy, in combination with other drugs for the management of chronic heart failure.

Adrenergic receptor antagonists - beta blockers

Beta blockers (β-blockers) are a class of drugs used for the management of hypertension, cardiac arrhythmias and cardioprotection after myocardial infarction. Beta blockers block the action of β-adrenergic receptors, associated with the sympathetic nervous system. There are three known types of beta receptors, designated β_1, β_2 and β_3. β_1-adrenergic receptors are located mainly in the heart and in the kidneys. β_2-adrenergic receptors are located mainly in the lungs, gastrointestinal tract, liver, uterus, vascular smooth muscle, and skeletal muscle. β_3-receptors are located in fat cells. Stimulation of β_1 receptors induces a positive effect on the heart and increases cardiac conduction velocity and automaticity. Stimulation of β_1 receptors on the kidney causes renin release. Stimulation of β_2 receptors induces smooth muscle relaxation (resulting in vasodilation and bronchodilation amongst other actions), induces tremor in skeletal muscle, and increases glycogenolysis in the liver and skeletal muscle. Stimulation of β_3 receptors induces lipolysis. Beta blockers inhibit these normal sympathetic actions, but have minimal effect on resting subjects. That is, they reduce the effect of excitement/physical exertion on heart rate and force of contraction, dilation of blood vessels and opening of bronchi, and also reduce tremor and breakdown of glycogen. There exist non-selective beta blockers like propanolol, beta-1 selective blockers like atenolol and beta-2 selective agents.

Adrenergic receptor agonist

The adrenergic receptors (or adrenoceptors) are a class of G protein-coupled receptors that are targets of the catecholamines. Adrenergic receptors specifically bind their endogenous ligands, the catecholamines adrenaline and noradrenaline (called epinephrine and norepinephrine in the United States), which leads to their activation. Many cells possess these receptors, and the binding of an agonist will generally cause a sympathetic response (i.e. the fight-or-flight response). For instance, the heart rate will increase and the pupils will dilate, energy will be mobilized, and blood flow diverted from other, non-essential, organs to skeletal muscle.

Diuretics

A diuretic is any drug that elevates the rate of urination (diuresis). There are several categories of diuretics. All diuretics increase the excretion of water from the body, although each class of diuretic does so in a distinct way. In medicine, diuretics are used to treat heart failure, liver cirrhosis, hypertension and certain kidney diseases. The antihypertensive

actions of some diuretics (thiazides and loop diuretics in particular) are independent of their diuretic effect. That is, the reduction in blood pressure is not due to decreased blood volume resulting from increased urine production, but occurs through other mechanisms and at lower doses than that required to produce diuresis. Indapamide was specifically designed with this in mind, and has a larger therapeutic window for hypertension (without pronounced diuresis) than most other diuretics. Chemically, diuretics are a diverse group of compounds that either stimulate or inhibit various hormones that naturally occur in the body to regulate urine production by the kidneys.

Calcium channel blockers

Calcium channel blockers are a class of drugs and natural substances with effects on many excitable cells of the body, like the muscle of the heart, smooth muscles of the vessels or neurons. The main action of calcium channel blockers is to decrease the blood pressure. It is for this action that it is used in individuals with hypertension.

Vasolidators

A vasodilator is a drug or chemical that relaxes the smooth muscle in blood vessels, which causes them to dilate. Dilation of arterial blood vessels (mainly arterioles) leads to a decrease in blood pressure.

Antibiotics

An antibiotic is a chemotherapeutic agent that inhibits or abolishes the growth of micro-organisms, such as bacteria. The term originally referred to any agent with biological activity against living organisms; however, "antibiotic" now refers to substances with anti-bacterial, anti-fungal, or anti-parasitical activity. The first widely used antibiotic compounds used in modern medicine were produced and isolated from living organisms, such as the penicillin class produced by fungi of the genus *Penicillium*, or streptomycin from bacteria of the genus *Streptomyces*. At the highest level, antibiotics can be classified as either bactericidal or bacteriostatic. Bactericidals kill bacteria directly where bacteriostatics prevent them from dividing. Antibiotics are only intended to be used in human medicine by a prescription. However, they are also used in other applications such as veterinary medicine, materials and product conservation etc.

Analgesics

An analgesic (painkiller) is any member of the diverse group of drugs used to relieve pain (achieve *analgesia*). The word *analgesic* derives from Greek *an-* ("without") and *-algia* ("pain"). Analgesic drugs act in various ways on the peripheral and central nervous systems; they include paracetamol (acetaminophen), the non-steroidal anti-inflammatory drugs (NSAIDs) such as the salicylates, narcotic drugs such as morphine, synthetic drugs with narcotic properties such as tramadol, and various others.

Non-steroidal anti-inflammatory drugs (NSAID)

NSAIDs are drugs with analgesic, antipyretic and anti-inflammatory effects - they reduce pain, fever and inflammation. The term "non-steroidal" is used to distinguish these drugs from steroids, which (among a broad range of other effects) have a similar anti-inflammatory action. The most prominent members of this group of drugs are aspirin, ibuprofen, and naproxen partly because they are available over-the-counter in many countries. NSAIDs are usually indicated for the treatment of acute or chronic conditions, where pain and inflammation are present. The anti-inflammatory action of NSAID rests in their ability to inhibit the activity of the cyclooxygenase (COX) enzyme, which in turn results in a diminished synthesis of proinflammatory prostaglandins (Burian, 2005). Prostaglandins act (among others) as messenger molecules in the process of inflammation. In the early 1990s, COX was demonstrated to exist as two distinct isoforms (Fu et al., 1990). COX-1 is constitutively expressed as a housekeeping enzyme in nearly all tissues, and mediates physiological responses (e.g., cytoprotection of the stomach, platelet aggregation). COX-2 is expressed in cells that are involved in inflammation (e.g., macrophages, monocytes, synoviocytes). It represents the isoform that is primarily responsible for the synthesis of the prostanoids involved in pathological processes, such as acute and chronic inflammatory states. Most NSAIDs act as non-selective inhibitors of the COX-1 and COX-2. NSAIDs act at the COX active site in several ways (Marnett et al., 1999). Aspirin irreversibly inactivates both COX-1 and COX-2 by a covalent modification of the active site. This modification interferes with the binding of the substrate (arachidonic acid) to the enzyme. By contrast, reversible competitive inhibitors of both isoforms (e.g., mefenamate, ibuprofen) compete with arachidonic acid for the COX active site. A third class of NSAIDs (e.g., flurbiprofen, indomethacin) causes a slow, time-dependent reversible inhibition of COX-1 and COX-2. This is caused by an induction of a conformational change of the enzyme.

Opiates and morphinomimetics

Morphine, the archetypal opioid, and various other substances (e.g. codeine, oxycodone, hydrocodone, diamorphine, pethidine) all exert a similar influence on the cerebral opioid receptor system. Dosing of all opioids may be limited by opioid toxicity (confusion, respiratory depression, myoclonic jerks and pinpoint pupils), but there is no dose ceiling in patients who tolerate this.

Opioids, while very effective analgesics may have some unpleasant side-effects, like nausea and vomiting. When used appropriately, opioids and similar narcotic analgesics are otherwise safe and effective, however there is a risk of addiction and the adaption of the body to the drug. Often the dose must be increased. In a chronic disease the no ceiling limit of the drug comes into play. However, there is still a toxic dose even if the body has become used to lower doses.

Statins

The statins (or HMG-CoA reductase inhibitors) form a class of hypolipidemic drugs used to lower cholesterol levels in people with or at risk of cardiovascular disease. They lower cholesterol by competitively inhibiting the enzyme HMG-CoA reductase, which is the rate-limiting enzyme of the mevalonate pathway of cholesterol synthesis (Endo, 1992). Inhibition of this enzyme in the liver stimulates LDL receptors, resulting in an increased clearance of low-density lipoprotein (LDL, so-called "bad cholesterol") from the bloodstream and a decrease in blood cholesterol levels (Ma et al., 1986). Most circulating cholesterol is manufactured internally, in amounts of about 1000 mg/day, via steroid biosynthesis through the HMG-CoA reductase pathway. Cholesterol, both from dietary intake and secreted into the duodenum as bile from the liver, is typically absorbed at a rate of 50% by the small intestines. Cholesterol is not water-soluble, and is therefore carried in the blood in the form of lipoproteins. The relative balance between these lipoproteins is determined by various factors, including genetics, diet, and insulin resistance. Low density lipoprotein (LDL) and very low density lipoprotein (VLDL) carry cholesterol toward tissues, and elevated levels of these lipoproteins are associated with atheroma formation (fat-containing deposits in the arterial wall) and cardiovascular disease. High density lipoprotein, in contrast, carries cholesterol back to the liver and is associated with protection against cardiovascular disease.

Fibrates

Fibrates are used for a range of metabolic disorders, mainly hypercholesterolemia (high cholesterol), and are therefore hypolipidemic agents. Fibrates are used as monotherapy or in combination therapy with statins. Although less effective in lowering LDL, fibrates improve HDL and triglyceride levels (i.e. increase HDL levels and decrease triglyceride levels). Although used clinically since at least the 1930s, the mechanism of action of fibrates remained undiscovered until, in the 1990s, it was discovered that fibrates activate peroxisome proliferator-activated receptors (PPAR), especially PPARα. The PPARs are a class of intracellular receptors that modulate carbohydrate, fat metabolism and adipose tissue differentiation. Therefore activation of PPARα signaling results in (1) increased fatty oxidation in the liver; (2) decreased hepatic triglyceride secretion; (3) increased lipoprotein lipase activity and thus increased VLDL clearance; (4) increased HDL and many additional effects.

Glucocorticoids

Glucocorticoids (GC) are a class of steroid hormones characterised by an ability to bind with the glucocorticoid receptor (GR) and trigger similar effects. Glucocorticoids are distinguished from mineralocorticoids and sex steroids by their specific receptors, target cells, and effects. Cortisol (or hydrocortisone) is the most important human glucocorticoid. It is essential for life, and regulates or supports a variety of important cardiovascular, metabolic, immunologic, and homeostatic functions. Glucocorticoids are used as therapeutics to suppress various allergic, inflammatory, and autoimmune disorders. They are also administered as posttransplantory immunosuppressants to prevent the acute transplant rejection and the graft-versus-host disease.

Thyroid hormones

The thyroid hormones**,** thyroxine (T4) and triiodothyronine (T3) are hormones produced by the thyroid gland. An essential component in the synthesis is iodine. The major form of thyroid hormone in the blood is thyroxine (T4). The thyroid hormones act on the body to increase the basal metabolic rate, affect protein synthesis and increase the body's sensitivity to catecholamines (such as adrenaline). The thyroid hormones are essential to proper development and differentiation of all cells of the human body. These hormones also

regulate protein, fat, and carbohydrate metabolism, affecting how human cells use energetic compounds. They also stimulate the metabolism of vitamins. Both excess and deficiency of thyroxine can cause disorders. T3 and T4 are used to treat thyroid hormone deficiency (hypothyroidism). Synthetic forms of T3 and T4 like Liothyronine or Levothyroxine can be used to replace thyroid hormones. Beside of this, natural thyroid drugs, made from desiccated thyroid glands of pigs, are used in replacement therapy. They are both absorbed well by the gut, so they can be given orally. Antithyroid drugs also called thionamides are used to treat hyperthyroidism (overactivity of the thyroid gland) in order to reduce the excessive thyroid activity. These drugs block the synthesis of thyroid hormones by the thyroid gland and therefore decrease the production and blood levels of T4 and T3.

Antithyroid drugs require at least three weeks (usually six to eight weeks or longer) to lower thyroid hormone levels because they only block synthesis of new T4 and T3; they do not alter the effects of the T3 and T4 that are already present in the thyroid and the blood stream. The two main antithyroid drugs currently available are: propylthiouracil (PTU) and methimazole (MMI, Tapazole).

Anti-diabetic drugs

Anti-diabetic drugs treat *diabetes mellitus* by lowering glucose levels in the blood. There are two different types of this disease. Diabetes mellitus type 1 caused by the lack of insulin and therefore insulin must be used as therapy by injection or inhalation. Diabetes mellitus type 2 is caused by insulin resistance of the cells. Treatments include agents which increase the amount of insulin secreted by the pancreas, agents which increase the sensitivity of target organs to insulin and agents which decrease the rate at which glucose is absorbed from the gastrointestinal tract.

Antidepressants

An antidepressant is a psychiatric medication or other substance (nutrient or herb) used for alleviating depression. Drug groups known as monoamine oxidase inhibitors, tricyclics and selective serotonin reuptake inhibitors are particularly associated with the term. Most antidepressants have a delayed onset of action and are usually taken over the course of weeks, months or years. Despite the name, antidepressants are often used in the treatment of other conditions, including anxiety disorders, bipolar disorder, obsessive compulsive

disorder, eating disorders and chronic pain. Some have also become known as lifestyle drugs or "mood brighteners".

The therapeutic effects of antidepressants are believed to be related to their effects on neurotransmitters. Monoamine oxidase inhibitors (MAOIs) block the break-down of monoamine neurotransmitters (serotonin and norepinephrine) by inhibiting oxidizing enzymes, thus leaving higher levels still active in the brain.

Tricyclic antidepressants (TCAs) prevent the reuptake of various neurotransmitters, including serotonin, norepinephrine, and dopamine. Selective serotonin reuptake inhibitors (SSRIs) prevent more specifically the reuptake of serotonin thereby increasing the level of active serotonin in synapses of the brain. Other novel antidepressants specifically affect serotonin and other neurotransmitters.

Selective serotonin reuptake inhibitors (SSRIs)

Selective serotonin reuptake inhibitors (SSRIs) are a family of antidepressants considered to be the current standard of drug treatment. It is thought that one cause of depression is an inadequate amount of serotonin, a compound used in the brain to transmit signals between neurons. SSRIs are said to work by preventing the reuptake of serotonin by the presynaptic neuron. This family of drugs includes fluoxetine (Prozac), paroxetine (Paxil), escitalopram (Lexapro, Esipram), citalopram (Celexa), and sertraline (Zoloft).

Serotonin-norepinephrine reuptake inhibitors (SNRIs)

Serotonin-norepinephrine reuptake inhibitors (SNRIs) such as venlafaxine (Effexor) and duloxetine (Cymbalta) are a newer generation of antidepressant that works on both norepinephrine and 5-hydroxytryptamine.

Noradrenergic and specific serotonergic antidepressants (NASSAs)

Noradrenergic and specific serotonergic antidepressants (NASSAs) form a newer class of antidepressants which purportedly work to increase norepinephrine and serotonin neurotransmission by blocking presynaptic alpha-2 adrenergic receptors, while at the same

time, minimizing serotonin related side-effects by blocking certain serotonin receptors. The only example of this class in clinical use is mirtazapine (Avanza, Zispin, Remeron).

Norepinephrine (noradrenaline) reuptake inhibitors (NRIs)

Norepinephrine (noradrenaline) reuptake inhibitors (NRIs) such as reboxetine (Edronax) act via norepinephrine (also known as *noradrenaline*). NRIs are thought to have a positive effect on concentration and motivation in particular.

Norepinephrine-dopamine reuptake inhibitors

Norepinephrine-dopamine reuptake inhibitors such as bupropion inhibit the neuronal reuptake of dopamine and norepinephrine (noradrenaline).

Tricyclic antidepressants (TCAs)

Tricyclic antidepressants are the earliest generation of these compounds and include medications such as amitriptyline and desipramine. Tricyclics block the reuptake of certain neurotransmitters such as norepinephrine (noradrenaline) and serotonin. They are used less commonly now due to the development of more selective and safer drugs.

Monoamine oxidase inhibitor (MAOIs)

Monoamine oxidase inhibitors (MAOIs) such as phenelzine (Nardil) may be used if other antidepressant medications are ineffective. Because there are potentially fatal interactions between this class of medication and certain foods (particularly those containing Tyramine), as well as certain drugs, classic MAOIs are rarely prescribed anymore. MAOIs work by blocking the enzyme monoamine oxidase which breaks down the neurotransmitters dopamine, serotonin, and norepinephrine (noradrenaline).

Psychoanaleptics

Psychoanaleptics, also known as stimulanting drugs that temporarily increase alertness and awareness. They usually have increased side-effects with increased effectiveness, and the more powerful variants are therefore often prescription medicines or illegal drugs.

Stimulants increase the activity of either the sympathetic nervous system, the central nervous system (CNS) or both. Some stimulants produce a sense of euphoria, in particular the stimulants which exert influence on the CNS. Stimulants are used therapeutically to increase or maintain alertness, to counteract fatigue in situations where sleep must be prevented (*e.g.* while operating vehicles), to counteract abnormal states that diminish alertness consciousness (such as in narcolepsy), to promote weight loss (phentermine) as well as to enhance the ability to concentrate in people diagnosed with attentional disruptions (especially ADHD). Occasionally, they are also used to treat depression. The euphoria produced by some stimulants leads to their recreational use, although this is illegal in the majority of jurisdictions.

Anticonvulsants

The anticonvulsants, also called antiepileptic drugs (abbreviated "AEDs"), belong to a diverse group of pharmaceuticals used in prevention of the occurrence of epileptic seizures. Anticonvulsants are also increasingly used for the treatment of bipolar disorder, since many seem to act as mood stabilizers. The goal of an anticonvulsant is to suppress the rapid and excessive firing of neurons that start a seizure. Failing this, a good anticonvulsant would prevent the spread of the seizure within the brain and offer protection against possible excitotoxic effects that may result in brain damage. Many anticonvulsants block sodium (Na^+) channels, calcium (Ca^{2+}) channels, AMPA receptors or NMDA receptors. Some anticonvulsants inhibit the metabolism of GABA or increase its release. Two examples of anticonvulants are described below.

Barbiturates

Barbiturates are drugs that act as central nervous system (CNS) depressants, and by virtue of this, they produce a wide spectrum of effects, from mild sedation to anesthesia.

Benzodiazepines

The benzodiazepines are a class of drugs with hypnotic, anxiolytic, anticonvulsive, amnestic and muscle relaxant properties. Benzodiazepines act as a central nervous system depressant. Long-term use can be problematic due to the development of tolerance and dependency.

Antineoplastic agents - Tumor chemotherapy

One of the challenges in today`s medicine is the fight against malignant tumors. Tumors are responsible for about 25% and 29% of deaths in females and males, respectively, in Switzerland, and similarly in other developed countries. The main characteristics of tumor cells (in contrast to healthy cells) are:

- they can grow independently from growth factors
- they are resistant to inhibitory growth signals
- they avoid apoptosis
- they show unlimited replication
- they induce agiogenesis
- they have a tissue invasive potential.

Chemotherapy in its most general sense refers to treatment of disease by chemicals that inhibit proliferation of cells, specifically those of microorganisms or tumors. In popular usage, it usually refers to antineoplastic drugs used to treat cancer or the combination of these drugs into a standardized treatment regime. There are two main ways to treat tumors; surgery/radiation therapy for localized tumors and chemotherapy. There are different types of chemotherapy, but the main goal of all of them is to inhibit proliferation of tumor cells or to drive them to apoptosis.

Main groups of chemotherapeutics and mode of action

There are many diverse modes of actions of anticancer drugs. Whereas classical drugs interfere with cell replication/division the new generation of drugs acts on angiogenesis and cell signaling. Moreover, antihormonal drugs are applied in cancers dependent on hormones for growth. The information to generate the categorization of cancer therapeutics was taken

from the book “Allgemeine und spezielle Pharmakologie und Toxikologie”, Urban und Fischer, 9. Auflage (2004) if not further specified. The following types of drugs are currently in use.

Alkylating antineoplastic agents

This group of drugs consists of chemicals that covalently bind to DNA bases, namely alkyl groups, and as a consequence, the DNA strands are unable to uncoil and to separate. Therefore the cell can no longer divide.

Examples of alkylating antineoplastic agents are:

- Ifosfamide: it is used for the treatment of a variety of cancers including testicular cancer, breast cancer and lung cancer. It is given intravenous.
- Darcarbazine: it is used to treat malignant melanoma and Hodgkin lymphoma. The administration to the patient is by injection or intravenous.
- Busulfan: it is in clinical use since 1959 for the treatment of chronic myelogenous leukemia. It is on the market under the brand name Myleran.

Platinum compounds

These agents interacalate with DNA strands and lead to the crosslinking of DNA strands causing the inhibition DNA replication and cell death. Platinum therapy is used for the treatment of specific cancers, including testicular, ovarian, lung, bladder, and head and neck cancers. Platinum compounds currently used are cisplatin, its derivative carboplatin and oxaliplatin, either used alone or in combination with other chemotherapy drugs. Oxaliplatin belongs to a new class of platinum based compound and has been found to be effective in various cancers that do not respond well to cisplatin.

Hydroxyurea

This compound inhibits the enzymes, which are responsible for the conversion of ribonucleotidediphosphates to desoxyribonucleotidediphosphates, and as a consequence, they inhibit the DNA synthesis. It is used for the management of melanoma, resistant chronic myelocytic leukemia, and recurrent, metastatic, or inoperable carcinoma of the ovary.

Antimetabolites

An antimetabolite is a chemical with a similar structure to a substance (a metabolite) required for normal biochemical reactions, yet different enough to interfere with the normal functions of cells, including cell division. Antimetabolites can be used in cancer treatment, as they interfere with DNA production and therefore cell division and the growth of tumors. Because cancer cells spend more time dividing than other cells, inhibiting cell division harms tumor cells more than other cells. Anti-metabolites masquerade as purine (azathioprine, mercaptopurine) or pyrimidine - which become the building blocks of DNA. They prevent these substances becoming incorporated into DNA during the S phase (of the cell cycle), stopping normal development and division. They also affect RNA synthesis. However, because thymidine is contained in DNA but not in RNA (where uracil is used instead), inhibition of thymidine sythesis via thymidylate synthase selectively inhibits DNA synthesis over RNA synthesis. Due to their efficiency, these drugs are the most widely used cytostatics.

Examples of antimetabolites (purine analogues) are:

- Thioguanine is used to treat acute leukemias and induction of remissions in acute granulocytic leukemias
- Pentostatin and cladribine are adenosine analogs which are primarily used to treat Hairy cell leukemia

Microtubuli inhibitors

These compounds bind to the cytosceleton protein tubulin and inhibit the polymerisation of tubulin to microtubuli. Therefore they inhibit the mitotic division of the cell, as chromosomes remain in the aequatorial plate of the metaphase.

Examples of microtubuli inhibitors:

- Vincristine and vinblastine are used to treat Hodgkin and non-Hodgkin lymphomas and for acute lymphocytic leukemia
- Paclitaxel (also known as taxol) is used to treat ovarian cancer, breast cancer and lung cancer

Topoisomerase inhibitors

The nuclear enzymes topoisomerase I and II are critical for DNA function and cell survival, they are responsible for the opening and closing of DNA strands during replication. As soon as these enzymes are inhibited by drugs the cells can not divide anymore. Therefore, these enzymes are promising cellular targets for anticancer drugs. Topoisomerase I inhibitors have shown significant activity against a broad range of tumours. Because of manageable toxicity and encouraging activity against solid tumors, topoisomerase I-active drugs offer promise in the clinical management of human tumours. Topoisomerase II inhibitors are also active against several types of tumors. However, treatment with these drugs often results in the development of the multi-drug resistance.

Examples for topoisomerases inhibitors are:

- Daunorubicin (belonging to the group of anthracyclines) is a topoisomerase II inhibitor used to treat leukemia
- Camptothecin is a topoisomerase I inhibitor and has a strong anticancer activity

Hormone-based tumor therapy

One category of hormone-based tumor therapy is build by the glucocorticoids. These drugs are used for the treatment of lymphoma. Sex hormones, another category, are used to treat prostate cancer and breast cancer. The growth of these cancers is often dependent on the presence of sex hormones. One group consists of the anti-estrogen drugs, competitive antagonist of the estrogen receptor. Another group is the anti-androgens which are applied to treat prostate cancer. Another category of drugs used in hormone-based tumor therapy, are the aromatase inhibitors. Aromatases are needed to convert androgens into estrogens and the inhibition of responsible enzymes has an anti-estrogenic effect. Therefore tumors that need estrogens for their growth will not proliferate. Aromatase inhibitors are increasingly used in breast cancer and ovarian cancer therapy.

- Prednison (glucocorticoid) is applied to treat leukemia.
- Tamoxifen (anti-estrogen) is currently the most common hormone treatment used to treat breast cancer. It is a competitive antagonist of the estrogen-receptor, which is often expressed in tumor cells that need estrogens as a growth signal.

- Flutamide, together with nilutamide and bicalutamide is the most used anti-androgenic drug, used for prostate cancer hormone therapy. Flutamide fits into the androgen receptors of the prostate cells and prevents the binding of testosterone, and therefore it inhibits the growth of cancer cells.
- Anastrozole, a drug belonging to the group of aromatase inhibitors, is used to treat breast cancer.
- Letrozole, another aromatase inhibitors, is used to treat postmenopausal women with early-stage, hormone responsive breast cancer after surgery. Letrozole did better to prevent a recurrence of disease - especially distant metastases - than the commonly tamoxifen.
- Exemestane is third member of the group of aromatase inhibitors that is often used to treat women with breast cancer.

Inhibitors of angiogenesis

Angiogenesis, the formation of new blood vessels, is a process controlled by certain factors (biomolecules). Some of them, like angiopoietin-1, basic fibroblast growth factor or vascular endothelial growth factor, stimulate cells to repair damaged blood vessels or form new ones. Other factors, called angiogenesis inhibitors, signal the process to stop. Angiogenesis plays an important role in the growth and spread of cancer. Once a nest of cancer cells reaches a certain size (1–2 mm in diameter), it must develop a blood supply in order to grow larger. Diffusion is no longer adequate to supply the cells with oxygen and nutrients and to take away metabolites. Therefore cancer cells secrete substances that promote the formation of new blood vessels. The inhibition of angiogenesis is a promising target for anticancer drugs.

Examples for inhibitors of angiogenesis are:

- Bevacizumab (also called Avastin) is used primarily in the treatment of colorectal cancer, but also in other types of cancer. It binds to vascular endothelial growth factor (VEGF), an important stimulator of angiogenesis, and stops the growth of new blood vessels needed for the tumor to grow.
- Suramin (also known as germanin) is used to treat prostate cancer. It inhibits angiogenesis induced by vascular endothelial growth factor and basic fibroblast growth factor.

Inhibitors of protein kinases

Protein kinases play important roles in regulating most cellular functions - proliferation/cell cycle, cell metabolism, survival/apoptosis, DNA damage repair, cell motility, response to the microenvironment - so it is no surprise that they are often themselves oncogenes. Kinases such as c-Src, c-Abl, mitogen activated protein (MAP) kinase, phosphotidylinositol-3-kinase (PI3K) AKT, and the epidermal growth factor (EGF) receptor are commonly activated in cancer cells, and are known to contribute to tumorigenesis. There are several ways to target these enzymes therapeutically, such as with antibodies or small molecules that block kinase-substrate interaction. A number of kinase inhibitors have therefore already been developed and approved for cancer treatment.

Examples for inhibitors of protein kinases are:

- Imatinib, also known as Clivec is a tyrosine kinase inhibitor. It is used to treat chronical myolid leukemia (CML). One hallmark of CML is the increased activity of the Abl protein tyrosine kinase leading to proliferation of cells. Imatinib inhibits the activity of this kinase and reduces cell proliferation.
- Gefitinib and cetuximab, inhibitors of the epidermal growth factor receptor, are used for the treatment of lung and colorectal cancer. Gefitinib is a selective inhibitor of epidermal growth factor receptor tyrosine kinase domain (EGFR). This target protein is also known as Her1 or ErbB-1. EGFR is overexpressed in the cells of certain types of human carcinomas leading to an inappropriate activation of the anti-apoptotic Ras signalling cascade resulting in uncontrolled cell proliferation. Gefitinib inhibits EGFR tyrosine kinase by binding to the adenosine triphosphate (ATP)-binding site of the enzyme. Thus the function of the EGFR tyrosine kinase in activating the Ras signal transduction cascade is inhibited.

Antibody based therapy

Human recombinant antibodies directed against proteins of cancer cells, although not tumor-cell-specific however, are a new way to treat cancer. There are different ways how antibodies fight against cancer cells. On one hand, antibodies make the cancer cell more visible to the immune system. The immune system attacks foreign invaders in the body, but it does not always recognize cancer cells as such. A monoclonal antibody can be directed to attach to certain parts of a cancer cell, thus marking the cancer cell and making it easier for the

immune system to identify it. Another way of how antibody-based therapy works is the inhibition of growth signals. Growth factors attach to receptors on the surface of normal cells and cancer cells, signalling the cells to grow. Certain cancer cells express extra copies of the growth factor receptor, thus leading to faster growth than in normal cells. Monoclonal antibodies can block these receptors and prevent the growth signal from getting through. Another method is the use of antibodies that can deliver radiation to cancer cells. By combining a radioactive particle with a monoclonal antibody, radiation can be delivered directly to the cancer cells. Instead of radioactive particles, also powerful anti-cancer drugs or toxins can be attached to monoclonal antibodies and be delivered directly to the target cancer cells.

Examples for antibody-based therapy are (www.mayoclinic.com/health/monoclonal-antibody):

- The monoclonal antibody drug rituximab (Rituxan) attaches to a specific protein (CD20) only found on B cells. Certain types of lymphomas arise from these same B cells. When rituximab attaches to the protein on the B cells, it makes the cells more visible to the immune system, which can then attack.
- Cetuximab (Erbitux), a monoclonal antibody approved to treat colon cancer and head and neck cancer, attaches to receptors on cancer cells that accept a certain growth signal (epidermal growth factor).
- Ibritumomab (Zevalin), approved for non-Hodgkin's lymphoma, combines a monoclonal antibody with two radioactive particles. The ibritumomab monoclonal antibody attaches to receptors on cancerous blood cells and delivers the radiation.
- Gemtuzumab (Mylotarg), approved for treating a certain type of acute myelogenous leukemia, is a monoclonal antibody attached to a potent anti-cancer drug derived from a bacterium. The monoclonal antibody in gemtuzumab attaches to specific receptors on leukemic cells. Then the anti-cancer drug enters the cancer cell and is activated, causing the cancer cell to die.

Immunomodulators and Cytokines

Cytokines (either proteins or glycoproteins), secreted by immune cells, are the messengers of the immune system. They have autocrine and paracrine functions, so that they function locally or at a distance to enhance or suppress immunity. In cancer therapy, cytokines, which

enhance immunity, are used. Cytokines regulate cells like natural killer (NK) cells, macrophages, and neutrophils belonging to the innate immune system. They also regulate the cells of the adaptive immune system (T and B cell).

Examples of cytokines used in cancer therapy

- Interleukin-2 (IL-2) and interferon-alfa 2b are two cytokines approved for the treatment of cancer. IL-2 has demonstrated activity against renal cell, melanoma, lymphoma, and leukemia. Interferon has activity in the same histologies but also in Kaposi's sarcoma, chronic myelogenous leukemia, and hairy cell leukemia.

- Interferon alpha, a family of molecules, binds to certain receptors on the surface of immune cells. It has profound and diverse effects on gene expression. It promotes the activity of B- and T-cells, and it also stimulates macrophages and dentritic cells. In cancer therapy it is used to treat various cancers like kidney cancer, metastatic melanoma, hairy cell leukemia and chronic myelogenous leukemia.

Environmental concentrations of antineoplastic drugs

There are over 50 cytotoxic drugs used routinely in chemotherapy in developed countries, but there exist more of these compounds. Around 75% of these drugs are administered in outpatient departments (patients come ambulant to see the doctor at the hospital) and the rest given to inpatients (patients are stationary at the hospital) (Johnson et al., 2008). The demand for chemotherapy treatment in developed countries continues to increase as cancer incidence is increasing due to a greater proportion of elderly people in the population. The majority of cytotoxic drugs are highly water soluble, with predicted $\log K_{OW}$ between -1 and 3 (Pruijn et al., 2004). Many of the cytotoxic drugs appear to be poorly biodegradable when incubated with activated sludge (Buerge et al., 2006). For example, Buerge et al. (2006) found no difference in cyclophasphamide and 5-fluorouracil (both antimetabolite anti-cancer drugs) concentration between influent and effluent in two Swiss sewage treatment plants. Reasons for this lack of removal are: (1.) Many cytotoxic drugs are hydrophilic and will not sorb to sludge, (2.) some of the antineoplastic drugs contain halogen atoms which are known to be critical for biodegradation and (3.) many of the chemotherapeutics could be toxic for sewage bacteria. Measurements for .cyclophosphamide and ifosfamide in sewage effluent of plants which receive waste from hospitals showed concentrations up to 100 ng/L (Kümmerer et al., 1997; Steger-Hartmann et al., 1997, 1996). In waters associated with a Swiss sewage treatment plant 0.15-0.17 ng/L cyclophosphamide were detected (Buerge et

al., 2006). Zuccato et al. found 2-10 ng/L of cyclophosphamide in the river Lambro close to Milan in samples collected 1997 (Zuccato et al., 2000). In conclusion, although fairly persistent, only low concentrations (ng/L range) of chemotherapeutics can be detected in the environment.

Potential effects of antineoplastic drugs on aquatic organisms

Many classical antineoplastic drugs used in cancer therapy have a high mutagenic and cancerogenic potential. Parent compounds are often bioactivated leading to formation of mutagenic metabolites. In case organisms in the environment are able to metabolize these pharmaceuticals, enhanced mutation frequencies, and subsequently, a cancer risk will result. But until now, it is uncertain that the activating enzymes for antineoplastic drugs are present in all eukaryotic organisms. Current evidence suggests that this is probable at last in higher eukaryotic organisms. One important goup of enzymes for the bioactivation of antineoplastic drugs is the cytochrom P450 family (Kang et al. 2008). Cytochrom P450 can be found in different vetebrate species (Fink-Gremmels 2008) and also in invertebrates (Snyder 2000). It is questionable that they would be similarly activated in prokaryotic cells. In addition, these drugs often have significant side effects in humans such as nausea, cytotoxicity, reduction in proliferation of cells in various tissues etc. Such side effects can also occur in non-mammalian species, as they have similar receptors (drug targets) than humans.
One would expect mutagenicity and cancerogenicity to occur in exposed aquatic organisms as well. Ideally, information would be available from experiments, where fish are tested against known concentrations of each cytotoxic drug of interest. However, to our knowledge, no such studies have yet been published. Therefore it is necessary to utilize other relevant information. It is well established that fish exposed to genotoxic xenobiotics develop DNA damage (Barsiene et al., 2006; Bolognesi et al., 2006; Palhares et al., 2002), which in turn can lead to an increased cancer incidence. In studies, where fish have been directly exposed to cytotoxic agents, including 5-fluorouracil and cyclophosphamide, genetic damage has been observed (Grisola et al., 2000; Grisolia, 2002). Therefore, it seems that cytotoxic drugs will cause the same types of genetic damage in fish as in mammals, and maybe also in invertebrates (Depledge, 1998). But it is currently unclear, which water concentration of these anti-cancer drugs will cause DNA damage in fish and other aquatic organisms. For estimation of potential toxic effects of antineoplastic drugs on aquatic organisms, already known toxic data from non-aquatic vertebrates can be used for extrapolation to aquatic organisms. It is known that 5-fluorouracil has the same toxic activity in rats (effects on development at concentrations 10-30 mg/kg body weight *in vivo* and at concentrations 0.15-

0.3 µg/mL in vitro) (Kuwagata et al., 1998), hamsters (effects on development at concentrations of 81 mg/kg body weight (Shah et al., 1989; Ben-Khaial et al., 1994), and in the fruitfly Drosophila (effects at concentration of 0.13 µg/mL) (Parente et al., 1980; Rizki et al., 1973) than in humans. Similar to these results, cyclophosphamide has been reported to be active in a similar range of other organisms including the insects *Drosophila* (DNA damage at concentration 140 µg/mL) (Siddique et al., 2005) and *Anopheles* (mutagenic effects at 20.7 µg/mL) ((Anderson et al., 1995). It was shown that a ten days exposure of the freshwater mollusk *Biomphalaria glabrata* with cyclophosphamide ($3.6x10^{-4}$ M) and mitomycin C (10^{-4} M) induces malformation of the offsprings (Nakano et al., 2003). Other ecotoxicological data on aquatic organisms are lacking in the literature. Therefore there is a need to perform pertinent experiments for use in risk assessment.

2. Identification of highly active pharmaceuticals

2.1 Identification of existing "however compounds"

One major challenge of the project was the identification of already existing "however" compounds. To obtain this information we performed an extensive search in available databases, summarized in Table 1, and existing literature.

Website	URL
PubMed	http://www.ncbi.nlm.nih.gov/sites/entrez
ScienceDirect	http://www.sciencedirect.com/
TOXNET	http://toxnet.nlm.nih.gov/index.html
Scirus	http://www.scirus.com/srsapp/
ECOTOX	http://cfpub.epa.gov/ecotox/
Drugsafety	http://drugsafetysite.com/

Table 1: Summary of used databases to identify "however" compounds.

Note that in the environmental risk assessment, the effect concentrations are divided by assessment factors (security factors) to take into accout that extrapolations are made between the laboratory and the environment, the variability within and between species, etc. Therefore, the investigation was not limited to effect values lower than 10 ng/L, but also to

consider additional effect data at low concentrations. So far, the only identified substances with effects at less than 10 ng/L are the synthetic sex hormone 17α-ethinylestradiol (EE2) (and mestranol, the analogous compound) contained in contraceptive pills, and the gestagene levonorgestrel (Table 2). Other compounds which exert adverse effects at very low concentrations, but above 10 ng/L are listed in Table 2. Based on the mechanisms of action and sorting the existing data we noticed that cancer chemotherapeutics may also be of potential environmental concern, and therefore, potential "HC". This group of drugs is characterized by a specific mode of action affecting key biological targets with major importance for the organism (e. g. cell proliferation, cell division, antihormonal action and cell signalling disruption). Therefore small alterations of the function of these key targets induced by chemotherapeutics can have major consequences on the organism. For example the breast cancer therapeutics tamoxifen, an anti-estrogen that binds as antagonist to the estrogen receptor, competes with the action of endogenous estrogens. This competition for the receptor binding can be of environmental concern, because tamoxifen has the potential to disturb the sex hormone system of aquatic organisms, because the homology between the human estrogen receptor and the fish estrogen receptors is up to 90% (see Table 7). In a partial full life cycle test with fathead minnow and the endpoints: development, growth and reproduction, the NOEC was 5.12 µg/L (Williams et al., 2007). But as cancer chemotherapeutics are regarded in a special report (Kümmerer et al., 2008), we did not go deeper in this direction. In general, there are two groups of pharmaceuticals that may have the potential to act as "HC": endocrine disruptors and highly active/selective compounds that act on key cellular targets. Endocrine disruptors (also referred to as hormonally active agents) are exogenous substances that act like hormones and have the potential to disrupt the physiological function of endogenous hormones. Since endogenous hormones are typically present in the body at very low concentrations, the exposure to small amounts of exogenous hormonally active substances can disrupt the proper function of the endocrine system. An endocrine disruptor might have the potential to elicit adverse effects at much lower concentrations than a toxic compound acting through an unspecific mechanism. Therefore, endocrine active substances can have effects on aquatic organisms at low concentrations. Examples for known endocrine disruptors are 17α-ethinylestradiol (EE2) and methyltestosterone. EE2 can induce feminisation in fish at concentrations of 1-5 ng/L (Länge et al., 2001). Additional data are summarized in Table 2. The androgen methyl testosterone can lead to sex reversal in fish at concentration 100 ng/L (Fujioka, 2002). Sex reversal in fish was also reported at concentrations of 10 ng/L (LOEC). The effect was elicited by a combination of methyl testosterone together with a specific water temperature (Fujioka, 2002). Methyl testosterone induced imposex and altered spermatogenesis in snails at 100 ng/L (Schulte-Oehlmann et al., 2004).

Highly active compounds, which belong to the second group with the potential to be "however" compound, are pharmaceuticals that have a specific mode of action and that target specific human receptors or enzymes. As these targets can be also found in other vertebrates, the risk to elicit an unwanted response in non-target species at low concentrations exists. Examples for such pharmaceuticals are the aromatase inhibitor fadrozole or the serotonin re-uptake inhibitor fluoxetine, both displaying unwanted effects on fish at low concentrations. Cytochrome P450 aromatase CYP19, the target of fadrozole, is a key enzyme in vertebrate steroidogenesis, catalyzing the conversion of C19 androgens to C18 estrogens such as beta-estradiol (E2). *Pimephales promelas* exposed to fadrozole for 21 days showed reduction in fecundity at concentrations of 2 µg/L. Additionally, there was a significant inhibition of brain aromatase activity in both male and female. In females, there was a decrease in plasma E2 concentration (Ankley et al., 2002). Exposure of *Oryzias latipes* to the serotonin reuptake inhibitor fluoxetine for 4 weeks lead to an increase in female plasma estradiol levels at fluoxetine concentrations of 0.1 and 0.5 µg/L. Additional developmental abnormalities were observed in offspring from fluoxetine exposed (0.1-5 µg/L) parents (Foran et al., 2004). In Table 2 and 3 substances are listed with effects in aquatic organisms at low concentrations. In addition to the endocrine active pharmaceuticals and highly active/selective pharmaceuticals, antibiotics may induce adverse effects at low concentrations. These effects concern in most cases plants and algae. For algae, the effect values are in the low µg/L concentration range. To obtain the information for Table 2 and 3, the databases PubMed and ScienceDirect were used. Some of the important key words for the performed search were: endocrine disruptors, serotonin reuptake inhibitors and aquatic organisms, sex reversal in fish, reproduction in fish and pharmaceuticals, aromatase inhibitors and fish. The choice of the used key words was mainly based on own toxicological knowledge.

Compound	Effect	LOEC	Organism	Reference
Effects below 10 ng/L				
17α–Ethinylestradiol EE2 (estrogen)	Feminisation Fecundity, fertility	4 ng/L 10 ng/L	*Pimephales promelas* *Oryzias latipes*	Länge et al. 2001 Hutchinson et al.

				2003
	Feminisation	5 ng/L	*Tilapia*	Shved et al. 2008
		4 ng/L	*Rutilus rutilus*	Lange et al. 2008
	Vitellogenin induction	2 ng/L	*Danio rerio*	Xu et al. 2008
		1 ng/L	*Pimephales promelas*	Jobling et al. 2003
	Increase in embryo production	1 ng/L	*Potamopyrgus antipodarum*	Jobling et al. 2003
Levonorgestrel	Reproductive success	0.8 ng/L	*Pimephales promelas*	Zeillinger et al. 2009
Effects at 10-100 ng/L				
17-Methyltestosterone (androgen)	Sex reversal	100 ng/L	*Pimephales promelas*	Fujioka 2002
	Sex-reversal, but temperature dependent	10 ng/L	*Carassius carassius*	Fujioka 2002
	Imposex	100 ng/L	*Marisa cormarietis*	Fujioka 2002
	Spermatogenesis	100 ng/L	*Marisa cormarietis*	Schulte-Oehlmann et al. 2004
Clotrimazole (fungicide)	Inhibition of algal 14α-demethylases	17 ng/L	Mixed algal population	Porsbring et al. 2009
Effects at concentrations > 100 ng/L				
Flutamide (antiandrogen)	Effects on spermatogenesis	10 µg/L	*Pimephales promelas*	Bayley et al. 2002
	Courtship behaviour, nest building	100 µg/L	*Gasterosteus aculeatus*	Sebire et al. 2008

Sertraline (SSRI)	Reproduction	9 µg/L	*Ceriodaphnia dubia*	Henry et al. 2004
Fluoxetine (SSRI)	Increased fecundity	56 µg/L	*Ceriodaphnia dubia*	Brooks et al. 2003
	Stimulation of reproduction	36 µg/L	*Ceriodaphnia dubia*	Brooks et al. 2003
	Developmental abnormalities	0.1-5 µg/L	*Oryzias latipes*	Foran et al. 2004
	Increased female estradiol level	0.1 µg/L	*Oryzias latipes*	Foran et al. 2004
	Longer half life than predicted from mammalian data	0.64 µg/L	*Oryzias latipes*	Paterson et al. 2008
Fadrazole (aromatase inhibitor)	Reduction in fecundity	2 µg/L	*Pimephales promelas*	Ankley et al. 2002
Carbamazepine (anti-epileptic)	Reproduction	25 µg/L	*Ceriodaphnia dubia*	Ferrari et al. 2003
Clofibric acid (anti-hyperlipoproteinemic)	Sex ratio	10 µg/L	*Daphnia magna*	Flaherty et al. 2005
Diazepam (anxiolytic)	Regeneration	10 µg/L	*Hydra vulgaris*	Pascoe et al. 2003
Amlodipine (Ca-channel blocker)	Regeneration	10 µg/L	*Hydra vulgaris*	Pascoe et al. 2003
Digoxin (digitalis medicine)	Regeneration	10 µg/L	*Hydra vulgaris*	Pascoe et al. 2003
Propranolol (β-Blocker)	Reproduction	0.5 µg/L	*Oryzias latipes*	Huggett et al. 2002
	Elevated estradiol/	1 µg/L	*Oryzias latipes*	Huggett et al. 2002

	testosterone levels in males			
Levofloxacin	Growth inhibition	EC(10): 13 µg/L	Plant (duckweed)	Brain et al. 2004
Lomefloxacin	Growth inhibition	EC(10): 8 µg/L	Plant (duckweed)	Brain et al. 2004
Ofloxacin	Growth inhibition	5 µg/L	Cyanobacteria	Ferrari et al. 2004
Sulfamethoxazole	Growth inhibition	5.9 µg/L EC(10): 11 µg/L	Cyanobacteria Plant (duckweed)	Ferrari et al. 2003/2004 Brain et al. 2004
Triclosan	Altered development	0.15 µg/L	*Xenopus laevis*	Veldhoen et al. 2006

Table 2: Summary of pharmaceuticals exerting effects at aquatic organisms at low concentrations, given are the lowest effect concentrations (LOEC).

In general, there are two groups of pharmaceuticals that may have the potential to act as "however" compounds: endocrine disruptors and highly active/selective compounds that act on key cellular targets. Endocrine disruptors (also referred to as hormonally active agents) are exogenous substances that act like hormones and have the potential to disrupt the hormone system.

Antibiotic	**Effect**	**LOEC and effect concentration**	**Organism**	**Reference**
Levofloxacin	Growth inhibition	EC(10): 13 µg/L	Plant (duckweed)	Brain et al. 2004
Lomefloxacin	Growth inhibition	EC(10): 8 µg/L	Plant (duckweed)	Brain et al. 2004
Ofloxacin	Growth inhibition	5 µg/L	Cyanobacteria	Ferrari et al. 2004
Sulfamethoxazole	Growth inhibition	5,9 µg/L EC(10): 11 µg/L	Cyanobacteria Plant (duckweed)	Ferrari et al. 2003/2004 Brain et al. 2004
Triclosan	Altered development	0.15 µg/L	*Xenopus laevis*	Veldhoen et al. 2006

Table 3: Summary of antibiotics displaying effects on organisms at low concentrations, given are the LOEC unless otherwise stated.

We hypothesize that only compounds with a specific mode of action can have critical effects at such low environmental concentrations, because compounds with a more general, unspecific mode of action display effects only at high environmental concentrations. Another reason is that targets of pharmaceuticals may be identical or similar in animals and lower organisms because receptors, biochemical pathways and enzymes are conserved in evolutionary terms.

This holds true for nuclear steroid receptors that are very similar in organisms of different evolutionary levels (Wilson et al., 2004), such as nuclear peroxisome proliferator-activated receptors (PPAR`s) (Escriva et al., 1997), adrenoreceptors such as β_1 and β_2-receptors (Nickerson et al., 2001). Also the insulin receptor, insulin-like growth factor and glucagon receptors are present in lower vertebrates and invertebrates (Garofalo, 2002). Compounds targeting these receptors could be "HC", because compounds interacting with these receptors induce important biological effects and necessary concentrations are very low.

2.2 Identification of important pathways of "HC"

As hormones play important roles in the development of organisms and in the regulation of various metabolic pathways, we investigated first hormonal pathways and second additional important pathways. We had a look at the working mechanisms, at the known homologies between humans and other vertebrates/ invertebrates, at known pharmaceuticals interfering with these pathways and at existing toxicological data. The information to make the following categorization was obtained from the textbook "Allgemeine und spezielle Pharmakologie und Toxikologie", Urban und Fischer, 9. Auflage (2004), if not otherwise stated. A summary of identified important pathways respectively receptors of "HC" is given in Table 4.

Compounds interacting with sex hormone system

In the following important hormones and their receptors are described along with their evolutionary conservation, some of the important pharmaceuticals used affecting this receptor system, and the potential ecotoxicological consequences.

Estrogen and the Estrogen receptor

Biology and physiology. Estrogens are a group of steroid compounds and function as the primary female sex hormone. Like all steroid hormones, estrogens readily diffuse across the

cell membrane; inside the cell, they interact with estrogen receptors. The three major naturally occurring estrogens in women are estrone (E1, produced during menopause), estradiol (E2, predominate form in nonpregnant females), and estriol (E3, the primary estrogen of pregnancy). In the body these are all produced from androgens through actions of enzymes. Estrogen is produced primarily by developing follicles in the ovaries, the corpus luteum, and the placenta. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulate the production of estrogen in the ovaries. Some estrogens are also produced in smaller amounts by other tissues such as the liver, adrenal glands, and the breasts. These secondary sources of estrogen are especially important in postmenopausal women. While estrogens are present in both men and women, they are usually present at significantly higher levels in women of reproductive age. They promote the development of female secondary sex characteristics, such as breasts, and are also involved in the thickening of the endometrium and other aspects of regulating the menstrual cycle. In males, estrogen regulates certain functions of the reproductive system important to the maturation of sperm (Nilsson et al., 2001).

Estrogens bind to the estrogen receptor. There are two different forms of the estrogen receptor, usually referred to as α and β, each encoded by a separate gene (ESR1 and ESR2 respectively). Hormone activated estrogen receptors form dimers, and since the two forms are coexpressed in many cell types, the receptors may form ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers. Estrogen receptor alpha and beta show significant overall sequence homology, and both are composed of seven domains. Due to alternative RNA splicing, several ER isoforms are known to exist. At least three ERα and five ERβ isoforms have been identified (Li et al., 2004; Nilsson et al.,2001). Only in fish, but not in humans, an ERγ receptor has been described (Hawkins et al., 2000). Different ligands may differ in their affinity for alpha and beta isoforms of the estrogen receptor: 17-beta-estradiol binds equally well to both receptors, estrone binds preferentially to the alpha receptor and estriol to the beta receptor (Ascenzi et al., 2006).

Evolutionary conservation. Estrogen receptors have been identified in all vertebrate groups from fish to mammals. The sequence homology between human and fish for example is up to 90% (Tchoudakova et al., 1999).

Pharmaceuticals: Since estrogen circulating in the blood can negatively feed-back to reduce circulating levels of FSH and LH, most oral contraceptives contain a synthetic estrogen, along with a synthetic progestin. Estrogen and other hormones are given as hormone replacement therapy to postmenopausal women in order to prevent osteoporosis as well as treat the symptoms of menopause such as hot flashes, vaginal dryness, urinary stress incontinence, chilly sensations, dizziness, fatigue, irritability, and sweating.

Ecotoxicological consequences. Due to the high conservation of estrogen receptors in fish, exposure of fish to 17 α-ethinylestradiol to a concentration below 10 ng/L induces feminisation and the expression of vitellogenin (Länge et al., 2001, 2008).

Progesterone and the Progesterone receptor

Biology and physiology. Progesterone (P4) is a steroid hormone that is synthesized by the ovary, with the amount of P4 secreted depending on the level of gonadotropin stimulation and the physiological status of the ovary. Moreover, granulosa cells, thecal/stromal cells, and luteal secrete P4 albeit at different levels (Duleba et al., 1999). Once secreted from the ovary, P4 acts at the level of 1) the hypothalamus-pituitary axis, to regulate gonadotropin secretion and mating behaviour; 2) the mammary gland and 3) the uterus. P4 influences numerous aspects of uterine physiology, including the differentiation of the endometrium and the development of the placenta (Spencer et al., 2004). There are at least three progesterone receptors that could regulate P4 action: PGR, members of the MPR family and PGRMC1. PGR clearly plays essential roles in ovulation and the induction of genes associated with the differentiation of luteal cells, and there are two potential mechanisms through which PGRs could act. The first mechanism involves the well-characterized ligand induction of transcriptional activity by the PGR (O'Malley, 2005). Recently, a second PGR-dependent mechanism has come to light. In this mechanism P4 binding can activate the SH3 domain within the PGR and, in turn, promote the binding and activation of Src kinases. By activating Src kinase, P4 has the capacity to influence numerous signal transduction pathways that ultimately regulate specific gene cascades (Shupnik, 2004). Members of the MPR family have been shown to be also P4 receptors. MPRs are expressed in luteal cells (Cai et al., 2005) and act to suppress intracellular cAMP levels and increase MAPK 3/1 activity (Zhu et al., 2003). The net effect of MPR activation is unknown. However, because a decrease in intracellular cAMP would suppress steroidogenesis (Peluso et al., 2004) and MAPK 3/1 activation is part of an apoptotic mechanism in many cell types (Peluso et al., 2005), expression and activation of MPR in the luteal cells could initiate changes that would make the luteal cell more susceptible to apoptosis and could promote the regression of the corpus luteum. The third P4-regulated signal transduction pathway involves the interaction of PGRMC1 and SERBP1. The role of SERBP1 in this complex is unknown but could involve localizing PGRMC1 to the plasma membrane and/or influencing whether the high-affinity and/or low-affinity binding sites within PGRMC1 are available to bind P4. PGRMC1 binds P4

and thereby promotes the activation of various kinases through an interaction with the Src homology domains that are present within its cytoplasmic tail.

Evolutionary conservation. Sex steroids and their receptors have been demonstrated in all vertebrate groups from agnatha to mammalia. The human sequence of the steroid hormones receptor, including progesterone, show strong similarity to the homologs in amphibian and fish (Baker, 1997). Recently, the presence of estrogen, androgen and progesterone in gonads of amphioxus *Branchiostoma belcheri* (phylum: Chordata) was confirmed (Mizuta et al., 2007).

Pharmaceuticals. Several synthetic ligands (both steroidal and non-steroidal) for PR have been developed which compete for binding with the natural hormone and are capable of inhibiting the receptor activity. Mifepristone (RU486) was the first of these PG antagonists that exhibited antiprogesterone activity in humans and has since been used in numerous clinical studies in the gynecologic and obstetrical fields. It was shown to be an effective abortifacient and postcoital contraceptive and has been used in the treatment of endometriosis, uterine myomas, and for meningiomas, which have large concentrations of PRs. Progestogenic hormones (progestins) are a component in oral contraceptives and hormone replacement therapies. Among others three progestins are currently marketed in contraceptive formulations, namely levonorgestrel, dienogest and drospirenolone.

Ecotoxicological consequences. Due to the sequence similarity between human and animal steroid hormone receptors, fish are also a target for human pharmaceuticals interacting with the progesterone receptor. It was shown, that Mifepristone inhibits the glucocorticoid receptor of rainbow trouts at a concentration of 14 µM (Mazon et al., 2004). The progestins lebonorgestrel, dienogest and drospirenolone caused an inhibition of reproductive success and hisological changes in gonads (Zeilinger et al., 2009). Levonorgestrel acted at extremely low concentrations of 1 ng/L, and dienogest at 6.5 µg/L.

Androgen and the Androgen receptor

Biology and physiology. Androgens play an important role not only in male sexual differentiation, puberty, sexual behavior and spermatogenesis, but also in the maintenance of bone architecture and muscle mass and strength. Androgen receptors (AR) belong to a superfamily of nuclear hormone receptors. They play an important role in the development and maintenance of male organs. These functions are largely attributed to the transcription factor functions of AR mediating the physiologic effects of androgens. By binding to specific

DNA sequences known as androgen-responsive elements (AREs), AR modulates the transcription of androgen-responsive genes. AR is nearly ubiquitous in mammalian tissues sequestered and stabilized in the cytoplasm bound to heat shock proteins. Upon binding testosterone or dihydrotestosterone, it undergoes a series of conformational changes that lead to translocation to the nucleus, allowing interactions with androgen response elements at various androgen target genes (Gelmann, 2002).

Evolutionary conservation. The AR genomic organisation is conserved throughout mammalian evolution from rodents to man. Conservation of segments of the *AR* gene throughout evolution implicates these regions as being critical for the activity of the molecule. The DNA binding domain of the AR is most highly conserved from *Xenopus* to human, and the ligand binding domain of the AR is also highly conserved.

Pharmaceuticals. Synthetic agonistic or antagonistic ligands of the AR are used in hormone therapy to treat hypogonadal men, osteoporosis, frailty and prostate cancer. Beside of this, steroidal androgens are abused by athletes as anabolic agents for enhancing physical performance. The major goal of androgen therapy is to achieve testosterone levels as close to physiological concentrations as possible. Methyltestosterone, an AR agonist, is used to treat men with testosterone deficiency and also to treat breast cancer, breast pain and swelling due to pregnancy in women. Flutamide, an oral antiandrogen drug, is primarily used to treat prostate cancer. It competes with testosterone and its metabolite dihydrotestosterone for the binding to the AR in the prostate gland. A newer antagonist of the AR is bicalutamide that has compared to flutamide a better side-effect profile.

Ecotoxicological consequences. Beside of binding to the human AR, synthetic androgens can bind to animal AR. Flutamide exposure (50-500 µg/l) of female fathead minnows induces an decrease in mature oocytes and exposure of male fathead minnows to the same flutamide concentrations induces degeneration and necrosis of spermatocytes (Jensen et al., 2004). Exposure of juvenile guppies to 10 µg/l flutamide results in the reduction in number of sperms. Methyltestosterone leads to masculinisation and intersex of Korean rockfish feeded with 0.05 µg/g (Bayley et al., 2002).

Thyroid Hormones

Biology and physiology. The thyroid hormones triiodothyronine (T3) and thyroxine (T4) are essential for growth and differentiation, for the regulation of energy metabolism, and for the physiological function of virtually all human tissues. The production of thyroid hormone is

regulated by the classic hypothalamus–pituitary–thyroid axis. T4 and T3 are secreted to the blood stream from the thyroid gland in response to thyroid stimulating hormone (TSH), produced by thyrotropes in the pituitary, which is released in response to thyrotropin releasing hormone (TRH) from the hypothalamus. Once secreted into the blood stream, THs are reversibly bound to plasma binding proteins (thyroxine-binding globulin, thyroxine-binding prealbumin or transthyretin, and albumin), which facilitate TH transport and entry into tissues including brain. T3 is the transcriptionally active TH, which is produced intracellularly from circulating T4 by the process of deiodination. Deiodination is the stepwise enzymatic removal of iodine from the TH molecule by enzymes known as deiodinases (Eales et al*.,* 1993). Once in the cell, T3 moves to the nucleus and binds to intracellular thyroid hormone receptors (TRs). TRs belong to the nuclear hormone receptor superfamily (Yen, 2001).

In mammals there are two main TR subtypes, TRα and TRβ, which are encoded by different genes. The TR binds the TRE (thyroid response elements) in the promoter of thyroid target genes generally as a heterodimer with a 9-*cis* retinoic acid receptor (RXR). Unliganded TR/RXR bound to a positively regulated TRE represses transcription by recruitment of co-repressor molecules, whereas once ligand binds to the TR/RXR, transcription is activated by recruitment of co-activator molecules. The opposite effect occurs on negatively regulated TREs (Yen, 2001).

Evolutionary conservation Thyroid hormones and thyroid hormone receptors can be found in all vertebrates. Beside of this, thyroid hormone receptor orthologues have been found in Plathylminths, Mollusca, Crustacea and Echinodermata (Wu et al*.,* 2007).

Pharmaceuticals. To treat thyroid gland malfunction, several pharmaceuticals are on the market. Levothyroxine is one of the most prescribed thyroid hormone replacement drug, used to treat hypothyroidism. It is a synthetic form of the natural T4. Another frequently used drug is liothyronine, a synthetic form of the T3. Additionally, natural thyroid drugs are made from the desiccated thyroid glands of pigs. To treat hyperthyroidism, antithyroid drugs like methimazole are on the market. This compound inhibits the synthesis of thyroid hormones.

Ecotoxicological consequences. Polychlorinated biphenyls (PCBs), hydroxylated PCBs, dibenzo-*p*-dioxins and dibenzofurans interact strongly with the mammalian TH transport protein, transthyretin (TTR). The interactions of these endocrine disrupting chemicals (EDCs) with TTR induced a rise in the plasma clearance rates of THs, inducing hypothyroxinemia in rat, seal and human (Crofton et al., 2005). The most characteristic feature of TTRs from non-mammalian vertebrates, such as birds, amphibians and fish, is their higher affinity for T3 than for T4 (Yamauchi et al., 1999). Therefore, chemicals interfering with T3-binding to TTR may

directly affect the free concentration of plasma T_3 and the plasma clearance rate of T3 in non-mammalian vertebrates.

The antibacterial compound triclosan (TCS) is a widespread environmental contaminant found in wastewater effluents (0.01 – 0.65 µg/l) in the US, UK, Japan and other countries. It has structural similarity to thyroid hormones. At high concentrations (up to 230 µg/l were used in this study) TCS has negative effects on tadpole activity and on final mass of *Xenopus laevis* (Fraker et al., 2004). At environmental relevant concentrations, TCS alters the rate of T3-induced metamorphosis. Veldhoen et al pretreated *X. laevis* tadpoles with 0.15 µg/l TCS for 4 days, afterwards they injected the thyroid hormone T3. With this treatment, they observed increased hind limb development and a decrease in total body weight. Additionally, they showed an altered TH receptor mRNA expression in *X. laevis* cells treated with 0.03 µg/l TCS before T3 treatment (Veldhoen et al., 2006)

Corticoid agonists and antagonists

Biology and physiology. The group of nuclear steroid receptors includes the corticosteroid receptors and their ligands. These ligands are steroid hormones, produced in the adrenal cortex from the precursor cholesterol. They display an important role in mediating stress responses, in regulating the immune response, anti-inflammatory processes, blood electrolyte levels and carbohydrate metabolism. There are mainly two groups of corticosteroids, the glucocorticoids with cortisol as one prominent member and the mineralcorticoids with aldosterone as one member. They are responsible for the control of the carbohydrate, fat and protein metabolism, respectively. They control the electrolyte and water levels, mainly by promoting sodium retention in the kidney. Important enzymes for the synthesis of corticosteroids out of cholesterol are the enzymes from the cytochrome P450 superfamily. The corticoid receptors form a complex with other proteins such as heatshock proteins in the cytosol. In this complex, the receptors are inactive. As soon as a corticosteroid enters the cytosol, it binds to the receptor. This binding induces a conformational change of the receptor, the complex comes apart and the free receptor translocates to the nucleus. There the receptor can function as a transcription factor by binding to the DNA and inducing gene transcription. Beside of this, the receptor can inhibit gene expression through interaction with other transcription factors.

Evolutionary conservation. Similarly to what has been reported in mammals, corticosteroids are major endocrine factors in teleost, involved in regulation of physiological functions, such as osmoregulation, respiration, immune responses, reproduction, growth,

and metabolism (Mommsen et al., 1999). The major corticosteroid released from the teleost interrenal is cortisol although, in a few species, small amounts of corticosterone, and other 18-hydroxylated steroids have been observed. There is a close similarity between fish and mammalian GR and MR (Fig.4). Due to the homology in the corticoid receptors, higher fish are a good target for human pharmaceuticals like corticoid receptor agonists and antagonists.

Pharmaceuticals. Based on the structure of cortisol, there are synthetic corticosteroids as pharmaceuticals on the market. The synthetic steroids display a higher affinity to the corticoidreceptor than the natural occurring ligands. These drugs are used as anti-inflammatory and immunosuppressive compounds. Beside these synthetic agonists, there are also some synthetic antagonists on the market.

Ecotoxicological consequences. It was shown that the synthetic agonist dexamethasone binds to the fish glucocorticoid receptor 1 and 2 with EC50 values of 1.4 nM and 0.35 nM (Bury et al., 2003). This group of pharmaceuticals could be a candidate to be “HC”.

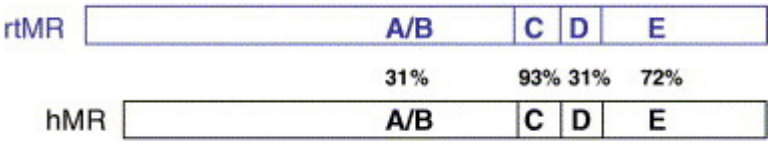

Fig.4: Amino acid identity between selected domains (A/B, C, D, and E) of the trout MR (rtMR) and human MR (hMR), (Prunet et al., 2006)(MR: mineralocorticoid receptor)

Parathyroid hormones

Biology and physiology. Parathyroid hormone (PTH) and PTH-related protein (PTHrP) are two factors that share amino acid sequence homology and act via a common receptor. In tetrapods, PTH is the main endocrine factor acting in bone and kidney to regulate calcium and phosphate. PTHrP is an essential paracrine developmental factor present in many tissues and is involved in the regulation of ossification, mammary gland development, muscle relaxation, and other functions. The actions of PTH and PTHrP are mediated by a G protein coupled receptor, referred to as PTH receptor 1 (PTHR1). Ligand binding to PTHR 1 stimulates G-mediated activation of adenylyl cyclase which stimulates cAMP production, and

subsequent activation of protein kinase (PKA). PTHR 1also stimulates G-mediated activation of protein kinase C (PKC).

Evolutionary conservation. Fish apparently lack an equivalent of the parathyroid gland, and therefore the existence of PTH-like factors in fish has been the subject of debate. However, final confirmation for their presence in fish came from the isolation of cDNAs for functional PTH receptors in zebrafish *Danio rerio* (Rubin et al., 1999). So far two forms of PTH, two of PTHrP and a protein with intermediated characteristic have been identified in teleost fish. Piscine and tetrapod PTHrP share high sequence homology at the N-terminus and its function in calcium metabolism also appears to have been conserved in vertebrates. Accessorily immunoreactive PTH-like peptides were detected in invertebrate ganglia (in the pond snail *Lymnea stagnalis*) (Hull et al*.,* 2006)

Pharmaceuticals. Synthetic parathyroid hormones are used to treat osteoporosis. Intermittent administration of parathyroid hormone stimulates bone formation by increasing osteoblast number and reduces the incidence of fracture in postmenopausal women, in elderly men, and in women with glucocorticoid-induced osteoporosis. Preotact belongs to this group of pharmaceuticals, and its structure is very close to the structure of natural occurring parathyroid hormones.Calcium is essential for a number of vital processes, from bone formation to blood clotting, regulation of enzymatic processes and modulation of permeability and excitability of plasma membranes. The physiological relevance of calcium requires that its concentrations in extracellular fluid are tightly controlled within a narrow range and mammalian species have developed a complex homeostatic system that includes parathyroid glands, kidneys, intestine and bones. Fish also maintain their circulating calcium levels within controlled limits, although the systems involved in this regulation are not totally clarified. In teleost fishes, a considerable part of the body calcium is stored in the scales, which are hard, generally flattened, skeletal elements found in the skin. Human pharmaceuticals interfering with the parathyroid receptors could alter this tightly regulated calcium homeostasis, and therefore they could be candidates for being “HC”.

Pharmaceuticals interacting with the cytochrome P450 system

Biology and physiology. The cytochromes P450 comprise one of the largest and most versatile protein families. P450 enzymes are particularly known to metabolize a variety of lipophilic xenobiotics e.g. drugs, pesticides, polycyclic aromatic hydrocarbons and plant allochemicals (Gonzalez., 2004). Many P450s have, however, endogenous functions, being specialized in the metabolism of signal molecules, such as steroid hormones (Werck-

Reichhart et al., 2000). P450s regulating physiologically important reactions usually have narrow substrate specificity and are often conserved between species and sometimes phyla to preserve important biosynthetic pathways. In contrast, many P450s that metabolize exogenous compounds have broader substrate specificities. Several metazoan genomes are now sequenced, revealing the presence of approximately 50–150 different P450 genes, including some pseudogenes, in species so distinct as nematodes, arthropods and mammals. Although the primary sequence identity between P450 proteins often is below 20%, highly conserved residues of the canonical P450 motifs ensure that the structural fold of the protein remains conserved (Werck-Reichhart et al., 2000).

Evolutionary conservation In marine invertebrates P450 genes and biochemical evidence of P450 enzyme activity have been demonstrated in Cnidaria, Annelida (Polychaeta), Mollusca, Arthropoda (Crustacea) and Echinodermata (Lee, 1998).

Pharmaceuticals. Human pharmaceuticals that affect the cytochrome P450 system, can display also effects on this system in lower vertebrates and invertebrates. Examples of such pharmaceuticals are:

- Barbiturates: Induction of cytochrome P450 enzymes in the liver
- Macrolide antibiotics: inhibit CYP3A4, a member of the cytochrome P450
- Inhibitors of the viral reverse transcriptase like Delaviridin: inhibit the monooxygenases CYP3A and CYP2D6
- Inhibitors of the cytochrom P450 aromatase (CYP19): inhibit synthesis of estrogen

Ecotoxicological consequences. Such compounds have the potential to be “however compounds”. For example it was shown that the CYP19 inhibitor fadrozole displays effects on the fathead minnow (*Pimephales promelas*) reproductive endocrinology and physiology in a short-term reproduction assay. A concentration-dependent reduction in fecundity was observed in fish exposed for 21 days to water concentrations of fadrozole ranging from 2 to 50 µg/l. There was a significant inhibition of brain aromatase activity in both male and female fathead minnows exposed to fadrozole at concentration of 50 µg/l. In females, this inhibition was accompanied by a concentration-dependent decrease in plasma E2 and vitellogenin concentrations at fadrozole concentrations of 10 µg/l and 2µg/l (Ankley et al., 2002).

Pharmaceuticals showing mutagenic activity

Zidovudine (AZT), lamivudine (3TC), and abacavir (ABC) are nucleoside reverse transcriptase inhibitors (NRTIs) often used as components in ‘‘highly active antiretroviral therapy’’ (HAART) designed to inhibit viral replication in HIV-infected patients. NRTIs are analogs of normal nucleosides that lack the 3`-OH of the deoxyribose sugar, and are therefore unable to extend the nascent DNA chain by forming a 5` to 3`-phosphodiester bond with the proceeding nucleic acid. They are able to inhibit viral replication by taking the place of endogenous nucleotides during reverse transcription of viral RNA and, thus, cause premature termination of proviral DNA synthesis. However, this mode of action also allows for the incorporation of these drugs into host cell nuclear DNA and mitochondrial DNA. Torres et al investigated the mutagenicity of AZT, 3T3 and ABC on human TK6 lymphoblastoid cells. They could show that exposure of the cells to 33 μM ABC increases the mutant frequency. AZT and 3T3 in a combined treatment, increases the mutant frequency also at 33 μM (Torres et al., 2007)

Immunosuppressive drugs such as cyclosporine A, mycophenolate mofetil, tacrolimus, and the immunosuppressive agent sirolimus are used effectively to prevent immunologic rejection after solid-organ transplantation. The most serious complication among patients undergoing immunosuppressive therapy is the risk of developing cancer. Oliveira et al investigated the mutagenicity of these compounds in human lymphocyte cultures. Addition of cyclosporine A to cultures led to a rise in mutagenicity at concentrations of 200 μg/L and 400 μg/L. The immunosuppressive drugs, sirolimus induced mutagenic activity at concentration 50 ng/mL (Oliveira et al., 2004)

This group of pharmaceuticals could also display effects at aquatic organisms, specially on such species which are exposed to this compound during their whole life.

Receptor	Ligand	Biological Functions
Hormone receptors		
Estrogen receptor α, β,(γ: only in fish)	Estrogen	primary female sex hormone, promotes development of female, secondary sex characteristics

Progesteron receptor	Progesterone	involved in female menstrual cycle and pregnancy, embryogenesis of humans and other species
Androgen receptor	Testosterone	principal male sex hormone, stimulates muscle growth and bone density
Thyroid hormone receptor α, β	Thyroid hormone	important for development/differentiation of cells, regulation of protein, fat and carbohydrate metabolism
Glucocorticoid receptor	Cortisol	involved in stress response, increases blood pressure, blood sugar levels, immunosuppressive action
Mineralocorticoid receptor	Aldosterone	reabsorption of sodium ions in the kidney, secretion of potassium ions, increase of the blood pressure
Retinoic acid receptor RAR α β, γ and retinoid X receptor RXR α, β, γ	Vitamin A and related compounds	form dimers with other nuclear receptors important for development
Other but minor important receptors		
Peroxisome proliferator-activated receptor PPAR α, β, γ, δ	free fatty acids	form dimers with other nuclear receptors like RXR, role in fat metabolism
α-Adrenoreceptor	Noradrenalin	smooth muscle contraction

β-Adrenoreceptor	Adrenalin	Increasing the heart beating rate,mobilizing energy
GABA receptors	Neurotransmitter GABA	inhibitory effects in CNS

Table 4: Important receptors and their ligands, which may be potential targets of “HC”.

3. Concepts for identification of “HC”

As already mentioned before, we think that only pharmaceuticals with the specific mode of action can elicit effects at concentrations close to 10 ng/L. Therefore we developed a concept based on the mode of action. Additionally we discuss, whether two other already existing approaches the fish plasma model concept and a QSAR concept, might be helpful in identifying compounds with effects at low concentrations.

3.1 Mode of action concept

The mode of action of pharmaceuticals can provide useful information concerning the potential toxic effects on environmental targets. In contrast to compounds acting non-specifically via a narcosis-type mechanism, drugs acting via specific mode of actions are potent enough to elicit responses at very low concentrations.

3.1.1 Consideration of toxicological data

Before considering the mode of action concept in detail, we wanted to gain a closer look at the existing toxicological data of pharmaceuticals that are generated during the registration process of each new drug. During the evaluation process of a new pharmaceutical, toxicological studies are performed to analyse for the potential risks of a new drug with mice and rats, and sometimes dogs. Such acute and chronic experiments performed for the registration of drugs are directed to clarify the three main questions: 1.Which doses are lethal? 2. Which doses elicit adverse chronic effects? 3. What are the chronic effects including reprotoxicity, mutagenicity, carcinogenicity and teratogenicity? These data may be an important source of information for the identification of the modes of action and the evaluation of possible targets not only in toxicology, but also in ecotoxicology.

Due to the lack of chronic data concerning the effects of pharmaceuticals on aquatic organisms, it is important to have a closer look on the existing toxicological data generated in toxicological experiments and studies with animals during the non-clinical testing and healthy volunteers or patients during the clinical evaluation. These data can possibly be used to identify targets and possible toxicological effects of the pharmaceuticals in question on aquatic organisms. Due to the lack or incompleteness of chronic toxicological data published on aquatic or terrestrial organisms, such toxicological data in mammals are valuable for potential use in ecotoxicological risk assessment.

Use of toxicological data for identification of "HC" compounds

During the non-clinical evaluation of new pharmaceuticals the pharmacology, pharmacokinetic and toxicology of the new drug is evaluated. Toxicological data serve as a basis for predicting potential adverse effects in humans. The onset, severity and duration of the toxic effects, their dose-dependency and degree of reversibility (or irreversibility), and species or gender-related differences should be evaluated during the non-clinical testing. Important endpoints of the non-clinical testing are:

- Pharmacodynamics
- Toxic signs
- Causes of death
- Pathological findings
- Genotoxic activity
- Carcinogenic potential
- Carcinogenic risk for humans
- Fertility and embryofetal development
- Effects on juvenile animals

One important outcome of such toxicological testing is the relation between the no observed adverse effect levels (NOAEL) and the toxic dose in relation to the maximum recommended human dose. Both, the NOEAL or the toxic dose can give useful information for the risk assessment. If the toxic dose of a pharmaceutical is close to its environmental concentration, there is a high risk that the pharmaceutical elicits unwanted effects in the environment. Taking into account that for most pharmaceuticals the environmental concentration is not known, the predicted environmental concentration PEC has to be taken for the risk assessment. The studies to determine the toxicity of a new pharmaceutical are mainly performed *in-vivo* in mice, rats, dogs, in some cases in non-human primates. Some tests are

performed *in-vitro* in cell culture systems (Table 5). Another point of such studies is the route of administration. For ecotoxicological assessments, the optimal case would be that the route mimics the way, how animals may be exposed to the drug in the environment. But also if this is not the case, the toxicological data can give useful hints for the risk assessment.

Application for ecotoxicological risk assessment

These data are important for the toxicological characterisation of drugs, particularly for identifying critical targets of effects (Table 6). However, an extrapolation of these toxicological data to aquatic organisms is difficult to perform. One major problem is that often only high doses are used in such experiments. They are justified to answer regulatory needs, but the problem of chronic effects at low concentration on aquatic organisms usually cannot be satisfactorily answered. Therefore, data from toxicological and pharmacological characterization of drugs are important to give hints for potential targets in organisms others than mammals. They may also point to the modes of action of these drugs. Pharmaceuticals having a toxic concentration or a NOAEL close to the concentration expected to occur in the environment (or PEC) must be considered as high risk compounds. However, target organs and potential toxicity in aquatic life can only be assessed by specific experiments with aquatic organisms.

Toxicology	Non-clinical guidelines*	Type of study	Endpoints
Single dose toxicity	3BS1A	Single-dose oral study in mice Single-dose oral study in rats Single-dose intravenous study in mice Single-dose intravenous study in rats	- Observed maximal non-lethal dose - Approximate lethal dose - Body weight - Noteworthy findings - Clinical observations
Repeated dose toxicity	ICHS4A	Two-week oral range finding study in rats Five-days oral range-finding study in monkeys Three-month oral study in rats One-month oral study in dogs	- No observed adverse effect level NOAEL - Body weight - Clinical observations - Died/sacrificed moribund - Food consumption - Hematology - Serum chemistry - Organ weights - Histopatology

		Nine-moth oral study in dogs Two-weeks palatability study in rats Three-month dietary range-finding study in mice Six-month oral study in rats	
Genotoxicity	ICHS2,2A,2B	Ames Test Human lymphocytes assay in-vitro Micronucleus test in rats	*In-vitro*: induction of reverse mutations in bacterial cells; - Induction of chromosome aberrations *In-vivo*: induction of bone-marrow micronuclei; - Induction of unscheduled DNA synthesis
Carcinogenicity	ICHSIB,SIA,S1C	Dietary carcinogenicity study in mice Oral carcinogenicity study in rats	- Died/sacrificed moribund - Survival - Body weight - Food consumption - Number of animals with neoplastic lesions in different tissues, for example hepatocellular adenoma
Reproductive and developmental toxicity	ICHS5A	Embryo-fetal development study in rats Embryo-fetal development study in rabbits Fertility and early embryonic development study in rats	- Fertility: number of sperm positive females number of pregnant females number of implantations, percentage of pre- and post-implantation loss - Early embryonic development/ Embryo-fetal development: - Number of litters, number of live fetuses and of dead fetuses, mean fetal body weight, fetal sex ratio, fetal abnormalies - Prenatal/postnatal development

Table 5: Summary of recommended toxicity tests during the non-clinical evaluation of new pharmaceuticals according to the common technical document for the registration of pharmaceuticals for human use, international conference on harmonisation of technical requirements for registration of pharmaceuticals for human use, 2002. Noteworth findings range from mild and moderate to marked. * Non clinical guidelines for human medicinal products according to the European Medicines Agency; www.emea.europa.eu/htms/human/humanguidelines/nonclinical/htm

The most critical endpoints of the toxicity tests for the identification of potential “HC” are:

- Death
- Effects on hormone system
- Effects on cell proliferation
- Alterations in the reproduction
- Developmental defects

All theses endpoints are critical for the individual because they affect important functions like survival. Beside the importance for the single organism some endpoints like reproduction and development are important for the whole population. As soon as the tested pharmaceuticals display adverse effects at these endpoints at concentrations close to the PEC, they are at the risk to be “HC”. But it is difficult to define a threshold or border area. Table 6 summarizes all important endpoints of the toxicological tests for the identification of potential “HC”.

Test/test number	Important endpoints	Actions
Single dose toxicity 3BS1A	> Death > Organ toxicity > Effects on hormone system	> If highly acute toxic than high probability to be “HC”
Repeated dose toxicity ICHS4A	> Death > Organ toxicity > Critical targets: hormone system, cell proliferation	> If highly acute toxic than probability to be “HC” > If hormone system/organs are affected, than high probability to be “HC”
Reproduction/ Development ICHS5A	> Reduced reproduction > Changed ratio males to females > Reduced viability or higher mortality of the off spring > Developmental defects	If fertility and reproduction affected, than high probability to be “HC”
Carcinogenicity ICHSIB,SIA,S1C	> Development of cancer	> Should be compared to other data on fertility and reproduction

Table 6: Identification of pharmaceuticals with high probability to be “HC” on the basis of toxicological data (important endpoints of EMEA-tests).

3.1.2 Description of the mode of action concept

Evolutionary receptor conservation and homologies

A specific mode of action includes, among others, binding of a drug to a specific human receptor. This binding can have agonistic or antagonistic effects on metabolic/endocrine reactions controlled through the targeted receptor. Interactions of drugs with specific target enzymes are an additional important mode of action leading to activation or inhibition of the enzyme. As a consequence this leads to activation or inhibition of certain metabolic pathways. A third mode of action is the interaction with cellular structures and biomolecules such as DNA, RNA, lipids and proteins. Compounds applied in cancer therapy are specifically directed to such targets in addition to their effects on cell signalling and cell replication. Oxidative stress is also directed in an unspecific manner to such target molecules.

Here we propose a pragmatic and stepwise approach based on the mechanisms of actions of drugs. In a first step of our concept to identify “HC”, pharmaceuticals that specifically interact with human receptors or enzymes, and therefore result in specific effects on key biological functions, are classified as potential “HC” (Fig. 5).

Receptors or enzymes that regulate important pathways are often conserved between the different vertebrate groups. Moreover homologies between vertebrate and invertebrate receptors and enzymes are often surprisingly high. In general, homologies of receptors in vertebrates (fish, amphibians, reptiles, birds) are higher than in invertebrates or plants, which is reasonable on an evolutionary basis. The so far known sequences homologies for receptors and enzymes in fish compared to humans may reach over 90%, in particular for nuclear hormone receptors (e.g. rainbow trout mineralocorticoid receptor (Prunet et al, 2006)). This similarity makes fish, amphibians, birds and reptiles potentially susceptible to similar biochemical and physiological mechanisms of activation/inactivation as are mammals.

A summary of homologies between human and fish for several important drug targets is given in Table 7. These data were obtained by the use of the database www.pubmed.gov and the function *Homologene*. This allowed first to search for human receptors, and in a second step, to display alignments with known, sequenced fish orthologues.

Gunnarsson et al predicted orthologes for 1318 human drug targets in 16 species of which several are relevant for ecotoxicity testing. In this study, zebrafish had orthologs to 85% of the drug target while only 61% were conserved in *Daphnia* and 35% in green alga (Gunnarsson et al., 2008). As a consequence chronic and target organ toxicity identified in mammalian safety assessments may be suitable to estimate potential toxic effects in fish and other vertebrates, but not on algae.

Data on invertebrates are much more limited and the fact that often similar enzymes and receptors display different functions in invertebrates than in mammals complicates the issue. One example for this phenomenon is the enzyme HMG-COA reductase. In invertebrates it plays an important role in juvenile hormone production. In plants it inhibits a key enzyme for biosynthesis, but in mammals it mediates the formation of cholesterol (for review: Fent et al., 2006).

Human receptor	**Fish species with homolog sequence**	**Identity to human sequence (%)**
Estrogen receptor 1 (alpha)	Zebrafish (*Danio rerio*) Rainbow-trout (*Oncorhynchus mykiss*) Salmon (*Salmo salar*) Killifish (*Fundulus heteroclitus*) Fathead minnow (*Pimephales promelas*)	53/56 57.1/48.3 56 50.4 58
Estrogen receptor 2 (beta)	Zebrafish (*Danio rerio*) Rainbow-trout (*Oncorhynchus mykiss*) Japanese Medaka (*Oryzias latipes*) Fathead minnow (*Pimephales promelas*)	56.2/68.8 54.8/62.5/58.9 53.8 52.5
Estrogen-related receptor alpha	Zebrafish (*Danio rerio*) Killifish (*Fundulus heteroclitus*) Japanese Medaka (*Oryzias latipes*)	67.9 60.5 61.4
Estrogen-related receptor gamma	Zebrafish (*Danio rerio*) Killifish (*Fundulus heteroclitus*) Rainbow-trout (*Oncorhynchus mykiss*) Japanese Medaka (*Oryzias latipes*)	64/72.2/86.9/93.6 76.7/71.9/78.2 79.8 81.8/91/71.8/75.3
Progesterone receptor	Zebrafish (*Danio rerio*)	64
Androgen receptor	Zebrafish (*Danio rerio*) Rainbow-trout (*Oncorhynchus mykiss*) Japanese Medaka (*Oryzias latipes*) Fathead Minnow (*Pimephales promelas*)	69.3/71.3/73.5/69.3 70.6/68.5 68.5 68.8
Thyroid hormone receptor alpha	Zebrafish (*Danio rerio*) Japanese Medaka (*Oryzias latipes*) Salmon (*Salmo salar*) Fathead minnow (*Pimephales promelas*)	88.2/90.2 83.9 84 84.1
Thyroid hormone receptor beta	Zebrafish (*Danio rerio*)	89
Glucocorticoid receptor	Zebrafish (*Danio rerio*) Rainbow trout (*Oncorhynchus mykiss*) Fathead minnow (*Pimephales promelas*)	49 53/52 50
Mineralocorticoid receptor	Zebrafish (*Danio rerio*) Rainbow trout (*Oncorhynchus*	58.8/50 57.6

	mykiss)	
Retinoic acid receptor RAR alpha	Zebrafish (*Danio rerio*) Japanese Medaka (*Oryzias latipes*) Fathead minnow (*Pimephales promelas*) Salmon (*Salmo salar*)	86/90.4/79.8 89 79.4 87.7
Retinoic acid receptor RAR beta	Zebrafish (*Danio rerio*) Japanese Medaka (*Oryzias latipes*)	74 89.5
Retinoic acid receptor RAR gamma	Zebrafish (*Danio rerio*) Japanese Medaka (*Oryzias latipes*)	85/90.8 88.5
Retinoid X receptor alpha	Zebrafish (*Danio rerio*) Rainbow trout (*Oncorhynchus mykiss*) Fathead minnow (*Pimephales promelas*)	86/91.1/92.6 89.7 93.2/84.5
Retinoid X receptor beta	Zebrafish (*Danio rerio*) Rainbow trout (*Oncorhynchus mykiss*) Japanese Medaka (*Oryzias latipes*) Fathead minnow (*Pimephales promelas*)	76/74/80.5 64.3 88.7/68 77.5
Retinoid X receptor gamma	Zebrafish (*Danio rerio*) Japanese Medaka (*Oryzias latipes*) Fathead minnow (*Pimephales promelas*) Salmon (*Salmo salar*)	74/76/82 83.5/92.8 61 82.3/82.7/92.9
Peroxisome proliferator-activated receptor alpha	Zebrafish (*Danio rerio*) Fathead minnow (*Pimephales promelas*)	72/76/74.6 74.3/60.7
Peroxisome proliferator-activated receptor gamma	Zebrafish (*Danio rerio*) Salmon (*Salmo salar*)	70/69.3 61.2
Peroxisome proliferator-activated receptor delta	Zebrafish (*Danio rerio*) Japanese Medaka (*Oryzias latipes*) Fathead minnow (*Pimephales promelas*) Salmon (*Salmo salar*)	71/79.6 75.4 73.5 77.8/73.6/76.8/74.6
Adrenoreceptor alpha 1A,1B and 1D	Zebrafish (*Danio rerio*) Rainbow trout (*Oncorhynchus mykiss*)	59/58/68/73/60 61/60/65/50
Adrenoreceptor beta	Zebrafish (*Danio rerio*) Rainbow trout (*Oncorhynchus mykiss*)	56/52 50
GABA A receptor	Zebrafish (*Danio rerio*) Japanese Medaka (*Oryzias latipes*) Fathead minnow (*Pimephales promelas*)	80/84/74/88/90 77.5 78
GABA receptors GABRR1-3, GABRQ	Zebrafish (*Danio rerio*) Japanese Medaka (*Oryzias latipes*)	71/74/76/66/77/78/80/87.5 76

Table 7: Homology between human and fish for several receptors that may serve as drug targets. (Data from www.pubmed.gov). For some receptors more than one value exists due to the fact that they were sequenced from different labs independently.

Furthermore, although pharmaceuticals may act on specific receptors, they may have different effects in invertebrates and algae: Beta-blockers may affect photosynthesis in algae besides general narcosis-type effects (Escher et al., 2006). It is also intriguing that highly conserved estrogen receptors are found in vertebrates, but are lacking in mussels. In *Mytilus spp*, only estrogen receptor-like proteins have been identified on the mRNA level and it is currently unknown whether they play a similar function as in vertebrates (Canesi et al., 2007).

Conclusions

Based on the evolutionary conservation of receptors, an important step to identify "HC" includes the evaluation of the degree of homology between the human target and the corresponding receptor/enzyme of aquatic organisms in case where this is known. The higher the degree of homology, the higher is the risk of the drug to elicit effects in aquatic organisms at low environmental concentrations. Based on the results displayed in Table 7, the analysed receptors can be separated into two groups according to the degree of homology to the human target. The first category consists of receptors having 60% or more identity, members of this group are at high risk to be affected by the human drug at low concentrations. The second category includes receptors having around 50% or less homology. For these receptors the risk to be effected by the human pharmaceutical is lower (Table 8). This categorization is based on pragmatic considerations.

High Risk	Estrogen related receptor gamma	up to 90%
	Thyroid hormone receptors	85-90%
	GABA receptors	80-90%
	RAR/RXR	70-90%
	Androgen receptors	70%
	PXR	70%
	Progesterone receptor	60%
	Estrogen receptors	up to 60%
	Adrenoreceptors	up to 60%
	Mineralocorticoid receptors	up to 60%
Low Risk	Glucocorticoid receptors	50%

Table 8: Risk of fish receptors according to the degree of homology to the human analogue.

3.1.3 Classification of regulated effects through conserved receptors

The basis to identify “however” compounds, is the classification of the effects related on the action of the receptor/enzyme. Figure 5 shows a flow scheme to be followed for a certain compound. Only pharmaceuticals that target receptors/enzymes regulating important biological pathways and target organs have the potential for being “HC”. Among them are seven critical target pathways and associated receptors identified in animals. If they are affected, negative implications for the maintenance of healthy populations are possible. Namely, targets are those regulating cell division or those that have developmental, hormonal, reproductive, immunological and neurological effects. For plants including algae, adverse effects on photosynthesis are most important.

One important step of the mode of action concept is the identification of homologous and conserved targets (receptors or enzymes) of the drugs in non-target organisms. As not all cellular pathways targeted by the pharmaceutical regulate important or essential processes for health and survival, a classification of critical conserved receptors or target biomolecular structures is needed. By applying a ranking of targets it is possible to identify receptors according to their importance. Drugs targeting critical pathways and important receptors and cellular events have a higher risk to be a “HC” than drugs acting unspecifically or interact with drug metabolizing enzymes, for instance.

Hence, we propose the following classification, which represents a pragmatic concept for use in regulation practice. We propose a hierarchical approach addressing the most important biological targets first with decreasing order of importance for health and survival. We assume that by following this pragmatic (and somewhat arbitrary) hierarchy, potentially

harmful compounds can better be identified that regarding all targets similarly at same weight and importance. For pragmatic and biological reasons, two main categories having different consequences are proposed:

1. All receptors/enzymes involved in the regulation of reproduction and development. Also included are all the receptors/enzymes regulating cell proliferation (including cell signalling), the nervous, endocrine and immune system and photysynthesis. Included are also inhibitors of key enzymes in hormone biosynthesis (e.g. inhibitors of aromatase)
2. Receptors/enzymes involved in the regulation of metabolism.

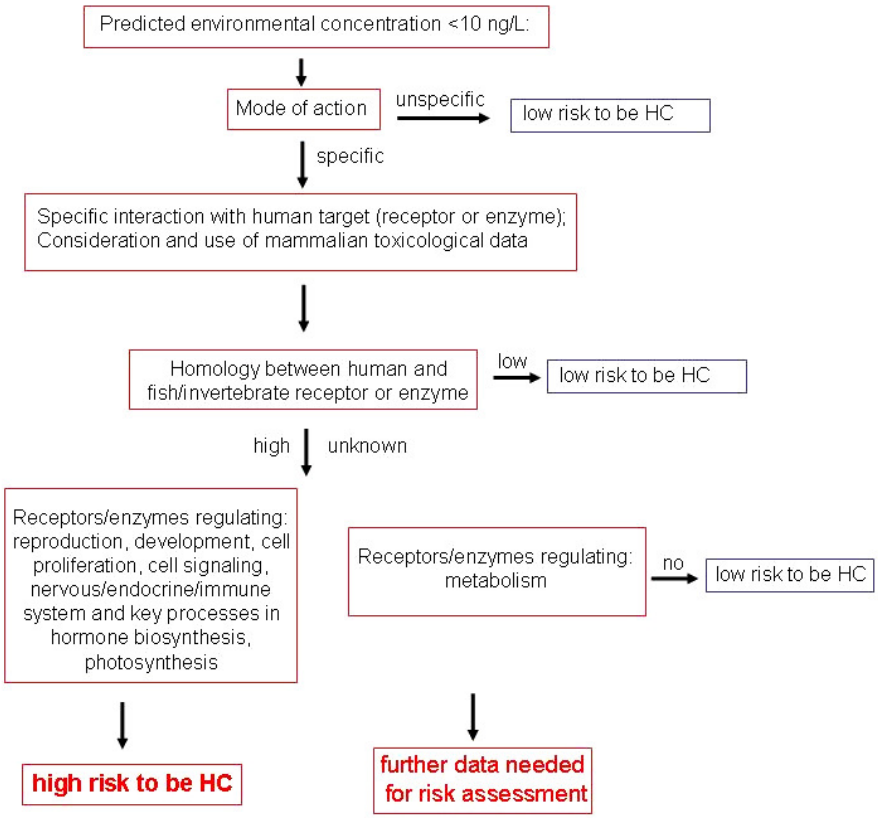

Figure 5: Flow scheme and criteria for the identification of “however” compounds (HC) among human pharmaceuticals in a stepwise mode of action concept.

In the first category, potential “HC” are found that affect critical targets and that may excert their adverse effects at low concentrations. In the second category, effect concentrations

may be higher as targets are not essential or very critical for the survival of the organism or the maintenance of a population. For such compounds additional ecotoxicological tests are necessary.

It has to be taken into account that this classification holds true for animals. For algae and plants it may look differently, because for these organisms photosynthetic production, growth, reproduction and survival are the most important processes. Therefore drugs targeting receptors/enzymes that are involved in humans and mammals in regulating specific effects may also unintentionally be involved in the regulation of photosynthetic production, growth and survival. A case in point are the HMG-CoA reductases, which are key enzymes for biosynthesis that have the highest risk to be “however” compounds for algae and plants (Fent et al., 2006).

Important aspects in the application of the mode of action concept

Knowledge of the modes of action in mammals is not a complete and reliable means of predicting effects in other organisms. This holds true in particular for organisms having larger biological distance to mammals such as invertebrates and plants. Receptors may be conserved in these species but their specific function, and hence, the effects of pharmaceuticals may differ from those in mammals and/or vertebrates. To date, not much is known on the evolutionary conservation of receptors (with the exeption of a few vertebrate species, enzymes and other biomolecules, which are important as targets of pharmaceuticals (for review Fent et al., 2006). Therefore, further investigations are necessary to elucidate the role of conserved mammalian or vertebrate receptors in invertebrates, similar to the work by Gunnarsson et al. 2008.

Further research is also required to extrapolate the modes of action concept to invertebrate species. There are several important points to be clarified before a reasonable extrapolation to the mechanisms of actions to invertebrate species or even plants is possible: The function of conserved receptors in non-target species has to be investigated, and additional effects, which cannot be predicted from the current approach, have to be identified by pertinent toxicological experiments. Furthermore, the extrapolation to algae and plants should be considered. In this case, negative effects on photosynthesis are critical in addition to cell proliferation, development and reproduction, which are key targets in animals too.

We propose that this mechanism-based concept outlined in Figure 5, should essentially be paralleled and complemented with additional concepts, as for many drugs the modes of actions are unknown. Therefore we propose to include additional, complementing approaches such as the fish plasma model.

3.1.4 Application of the mode of action concept for selected pharmaceuticals

A few pharmaceuticals were selected for analysis using the mode of action concept for their potential to be "HC" (Table 9). For these substances, some information on ecotoxicological effects does exist. The group of analyzed pharmaceuticals includes the known "HCs" EE2 and levonorgestrel, important pharmaceuticals as suspected "HC" and the non "HC" pharmaceutical paracetamol. The three main questions of the concept were answered by using the following sources: www.drugbank.ca, www.pubmed.gov and own categorization/priorisation of signalling pathways according to Figure 5.

The following three questions are answered:

1. Specific mode of action: yes or no?
2. How strong is the homology between human drug target and fish homologe?
3. How important is the regulated pathway?

Compound	**Mode of action concept**	**Outcome**
Ethinylestradiol (EE2)	1. Mode of action: specific binding to estrogen receptor 2. Homology human target versus fish receptor: up to 90% > high 3. Receptor function: reproduction > regulates important pathways for fertility and reproduction	High risk to be HC
Levonorgestrel	1. Specific binding to estrogen and progesterone receptor 2. Homology from 64 up to 90% > high 3. Receptor function: reproduction > regulates important pathways for fertility and reproduction	High risk to be HC
Tamoxifen	1. Specific binding to estrogen receptor 2. Homology up to 90% > high 3. Receptor function: reproduction > regulates important signalling pathways for fertility and reproduction	High risk to be HC
Propranolol	1. Specific binding to adrenoreceptor 2. Homology: 50-60% > high 3. Receptor function: effects at heart rate, cardiac output, blood pressure, muscle relaxation > regulated pathways minor important	Low risk to be HC
Fluoxetine	1. Specific: SSRI 2. Homology: over 70% > high 3. Functions: effects at CNS, effects on behaviour > regulation of important pathways	Moderate to high risk to be HC
Paracetamol	1. Specific inhibition of Cox-enzymes 2. Homology: around 60%: high 3. Function: effects on muscles, neurons, platelets and cell growth > pathways of minor importance	Low risk to be HC
Diclofenac	1. Specific inhibition of Cox-enzymes	Low risk to be

	2. Homology around 60%: high 3. Function: effects on muscles, neurons, platelets and cell growth > pathways of minor importance	HC
Diazepam	1. Specific binding to GABA receptors 2. Homology: 80-90% > high 3. Function: effects on neuronal activity, sedative properties, muscle relaxant, anticonvulsant > functions of minor importance	Low risk to be HC

Table 9: Analysis of pharmaceuticals using the mode of action concept for their potential to be “HC”.

The key criteria for the identification of potential “HC” are:
- Mode of Action: the more specific the mode of action of a drug is the higher is the risk of the drug to elicit unwanted effects in aquatic organisms at low concentrations.
- Degree of homology between the human drug target and the potential target in the aquatic organism. A high amount of sequence homology increases the risk of unwanted effects in aquatic organisms.
- Importance of effected pathway. If the drug affects an important pathway like reproduction than the risk of the drug to be “HC” is high.

The obtained results by using the mode of action concept are in accordance to already known data. For example, our concept classified EE2 and levonorgestrel, the already known “HCs”as high risk compounds. This also holds true for tamoxifen and fluoxetine. However, for these two compounds, the NOEC lies over 10 ng/L. In contrast, paracetamol was also identified by our concept as a compound with low potential to be “HC”. So far our concept seems to be useful to identify “HC”. But further analysis, with a lager set of pharmaceuticals, considering PEC and NOEC, has to be performed.

3.2 The fish plasma model concept – an additional/complementary model to identify “HC”

For the risk assessment of pharmaceuticals, it would be helpful to know the fish plasma concentration of pharmaceuticals additionally to the PEC. By comparing the fish plasma concentration with the human therapeutic plasma concentration respectively with toxic plasma concentration obtained during the toxicological tests, the potential risk of the pharmaceutical for the fish could be estimated. But so far, data about fish plasma concentrations are rare. But there exists a theoretical approach developed by Huggett to calculate the fish plasma concentration based on environmental concentrations and water solubility of the pharmaceutical. The fish plasma model (Huggett et al., 2003) estimates fish

plasma concentrations of drugs, which are used to define the need for further investigations of chronic effects in fish according to calculated values. The model can be summarized as follows: In case the estimated fish plasma concentration of a pharmaceutical is close to its human therapeutic plasma concentration, the compound is assumed to have potential adverse effects in aquatic organisms.
The model uses the information about concentrations of drugs in human plasma. Moreover it is based on the hypothesis that the main targets of the drug (receptors and enzymes) are similar between humans and fish, and so are the many physiological processes. In the fish-plasma model the extensive scientific and mechanistic understanding of pharmaceuticals in mammalian systems is used for the selection of pharmaceuticals with potential (chronic) risks for aquatic organisms. The main concept behind the model is the assumption of the similarity of the (wanted) plasma concentration of the drugs in humans and the (unwanted) effect concentrations in fish. Therefore pharmaceuticals with the highest potential for chronic effects are those for which the predicted plasma concentration in fish is close to the plasma concentration in humans.

3.2.1 Description of the model

As mentioned, the key assumption for the fish-plasma model is the similarity of many receptors and enzymes across mammalian and even non-mammalian species. The best model would be based on the highest human plasma steady state concentration (drug effect concentration) that corresponds to a NOEC (H_{SS}PC NOEC). This concentration could than be compared to a measured fish steady state plasma concentration (F_{SS}PC). The ratio of these two plasma concentrations could than be expressed as a margin of safety:

$$\text{Margin of Safety} = H_{SS}\text{PC NOEC}/F_{SS}\text{PC}$$

This margin of safety should be as large as possible for human pharmaceuticals. But unfortunately on one side drug levels in humans are reported as the recommended dose and not as the NOEC. On the other side, there are very limited data on steady state fish plasma concentrations for pharmaceuticals. As an alternative to this approach, an effect ratio (ER) can be calculated. As the human therapeutic plasma concentration H_TPC at the maximum dose of a drug is available it can be used instead of the H_{SS}PC NOEC. The fish plasma level F_{SS}PC can be calculated by using the predicted or measured environmental concentration of a certain drug and its Log K_{OW}. These two values can be applied to calculate the ER:

$$ER = H_T\text{PC}/F_{SS}\text{PC}$$

As with the margin of safety, the ER ratio should be as large as possible. If the ER is lower or equal to 1, than there is a potential for receptor mediated responses in fish. The lower the ER is, the greater the need for additional chronic investigations in fish.

H_TPCs are investigated as a standard value during the drug development process. They can be expressed as a single point in time (Cmax) or as a function of time (AUC). Both values can be obtained from databases (for example: Online Physicians Desk References (www.pdr.net)).
F_{SS}PC can be estimated by using the Log K_{OW} value and a measured or predicted environmental concentration. Accumulation via food chain is not considered in this assumption. The partitioning of a substance between the aqueous phase and the arterial blood in trout is estimated by Fitzsimmons (Fitzsimmons et al, 2001):

$$\text{Log } P_{Blood:Water} = 0.73*\text{Log } K_{OW}-0.88$$

Using these relationships, drug partitioning between blood and water for a given environmental concentration can be equated to a fish steady state plasma concentration:

$$F_{SS}PC = EC*(P_{Blood:Water})$$

The major driving force in this model for a compound crossing from water into the blood of fish is the hydrophobicity. It should be noted that in this model metabolism, excretion or protein binding of the drug in fish is not taken into account. Therefore the model can be regarded as a worst case scenario in that it is assuming maximal blood levels. In addition, calculation of bioconcentration factors based on the sugggested formula using the Kow is not validated for ionizable compounds like pharmaceuticals.

Huggett suggested the use of safety factors to refine the degree of uncertainty expressed in the model. As an initial approach, a safety factor of 1000 is reasonable. This factor is derived by applying a 10-fold factor for extrapolation of humans to animals, a 10-fold factor for sensitivity differences and a 10-fold factor for extrapolating from mammalian to non-mammalian species. Therefore, compounds with an effect ratio lower than 1000 are regarded as critical for potential adverse effects, needing additional assessment in fish.

3.2.2 Application for selected pharmaceuticals

To evaluate the fish plasma model, we calculated the fish plasma concentration (Table 10) and the effect ratio, without safety factors (Table 11) applying the mathematical formula of Huggett (Huggett et al., 2003) for some pharmaceuticals including the known "HCs" EE2 and levonorgestrel. We did not apply safety factors (or application factors) to keep the analysis as simple as possible. By this analysis we wanted to get a first idea whether or not the fish plasma model might be a useful model for such purposes. We did not want to conduct a datailled analysis of the fish plasma model. For levonorgestrol, no information about environmetal concentrations is available. Therefore we used 10 ng/L (according to the EMEA guideline) and 100 ng/L as environmental concentrations to calculate the fish plasma model. Considering estimated fish plasma concentrations of certain pharmaceuticals, we calculated the effect ratio for the pharmaceuticals according to (Huggett et al., 2003) (Table 11). Compounds with an effect ratio below 1 have a potential risk to be effective in fish resulting in adverse effects. Fig. 6 shows the correlation between human and fish plasma concentration. The following critical drugs for which fish plasma concentrations are close to human plasma concentrations or even above human plasma concentrations, were identified: Propranolol, tamoxifen, levonorgestrel and EE2. For three additional compounds, diclofenac, metoprolol and fluoxetine, fish plasma concentrations are close to human plasma concentrations and must therefore be considered as potential critical drugs. For all other drugs estimated fish plasma concentrations are much below the human plasma concentrations and can therefore be considered as compounds not relevant for the discussion of effects at low concentrations.

Pharmaceutical	**Log K_{OW}**	**Log $P_{blood:water}$**	**$P_{blood:water}$**	**Environmental conc. (ng/L)**	**$F_{SS}PC$ (µg/ml)**	**H_TPC (µg/ml)**
Acetylsalicylic acid	1.19	-0.0113	0.97	500	$4.85*10^{-5}$	20-200
Salicylic acid	2.26	0.7698	5.89	5000	0.029	20-200
Dextropropoxyphene	4.90	2.697	497.7			0.05-0.3
Diclofenac	4.22	2.2	158	1100	0.173	0.5-3
Fenoprofen	3.64	1.77	58.9	200	0.012	30-60
Ibuprofen	3.97	2	100	6000	0.6	15-30
Indometacin	3.66	1.8	63.1	400	0.025	0.3-1
Ketoprofen	3.56	1.72	52.5	200	0.011	1-6
Mefenamic acid	4.04	2.1	126	500	0.063	2-10
Naproxen	3.31	1.5	31.6	800	0.0252	20-50
Paracetamol	0.46	-0.5	0.32	10000	$3.2*10^{-3}$	10-25
Phenazone				1000		5-25
Acebutolol	2.36	0.84	6.9			0.2-2
Metoprolol	2.48	0.93	8.5	1400	0.012	0.035-0.5
Nadolol	1.40	0.14	1.38			0.01-0.25

Oxprenolol						0.05-0.3
Propranolol	3.59	1.8	63.1	600	0.038	0.02-0.3
Bezafibrate				1400		-15
Clofibric acid	3.58	1.7	50.1	700	0.035	
Fenofibrat	5.58	3.2	1584			5-30
Gemfibrazol	4.77	2.60	398.11	900	0.358	0.250
Carbamazepine	2.45	0.91	8.1	1000	$8.1*10^{-3}$	2-8
Diazepam	2.82	1.18	15.13	5	$7.5*10^{-5}$	0.2-2
Fluoxetine	5.37	3.04	1096.5	60	0.066	0.16-0.5
Cyclophsophamide						10-25
Ifosfamide	-0.01	-0.89	0.13			
Tamoxifen	6.59	3.9	7943	400	31.77	0.05-0.5
Albuterol	1.31	0.08	1.2	50	$6*10^{-5}$	0.01-0.02
Caffeine	-0.07	-0.93	0.12	6000	$7.2*10^{-4}$	4-10
Ethinylestradiol EE2	3.63	1.77	58.9	0.5	$2.9*10^{-5}$	0.00001
Estradiol	3.57	1.73	53.7	0.5	$2.6*10^{-5}$	
Levonorgestrel	3.8	1.894	78.34	10	$7.834*10^{-4}$	0.002-0.02
				100	$7.834*10^{-3}$	

Table 10: Application of the fish plasma model for estimation of the fish plasma concentrations of various pharmaceuticals with known environmental concentrations. Log $P_{blood:water}$ and $F_{SS}PC$ were calculated according to the fish-plasma model (Huggett et al, 2003). Values for environmental concentrations were taken from literature (Fent et al., 2006), log K_{OW} values were taken www.drugbank.ca., and information about the human therapeutic plasma concentrations from medical internet sources www.drugs.com, www.drugbank.ca and www.drugsafetysite.com.

Pharmaceutical	**Human plasma concentration (HPC, µg/mL)**	**Estimated fish plasma conc. (FPC, µg/mL**	**Effect ratio (HPC/FPC)**
Acetylsalicyclic acid	20	0.0000485	41237
Salicyclic acid	20	0.029	690
Diclofenac	0.5	0.173	3
Fenoprofen	30	0.012	2500
Ibuprofen	15	0.6	25
Indometacin	0.3	0.025	12
Ketoprofen	1	0.011	91
Mefenamic acid	2	0.063	32
Naproxen	20	0.0252	794
Paracetamol	10	0.0032	3125
Metoprolol	0.035	0.012	3
Propranolol	0.02	0.038	0.5
Carbamazepine	2	0.0081	247

Diazepam	0.2	0.000075	2667
Fluoxetine	0.16	0.066	2.4
Tamoxifen	0.05	31.77	0.002
Albuterol	0.01	0.00006	167
Caffeine	4	0.00072	5556
Ethinylestradiol EE2	0.00001	0.000026	0.4
Levonorgestrel	0.002	0.00078	2.56
		0.0078	0.256

Table 11: Human plasma concentrations (HPC), estimated fish plasma concentration (FPC) and effect ratios for estimation of potential effects. To calculate the effect ratio according to fish plasma model (Huggett et al., 2003) the minimal effect concentration for the HPC was used to analyse for a worst case scenario.

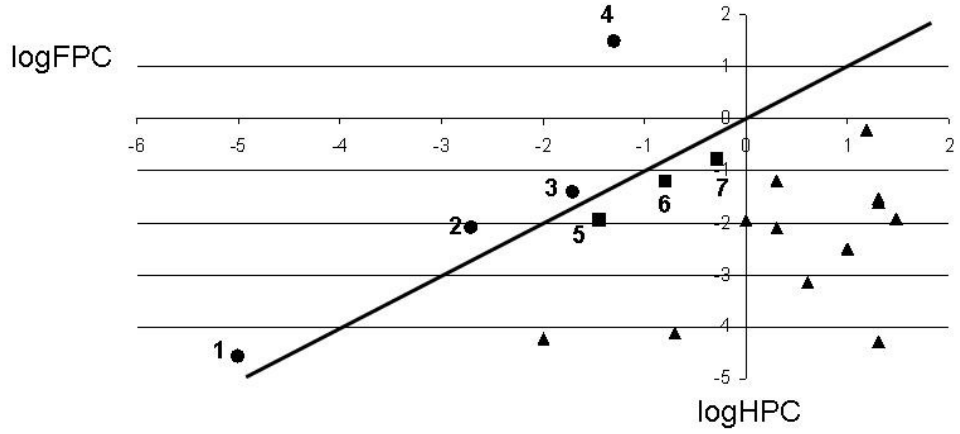

Figure 6: Correlation between the estimated fish plasma concentration (FPC) and human therapeutic plasma concentrations (HPC) of selected pharmaceuticals. All dots in the area above the line (1: EE2, 2: levonorgestrel, 3: propranolol, 4: tamoxifen) are compounds having a potential risk for fish. Squares which are near the line but below (5: metoprolol, 6: fluoxetine, 7: diclofenac) are compounds close to the cut off of 1. These drugs may have the potential for adverse effects in fish. The other triangel refer to pharmaceuticals with lower potential to display a risk for fish. But still, a risk for fish can not be excluded. Due to graphical reasons, FPC and HPC were converted to logarithmic values.

3.2.3 Comparison of fish plasma model and mode of action concept

To further evaluate the usefulness of the developed/described models, we compared the outcome of the mode of action concept with the outcome of the fish plasma model (Table 12).

Compound	Fish plasma model	Mode of action
Ethinylestradiol	HC	HC
Levonorgestrel	HC	HC
Tamoxifen	HC	HC
Fluoxetine	HC	HC
Diclofenac	HC	-
Propranolol	HC	-
Diazepam	-	-
Paracetamol	-	-

Table 12: Comparison of the results of the two different models.

Table 12 shows that the outcome of the analysis for the pharmaceuticals applying the two different models is not identical. The fish plasma model identifies diclofenac and propranolol as potential compounds of potential risk to the environment, which were not identified by using the mode of action concept. The reason for the classification of these two compounds as high risk compound by the fish plasma model is due to the high environmental concentration of both compounds (diclofenac up to 1100 ng/L and propranolol up to 600 ng/L). For levonorgestrel, only the higher environmental concentration (100 ng/L) gave an effect ratio below 1, but the lower environmental concentration (10 ng/L) gave an effect ratio above 1. The mode of action concept classified levonorgestrel as potential “HC”. This outcome is in accordance to experimental data that showed effects of levonorgestrel on fish at concentrations below 10 ng/L (Zeillinger et al., in 2009). These examples demonstrate that the outcome of fish plasma model depends on environmental concentrations of a compound. In this point, the mode of action concept is more conservative and more robust in identification of potential “HC” because it is based on a mechanistic point of view and the environmental concentration is not taken into account. However, PECs for the two identified compounds, diclofenac and propranolol, are above the action limit of 10 ng/L and therefore experimental fate and effect testing has to be performed anyway.

3.4 The QSAR concept

3.4.1 Quantitative Structure-Activity Relationships QSAR

More than a century ago, Crum-Brown and Fraser expressed the idea that the physiological action of a substance was a function of its chemical composition and constitution (Crum-Brown, 1868). Some 40 years ago, Hansch began quantifying relationships between a compound's physicochemical properties and its biological activity (Fujita et al., 1964). His series of papers laid the foundations for a concept later referred to as quantitative structure-activity relationships (QSAR). Since then, researchers have widely employed QSAR, for example, to predict the activity of new drugs or the toxicity of environmental chemicals. The aim of QSAR is to predict properties of molecules or classify molecules based on structural features. Properties can be physical properties like boiling point or aqueous solubility or biological activities like carcinogenicity or LD_{50}. To perform QSAR analysis, a large group of descriptors (topological, geometric and electronic properties of the molecule) and known biological activities/physical properties for a molecule are needed. With the help of mathematical calculations, correlations between molecule structure and activities/properties are modelled. This allows predictions about biological activities or physical properties of a new molecule just by comparing the descriptors of this unknown molecule with the descriptors of the already known and analysed compounds. An illustration of the model is shown in Figure 7.

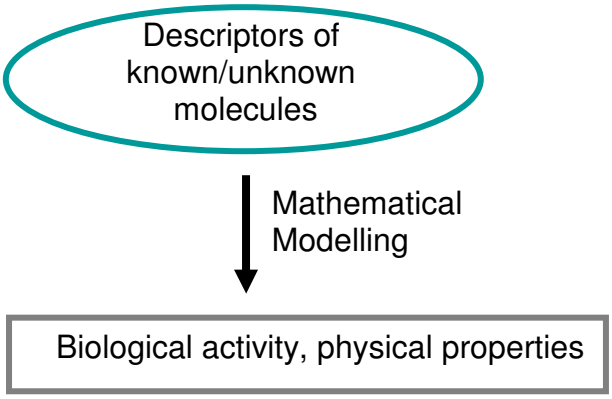

Figure 7: Schematic summary of the QSAR model.

3.4.2 Analysis of binding affinities of chemicals or pharmaceuticals to specific human receptors using VirtualToxLab

In silico techniques for the prediction of toxicological endpoints are extremely appealing because of their inexpensiveness and the short processing time to generate a big amount of data. Numerous commercially available and free web-based programs for toxicity prediction are available; some of them are listed and briefly described below (Muster et al., 2008).

- Classical QSAR approaches: Correlate structural or property descriptors of compounds with biological activities, QSARs for various endpoints published

- ToxScope: ToxScope correlates toxicity information with structural features of chemical libraries, and creates a data mining system

- COMPACT: COMPACT is a procedure for the rapid identification of potential carcinogenicity or toxicities mediated by CYP450s

- MDL QSAR: QSAR modeling system to establish structure-property relationships, create new calculators and generate new compound libraries

- PASS: comparison of new structures with structures of well-known biological activity profiles by using structure descriptors

- MetaDrug: Assessment of toxicity by generating networks around proteins and genes (toxicogenomics platform)

- CADD: Computer-aided drug design (CADD) by multi-dimensional QSARs applied to toxicity-relevant targets

- PreADMET: Calculation of important descriptors and neural network for the construction of prediction system

- Admensa Interactive: QSAR-based system primarily for ADME (absorption, distribution, metabolism and excretion) optimization

- BfR decision support system: Rule-based system using physicochemical properties and substructures

The *VirtualToxLab*, University of Basel (www.biograf.ch), is also an *in silico* tool for predicting the toxic potential of existing and hypothetical compounds (drugs and environmental

chemicals) by simulating and quantifying their interactions with human receptors at the molecular level using automated multi-dimensional QSAR. Currently, it includes 11 validated models for the aryl hydrocarbon, estrogen α/β, androgen, thyroid α/β, glucocorticoid, mineralocorticoid and peroxisome proliferator-activated receptor γ as well as for the enzymes CYP450 3A4 and CYP450 2A13. The models were trained using a representative selection of 667 substances and validated with 207 compounds different there from. This model could be useful for the identification of potential "HC". As it predicts the affinity respectively the potential binding of pharmaceuticals to human receptors, it could be a good additional model beside the mode of action concept.

3.4.3 Dimensionality of QSAR approaches

The dimensionality of QSAR analysis describes how many parameters are used to calculate respectively to model the binding affinity of a compound to a selected receptor. The first QSAR analysis (1D-QSAR) used only a few parameters like logP, hydrophobicity, electronic properties and steric parameters (Vedani et al., 2006). The most recently developed QSAR today, is the 6D-QSAR. There beside the use of the 5D-QSAR, different solvation scenarios are taken into account (Vedani et al., 2006). A detailed description of all existing QSAR levels is shown in Table 13.

Dimension	**Method**
1D-QSAR	Affinity correlates with pKa, logP, electronic properties etc.
2D-QSAR	Affinity correlates with structural patterns
3D-QSAR	Affinity correlates with three-dimensional structure of the ligands
4D-QSAR	Ligands are represented as an ensemble of conformers, orientations, protonation states and stereoisomeres
5D-QSAR	Like 4D, additionally representation of different induced-fit models
6D-QSAR	Like 5D, additionally representation of different solvation scenarios

Table 13: Different levels of QSAR analysis according to the used parameters (Vedani et al., 2006). The induced-fit is the change in the binding pocket of the receptor induced by the ligand to facilitate the binding between receptor and ligand. The induced-fit represents a local phenomenon including for example the subtle rearrangement of few side chains. It may not

only alter the topology of the binding pocket but the character (hydrophobic/hydrophilic, dielectric properties) of subpockets or the solvent accessibility of the binding site.

By using these models the "toxic potential" could be calculated. The "toxic potential" of the analysed compounds is expressed as weighted toxic potential (WTP). It derives from the calculated binding affinities towards the 11 target proteins presently comprised in the *VirtualToxLab*. The WTP as derived from the *VirtualToxLab* is based solely on thermodynamic considerations. It does not include any toxicokinetic processes such as adsorption, distribution, metabolism, elimination or target tissues. It is assumed that the only important parameter to predict toxicity of a compound is its binding affinity to the analysed receptor (thermodynamic analysis). The WTP ranges from 0.0 (none) to 1.0 (extreme) and has arbitrarily been split into five classes: WTP > 0.85 (****, extreme risk), WTP > 0.675 (***, high risk), WTP > 0.5 (**, risk), WTP > 0.325 (*, low risk) and WTP < 0.325 (–, no risk). These classes are solely directed to humans and mammals. Table 14 gives examples of pharmaceuticals belonging to different categories with yielded WTP. High numbers refer to probable interactions with important receptors. The higher the WTP, the more probable is the interaction. The high potential to interact with hormone receptors means that these pharmaceuticals are probable candidates for being "HC" compounds.

Compound	**Weighted Toxic Potential**	**WTP Class**	**Weighted main target(s)**
WTP * **			
Bicalutamide	0.678	***	→ GR, ERβ
Budesonide	0.722	***	→ GR, ERβ
Ezetimibe	0.836	***	→ GR, ERα, ERβ
Fluticasone	0.777	***	→ GR, ERα, ERβ, AR
Diethylstilbestrol	0.827	***	→ ERα, ERβ
Bendroflumethiazide (R/S)	0.690/ 0.540	***	→ GR, ERα
Dehydroepiandrosterone	0.792	***	→ ERα, ERβ, AR
Finasetride	0.852	***	→ GR, ERα, ERβ, AR
Prednisone	0.704	***	→ ERβ, AR

Spironolactone	0.788	***	→GR, ERα, AR
Ulobetasol	0.720	***	→ GR, ERβ, AR, AhR
Drugs used in the analysis			
Ethinylestradiol	0.834	***	→ ERα, ERβ, AR
Levonorgestrel	0.801	***	→ ERα, AR
Tamoxifen	0.621	**	→ GR, AR
Fluoxetine (R/S)	0.519/ 0.401	**/*	→ GR, AR
Diclofenac	0.131	-	
Propranolol (R/S)	0.344/ 0.397	*/*	→ GR, AR
Paracetamol	0.328	-	→ AR, ERβ

Table 14: The "toxic potential" of compounds calculated using multi-dimensional QSAR. The more asterisks are, the higher the toxic potential of the different compounds is, according to the Virtual ToxLab program (www.biograf.ch). Weighted main targets are estrogenreceptor α,β (ERα,β), androgenreceptor (AR), thyroid receptor α,β (TRα,β) and aryl hydrocarbon receptor (AhR).

Most recently, an Internet Portal to the VirtualToxLab was developed (www.biograf.ch). This allows easy access to the technology. Upon uploading the 3D coordinates of a compound of interest, the compound will be automatically processed and tested against the selected virtual test kits. Whether this tool is actually applicable to ecotoxicology and other animals than mammals has to be evaluated by further investigations. Moreover it must be evaluated whether the lack of toxicokinetics in this model hampers its usefulness. This new tool is, however, a useful contribution as a first screening tool if no other data are available. In case compounds ingestions are identified to interact strongly with important hormone receptor, they may be regarded as potential "HC".

3.4.4 ECOSAR

ECOSAR (Ecological Structure Activity Relationships) is a computer software program that is used to estimate the toxicity of chemicals used in industry and discharged into water (U.S. environmental protection agency). The program predicts the toxicity of industrial chemicals to

aquatic organisms such as fish, invertebrates, and algae by using Structure Activity Relationships (SARs). SAR, is a technique routinely used to estimate aquatic toxicity of chemicals. The program estimates a chemical's acute (short-term) toxicity and, when available, chronic (long-term or delayed) toxicity. ECOSAR uses SARs to predict the aquatic toxicity of chemicals based on their structural similarity to chemicals for which aquatic toxicity data are available. SARs express the (assumed) correlations between a compound's physicochemical properties and its aquatic toxicity. SARs measured for one compound can be used to predict the toxicity of similar compounds belonging to the same chemical class. ECOSAR also allows access to over 100 SARs developed for 42 chemical classes, not including pharmaceuticals. The SARs contained within the program are based on test data. Many of the SAR predictions have been validated. Most SAR calculations in the ECOSAR programs are based upon the octanol/water partition coefficient (K_{OW}). ECOSAR is used by the U.S. Environmental Protection Agency (EPA) to predict the aquatic toxicity of new industrial chemicals in the absence of test data. The use of SARs in the U.S.A. is a popular practice for estimating ecotoxicity for many chemicals. A disadvantage of such models based on physicochemical properties is the occurrence of compounds that have a specific mode of action. In these cases, false results may result. As the predictions are based on the K_{OW}, the risk of compounds with a specific mode of action and therefore with the potential to elicit unwanted effects at low concentrations, but a low K_{OW} could be underestimated. Additionally, the specific interaction of such compounds with receptors and key biological processes is not modelled in ECOSAR. ECOSAR is thus considered not suitable for evaluation of effects of pharmaceuticals at low concentrations.

3.5 Comparison of fish plasma model, mode of action concept and VirtualTox lab

To further evaluate the usefulness of the developed/described models, we compared the outcome of the three models. The mode of action concept is compared to the fish plasma model and the VirtualTox lab model (Table 15).

Table 15 shows that the outcome of the analysis for the pharmaceuticals applying the three different models is not identical. The fish plasma model identifies diclofenac and propranolol as potential “HC”, which were not identified by using the mode of action concept and the VirtualTox lab. The reason for the classification of these two compounds as high risk compound by the fish plasma model is due to the high environmental concentration of both compounds (diclofenac up to 1100 ng/L and propranolol up to 600 ng/L).

Compound	Fish plasma model	Mode of action concept	VirtualTox lab
Ethinylestradiol	HC	HC	High Risk (WTP:0.834)
Levonorgestrel	HC	HC	High Risk (WTP:0.801)
Tamoxifen	HC	HC	Risk (WTP: 0.621)
Fluoxetine	HC	HC	Risk (WTP: 0.519)
Diclofenac	HC	-	No risk (WTP: 0.131)
Propranolol	HC	-	Low risk (WTP: 0.397)
Paracetamol	-	-	No risk (WTP: 0.328)

Table 15: Comparison of the results of the three different models.

These examples demonstrate that the outcome of fish plasma model depends on environmental concentrations of a compound. In this point, the mode of action concept and the VirtualTox lab are more conservative and more robust in identifying potential “HC”, because it is based on a mechanistic point of view and the environmental concentration is not taken into account. It should be noted that the predicted environmental concentrations of the two identified compounds, diclofenac and propranolol, are higher than the action limit of 10 ng/L, and therefore, additional testing has to be performed anyway.

3.6 Conclusions

We propose a stepwise approach for the identification of “HC” taking the different concepts into account. A key role plays the mode of action concept with the three major steps: (1) identification of the mode of action taking the toxicological information into account; (2) identification of effected/regulated pathways and (3) classification in minor and major important pathways. Beside of these three major criteria for the identification of “HC”, we propose to include the information about target homology and the toxicological data. The target homology can be helpful to estimate the risk that a compound will display effects in other species than humans. The toxicological data can be helpful for the identification of additional (unwanted) target pathways of the pharmaceutical that than also have to be included in the analysis. Additionally, we propose to include the VirtualToxLab model to support the mode of action concept and for the identification of additional targets of the pharmaceutical. The fish plasma model is taken into consideration in addition for prediction of the fish plasma concentration of a pharmaceutical for comparision with the human therapeutic plasma concentration, and therefore to complement the identification. However, is should be emphasised that the estimated fish plasma concentration depends largely on

the compound´s K_{OW} and on the estimated environmental concentration and it is thus considered less useful. A summary of our proposed combined approach is shown in Figure 8.

Following the evaluation of the proposed approach on model compounds, the applicability of the mode of action concept and the other concepts discussed needs to be tested by regulators, contract laboratories, and the industry during environmental risk assessments. Further discussion on which approach or combination of approaches are best suited to identify all substances with effects at low concentrations is needed.

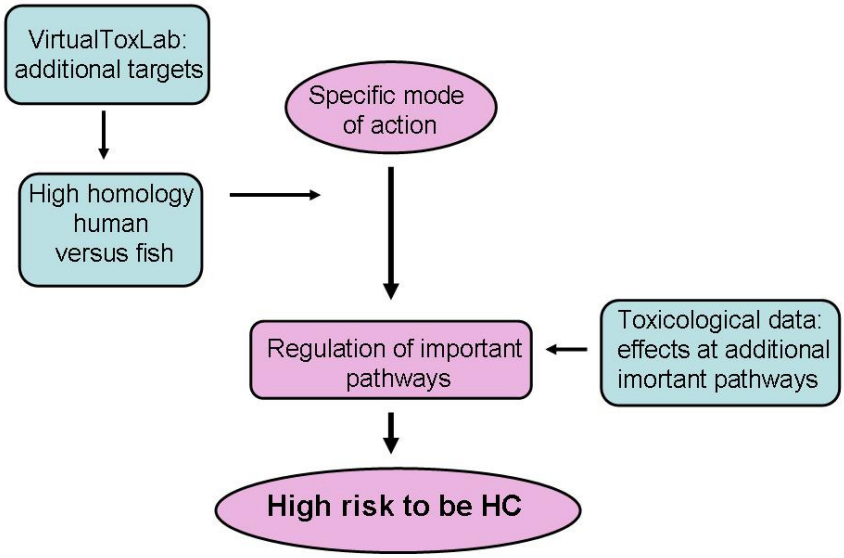

Fig. 8: Summary of our proposed approach for the identification of “HC”.

4. Environmental risk assessment: Phase II: environmental fate and effects analysis

For highly active substances (HC), the EMEA guideline recommends a tailored risk assessment stategy that addresses the specific mechanism of action of the compound. The project also aimed at addressing the question whether effects at low concentrations would be detected by the standard tests required in Tier A or if an alternative testing strategy is necessary.

Phase II, Tier A of the EMEA guideline requires the activated sludge, respiration inhibition tests (OECD 209), the algae growth inhibition test (OECD 201), the *Daphnia sp.* reproduction test (OECD 211), and the fish early life stage toxicity tests (OECD 210) for the aquatic compartment.

OECD 201

Alga, Growth Inhibition Test

The purpose of this test is to determine the effects of a substance on the growth of freshwater microalgae and/or cyanobacteria. Exponentially growing test organisms are exposed to the test substance in batch cultures over a period of normally 72 hours. In spite of the relatively brief test duration, effects over several generations can be assessed. The system response is the reduction of growth in a series of algal cultures (test units) exposed to various concentrations of a test substance. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures. For full expression of the system response to toxic effects (optimal sensitivity), the cultures are allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time to measure reduction of the specific growth rate. Growth and growth inhibition are quantified from measurements of the algal biomass as a function of time. Algal biomass is defined as the dry weight per volume, e.g. mg algae/litre test solution. The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of growth rate (e.g. 50%) is determined and expressed as the ErCx (e.g. ErC50). In addition, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined. Several species of non-attached microalgae and cyanobacteria may be used:
Green algae: *Pseudokirchneriella subcapitata,* (formerly known as *Selenastrum capricornutum*), ATCC 22662, CCAP 278/4, 61.81 SAG; *Desmodesmus subspicatus* (formerly known as *Scenedesmus subspicatus*) 86.81 SAG
Diatoms: *Navicula pelliculosa,* UTEX 664;
Cyanobacteria: *Anabaena flos-aquae,* UTEX 1444, ATCC 29413, CCAP 1403/13A; *Synechococcus leopoliensis,* UTEX 625, CCAP 1405/1

OECD 210

Fish, Early-Life Stage Toxicity Test

Tests with the early-life stages of fish are intended to define the lethal and sub-lethal effects of chemicals on the stages and species tested.

Principle of the test: The early-life stages of fish are exposed to a range of concentrations of the test substance dissolved in water, preferably under flow-through conditions, or where appropriate, semi-static conditions. The test is begun by placing fertilised eggs in the test chambers and is continued at least until all the control fish are free-feeding. Test duration will depend upon the species used, examples for different species are shown in Table 18. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration and hence the no observed effect concentration. The number of fertilised eggs at the start of the test should be sufficient to meet statistical requirements.

Species	Recommended test duration
Oncorhynchus mykiss	2 weeks after control are free-feeding or 60 days post-hatch
Pimephales promelas	32 days from start of the test or 28 days post-hatch
Danio rerio	30 days post-hatch
Oryzias latipes	30 days post-hatch

Table 18: Recommended durations of the fish early stage test for different fish species.

Test endpoints: Abnormal appearance: the number of larvae or fish showing abnormality of body form should be recorded at adequate intervals depending on the duration of the test and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several percent in the control(s) in some species.

Abnormal behaviour: abnormalities, e.g. hyperventilation, unco-ordinated swimming, atypical quiescence and atypical feeding behaviour should be recorded at adequate intervals depending on the duration of the test.

Weight: at the end of the test all surviving fish must be weighed.

Length: at the end of the test, measurement of individual lengths is recommended.

These observations will result in the following data being available for statistical analysis:

- Cumulative mortality;

- Numbers of healthy fish at end of test;
- Time to start of hatching and end of hatching;
- Numbers of larvae hatching each day;
- Length and weight of surviving animals;
- Numbers of deformed larvae;
- Numbers of fish exhibiting abnormal behaviour.

Recommended species are among freshwater fish: *Oncorhynchus mykiss, Pimephales promelas, Danio rerio*, *Oryzias latipes*
Saltwater fish:
Cyprinodon variegatus

OECD 211
Daphnia magna Reproduction Test

Principle of the test: The primary objective of the test is to assess the effect of chemicals on the reproductive output of *Daphnia magna*. To this end, young female *Daphnia* (the parent animals), aged less than 24 hours at the start of the test, are exposed to the test substance added to water at a range of concentrations. The test duration is 21 days. At the end of the test, the total number of living offspring produced per parent animal alive at the end of the test is assessed. This means that juveniles produced by adults that die during the test are excluded from the calculations. The reproductive output of the animals exposed to the test substance is compared to that of the control(s) in order to determine the lowest observed effect concentration (LOEC), and hence, the no observed effect concentration (NOEC). In addition, and as far as possible, the data are analysed using a regression model in order to estimate the concentration that would cause a x % reduction in reproductive output, i.e. ECx (e.g. EC50, EC20 or EC10).

OECD 209
Activated Sludge, Respiration Inhibition Test
The method described in this test guideline assesses the effect of a test substance on micro-organisms by measuring the respiration rate under defined conditions in the presence of different concentrations of the test substance. The purpose of this test guideline is to provide a rapid screening method whereby substances which may adversely affect aerobic microbial treatment plants can be identified and to indicate suitable non-inhibitory concentrations of test substances to be used in biodegradability tests. The respiration rate is the oxygen consumption of aerobic sludge or waste-water microorganisms expressed generally as mg

per litre per hour. EC 50 in this Test Guideline is the concentration of the test substance at which the respiration rate is 50% of that shown by the control under conditions described in this guideline.

Principle of the test: The respiration rate of an activated sludge fed with a standard amount of synthetic sewage feed is measured after a contact time of 30 minutes or 3 hours, or both. The respiration rate of the same activated sludge in the presence of various concentrations of the test substance under otherwise identical conditions is also measured The inhibitory effect of the test substance at a particular concentration is expressed as a percentage of the mean respiration rates of two controls. An EC 50 value is calculated from determinations at different concentrations. Activated sludge from a sewage treatment plant is normally used as the microbial innoculum for the test.

Recommended tests and the identification of potential "HC"

The characteristics of "HC" are first, they elicit effects on aquatic organisms at very low concentrations, and second, important pathways such as reproduction, development or cell proliferation are affected. To obtain data that could give hints to such properties of a test substance, the endpoints of the appropriate test should include reproductive success, embryonic development and cell proliferation. In the Tier A assessment, tests determining effects on reproduction are relevant in identifying "HC". The Daphnia reproduction test (OECD 211), gives information on the chronic potential of a test substance and its effect on reproduction. Acute and subacute toxicity and developmental effects may be detected in the fish early life stage test (OECD 210). During the Tier A assessment, the test on algae growth (OECD 201) may point to inhibitory effects on cell proliferation. If a substance elicits growth inhibition of algae, and affects the respiratory rate in activated sludge (OECD 209) at very low concentrations, this would be detected in the standard tests.

Is the fish early life stage testing ELS sufficient enough for the identification of "HC"?

An extended early life stage test with zebrafish (McAllister and Kime, 2003) showed in case of tributyltin (TBT) that effects on zebrafish in early development became apparent at

adulthood and were irreversible even after fish were held for 5 months in clean water. Exposure up to 70 days post hatch to 0.1 ng/L TBT yielded a male biased population and resulted in sperms lagging flagella. Exposure to 1 ng/L reduced sperm motility and exposure to 10 ng/L resulted in a complete loss of sperm flagella. But the alterations in sperm motility and assembly were examined 3 to 5 months after exposure to TBT. These results clearly show that effects induced through substances can be missed by performing fish early life stage tests only. In addition, this test last only a shorter period of time.

Bogers (Bogers et al., 2006) performed an extended fish early life stage test with fathead minnow exposed to methyldihydrotestosterone (MDHT). The no observed effect concentration (NOEC) for vitellogenin induction in males was above 1 µg/L MDTH for fish exposed 63 days post hatch. This NOEC was reduced by a factor of more than 3 when the fish were exposed for 114 days post hatch. For gonadal attachments the NOEC dropped from above 1 µg/L MDTH to below 100 ng/L when exposed 114 days instead of 63 days post hatch. This is another example showing that the fish early life stage test is not sufficient to monitor all relevant effects of substances. In particular, adverse effects on fertility and reproduction cannot be assessed by this short-term test.

In a prolonged ELS test with the marine flatfish sole (*Solea solea*), exposed to PCB 126 (pentachlorobiphenyl), Foekema (Foekema et al., 2008) showed that the fish early life stage test may underestimate the toxic potential of compounds with low acute toxicity such as PCB congeners. In a first experiment, eggs and larvae were exposed to PCB 126 until 15 days post fertilisation (15 dpf), the moment that all fish had become free-feeding. At the moment when all fish were free-feeding the LC_{50} ranged between 39 and 83 ng PCB 126/L. In a second experiment, eggs and larvae were exposed until 4, 8, 10 and 15 days after fertilisation (DPF) with PCB 126. Subsequently the development of the larvae was registered under further unexposed conditions.). With this prolonged observation period, the LC_{50} ranged from 1.7-3.7 ng/L PCB 126 for the groups exposed for 4, 8 and 10 dpf, respectively. This study indicates that ELS fish tests that are terminated shortly after the fish becomes free-feeding, underestimate the toxic potential of compounds with low acute toxicity such as PCBs.

Liao (Liao et al., 2008) showed that the sensitivity of fish to 17 beta-estradiol (E2) varies with different life stages. Rare minnows were exposed to E2 at environmentally relevant (5-100 ng/L) and high (1000 ng/L) concentrations and induction of vitellogenin (VTG) and gonad development were assessed. The LOEC for VTG induction in the adult stage was 25 ng/L E2, but in the larval and juvenile stage the LOEC was 100 ng/L. This clearly demonstrated that the VTG induction is more sensitive in the adult stage than in the larval/juvenile stage.

These data shows that the ELS test is not sufficient enough to monitor all effects of pharmaceuticals/chemicals on fish.

Conclusions

This study indicates among others that the toxicity is not only dependent on the exposure duration, but also on the life stage. The early life stage is not necessarily the most sensitive life stage. Hence, chronic fish toxicity tests and tests on fish fertility and reproduction are necessary for clear identification of “HC”.

Summary

The goal of the presented study was to develop a concept that enables the regulators to identify potential highly active compounds “HC” with the help of existing data. We discussed shortly the case of EE2 and gave a short description of the EMEA guideline.
To have a better overview about the existing pharmaceuticals, we made a catagorisation according to the mode of action of existing pharmaceuticals.
During the next step, we search the literature and data bases for existing “HC”. We made a summary of found pharmaceuticals that display effects at aquatic organisms at low concentrations. Following this, we identified important pathways and receptors that could be effected by “HC”. The main questions were: biological function of the pathway, evolutionary conservation of the receptor, existing pharmaceuticals that display effects at these pathways and ecotoxicological effects.
The main time, we spent for the identification respectively the development of concepts that enables the regulators to identify potential “HC”. We developed the mode of action concept with the main questions: what is the mode of action of a pharmaceutical, what is the degree of homology between the human drug target and the potential drug target in non-human species and how important for the organisme is the effected pathway. During the development of this concept, we discussed also the usefulness of existing toxicological data for the identification of “HC”. We made a detailed investigation of receptor aminosequence homology between human and non-human species. And we applied the mode of action concept using selected drugs to identify potential “HC”. In a next step, we described the fish plasma model developed by Huggett and we applied this model for selected pharmaceutical.

We also investigated whether QSAR models would be useful for the identification of HC. From the existing QSARs we described the VirtualTox lab in detail. We also used some pharmaceuticals to apply this QSAR model.. At the end, we compared the outcome of all three described concepts. The mode of action concept and the VirtualTox lab gave the same results. The fish plasma model identified two compounds as “HC” which were not identified by the other two models. But the reason for this, is the high environmental concentration of both compounds.

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