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Analysis of the Frequency of Kidney Toxicity in Preclinical Safety Studies using the eTOX Database

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1 INTRODUCTION

1.1 Introduction

While toxicity observed during preclinical stages of drug development continues to be the leading cause of drug attrition (1), a systematic evaluation of the frequency of a particular treatment-related toxicity finding across studies, the concordance between histopathological and clinical chemistry findings, the impact of study duration, species-specificity of a particular toxicity finding, and finally incidences in control animals, has been hampered by the lack of access to sufficiently large and representative data sets of high-quality in vivo preclinical safety data generated by pharmaceutical companies during drug development.

The Innovative Medicines Initiative IMI eTOX project was initiated to develop innovative strategies and novel software tools to better predict the safety and the side effects of new candidate medicines (2). Core to this project, the eTOX database was established by extracting in vivo preclinical safety data from systemic toxicity reports of the participating pharmaceutical companies. The IMI eTOX database, currently the largest and most detailed preclinical database, provides a unique opportunity to systematically analyze preclinical toxicity data across multiple companies and extract information, which may help improve testing and decision making in drug development.

The kidney is known as a main target of toxicity in preclinical studies and in the clinical setting. However, early detection of renal damage still poses a big problem to researchers and clinicians (3). This is especially striking when looking at the importance of kidney toxicity in clinical daily life and the massive public healthcare burden kidney injury and its related comorbidities impose (4-7).

1.2 The process of drug development

The process of drug development describes the procedure of identifying and validating a small molecule or biologic with the capability to lessen human disease, and subsequently introducing it to the market for clinical application. Research for new potentially promising molecules starts with the identification of substances interfering with a biological pathway associated with a particular

disease. These lead compounds can be identified from naturally occurring substances in plants, humans or animals as well as from targeted chemical synthesis as analogs of other compounds that are known to be effective against a particular disease or from random or receptor-targeted high-throughput screening (8).

Before testing the compound in humans, in vitro and in vivo assays, including biochemical, cell and animal models, are necessary to determine the efficacy and safety of the potential drug. Regulatory requirements call for in vivo safety studies in at least two animal species: rodent (rat or mouse) and non-rodent (dog, rabbit, nonhuman primate, or other suitable species). Typically, three dose groups are formed, with a low dose close to the pharmacologically effective dose with the aim of determination of a no observable effect level (NOEL) or no observed adverse effect level (NOAEL). A safety margin is established based on the NOAEL in the "most sensitive" of the tested species in order to account for differences between laboratory species and humans. Besides central nervous system, respiratory and cardiovascular monitoring of experimental animals, an extensive pathological assessment is carried out, involving organ systems such as heart, liver, kidneys, spleen, brain, etc. In preclinical testing, the route of administration for clinical studies has to be identified as it must match the route of administration in the definitive preclinical studies. Routes of administration can be divided into enteral (oral, buccal, and rectal) and parenteral administration, including injectable (intravenous, intramuscular, and subcutaneous), topical, and inhalational routes. If the proposed route is oral, the drug is typically administered as a bolus via gavage to rats and via gavage or capsule to dogs. The duration of administration and dose regimen must, at a minimum, match with the planned clinical protocol. Preclinical testing is conducted according to good laboratory practice (GLP) guidelines, which regulate how laboratory studies are performed (9-12). The preclinical phase of development alone lasts on average six to ten years (13).

The third stage of the drug development process involves clinical trials in humans, where tolerability, efficacy and safety of the compound are tested. This

stage can be divided into four phases: Phase I starts with tests of the drug in a small number of healthy volunteers in order to evaluate safety and the ideal dosage. Phase II trials involve a population of patients with the disease with the purpose of investigation of efficacy and safety of the agent in the target population. The final stage of development, phase III and IV, comprise studies in larger patient populations to further specify adverse reactions and efficacy (12, 14). When these studies are completed and safety and efficacy has been demonstrated in the intended patient population (i.e. the drug's benefits outweigh its risks), the pharmaceutical company can request approval from the regulatory agencies. From approximately 5,000 to 10,000 active substances under consideration in phase I, from a statistical view only one compound will receive approval after completion of the final clinical phase (15). However, even after authorization, drugs may be withdrawn from the market due to safety reasons. In total 464 drugs were withdrawn from the pharmaceutical markets between 1950 and 2017 (16). Figure 1 gives a graphic overview over the process of drug development.

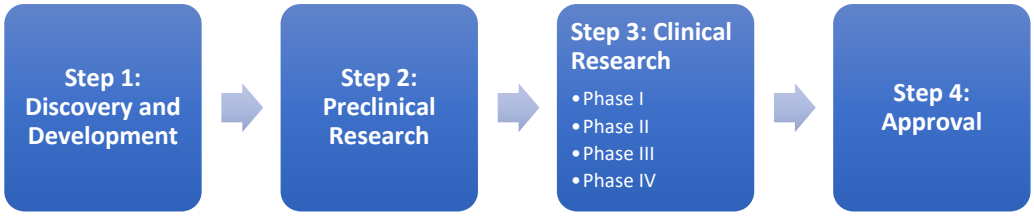


Figure 1 - The process of drug development (9-12). The procedure of developing a drug can be divided into four general steps. It starts off with the development of a chemical structure and trials with the compound in preclinical animal research. After that the substance has to pass through four clinical phase trials before it can be approved.

1.3 Challenges in current drug development

There is ample evidence of the value of pharmaceutical research and development of new drugs in order to help treat and cure diseases: For instance, since peaking in the 1990s, cancer death rates have declined 23% (17). Approximately 83% of survival gains in cancer are attributable to new treatments, including medicines (18). Similarly impressive numbers are available for AIDS: Since the introduction of highly active antiretroviral treatment (HAART), the AIDS

death rate has dropped by 87% (19). As a consequence of HAART and all the medical innovations that followed, approximately 862,000 premature deaths were avoided in the United States alone (20).

Despite these achievements, a considerable decline in success rates in clinical testing over the last decades can be observed, even with the industry's increased efforts to address those low success rates (21).

Currently, drug discovery and development is very costly, time consuming and inefficient: From thousands of new chemical leads, only a few will result in approval by the end of development (10). The average time to develop a new drug is estimated between 10 to 15 years (22), with companies typically spending tens to hundreds of millions of U.S. dollars for each drug launch on the market (23). Even after approval only 2 out of 10 marketed drugs are reported to return revenues that match or exceed research and development costs (24). In Germany alone, research-based pharmaceutical companies spend around 5 billion euros per year on research and development, equaling almost 14 million euros daily (15). In fact, eight of the 20 companies worldwide with the highest research expenditures are in the pharmaceutical industry (15).

Although research expenditures of pharmaceutical companies continue to mount, there is no considerable increase in the number of approvals of new drugs in the last two decades (Figure 2: total research and development costs, PhRMA member companies (25) vs. number of FDA approved drug products (26)). The potential reasons for the decline in research and development productivity are intricate: *Cook et al.* name for instance rising clinical trial expenditures and adjustments in the regulatory landscape, an increased aversion to risk, a challenging reimbursement and payer environment, and the fact that companies are attempting to find medications for more complex and difficult-to-treat illnesses, and/or improve on existing therapies that already have substantial efficacy (27). Besides lack of efficacy, drug safety remains a major cause of drug attrition. Approximately one third of all drug development projects already fail in the preclinical stage due to toxicity issues (28): The most frequent drug safety

issues during preclinical development are cardiovascular toxicity (17%), hepatotoxicity (14%) and renal toxicity (8%) (29, 30).

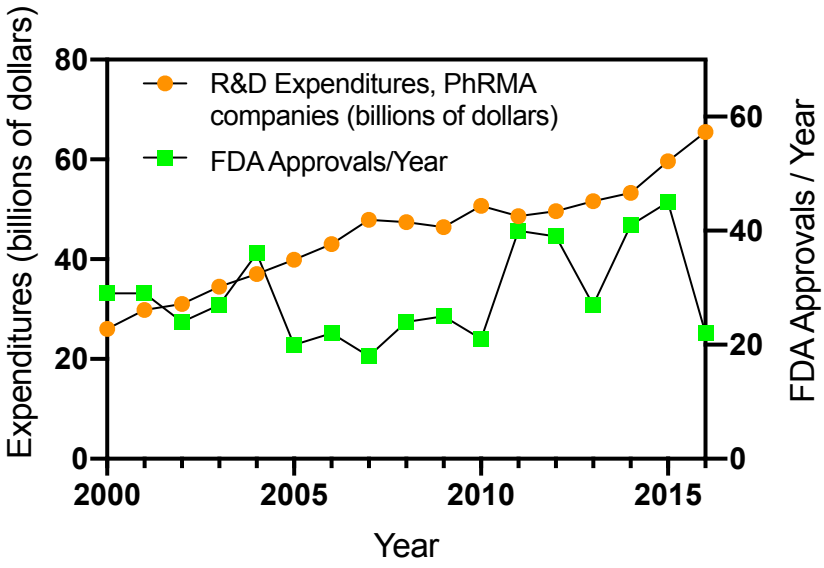


Figure 2 - Total research and development costs, PhRMA member companies vs. Number of FDA approved drug product. The graph compares the yearly amount of money put into research and development by the PhRMA (Pharmaceutical Research and Manufacturers of America) companies (in billions of dollar) with the yearly number of drug approvals by the American Food and Drug Association (FDA) from the year 2000 until 2016. It reveals that even though research expenditures have recently been consistently raised the number of approvals does not seem to increase.

These facts and figures show that new approaches to improve knowledge, understanding and prediction of organ toxicity in preclinical research are critically needed. In order to “tackle” this problem, a large amount of preclinical safety data is needed. This is where the eTOX database tries to break ground through merging the poorly used but highly relevant preclinical drug safety data of compounds into one large scale database to enable closer insight and investigation.

1.4 The IMI eTOX database and its objectives

Currently, the safety aspects of drug development are addressed by running numerous animal studies. In recent decades the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has produced a set of guidelines describing the requirements in pharmaceutical drug safety testing and the extent of toxicity data needed for approval.

The exact requirements depend on many factors such as drug type (biotechnology-derived, anticancer-pharmaceutical, gene therapy product), target group (children, pregnant women) and planned duration of drug administration (chronic toxicity testing, carcinogenicity testing). Depending on these factors, an array of studies is to be conducted.

The ICH guideline M3 (R2) on “non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals” describes, inter alia, the regularly needed sets of preclinical studies in order to begin with clinical trials: These requirements encompass a “core battery of safety pharmacology studies includ[ing] the assessment of effects on cardiovascular, central nervous and respiratory systems, and should generally be conducted prior to human exposure” (31). Likewise, “in vitro metabolic data for animals and humans, and exposure data in animals should be evaluated prior to initiating human clinical trials” (31). In order to support clinical development trials up to two weeks in duration, repeated dose toxicity studies in two species (one non-rodent) are to be carried out for a minimum duration of two weeks. The maximum duration of a clinical trial between two weeks and six months should not exceed the minimum duration of the preclinical study duration in neither rodent nor non-rodent. It is furthermore described as “preferable to evaluate local tolerance by the intended therapeutic route as part of the general toxicity studies” (31). In order to estimate a first maximum dose in human trials, the No Observed Adverse Effect Level (NOAEL) needs to be determined in nonclinical safety studies in the most sensitive and relevant animal species. Additionally, “all new human pharmaceuticals should be evaluated for the potential to produce immunotoxicity using standard toxicity studies and additional immunotoxicity studies“ (31).

Requirements prior to marketing authorization depend on the duration of the planned maximum treatment time (Table 1). For example, the allowed use of a pharmaceutical can be a maximum of two weeks, if the duration of repeated dose toxicity studies equals one month in rodents and non-rodents (31).

Table 1 - Duration of preclinical repeated dose toxicity studies to support marketing in all regions.

Duration of Indicated Treatment in Humans	Minimum treatment duration in rodent	Minimum treatment duration in non-rodent
Up to 2 weeks	1 month	1 month
>2 weeks to 1 month	3 months	3 months
>1 month to 3 months	6 months	6 months
>3 months	6 months	9 months

Moreover, the ICH-guideline states that carcinogenicity studies "should be performed for any pharmaceutical whose expected clinical use is continuous for at least 6 months" (32). The objective of carcinogenicity studies is the identification of "a tumorigenic potential in animals and to assess the relevant risk in humans" (32). For carcinogenicity studies, the basic scheme comprises one long-term rodent study (with choice of species based on considerations including pharmacology, repeated-dose toxicology, metabolism, toxicokinetics and route of administration) plus one additional in vivo test (either "Short or medium-term in vivo rodent test systems" or "A long-term carcinogenicity study in a second rodent species") (32). "In the absence of clear evidence favoring one species, it is recommended that the rat be selected" for the long-term rodent carcinogenicity study (32). Route of exposure in animals is recommended to match the intended clinical route (32).

ICH-guidelines for safety pharmacology studies suggests the use of animal models as well as ex vivo and in vitro preparations as test systems (33). Concerning the experimental design, the "size of the groups should be sufficient to allow meaningful scientific interpretation of the data generated", it should include control groups, "the expected clinical route of administration should be used when feasible" and "exposure to the parent substance and its major metabolites should be similar to or greater than that achieved in humans" (33). Dose levels "should include and exceed the primary pharmacodynamic or therapeutic range" (33). The duration of safety pharmacology studies should be based on the results of pharmacodynamic effects in previous safety pharmacology studies. Safety testing should always include the vital organ systems such as the cardiovascular, respiratory and central nervous system plus additional possibly relevant, individual organ sites. In order to ensure the quality and reliability of

non-clinical safety studies all studies should be conducted in compliance with GLP (good laboratory practice) (33).

Concerning the renal/urinary system the ICH-guideline for safety pharmacology states that “the effects of the test substance on renal parameters should be assessed (33). For example, urinary volume, specific gravity, osmolality, pH, fluid/electrolyte balance, proteins, cytology, and blood chemistry determinations such as blood urea nitrogen, creatinine and plasma proteins can be used” (33).

The Europeans Medicines Agency “Guideline on repeated dose toxicity” includes the following statements (34). Concerning the animal species, it is specified that “Within the usual spectrum of laboratory animals used for toxicity testing, the species should be chosen based on their similarity to humans with regard to pharmacokinetic profile including biotransformation (34). [...] In general, repeated dose toxicity studies shall be carried out in two species of mammals, one of which must be a non-rodent” (34). Furthermore “The dose regimen and route of administration should be chosen based on the intended clinical use with the aim to obtain sufficient exposure of the animals to the substance and its metabolites” (34). Regarding the different dose levels during repeated toxicity testing, guideline recommendations are as follows:

“In general, the treatment should include:

- appropriate control group(s); in special cases a positive control group may be necessary
- a low dose, sufficient to produce a pharmacodynamic effect or the desired therapeutic effect, or result in systemic exposure comparable with that expected at the intended clinical use
- a high dose, selected to enable identification of target organ toxicity or other non-specific toxicity, or until limited by volume of dose
- an intermediate dose, such as the geometric mean between the high and the low dose (34).”

These studies, using many laboratory animals ranging from rat to dog and monkey, are time consuming and are performed before and in parallel to human

clinical studies. Toxicological and pharmacokinetics observations in animal studies are interpreted and extrapolated to human, which decide on start and continuation of clinical studies with the ultimate goal of providing new, more efficacious drugs to the human population. The discovery of unexpected or unacceptable toxicological side effects can either greatly delay the development of new drugs or even stop it altogether, wasting years of research, vast amount of resources, time and money.

Over the last few decades, the pharmaceutical industry has routinely produced preclinical in vivo toxicity data in order to assess the safety of their proprietary drug candidates. Irrespective of whether or not the drug candidate ultimately made it to the market, these data are usually not made available to the public and rest in company archives.

> "This is despite the fact that there is a lot of knowledge to be extracted from it, and this knowledge can be used to improve the process and the speed with which we develop new drugs. eTOX was about finding a way to reuse this wealth of data" (35).
>
> *Francois Pognan of Novartis, eTOX project coordinator*

In a joint effort the eTOX project[1] was initiated in order to establish a large, comprehensive database of high-quality in vivo preclinical toxicity data (2) with the intention of taking advantage from this extensive amount of raw data.

The project, funded by the European Union's Innovative Medicines Initiative (IMI), started in 2010 and was completed by the end of 2016. In total thirteen pharmaceutical companies, eleven academic institutions and six small- and mid-sized enterprises collaborated (Table 2) for the purpose of building the database called "eTOX sys" (2).

[1] Full title: "Integrating bioinformatics and chemoinformatics approaches for the development of expert systems allowing the in-silico prediction of toxicities"

Table 2 - Partners of the eTOX project. The eTOX partners can be divided into private EFPIA (European Federation of Pharmaceutical Industries and Associations) companies and into a public sector comprising universities, research organizations, public bodies and non-profit groups as well as small and medium-sized enterprises (SMEs).

Private	Public	
EFPIA Companies	**Academic Institutions**	**SMEs**
Novartis Pharma AG (project leader)	Fundació Institut Mar d'Investigacions Mèdiques (project management, co-ordination of public partners)	Lhasa Limited
AstraZeneca AB	Fundación Centro Nacional de Investigaciones Oncológicas Carlos III	Inte:Ligand GmbH
Bayer Pharma AG (deputy project leader)	European Molecular Biology Laboratory	Molecular Networks GmbH
Boehringer Ingelheim International GmbH	Liverpool John Moores University	Chemotargets SL
Esteve	Technical University of Denmark	Lead Molecular Design S.L.
GlaxoSmithKline Research and Development LTD	Universität Wien	Synapse Research Management Partners SL
Janssen Pharmaceutica NV	VU University Amsterdam	
H. Lundbeck A/S	Fraunhofer Gesellschaft Zur Foerderung Der Angewandten Forschung E.V., München, Germany	
Pfizer Limited	Erasmus Universitair Medisch Centrum Rotterdam	
F. HOFFMANN-LA ROCHE AG	University of Leicester	
UCB Pharma SA	Universitat Politècnica de Valencia	
Sanofi-Aventis Deutschland GmbH		
Institut de Recherches Internationales Servier		

Establishment of the eTOX database required proprietary company data to be protected and nomenclature to be standardized (e.g. 181 terms for Phosphorous including: Inorganic phosphorous, Phospho-rous, Phosphate, Phos, PO4-IN, IN_PHOS, Phosporous (P3-), INP: Inorg. phosphate) (36).

The eTOX database currently contains over 1,900 compounds and 8,000 associated repeat dose studies derived from proprietary data provided by the pharmaceutical eTOX partners (37). Additionally, the database is supplemented by incorporation of publicly available, high quality toxicology data. The IMI eTOX database includes toxicity data (body weight, clinical signs, clinical chemistry, hematology, hemostasis, organ weight, gross pathology, histopathology, urinalysis) from subacute, subchronic and chronic toxicity studies conducted in different species, including rats, mice, dogs, monkeys and minipigs.

1.5 Drug-induced renal toxicity

Drug toxicity in general has been reported to account for more than 30 percent of the attrition of substances in drug development in preclinical animal studies (1). In a strategic review of its small-molecule drug portfolio undertaken by AstraZeneca in 2011, the company attributed the majority of preclinical safety closures to specific organ toxicities with renal toxicity (8%) being the third most common cause for such closure. This was only exceeded by cardiovascular toxicity (17%) and hepatotoxicity (14%) (29). The reasons for safety failure were described to be consistent with those observed by others in industry (30). These numbers underline the contribution of kidney toxicity observed in preclinical toxicity studies as a major road-block to development of new drugs.

Reasons for the difficulty with the issue of nephrotoxicity in preclinical development and subsequently in clinical trials are diverse: A significant problem is caused by the fact that adverse findings observed in one species are not always evident in other species. It has been reported that only 40 to 60 percent of adverse findings in animals are predictive of toxicities in human trials. Another obstacle to overcome is the lack of monitoring for early indications of kidney injury (except renal biopsies) in human subjects during clinical development. This inability can possibly lead to the suspension of the development of compounds found to cause renal damage in preclinical animal studies, even if the relevance of the findings to humans has not been established (38, 39).

Drug-induced nephrotoxicity not only is a common problem in preclinical development of future drugs, but also for human patients in hospitals. Due to the kidney's disproportionately large blood flow (20–25% of resting cardiac output) and its function to metabolize and excrete waste products and xenobiotics, the occurrence of kidney injury in patients is often caused through the interaction of medications with various parts of the nephron (40-43). In fact, potentially nephrotoxic marketed drugs are contributing factors in at least 25 percent of cases of acute kidney injury in critically ill humans (44).

1.6 Renal toxicity findings

The following paragraphs summarize renal histopathological findings frequently observed in toxicity studies in experimental animals.

1.6.1 Accumulation, hyaline droplets

Hyaline droplets are abnormally large, dense secondary lysosomes (45) that are normally present in the proximal tubule epithelia of young, mature male rats (46). However, their biological importance is not well understood (47). The number of hyaline droplets has been described to increase in rats after treatment with substances which can interfere with renal protein secretion and protein metabolism (48). Moreover, the occurrence of hyaline droplets has been linked with the development of renal tubular necrosis in humans and has been suggested as a possible marker (47). They also have been found in the proximal tubular epithelium in biopsies from patients with general renal disorders (47).

1.6.2 Atrophy, tubular

The term “tubular atrophy” refers to the loss of either entire tubules or single tubular epithelial cells (49). In rats and mice with progressive renal failure it is known as a constant feature and often is observable in late stage chronic progressive nephropathy. According to *Frazier et al “*apoptosis of the renal tubular epithelium has been shown to be an integral factor involved in the pathogenesis of tubular atrophy in rodent models of progressive renal failure. Increased reactive oxygen species and a renal environment favoring proapoptotic signals contribute to cell death” (50). Furthermore, transforming growth factor beta has been demonstrated to play a vital role in the induction of fibrosis accompanying tubular atrophy (51).

1.6.3 Basophilia, tubule

Tubular Basophilia is considered among the most frequently encountered manifestations of toxic damage in preclinical studies. It is known to represent initial and some of the earliest evidence of nephron injury. Histologically it presents as a tinctorial change in the epithelial cytoplasm (52). Tubular regeneration

may be reflected by an increase in tubular basophilia, but tubular basophilia might also be a sequel to degenerative conditions, for instance after application of nephrotoxic agents, especially those that cause acute necrosis (53). Under regenerative circumstances the basophilia disappears, as the cell becomes differentiated renal tubular epithelium after the repairing process (54). While the incidence and severity of tubule basophilia can increase in response to various chemical agents (54), it may also occur spontaneously in untreated animals. There is also evidence that it is one of the precursor lesions and main features of chronic progressive nephropathy in the rat kidney (55). Moreover, an increased appearance of tubular basophilia has been linked with several other kidney diseases such as pyelonephritis and kidney nephroblastoma (50). Tubular basophilia may also occur as a minimal focal lesion in up to 40% of control beagle studies (50).

1.6.4 Dilation

Renal tubular dilation is an expansion of the renal tubules. This may be due to a direct toxic effect of a xenobiotic agent on the renal epithelium. Tubular dilation often accompanies other forms of renal damage (e.g., necrosis or degeneration). In rats, for example, chronic hypokalemia has been linked to renal tubular dilatation as a consequence of luminal obstruction by hypertrophy and hyperplasia of collecting duct epithelium, while tubular dilation has also been associated with the occurrence of renal ischemia (56). The existence of some dilated tubules in rodents is histologically normal (48, 57). In male and female laboratory rats dilated tubules are part of the spontaneous rodent-specific, age-related disease called chronic progressive nephropathy that can represent a significant confounder for analysis of renal histopathology in toxicology studies. Male gender poses a primary risk factor for the occurrence of the disease complex of chronic progressive nephropathy (55).

1.6.5 Hyperplasia

The term “kidney hyperplasia” denotes the proliferation of cells in the kidney. There are two types of renal tubular hyperplasia: Simple tubular hyperplasia

and atypical hyperplasia. Simple tubular hyperplasia is characterized by an increased number of epithelial cells limited to a single cell layer. In contrast, atypical hyperplasia refers to two or more cell layers (48). In humans, contralateral hyperplastic kidney growth can be linked to increased renal function and has been observed in cases of post-obstructed, poorly functioning kidneys as well as after unilateral nephrectomies (58, 59).

1.6.6 Inflammation, neutrophilic

In rodents, inflammation can be divided into five different categories: acute, suppurative, chronic, chronic active, and granulomatous. The classification of different types of inflammation is based on the immune cell type, which infiltrates primarily into the affected tissue. Neutrophilic granulocytes are predominantly present in acute and suppurative inflammation (60).

1.6.7 Necrosis

Morphologic presentation of nephrotoxicity at the cellular level can occur as necrosis, apoptosis, or hyperplasia (55). Necrosis refers to the death of cells in an organ or tissue, according to *Walker et al.* (61). In necrosis, loss of cell volume homeostasis leading to a swelling of plasma and organelle membranes provokes a rupture and dissolution of organized cell structure. Necrotic cells are typically enlarged and less intensely eosinophilic than normal (55). Necroptosis, a programmed form of necrosis, has been detected in experimental acute kidney injury caused by renal ischemia-reperfusion injury, rhabdomyolysis, and nephrotoxicity caused by cisplatin or crystals (62-66).

The distinction between certain types of necrosis is essential for the content of this work:

Acute alteration of proximal tubular epithelium, induced by xenobiotics in the human and laboratory animals, can lead to acute tubular necrosis. Patterns of alteration in the cell depend on the toxic agent and thus on its mechanism: Causes vary from clear direct toxic effect (gentamicin, cisplatin), and hypoxic damage induced by altered medullary microcirculation or enhanced metabolic

requirements (NSAIDs, radiocontrast agents, cyclosporine) or both, to injury caused by free-radical generation (55).

Renal papillary necrosis histologically is marked by coagulative necrosis of the renal papilla and the medullary pyramids. This can lead to fibrosis and calcification and eventually to renal failure (67). In preclinical safety testing, renal papillary necrosis is a relatively common form of toxicity encountered, also being encompassed under the term analgesic nephropathy (55). In rats, this form of necrosis has been linked to diabetes induced by streptozotocin (68) as well as to the administration of NSAIDs and analgesics (55). Even though renal papillary necrosis risk can effectively be determined through the measurement of monoclonal antibodies to rat renal papillary tissue (RPA-1 and RPA-2) in the urine of laboratory rats, histopathology is the commonly used method in routine toxicology testing for identification of renal papillary necrosis in rats (55). In human patients main causes for this type of necrosis include analgesic nephropathy (69, 70), the prolonged use of NSAIDs (71), sickle cell nephropathy (72, 73), as well as diabetes mellitus (67).

The term “single cell necrosis” is a general designation that describes the occurrence of noncontiguous cells in a tissue that are characterized by cell and nuclear swelling and pale cytoplasm (74).

1.6.8 Glomerulosclerosis

Glomerulosclerosis (GS) refers to scarring or hardening of the glomeruli in the kidney. Damaged glomeruli cannot filter the plasma adequately in order to produce glomerular filtrate. As a result, proteins from the blood leak into the urine rather than remaining in the bloodstream, which leads to proteinuria. The level of protein in the blood declines and the “decrement in oncotic pressure leads to excess filtration of fluid from the intravascular space to the interstitial space”, causing the development of edema and an increase of body weight (75). Urinary protein loss also stimulates an increased LDL synthesis by the liver (76). An increase in serum triglycerides has also been linked to GS (77). Furthermore, red blood cells can escape through the injured glomerular filtration barrier leading to hematuria (78).

In rats, GS is considered as part of the disease pattern called chronic progressive nephropathy (CPN): Whereas the precise etiology of CPN and the mechanism(s) underlying its pathogenesis remain unknown, it is acknowledged as a rodent-specific entity, and not only a degenerative disease, but also has regenerative aspects with a high cell proliferative rate in affected tubules.
CPN moreover has been described as an age-related disease modulated by several extrinsic aspects including diet and chemical exposures. A number of factors, such as dietary manipulations, have been demonstrated to significantly modify the expression of CPN (55). Amongst the chemicals exacerbating CPN are hydroquinone (79), ethyl benzene (80) and quercetin (81).

In humans, sclerosis of the glomerulus is a common histopathological and structural feature of chronic kidney disease (82).
In the US alone, about 20 million people suffer from kidney disease. Even though causes of renal failure vary, the glomerular filtration barrier repeatedly is the target of injury (83). Proteinuria alone has been shown to be an independent factor of risk for cardiovascular morbidity and mortality, visible through an almost 6-fold increased incidence of myocardial infarction in such patients (84).

Both children and adults can develop GS possibly resulting from various types of kidney conditions. One frequently encountered type of GS is called focal segmental glomerulosclerosis (FSGS). FSGS, the most common cause of nephrotic syndrome in US adults, accounts for about 4% of end-stage renal disease (85). This chronic kidney condition has been linked to infections, such as HIV infection (86) and Parvovirus B19 (87), but also to drug use: FSGS has been reported in patients receiving exogenous interferon therapy (88) or patients treated with pamidronate, a member of the class of bisphosphonates (89). In addition, FSGS has been observed after treatment with sirolimus, an inhibitor of the mammalian target of rapamycin (mTOR) pathway (90).

1.7 Clinical chemistry parameters relevant to nephrotoxicity testing

In preclinical toxicology testing, microscopic assessment of the kidney continues to be the gold standard. However, it has the limitation, that kidney

function cannot be monitored adequately over a certain amount of time. There is a wide range of biochemical markers, which have been used as parameters for the evaluation of nephrotoxicity in preclinical testing in the last decades. In general terms, a safety biomarker is defined as any analyte that can be quantified to indicate an adverse response to a test agent. Biochemical parameters of renal injury are valuable as they empower researchers and doctors not only to monitor kidney function or injury repeatedly. Essential qualities for renal biomarkers are non-invasiveness, specificity for kidney, reliability, inexpensiveness and low inter-individual variability (55). Biomarkers of nephrotoxicity comprise on one side traditional markers, which have been in use for many years in laboratory animals and humans and can be evaluated in routinely collected serum and urine samples. Amongst those biomarkers are serum creatinine, blood urea nitrogen, glucose, leakage of enzymes including urinary N-Acetyl-β-(D)-glucosaminidase and glutathione-S-transferases, total urinary protein and albumin, or low molecular weight proteins such as β2-microglobulin.

On the other side there are the so called “novel biomarkers” such as kidney injury molecule-1 (KIM-1), clusterin, cystatin C, trefoil factor 3 (TFF3), neutrophil gelatinase-associated lipocalin (NGAL), and liver-type fatty acid- binding protein (L-FABP) (55). Specifically, combinations of these novel biomarkers have been demonstrated to show high potential and improved sensitivity in preclinical safety assessments for evaluation of kidney function in comparison to traditional renal biomarkers (91). In fact, in 2010, seven renal safety biomarkers including kidney injury molecule-1, clusterin, albumin, total protein, β 2-microglobulin, cystatin C and trefoil factor 3 in urine have already been qualified for limited use in nonclinical and clinical drug development to help guide safety assessments (92). However, these novel biomarkers are not yet routinely used (93), and were not contained in the eTOX database. Thus, an evaluation of these novel biomarkers in relation to the different toxicity findings could not be included in this work.

1.7.1 Serum urea

Urea, a nitrogenous waste product produced from protein breakdown, is nearly completely eliminated by the kidneys in urine (94). Similar to serum creatinine (chapter 1.7.2), serum urea is routinely used as a parameter to detect acute kidney injury as well as chronic kidney disease. However, at the same time it is recognized as a suboptimal serum marker due to the fact that serum urea lacks sensitivity: In the clinic, serum urea is considered only a late indicator of a decline in renal function through renal injury as it does not become significantly elevated until at least one-half of the kidney mass has been comprised (55, 95). Moreover, the rate of urea production is not constant and is influenced by many physiological functions independent of kidney function (e.g.: upper gastrointestinal hemorrhage, protein intake, high-dose steroid therapy, catabolic state, volume status) (96-98).

1.7.2 Serum creatinine

Creatinine is derived from the metabolism of skeletal muscle and from dietary meat consumption (95). Just like serum urea, serum creatinine is routinely used as the main serum marker to monitor kidney function (99), but is known to lack sensitivity as well as predictive value (93, 100). In a recent study, *Steubl et al* reported that serum creatinine levels were increased only after a reversible or irreversible damage of about 40–50 percent of the renal parenchyma (101). Serum creatinine is also known to be influenced by nonrenal factors independent of kidney function, for instance: age, sex, race, muscular mass, nutritional condition, infection and volume of distribution (102, 103). Despite these limitations, serum creatinine is still considered to be one of the most important renal markers in preclinical toxicology (55). Moreover, in the absence of (not yet) routinely used and cost-effective markers with improved predictivity, its clinical relevance in human populations is immense as an increase in serum creatinine has been shown to be a risk factor for mortality in hospitalized (104) as well as in intensive care unit patients (105). Even a small acute increase in serum creatinine has been demonstrated to have lasting impact on long-term mortality (106).

1.7.3 Serum albumin

Synthesis of albumin takes place in the hepatocytes. It is the most common plasma protein. Serum albumin provides oncotic support, acts as a transport protein and is an essential amino acid reservoir (107, 108). A decreased renal reabsorption of albumin due to impaired kidney function results in a higher excretion of albumin in the urine. In severe cases, this may also result in lower serum albumin levels (55). In rats, lower levels of serum albumin have been associated with the development of nephrotic syndrome after repeated administration of haemophilus pertussis vaccine within toxicity studies (109).

In humans, hypoalbuminemia has not only been described as an independent predictor of acute kidney injury and of death following acute kidney injury (110), but is also associated with increased morbidity and mortality (111). Lower serum albumin levels moreover have been observed to be strongly and independently linked with a decline of kidney function in elder humans (112). A reduction in serum albumin levels can also have extrarenal causes: Liver diseases such as cirrhosis or hepatitis lead to decreased albumin synthesis in the liver and thus to reduced serum albumin levels (108).

1.7.4 Serum phosphate

Phosphate is a macronutrient that is indispensable for human metabolism since it is needed for several cellular functions, for instance structure, signaling pathways, skeletal mineralization, and energy production (113). Inorganic phosphorus for example is needed in biological molecules such as DNA and RNA, where it forms part of the structural framework of these molecules. Furthermore, phosphate is made use of in living cells in order to transport cellular energy via adenosine triphosphate (ATP) (114). Additionally, it plays a central role in the pentose phosphate pathway, which leads to the production of NADPH in mammalians in order to protect against the toxicity of reactive oxygen species (115). Phosphate also is needed for cellular signal transduction, for example activation of the extracellular-signal-regulated kinase (ERK) pathway (as one of the major signaling cassettes of the mitogen activated protein kinase [MAPK] signaling pathway) is induced by an increase in extracellular phosphate (116).

For the maintenance of normal serum phosphate levels, many factors are in play. They include nutrition and absorption of dietary phosphate in the gastrointestinal system as well as reabsorption and excretion in the kidney (113). In healthy rat kidneys, phosphate balance is determined through active transport in the proximal tubule. The phosphate glomerular filtrate concentration is 90 percent of the plasma level: Two thirds of filtered phosphate are reabsorbed in the proximal tubule, whereas 20 percent usually is cleared in the urine (55). Additionally, phosphate reabsorption is highly influenced by parathyroid hormone, fibroblast growth factor 23 and dietary phosphate (113).

Neves et al. linked hyperphosphatemia in rats with myocardial hypertrophy, impaired renal function, and adverse effects on bone remodeling (117). More recently *Zhang et al.* again demonstrated that elevated serum phosphate levels were found to significantly influence renal function and bone density causing chronic renal failure alongside a lower osseous density (118).

In healthy individuals even slight hyperphosphatemia has been related to increased morbidity and mortality (study with 60 months of follow-up) (119), while at the same time elevated serum phosphate levels have been linked with an increased risk of cardiovascular events and mortality in patients with chronic kidney disease (120, 121). Hyperphosphatemia has been observed in patients with stage four chronic kidney disease and is known to intensify further in end-stage kidney disease (chronic kidney disease, stage 5) (122). Also, levels of increased phosphate are associated with acute renal failure caused by an inability of the kidneys to excrete phosphate load (123). Hypophosphatemia, for example possibly caused through a global defect in the tubule within the Fanconi syndrome, can lead to osteomalacia in adults and to rickets in children (124). Also, a number of therapeutic drugs are toxic to the kidney proximal tubule and can cause the renal Fanconi syndrome (cisplatin, ifosfamide, tenofovir, sodium valproate, aminoglycoside antibiotics, deferasirox) (126). The loss of water and electrolytes observable in this condition causes symptoms including thirst, fatigue, weakness, and polyuria. A wide variety of signs and neuromuscular symptoms such as paresthesia, tremor, and muscle weakness are accompanied by

hypophosphatemia, particularly with serum phosphorus values lower than 1 mg/dL. Furthermore, impaired myocardial contractility can go along with severe hypophosphatemia and even cases of rhabdomyolysis can rarely be brought about through hypophosphatemia in humans (125). In chronic kidney disease renal tubular reabsorption of phosphate has been stated as an effective marker to monitor specifically the function of the proximal tubule (126). In clinical practice hypophosphatemia is commonly missed due to nonspecific signs and symptoms, however it has been described to cause considerable morbidity and to contribute to mortality (127, 128).

1.7.5 Serum triglycerides, cholesterol and protein

If kidney damage or kidney disease causes an increased permeability of the glomerulus, protein loss increases, and proteinuria occurs. Initially this results in an increase in lipoprotein synthesis as a reactive compensation mechanism. At the same time, there is a loss of lipoprotein lipase, an enzyme which catalyzes the breakdown of triglycerides from lipoproteins. These processes lead to hyperlipidemia and dyslipidemia. While protein loss leads to reduced serum protein levels, cholesterol and triglycerides increase due to the shift in the ratio of blood lipids (129).

1.8 Animal species

In preclinical toxicology testing, safety studies typically follow the two-species approach, consisting of rodents (typically rats) and a non-rodent species, such as dogs, non-human primates, rabbits and minipigs. The two-species approach is widely considered to increase the predictivity of human toxicity based on animal studies. For instance, *Olson et al* reported that non-rodent data from either non-human primates or dogs taken together with rodent data predicted human toxicity in 71% of all cases, while rodent data alone only predicted 43% of human toxicities (14). However, there are also studies that suggest that the two-species approach or generally animal studies in non-rodents do not afford any statistically measurable advantages. *Matthews* (2008) concluded that “the data provide no statistically credible evidence that these animal models

[dogs and monkeys] contribute any predictive value, either separately or in combination" (130).

Nevertheless, the dog is commonly regarded as the first choice of non-rodent species. Within the species of dogs, the beagle continues to be the main breed used in laboratories for preclinical toxicity studies. This is because beagles are purpose-bred and easily available and at the same time knowledge on their physiology is extensive (131).

Wistar rats encompass a number of rat substrains and are counted amongst the currently most frequently used rat strains for laboratory research (132). One of the advantages of the use of Wistar rats in preclinical development is that the background incidence of microscopic changes in Wistar rats is significantly lower than that of others (e.g.: Sprague-Dawley rats), in part due to the lower incidence of chronic progressive nephropathy in this rat strain (55). The outbred Wistar and Wistar Han strains are utilized extensively in Europe for preclinical development studies even though for neither of those strains a considerable amount of published data of historical control pathology and clinical pathology is available (133). Even within the Wistar strain, laboratory parameters and behavior has been demonstrated to vary (132).

1.9 Specific objectives and description of work

The overall objective of this dissertation is to contribute further knowledge in the field of preclinical compound-induced nephrotoxicity through mining the eTOX database and its preclinical studies regarding the occurrence of frequent renal histopathological findings (necrosis, glomerulosclerosis, etc.) in order to better understand and manage the development of certain toxicity findings and safety liabilities and their mechanisms of toxicity in preclinical development.

This work aimed at detecting the frequency of specific compound-induced nephrotoxicities in beagle dog and Wistar rat oral gavage studies and further to compare these frequencies with the incidence of histopathological findings in control animals in 28-day oral gavage studies. The exact background incidences of the selected renal findings were determined through database searches revealing the total number of control animals in four-week studies in

Wistar rats and beagle dogs each, followed by searches for the specific histopathologies in control animals of these studies before being able to calculate the respective background incidences. Information on the background incidence makes a crucial difference if a renal lesion occurs after administration of a compound. This is due to the fact that the study pathologist has to evaluate whether a histopathological lesion occurred spontaneously or whether it was provoked through administration of a (potentially toxic) substance. Furthermore, the knowledge of the background incidence of a histopathological lesion is essential for the establishment of the nature and cause of morbidity in an individual animal, which represents an integral assignment for the practicing toxicologic pathologist. Also, expression of nephrotoxicity simply might occur as a slight exacerbation of the severity of a spontaneously occurring lesion. Spontaneous disease may also mask or mimic a toxicologic response in preclinical trials.

Moreover, this dissertation intends to give insight into the relationship between the development of renal toxicity findings and clinical chemistry parameters indicative of nephrotoxicity. In order to classify concentrations of clinical chemistry parameters as physiological or unphysiological reference intervals will be determined before plotting clinical chemistry values into the respective graphs. The aim is to assess the value of the examined clinical chemistry parameters for distinct study duration endpoints in Wistar rats.

A further objective is to learn about the selected histopathological lesions and their relation to treatment duration, including consideration of dose in preclinical animal testing. This was achieved through comparing the frequency of a toxicity finding in 28-day oral gavage rat studies with the frequency of the toxicity finding in rat studies after administration of the same compound over a different study duration. The knowledge of histopathological effects and their occurrence over different treatment duration can potentially demonstrate transience of histopathological effects. For example, if a histopathological lesion frequently manifests in acute studies after administration of a specific compound but is not identifiable in subacute and chronic toxicity studies after administration of the same dose, its impact is lower than a finding constantly visible over several study

durations. Moreover, information on how long a substance can be administered to an animal without serious toxicity findings plays a crucial role for future application of the new medication. Detailed knowledge about the consistency of adverse reactions of drugs obviously is amongst the key information that researchers have to identify and comprehend before moving from preclinical testing to the clinical stage.

Knowledge of cross-species consistency is of vital importance in preclinical research. In order to obtain data concerning this cross-species consistency, the frequency of the different histopathological treatment-related renal toxicity findings was compared between the species of Wistar rat and beagle dog specifically in 28-day oral gavage studies. The cross-species consistency plays a vital role in regard to the application and transferability of the results to human beings. Specifically, this means that a nephrotoxic reaction observable in rodent *and* non-rodent is much more probable to manifest in human populations compared to a nephrotoxic effect only recognized in rats.

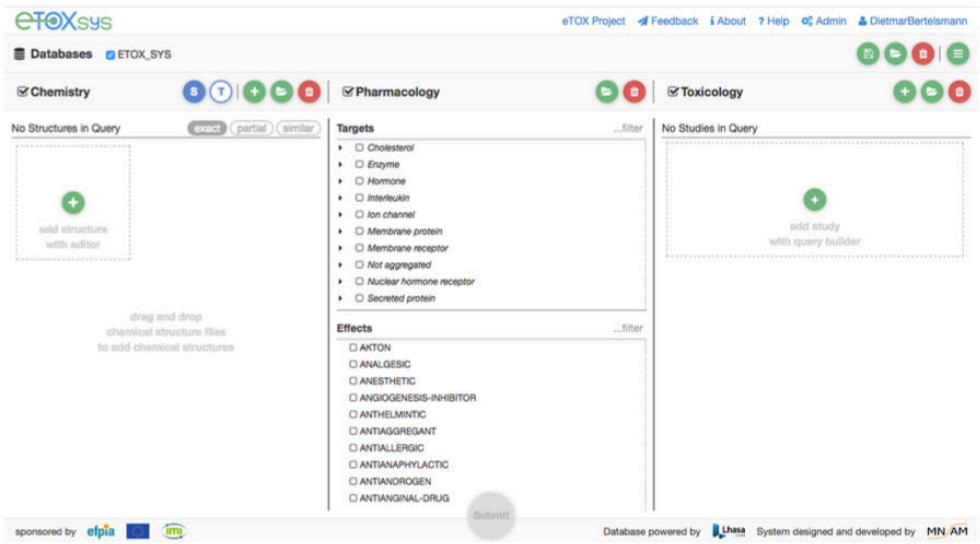
Ultimately, the overarching goal of this work is to possibly assist in contributing to a reduction of the drug attrition rate in preclinical development through the availability of new detailed information on the occurrence of renal toxicity findings.

2 MATERIAL AND METHODS

2.1 eTOX database queries and organization of relevant data

The following section summarizes the procedure of data queries using the eTOX software. As can be seen from the screenshot shown in Figure 3 A, the eTOX database can be mined for either chemical structures, pharmacological targets and/or toxicological findings. In order to identify compounds that induce histopathological findings in kidney, the first step was to select animal species, route of administration, study duration and possibly sex specificity. Next the query was further specified by selecting the search preferences (clinical chemistry, gross pathology, histopathology, urinalysis, etc. – in this work always histopathology) (Figure 3 B). Subsequently, selection of the specific site of histopathological effect as well as possibly severity and relevance of the histopathological effect was made (Figure 3 C, D). The results were displayed in a list of compounds (Figure 3 E), which were downloaded as individual “Excel spreadsheets”.

A


eTOXsys
eTOX Project
Feedback
About
Help
Admin
DietmarBertelsmann
Databases
ETOX_SYS
Chemistry
Pharmacology
Toxicology
No Structures in Query
exact
partial
similar
add structure with editor
drag and drop chemical structure files to add chemical structures
Targets
...filter
Cholesterol
Enzyme
Hormone
Interleukin
Ion channel
Membrane protein
Membrane receptor
Not aggregated
Nuclear hormone receptor
Secreted protein
Effects
AKTON
ANALGESIC
ANESTHETIC
ANGIOGENESIS-INHIBITOR
ANTHELMINTIC
ANTIAGGREGANT
ANTIALLERGIC
ANTIANAPHYLACTIC
ANTIANDROGEN
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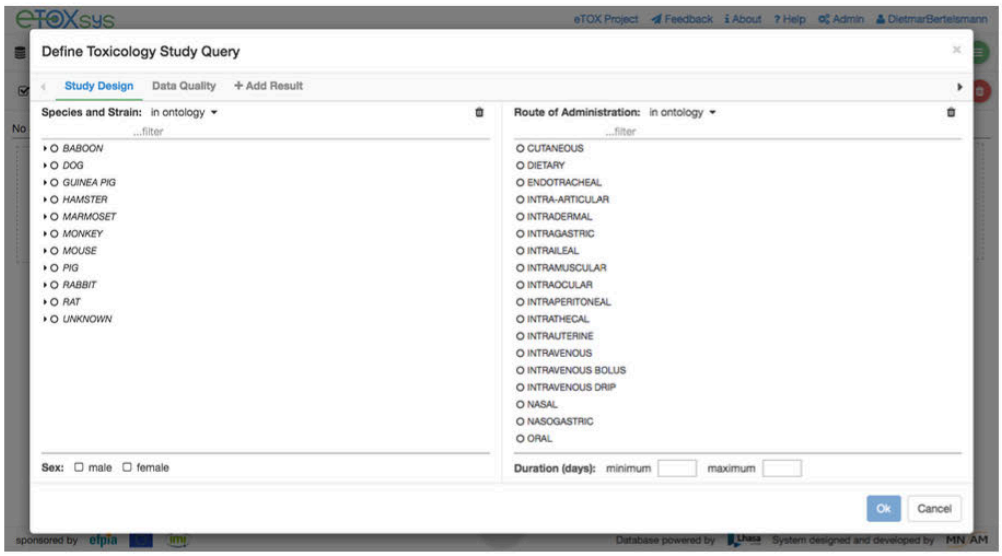
No Studies in Query
add study with query builder
sponsored by efpia
Database powered by Lhasa
System designed and developed by MN.AM

B


Define Toxicology Study Query
Study Design
Data Quality
+ Add Result
Species and Strain: in ontology
...filter
BABOON
DOG
GUINEA PIG
HAMSTER
MARMOSET
MONKEY
MOUSE
PIG
RABBIT
RAT
UNKNOWN
Route of Administration: in ontology
...filter
CUTANEOUS
DIETARY
ENDOTRACHEAL
INTRA-ARTICULAR
INTRADERMAL
INTRAGASTRIC
INTRAILEAL
INTRAMUSCULAR
INTRAOCULAR
INTRAPERITONEAL
INTRATHECAL
INTRAUTERINE
INTRAVENOUS
INTRAVENOUS BOLUS
INTRAVENOUS DRIP
NASAL
NASOGASTRIC
ORAL
Sex: male female
Duration (days): minimum maximum
Ok
Cancel

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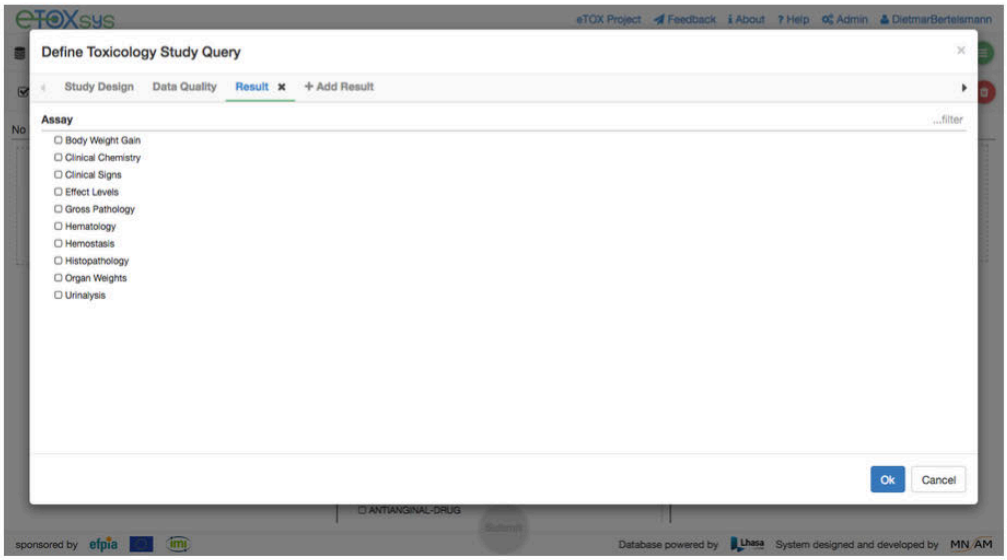

Define Toxicology Study Query
Study Design
Data Quality
Result
+ Add Result
Assay
...filter
Body Weight Gain
Clinical Chemistry
Clinical Signs
Effect Levels
Gross Pathology
Hematology
Hemostasis
Histopathology
Organ Weights
Urinalysis
Ok
Cancel

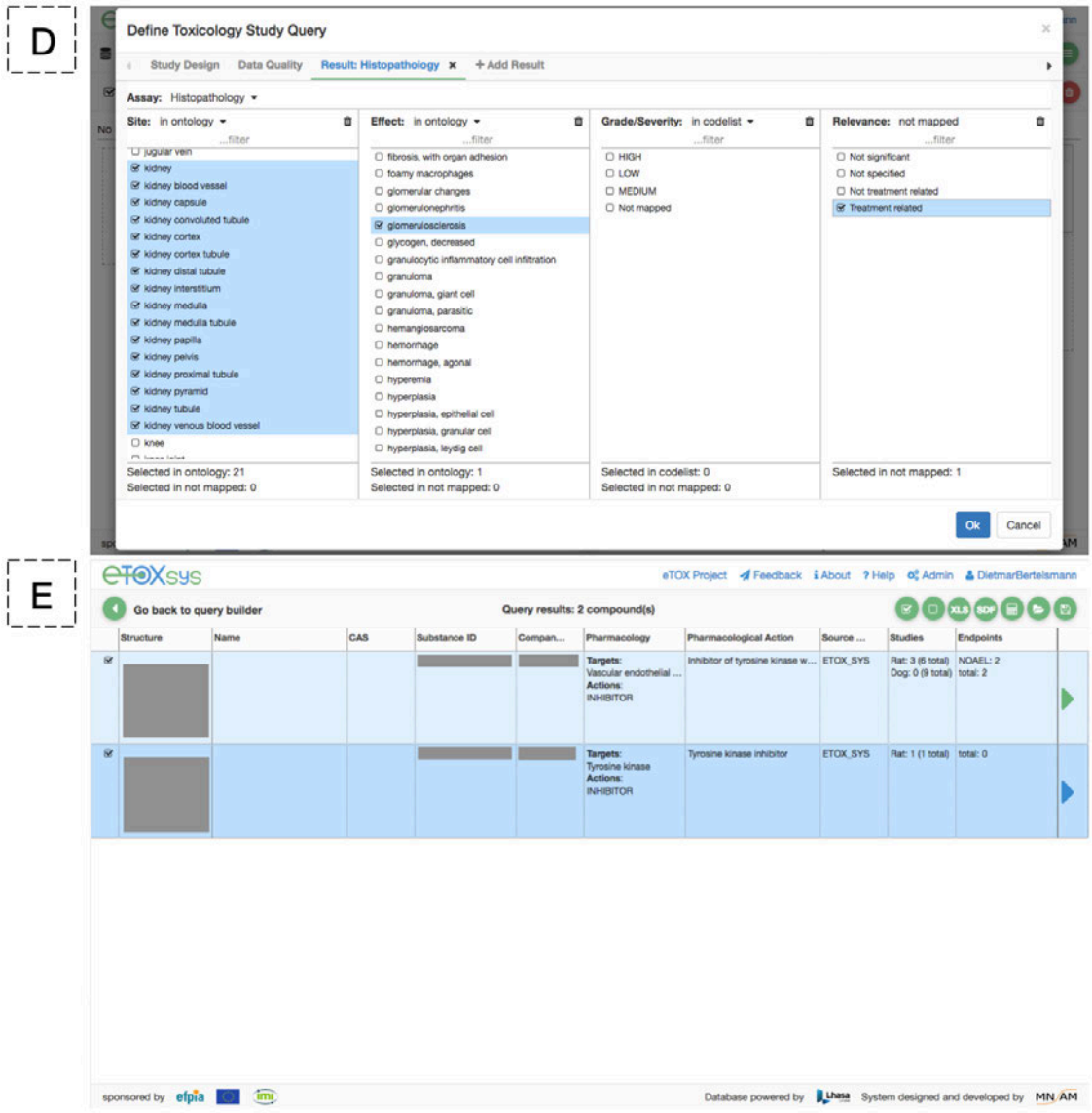


Figure 3 - Sequence of eTOX database search result screenshots. A Screenshot 1: Possible election of a database query between chemistry, pharmacology and toxicology. **B** Screenshot 2: Choice between species, various routes of administration and study duration. **C** Screenshot 3: Definition of study result (e.g. histopathology). **D** Screenshot 4: Choice between organ site, effect, severity and relevance. **E** Screenshot 5: List of matching compounds.

Search criteria were strictly defined: The eTOX database was mined specifically for 28-day studies in the species of Wistar rat or Wistar Han rat, which were fed via oral gavage. 28-day studies are commonly used in preclinical research as they are indispensable for marketing authorization of a pharmaceutical substance with a planned maximum treatment duration of two weeks, as stated by the ICH guidelines (31). As the route of application, oral gavage as the most frequently used route in preclinical studies was chosen. This has the reason that

pharmaceutical substances are most often administered orally in human populations, and the ICH recommends a similar way of administration in preclinical studies. In order for the compound to be listed, a certain renal toxicity finding had to be present in at least one treated animal and had to be marked as treatment related by the study directors in the eTOX database.

The data bank searches for each histopathological toxicity finding are summarized in Tables 3-10. Eight histopathological endpoints were chosen because of their high relevance in preclinical renal safety assessment: Accumulation, hyaline droplets, tubular atrophy, tubular basophilia, renal dilation, hyperplasia, neutrophilic inflammation, necrosis and glomerulosclerosis.

Table 3 - Search parameter employed to identify compounds associated with renal accumulation of hyaline droplets in 28-day oral gavage studies in Wistar/Wistar Han rats

	Chosen Search Criteria
Species	Wistar; Wistar Han
Route of administration	Oral gavage
Duration	28-32 days
Result	Histopathology
Site	Kidney
Effect	Accumulation, hyaline droplets
Relevance	Treatment-related

Table 4 - Search criteria used to identify compounds associated with tubular atrophy in 28-day oral gavage studies in Wistar/Wistar Han rats

	Chosen Search Criteria
Species	Wistar; Wistar Han
Route of administration	Oral gavage
Duration	28-32 days
Result	Histopathology
Site	Kidney
Effect	Tubular atrophy
Relevance	Treatment-related

Table 5 - Search criteria used for determination of substances associated with treatment-related tubular basophilia in 28-day oral gavage studies conducted in Wistar/Wistar Han rats

	Search Criteria
Species	Wistar; Wistar Han
Route of administration	Oral gavage
Duration	28-32 days
Result	Histopathology
Site	Kidney
Effect	Basophilia, tubule
Relevance	Treatment-related

Table 6 - Search parameter utilized to identify compounds associated with treatment-related renal dilation in 28-day oral gavage studies in Wistar/Wistar Han rats

	Chosen Search Criteria
Species	Wistar; Wistar Han
Route of administration	Oral gavage
Duration	28-32 days
Result	Histopathology
Site	Kidney
Effect	Dilation
Relevance	Treatment-related

Table 7 - Search criteria applied to determine substances associated with renal hyperplasia in 28-day oral gavage studies in Wistar/Wistar Han rats

	Chosen Search Criteria
Species	Wistar; Wistar Han
Route of administration	Oral gavage
Duration	28-32 days
Result	Histopathology
Site	Kidney
Effect	Hyperplasia
Relevance	Treatment-related

Table 8 - Search criteria used to identify compounds associated with renal neutrophilic inflammation in 28-day oral gavage studies in Wistar/Wistar Han rats

	Chosen Search Criteria
Species	Wistar; Wistar Han
Route of administration	Oral gavage
Duration	28-32 days
Result	Histopathology
Site	Kidney
Effect	Inflammation, neutrophilic
Relevance	Treatment-related

Table 9 - Search parameter employed for determination of compounds associated with renal necrosis in 28-day oral gavage studies in Wistar/Wistar Han rats

	Chosen Search Criteria
Species	Wistar; Wistar Han
Route of administration	Oral gavage
Duration	28-32 days
Result	Histopathology
Site	Kidney
Effect	Necrosis
Relevance	Treatment-related

Table 10 - Search criteria selected to identify substances associated with glomerulosclerosis in 28-day oral gavage studies in Wistar/Wistar Han rats

	Chosen Search Criteria
Species	Wistar; Wistar Han
Route of administration	Oral gavage
Duration	28-32 days
Result	Histopathology
Site	Kidney
Effect	Glomerulosclerosis
Relevance	Treatment-related

The output of these searches was a list of compounds for each search. For each compound identified as inducing a particular toxicity finding in kidney,

a separate excel file was downloaded. These excel files were comprised of several sheets depending on the total number of studies that had been conducted with the particular compound and the number of endpoints investigated in each study (gross pathology, histopathology, clinical signs, clinical chemistry, hematology, general effects, toxicokinetics, etc.). For some compounds, inter alia, a range of additional studies were available, which did not match the search criteria (e.g. toxicokinetics studies, short-term studies, long-term studies, studies in other animals than Wistar/Wistar Han rat). The next step was to filter out relevant information needed for the purpose of this work through separation of studies for each individual compound that matched the search criteria (i.e. appropriate study species, study duration, etc.). Raw data relevant for the renal histopathological toxicity finding were then transferred to a new excel sheet (Figure 4), which facilitated detailed comparison between the single relevant studies. Only those studies were included, which matched search criteria (28-day oral gavage studies in Wistar/Wistar Han rats) or were needed for further analysis and comparison (28-day oral gavage studies in beagle dogs; studies in rats over a time duration other than 28 days). For example, 14 different studies had been carried out for compound “AZ_GGA_200009505”. Of these, six were relevant this work: Whereas studies 1, 2, 3, 4 and 6 were Wistar/Wistar Han rat studies of different time duration, study 12 was a 28-day oral gavage study carried out in the species of beagle dog. Excluded were the following studies: Study 5 (in which kidney histopathology had not been conducted), 6 (1-day toxicokinetics study), 7, 8, 9, 10, 11, 13 and 14 (studies in beagle dogs with a time duration other than 28 days).

In the separate, newly created excel sheets detailed information is collected for each of the identified compounds and their respective relevant studies. Within the single studies a distinction was made between the separate treatment groups (male low-dose, female low-dose, male mid-dose, female mid-dose, male high-dose & female high-dose).

The created excel sheet (e.g. Figure 4) provides information on the name of the compound, the pharmacological mechanism, animal species, duration of

the study as well as the route of administration. Furthermore, the size of the specific treatment groups, their gender and the administered dose is depicted. Also, information is shown on the specific toxicology finding (e.g. glomerulosclerosis) including its frequency, relevance and severity. Concerning the relevance, a distinction was made between treatment-related (TR), not treatment-related (nTR) and between no relevance given (nR). Designations of histopathological findings as “treatment-related” indicate that the study directors considered that a cause-effect relationship between compound administration and the renal changes exists. Degrees of severity of the specific toxicity finding were divided into low, medium and high. Additionally, mean clinical chemistry values of each dose group (including concentrations of serum urea, serum creatinine, serum albumin, serum cholesterol, serum triglycerides, serum protein, protein in the urine and serum inorganic phosphate), further histopathology toxicity findings and renal gross pathology findings were listed.

	Compound	Pharma-cology	Study	Duration (days)	Oral Gavage	Animals /Group	Sex	Dosage (mg/kg)	Glomerulo-sclerosis findings	Relevance	Low Grade	Med. Grade	High Grade	Urea (mmol/l)	Creatinine (umol/l)	Albumin (g/l)	Cholesterol (mmol/l)	Triglycerides (mmol/l)	Serum Protein (g/l)	Protein in Urine (mg/dl)	Inorganic Phosphate (mmol/l)	More Kidney Histopathology findings	Gross Pathology
1																							
2																							
3						6	M	0	0					6,7	58	33	2,5	2,21	67	87,67	2,87	4/6 Tubular Basophilia (nR)	-
4						6	F	0	0					6,8	55	34	2,5	1,37	65	16,17	2,45	1/6 Tubular Basophilia (nR)	-
5						6	M	1,5	0					7	58	34	2,5	1,4 (decr. TR)	67	53,33	2,65	5/6 Tubular Basophilia (nTR)	-
6						6	F	1,5	0					6,4	51	32	2,4	1,16	59 (decr. TR)	11,83	2,78	4/6 Tubular Basophilia (nR)	-
7			2) Wistar Rat	29	Yes	6	M	3	0					7	56	34	2,5	1,37 (decr. TR)	65	123,5	2,61	1/6 Tubular Basophilia (TR)	-
8						6	F	3	0					5,9	52	31 (decr. TR)	2,5	1,24	57 (decr. TR)	13,2	2,5	2/6 Tubular Basophilia (TR)	-
9						6	M	6	4	TR	4	-	-	5,9	59	33	3,1 (incr. TR)	1,4 (decr. TR)	65	82,67	2,65	2/6 Tubular Basophilia (TR)	-
10						6	F	6	6	TR	1	5	-	7,7	53	25 (decr. TR)	3,9 (incr. TR)	1,97	50 (decr. TR)	178,67 (incr. - TR)	2,56	6/6 Tubular Basophilia (TR)	2/6 Pale di
11																							
12						10	M	0	0					6,4	57	32	2,4	1,42	68	41,54	2,45	4/10 Tubular Basophilia (nR)	nD
13						10	F	0	0					6,8	60	35	2,2	1,04	71	5,28	2,17	5/10 Tubular Basophilia (nR)	nD
14						10	M	1	0					6,7	59	33	2,6	1,45	70	35,26	2,43	5/10 Tubular Basophilia (nR)	nD
15		VEGFR-Inhibitor	3) Wistar Rat	30	Yes	10	F	1	0					6,6	59	32 (decr. nR)	2,3	1,06	65 (decr. nR)	5,39	2,38	5/10 Tubular Basophilia (nR)	nD
16	AZD9935					10	M	2,5	0					6,7	59	32	2,3	1,3	65	40,69	2,56	5/10 Tubular Basophilia (TR)	nD
17						10	F	2,5	2	TR	2	-	-	7	61	31 (decr. nR)	3 (incr. nR)	0,96	66 (decr. nR)	8,14 (incr. nTR)	2,33	2/10 Tubular Basophilia (TR)	nD
18						10	M	5	0					6,5	60	32	3,1 (incr. nR)	1,91 (incr. nR)	68	16,24	2,49	10/10 Tubular Basophilia (TR)	nD
19						10	F	5	10	TR	6	4	-	6,8	59	28 (decr. nR)	2,7 (incr. nR)	1,13	58 (decr. nR)	12,16 (incr. nTR)	2,17	3/10 Tubular Basophilia (TR)	nD
20																							
21						3	M	0	0					5,1	83	30	3,5	0,55	58	20,39	1,24	-	-
22						3	F	0	0					5,6	78	31	4,4	0,79	60	6,37	1,28	-	-
23						3	M	0,1	0					5,7	81	31	3,5	0,51	61	44,96	1,27	-	-
24						3	F	0,1	0					5,9	81	31	3,9	0,45	59	6,26	1,36	several findings of calculi (nR)	-
25			8) Beagle	31	Yes	3	M	0,25	0					7,2	83	30	3,7	0,57	61	42,9	1,44	-	-
26						3	F	0,25	0					4,5	74	31	4,6	0,78	66	5,91	1,51	-	-
27						3	M	0,5	0					5	66 (decr. nR)	27 (decr. nR)	6,6 (incr. nR)	0,52	68 (decr. nR)	16,74 (decr. nR)	1,77	-	-
28						3	F	0,5	0					4,5	67	27	5,7	0,69	62	65,33	1,48	-	-
29																							
30																							
31																							
32						6	M	0	0					6,7	58	33	2,5	2,21	nD	87,67	2,87	NAD	nD
33						6	F	0	0					6,8	55	34	2,5	1,37	nD	16,17	2,45	NAD	nD
34						6	M	20	0					7,2	62	34	2,6	1,73	nD	45,5	2,55	3/6 Tubular Basophilia (nTR)	nD
35						6	F	20	0					7,2	56	34	2,5	1,03	nD	13,5	2,77	2/6 Tubular Basophilia (nR)	nD
36						6	M	40	7	TR	2	0	-	7	59	33	3,3	1,38	nD	62,83	2,7	3/6 Tubular Basophilia (TR)	nD
37		M596380	1) Wistar Rat	29	Y	6	F	40	1	TR	1	0	-	6,6	60	33	2,6	1,14	nD	15,83	2,64	1/6 Tubular Basophilia (TR)	nD
38						6	M	80	6	TR	2	4	-	6,4	54	29 (decr. TR)	3,2 (incr. TR)	1,56 (decr. TR)	nD	95,4	2,28 (decr. TR)	3/6 Tubular Basophilia (TR)	nD
39			Tyrosine-Kinase-Inhibitor			6	F	80	5	TR	2	3	-	6,2	54	32	2,9	1,36	nD	13	2,64	4/6 Tubular Basophilia (TR)	nD
40																							

Figure 4 – Example screenshot from excel sheet with the purpose of comparing glomerulosclerosis toxicity findings. The excel sheet shows specific information of single dose groups in each row. Included were only those studies which matched search criteria (28-day oral gavage studies in Wistar/Wistar Han rats) or were needed for further analysis and comparison (28-day oral gavage studies in beagle dogs; studies in rats over a time duration other than 28 days). The treatment groups, associated with a specific compound and specific study, consist either of male or female animals. The respective study can be seen in the third column, as well as the used species. The fourth column displays the corresponding study duration, the fifth column the way of administration and the sixth column reveals the size of the specific treatment group. Furthermore, the administered dosages, the amount of registered cases of glomerulosclerosis and their corresponding relevance and grade are given. Concerning the relevance of the renal histopathological findings, a distinction was made between treatment-related (TR), not treatment-related (nTR) and between no relevance given (nR). The further brackets, inter alia, show the treatment groups' respective clinical chemistry.

Subsequent to the organization of data in excel sheets, data graphs were developed in order to illustrate data. Creation of the graphs was performed using Microsoft excel 2020 software. In order to adequately depict clinical chemistry values, scatter charts were chosen: Scatter plots were created by plotting the mean clinical chemistry values (as stated in the eTOX database) of the control-, low-dose-, mid-dose-, and high-dose group for each study for each gender. Moreover, reference intervals were calculated for each clinical chemistry parameter based on clinical chemistry data of male and female control animals from 28-day oral gavage studies and included in the scatter charts. The calculation of these reference intervals is explained in detail in chapter 2.3. For the presentation of the consistency of the occurrence of histopathological findings at different treatment durations as well as for the consistency across species, the use of bar charts was selected. Regarding consistency of the occurrence of histopathological findings at different treatment durations it was distinguished between studies with time durations varying between <28 days, 28-32 days, 88-92 days and 26 weeks as well as between low-, mid- and high-dose groups of each study. The “bars” demonstrate the percentage of affected animals (e.g. 67% [4/6] of male animals were identified with glomerulosclerosis in the high-dose group in study 2 of compound “AZD9935” after 28 days). A color code was introduced in order to indicate the corresponding dose-range in each dose group. For the presentation of consistency across species the bar charts distinguished between 28-day studies (low-, mid- and high-dose groups respectively) conducted in Wistar rats and beagle dogs. Again, a color table was used in order to illustrate the corresponding doses.

2.2 Determination of background incidences in control Wistar rats and beagle dogs

In order to determine the spontaneous incidence of a specific renal histopathological finding, the first step involved a database search, which revealed the total number of control animals in four-week studies contained in the database. This was carried out through searching the eTOX database with the search criteria shown in Table 11 to identify 28-32 day oral gavage studies in which

kidney histopathology had been conducted. Calculation of the background incidence was carried out for each of the eight histopathological findings (accumulation, hyaline droplets; tubular atrophy; tubular basophilia; dilation; hyperplasia; neutrophilic inflammation; necrosis; glomerulosclerosis). Since background incidences were determined for both rat and dog, the search was carried out for each species separately, i.e. by including the additional search terms “Wistar rat” and “beagle dog”. In order to determine the total number of control animals, the respective number of control animals was registered for each study and compound before finally being added up. A gender-specific distinction was made between male and female animals of each species.

Table 11 - Search parameters selected to identify the total number of control animals within 28-32 day oral gavage studies in rats and dogs, respectively, in which kidney histopathology was conducted. This was needed in order to calculate the background incidence for each histopathological finding.

	Chosen Search Criteria
Species	Wistar rat; Beagle dog
Route of administration	Oral gavage
Duration	28-32 days
Result	Histopathology
Site	Kidney

In total 894 male and 889 female control animals were identified from 28-32 day oral gavage studies in Wistar rats. For the species of beagle dogs 270 male and 279 female control animals were identified.

Next, the frequency of the respective histopathological effects in the identified control animals was determined. In order to achieve this, the corresponding searches with individual histopathological findings (necrosis, glomerulosclerosis, etc.) were performed. Identical search parameters were used as described above, with the single difference that a histopathological result was added in the search (necrosis, glomerulosclerosis, etc.). Now, studies were individually checked for the number of control animals with the specific histopathological result per study and compound. Again, the numbers were added up for each histopathological finding and separately for each sex, resulting in the total number of toxicity findings in male and female control animals.

2.3 Determination of the frequency of specific histopathological endpoints in <28-day, 28-day, 90-day and >90-day studies in Wistar rats and beagle dogs

In order to compare the frequency of various treatment-related and not treatment-related renal histopathology findings between Wistar rats and beagle dogs, including consideration of study duration, a series of database searches was conducted (Table 12). This resulted in the absolute number of compounds in which selected histopathological finding were observed in oral gavage studies in Wistar rat or beagle. The resulting data was then transferred and visualized in bar charts (Figure 8) through the use of Microsoft Excel.

Table 12 - Search criteria used to identify the frequency of selected treatment-related and not treatment-related histopathological endpoints in oral gavage studies of different treatment duration in Wistar rat and beagle dog

	Chosen Search Criteria
Species	Wistar rat/beagle dog
Route of administration	Oral gavage
Duration	0-27; 28-32; 33-87; 88-92 days
Result	Histopathology
Site	Kidney
Effect	Dilation; basophilia, tubule; neutrophilic inflammation; necrosis; glomerulosclerosis; accumulation, hyaline droplets; atrophy; hyperplasia
Relevance	Treatment-related / not treatment-related

In order to obtain the frequency of renal findings in preclinical studies in Wistar rat or beagle dog studies, another search had to be conducted to reveal the total number of compounds for which oral gavage Wistar rat/beagle dog studies and kidney histopathology for each of the four different study durations were available (Table 13). These results are shown in table 18 in the results section in chapter 3.4.2.

Table 13 - Search criteria for detection of the total number of compounds with oral gavage Wistar rat/beagle dog studies of different treatment durations, in which kidney histopathology was conducted

	Chosen Search Criteria
Species	Wistar rat/beagle dog
Route of administration	Oral gavage
Duration	0-27; 28-32; 33-87; 88-92 days
Result	Histopathology
Site	Kidney

The frequency was then calculated for each endpoint separately: For example six compounds were identified in the eTOX database with 28-day oral gavage studies conducted in Wistar rats with the histopathological result of treatment-related renal dilation. At the same time there are in total 79 compounds in the eTOX database with 28-day oral gavage Wistar rat studies in which renal histopathology was conducted. Division of the number of compounds with 28-day oral gavage studies conducted in Wistar rats with the histopathological result of treatment-related renal dilation (=6) through the total number of compounds in the eTOX database with 28-day oral gavage Wistar rat studies, in which renal histopathology was conducted (=79), resulted in the frequency: 24/79 = 0,759 = 7,6 %. The calculated results were again transferred and visualized in a set of bar charts (Figure 9) through the use of Microsoft Excel.

2.4 Determination of reference intervals for selected clinical chemistry parameters indicative of nephrotoxicity

Determination of reference intervals for clinical chemistry parameter indicative of nephrotoxicity was carried out by Christine Kalisch.

In order to improve the evaluation of the consistency between renal histopathological findings and clinical chemistry, it is important to establish reference intervals for each relevant clinical chemistry parameter. Reference intervals are used for comparison with measured values to detect a disease or to monitor the course of a disease. A reference interval depicts the range of laboratory values of healthy animals. This range contains 95% of the values. A higher or lower value is found in 5% of healthy animals. When establishing reference intervals,

differences in terms of species, breed and sex have to be considered. The aim was to analyze data contained within the eTOX database to establish reference intervals for renal clinical chemistry parameters in male and female Wistar rats, including serum creatinine, urea, albumin, inorganic phosphate, serum protein, cholesterol and triglycerides. These reference intervals are needed in order to identify values, which are not deemed as normal for a physiologic measurement in healthy animals. In order to know which values are to be considered as un-physiological, reference intervals are necessary. Unphysiological values are those that do not lie within these reference limits and may indicate a pathological finding. The calculated reference intervals specifically refer to the content of the eTOX database and is not data universally valid.

A five-step procedure for the creation of reference intervals was followed:

(1) Data collection
(2) Identification and elimination of outliers
(3) Analysis of data distribution
(4) Selection of the method for determination of reference intervals
(5) Calculation of reference values

2.4.1 Data collection

For the determination of reference intervals in Wistar rats, only data from healthy animals were used. Clinical chemistry data of male and female control animals from 28-day studies (route of administration: oral gavage) were collected and evaluated per sex.

2.4.2 Identification and elimination of outliers

Calculation of the limits of the reference intervals is based on the largest and smallest value of the data set. An undetected outlier for example due to random errors or a single animal in poor health can severely skew the results and cause a larger range of intervals.

To identify outliers, the method by Henry and Reed was used: For this method, all values which are contained within the respective data set, must be

sorted in ascending order. This had to be performed for each clinical chemistry parameter sex-specifically.

$$r = \frac{x_{(n)} - x_{(n-1)}}{x_{(n)} - x_{(1)}} > \frac{1}{3}$$

If the distance between the largest value within the respective data set ($x_{(n)}$) and the second largest value ($x_{(n-1)}$) is more than 1/3, $x_{(n)}$ can be treated as an outlier.

$$r = \frac{x_{(2)} - x_{(1)}}{x_{(n)} - x_{(1)}} > \frac{1}{3}$$

If the distance between the smallest value ($x_{(1)}$) and second smallest value ($x_{(2)}$) is more than 1/3, $x_{(1)}$ can be treated as an outlier (134). After eliminating the detected outliers, the distribution of data can be analyzed.

2.4.3 Analysis of data distribution

Biological medical data rarely follows a normal distribution. Nevertheless, it is important to test for normal distribution in order to subsequently select a suitable method for determination of reference intervals. In order to analyze whether the present data follows a normal distribution, the Kolmogorov-Smirnov test with Lilliefors significance correction was used.

The null hypothesis of the Kolmogorov-Smirnov test indicates that the data is normally distributed. A p-value of 0.05 or less, indicates that the data is not normally distributed and thus the null hypothesis is rejected. The outcome of the evaluation of data distribution is demonstrated in the results section in part 3.3.

2.4.4 Selection of the method for determining reference intervals

When establishing reference intervals, a distinction is made between parametric and non-parametric methods. Parametric methods are used to evaluate normally distributed datasets. These include the calculation of parametric tolerance intervals and the parametric percentile estimate. For the evaluation of the two normally distributed datasets, the classical method of parametric tolerance intervals was employed. In this case, a prescribed proportion of the data (95%) with a defined probability (P 0.90) is included in the limits.

$$L_1 = \bar{x} - ks$$

$$L_2 = \bar{x} + ks$$

L_1 and L_2 are the lower and upper limit of the reference interval. The value of k was determined from table values published in "Tables of Tolerance-Limit Factors for Normal Distributions" by *Weissberg et al* (135).

Non-parametric methods include the calculation of non-parametric tolerance intervals and non-parametric percentile estimation and are used to evaluate not normally distributed datasets, because they are independent of the type of distribution. Clinical chemistry reference ranges for all not normally distributed datasets were calculated as the 2.5th and 97.5th percentiles. This method is rank based so for the data with a sample size greater than 120, a 90% two-sided confidence interval of the limits was calculated (136).

$$r_u = 0.025 \times (n - 1)$$

$$r_o = 0.975 \times (n + 1)$$

The limits r_u and r_o are the 2.5 and 97.5 percentiles. The variable n describes the number of measured values. The determination of reference intervals for clinical chemistry parameters indicative of nephrotoxicity in 28-day oral gavage studies in Wistar rats will be described in the results section in chapter 3.3.

2.5 Methodological approach to analyse the consistency of histopathological effects in relation to treatment duration, including consideration of dose

The consistency of the occurrence of histopathological findings across different treatment durations was visualized by plotting the percentage of animals affected with a certain histopathological finding in the respective (low-, medium- and high-dose) group against the treatment duration of the respective study. A distinction was made between treatment durations of <28 days, 28-32 days, 88-90 days and 26 weeks. The data was taken from the prepared excel sheets (see chapter 2.1). For the analysis of the consistency of histopathological effects in relation to treatment duration only studies, conducted in the species

of rat (Wistar, Wistar Han, strain unassigned) were included. Besides oral gavage studies, routes of administration labelled as “oral” or “intravenous drip” were utilized for analysis. Furthermore, one-day toxicology studies were excluded from analysis. Separate bar charts were created for male and female animals. The graphs were created using Microsoft Excel 2020 software. A color code was used to highlight the dose range (rather than the exact dose) in the respective treatment groups.

2.6 Methodological approach to analyse the consistency of histopathological effects across species, including consideration of dose

Bar graphs in order to analyse the consistency of histopathological effects across species were created in a similar way as those for the analysis of the consistency across different treatment durations: The percentage of animals affected with a certain histopathological finding in the respective group was plotted against the respective species (rat or beagle) by the use of Microsoft excel 2020 software and a color code was used to depict the corresponding dose ranges. Data was taken from the corresponding excel sheets: Only 28-day studies conducted in rats or beagle dogs were selected. Besides “oral gavage”, “oral” or “intravenous drip” were considered as route of administration. Charts were created for male and female animals separately.

3 RESULTS

3.1 The structure of the eTOX database

The software platform “ETOXSYS” was used to access the eTOX data. This platform currently contains 1395 compounds and 6501 associated preclinical safety studies.

As can be seen from Figure 5 A, the majority of the studies contained in the eTOX database was carried out in rats (1217 studies) and dogs (592 studies). The most common rat strains were “Wistar Han” (430 studies) and “Wistar” (408 studies), both accounting for approximately a third of the studies with rats as study species.

Irrespective of species, the most common route of administration was “oral gavage” (836 studies) followed by “oral” (575 studies) and “intravenous” (408 studies) (Figure 5 B).

Study duration was predominantly < 28 days (1017 studies), followed by 4-week studies (28-32 days) that constituted a third of the studies (683 studies) in the database (Figure 5 C).

Analysis of the frequency of histopathological findings per target organ in 28-day oral gavage studies in Wistar revealed liver (78) and kidney (75) as the most common target organs (Figure 6). Other frequent target organs were lung (69 studies), spleen (68 studies), cardiovascular system (65 studies) and gastrointestinal tract (63 studies) (Figure 6).

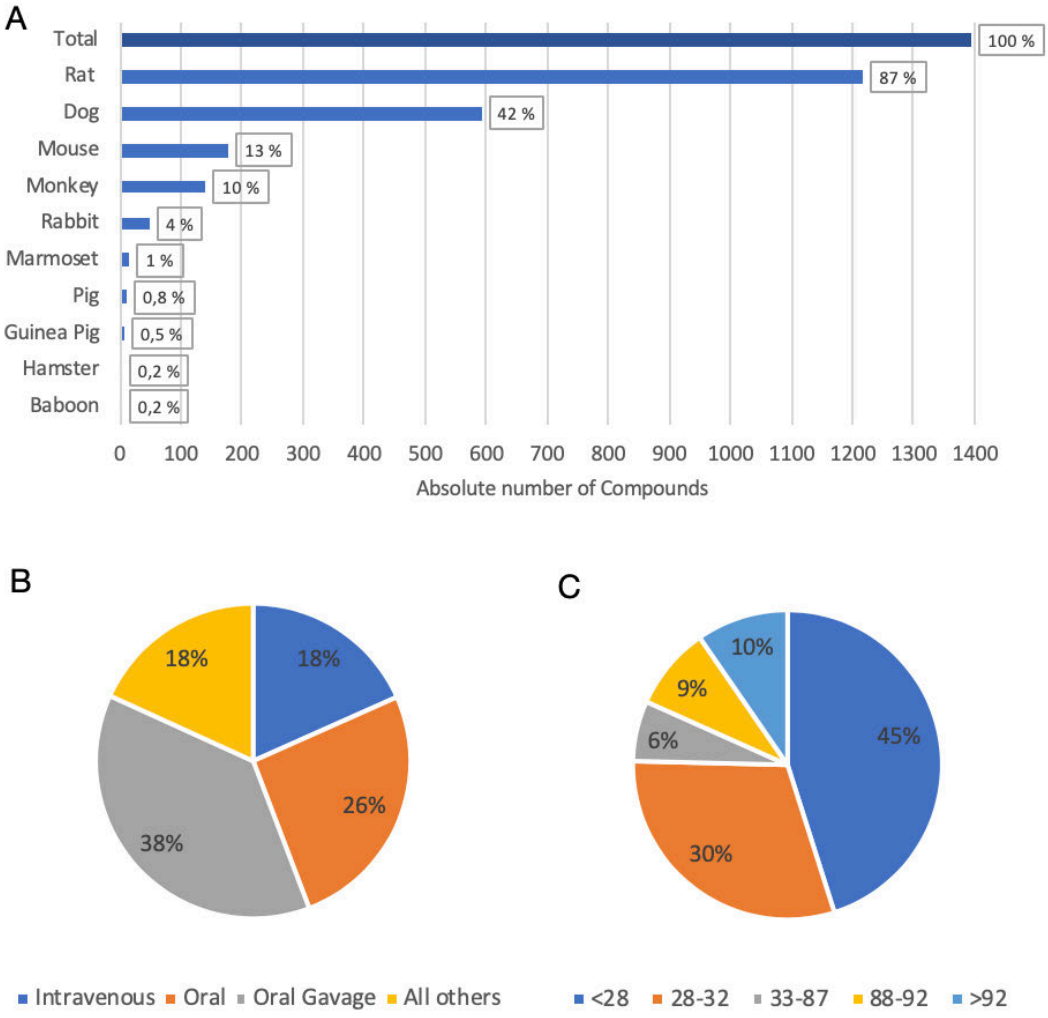


Figure 5 – General distributions of species, routes of administration and study durations in the eTOX database. A Distribution of animal species in the eTOX database. **A** displays the absolute number and the percentage of compounds of all species included in the database. It is visible that the great majority of studies was conducted in rats and dogs. **B** Distribution of different routes of administration. Figure B shows that “oral gavage”, “oral” and “intravenous” – in descending order - are the three most commonly used types of administration routes. **C** Distribution of study durations in days. This pie chart highlights the fact that the most frequent study types in the eTOX database are acute and subacute studies with a time period below 28 days, accounting for approximately three quarters of all studies in the database.

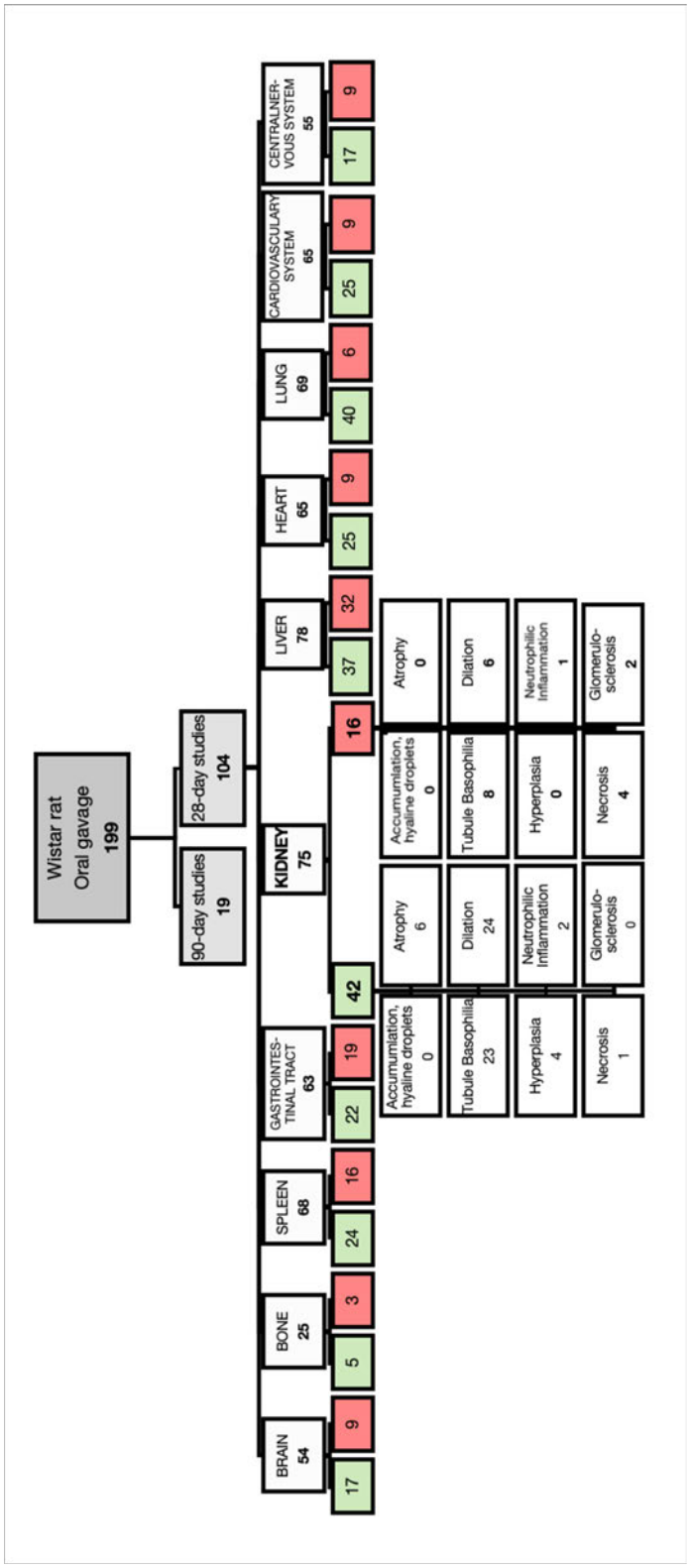


Figure 6 – Frequency of treatment-(red boxes) and non-treatment-related (green boxes) histopathological findings in different target organs in 28-day gavage studies in Wistar rats contained in the eTOX database in which histopathology of the respective target organ was evaluated. *The numbers in the boxes indicate the number of compounds identified in each specific query. E.g. the search identified 199 compounds for which oral gavage studies in Wistar rats were available. Of these, 104 were 28-day oral toxicity studies. In 75 of these, renal histopathology was assessed.*

3.2 Renal histopathology findings in 28-day oral gavage Wistar rat and Wistar Han studies contained in the eTOX database

While there is a vast amount of search options in the eTOX database, three basic search terms were used for every database search in this work (Table 14) in order to identify 28-day oral gavage Wistar rat and Wistar Han studies.

Table 14 - Basic search terms across all database searches

Species:	Rat → Wistar or Wistar Han
Way of administration:	Oral gavage
Study duration:	28 – 32 days

Table 15 shows that the searches were subsequently narrowed down by including additional search criteria. Not all renal findings were considered treatment-related by the respective study pathologists.

Table 15 - Number of compounds associated with basic search parameters in the eTOX database

Search Criteria	Wistar	Wistar Han	Total
Wistar Rat:	408	430	838
Wistar Rat and oral gavage:	199	298	497
Wistar Rat, oral gavage and 28-32 days:	104	154	258
Wistar Rat, oral gavage, 28-32 days and histopathological effect in kidney:	79	129	208
Wistar Rat, oral gavage, 28-32 days and treatment-related histopathological effect in kidney:	16	33	49

Of the selected renal histopathological findings, the most frequent finding observed in 28-day oral gavage studies in Wistar rats was the toxicity finding of renal dilation, followed by tubular basophilia (Table 16). Tubular basophilia and dilation were also the most frequent treatment-related histopathology findings, followed by renal necrosis, glomerulosclerosis and neutrophilic inflammation. Hyaline droplets accumulation, renal atrophy and renal hyperplasia were not identified in 28-day oral gavage studies in Wistar rats in the eTOX database.

Table 16 – Number of compounds associated with the different types of histopathological findings in the eTOX database. A distinction was made between compounds with histopathological effect and compounds with treatment-related histopathological effect. Included are only those compounds associated with 28-32 oral gavage studies conducted in Wistar (and Wistar Han rats). Numbers marked with an asterisk include the strain of Wistar Han.

Histopathological Effect	Number of compounds with histopathological effect	Number of compounds with treatment-related histopathological effect
Accumulation, hyaline droplets	0	0
Atrophy	7	0
Basophilia, tubule	34	8 (22*)
Dilation	47	6 (14*)
Hyperplasia	7	0
Inflammation, neutrophilic	4	1
Necrosis	11	9*
Glomerulosclerosis	3	3*

3.3 Determination of reference intervals for clinical chemistry parameters indicative of nephrotoxicity in 28-day oral gavage studies in Wistar rats

3.3.1 Analysis of data distribution

Analysis of data distribution according to Kolmogorov-Smirnov with Lilliefors significance correction revealed normal distribution for the parameters of inorganic phosphate and serum protein in male rats. The data was not normally distributed for the following parameters: Creatinine, urea, albumin, cholesterol and triglycerides each in both sexes, as well as for inorganic phosphate and serum protein in female rats (Table 17).

Table 17 - Results of the statistical evaluation concerning the normal distribution test according to Kolmogorov-Smirnov with Lilliefors significance correction. *The asterisk (*) marks the limit of true significance. The rows marked in grey indicate the cases with a normal distribution. Df = Degrees of freedom.*

Parameter	Sex	Statistic	Df	Significance
Creatinine	Male	0.113	130	0.000
	Female	0.135	129	0.000
Urea	Male	0.14	128	0.000
	Female	0.202	127	0.000
Albumin	Male	0.168	99	0.000
	Female	0.098	98	0.021
Inorganic phosphate	Male	0.064	114	0.200*
	Female	0.075	112	0.164
Serum protein	Male	0.071	116	0.200*
	Female	0.091	114	0.021
Cholesterol	Male	0.092	117	0.016
	Female	0.092	116	0.017
Triglycerides	Male	0.083	97	0.096
	Female	0.082	96	0.111

3.3.2 Establishment of clinical chemistry parameter reference ranges and comparison with literature

Tables 18 and 19 show the calculated reference ranges for the selected clinical chemistry parameters.

Table 18 - Reference ranges of normally distributed clinical chemistry parameters relevant to nephrotoxicity calculated as parametric tolerance intervals.

Parameter	Sex	Reference range	Standard error of average value
Inorganic phosphate (mmol/l)	Male	1.89 – 3.56	0.03627
Serum protein (g/l)	Male	56.56 – 70.79	0.30469

Table 19 - Reference ranges of not normally distributed clinical chemistry parameters relevant to nephrotoxicity calculated as 2.5th and 97.5th percentile intervals.

Parameter	Sex	Reference range	0.90 confidence interval
Creatinine (µmol/l)	Male	20.9 – 65.5	8.8 – 30.6; 62.0 – 75.9
	Female	23.2 – 78.6	17.7 – 34.6; 63.7 – 83.7
Urea (mmol/l)	Male	2.1 – 8.7	1.8 – 2.3; 8.2 – 9.9
	Female	2.0 – 8.8	1.9 – 2.5; 8.3 – 9.4
Albumin (g/l)	Male	25.3 - 46.0	
	Female	26.4 – 50.1	
Inorganic phosphate (mmol/l)	Female	1.6 – 3.1	
Serum protein (g/l)	Female	55.8 – 71.3	
Cholesterol (mmol/l)	Male	0.9 – 2.7	
	Female	0.8 – 2.7	
Triglycerides (mmol/l)	Male	0.4 – 2.4	
	Female	0.2 – 1.8	

Table 20 summarizes the reference intervals calculated from 28-day oral gavage studies in male and female Wistar rats contained in the eTOX database and compares them with results from literature (137, 138): Comparison between calculated intervals from 28-day oral gavage studies in male and female Wistar rats contained in the eTOX database and intervals reported in the literature demonstrate high concordance for serum creatinine, serum protein and cholesterol in males and females and triglycerides in males. Considerable discrepancies were identified for the serum parameter urea, albumin and phosphate in male and female animals and for triglycerides in female animals. Values of the determined 28-day oral gavage Wistar rat reference intervals for inorganic phosphate in males and female were exceedingly higher than values derived from literature. In contrast, reference values established for serum urea and serum albumin in male and female animals and for triglycerides in females based on 28-day studies in Wistar rats contained in the eTOX database were considerably lower as compared to reference ranges reported in literature.

There are multiple possible reasons for a deviation of the calculated values from values reported in literature which derive primarily from variation in study design and analytical methods across companies and sites. These include different housing-conditions (climate, hygienic conditions, cage design), utilization of different methods for analysis of clinical chemistry parameters, fasting or non-fasting prior to blood tests and even minor procedural details such as animal handling. For example *Swaim et al.* showed that significant changes in the

serum hepatic enzyme alanine transaminase can occur if rodents are handled by the body instead of the tail (139). Another report highlighted the impact of method and site of blood removal in rodents on the concentration of some parameters, such as cytokines (140). According to *Sorge et al*., even the sex of the study personnel may affect the outcome of certain rodent studies through olfactory cues (141).

Animals from referenced literature studies were given free access to food and water ad libitum (142, 143). In contrast to literature studies, studies contained in the eTOX database lack information on animal housing, food composition and food restriction prior to blood and urine sampling. Furthermore, data contained in the eTOX database were contributed by different companies and may have been obtained using different methodologies, possibly explaining the wider reference ranges.

Table 20 - Reference intervals of serum clinical chemistry parameters relevant to nephrotoxicity calculated from 28-day oral gavage studies in male and female Wistar rats contained in the eTOX database vs. literature data.

Analyte	Sex	Results	Literature
Creatinine (µmol/l)	Male	20.9 – 65.5	31 – 48
	Female	23.2 – 78.6	37 – 53
Urea (mmol/l)	Male	2.1 – 8.7	4.0 – 9.3
	Female	2.0 – 8.8	6.8 – 11.3
Albumin (g/l)	Male	25.3 – 46.0	44.4 – 58.4
	Female	26.4 – 50.1	
Inorganic phosphate (mmol/l)	Male	1.9 – 3.6	0.73 – 1.95
	Female	1.6 – 3.1	
Serum protein (g/l)	Male	56.7 – 70.8	40 – 60
	Female	55.8 – 71.3	40 – 80
Cholesterol (mmol/l)	Male	0.89 – 2.80	1.1 – 2.0
	Female	0.80 – 2.72	0.7 – 2.5
Triglycerides (mmol/l)	Male	0.42 – 2.38	0.4 – 2.1
	Female	0.22 – 1.80	0.4 – 3.4

3.4 Analysis of the frequency and consistency of renal toxicity findings across species

3.4.1 Spontaneous renal histopathological findings in 28-day oral gavage studies in control Wistar rats and beagle dogs

In order to contribute to a better understanding of renal adverse reactions of drugs in preclinical research, spontaneous renal histopathological findings

occurring in 28-day oral gavage studies in Wistar rat and beagle dog as the two most common rodent and non-rodent species were determined. The improved availability of detailed information on background incidences helps the study pathologist to evaluate if a histopathological finding occurred spontaneously or whether it was provoked through administration of a (potentially toxic) compound.

In both species and both genders tubular basophilia showed the highest spontaneous incidence, with 12,4% in male and 10,7% in female rats as well as with 5,6% in male and 1,8% in female beagle dogs. In contrast, the background incidences of hyaline droplet accumulation, neutrophilic inflammation and necrosis were below one percent in both species. Glomerulosclerosis was not recorded as a background finding in control animals of either species.

Considerable sex-differences in the background incidence of renal dilation in rats (male: 8,1% vs. female: 2,7%) as well as tubular basophilia (male: 5,6% vs female: 1,8%) were observed in beagle dogs.

With few exceptions, higher spontaneous incidences of renal lesions were typically found in rats as compared to beagle dogs. Renal hyperplasia and hyaline droplets accumulation (females only) exhibited higher background incidence in the beagle dog as compared to the Wistar Rat. Similarly, neutrophilic inflammation and necrosis (males only) showed a slightly higher spontaneous occurrence in beagle dogs in comparison to Wistar rats.

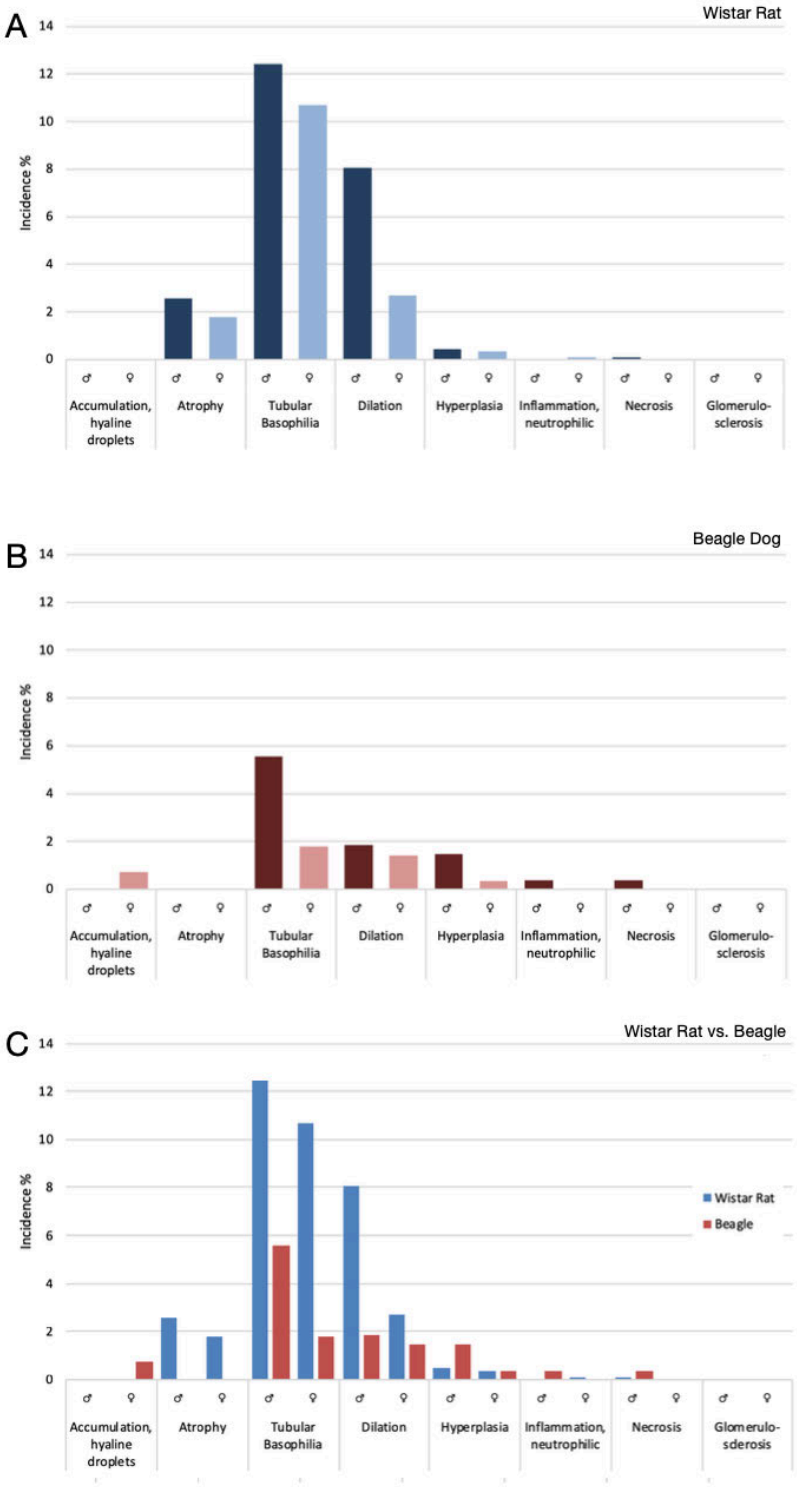


Figure 7 - Background incidences of renal histopathological findings in control Wistar rat and beagle dogs in 28-day preclinical toxicity studies. A Background incidence in male and female Wistar rats. **B** Background incidence in male and female beagle dogs. **C** Comparison between male and female background incidence in Wistar rat and beagle dog.

3.4.2 Frequency of renal histopathological endpoints in <28-day, 28-day, 90-day and >90-day studies in Wistar rats and beagle dogs

Comparison of the total number of various treatment-related and non-treatment-related renal histopathology findings between Wistar rats and beagle dogs across study durations reveals that the eTOX database includes considerably more subacute (≤28 days) studies in which renal effects were observed as compared to subchronic and chronic (≥90 day) studies in both rats and dogs (Table 21). The total number of studies that included histopathological evaluation of kidney tissue was significantly higher for dog as compared to the rat (Table 21). However, renal histopathological effects were identified in the dog less frequently (Figure 8 and Figure 9).

Table 21 – Total number of compounds with kidney histopathology conducted in oral gavage studies of different treatment durations in Wistar rats and beagle dogs

Study duration	Wistar Rat	Beagle dog
< 28 days	39	116
28-32 days	79	104
88-92 days	17	19
> 92 days	20	44

The absolute numbers (Figure 8) as well as the relative numbers of renal histopathological findings in Wistar rats and beagle dogs (Figure 9) reveal that irrespective of the study duration, dilation and tubular basophilia were the most frequent renal lesions identified in both Wistar rat and beagle dog, but were frequently denoted as non-treatment related by the study pathologist. In contrast to these lesions, which also exhibit high background incidences (Figure 7), necrosis and glomerulosclerosis were less frequently observed and were predominantly considered treatment related.

It should be noted that no rational was provided as to why a particular finding was considered treatment or non-treatment related.

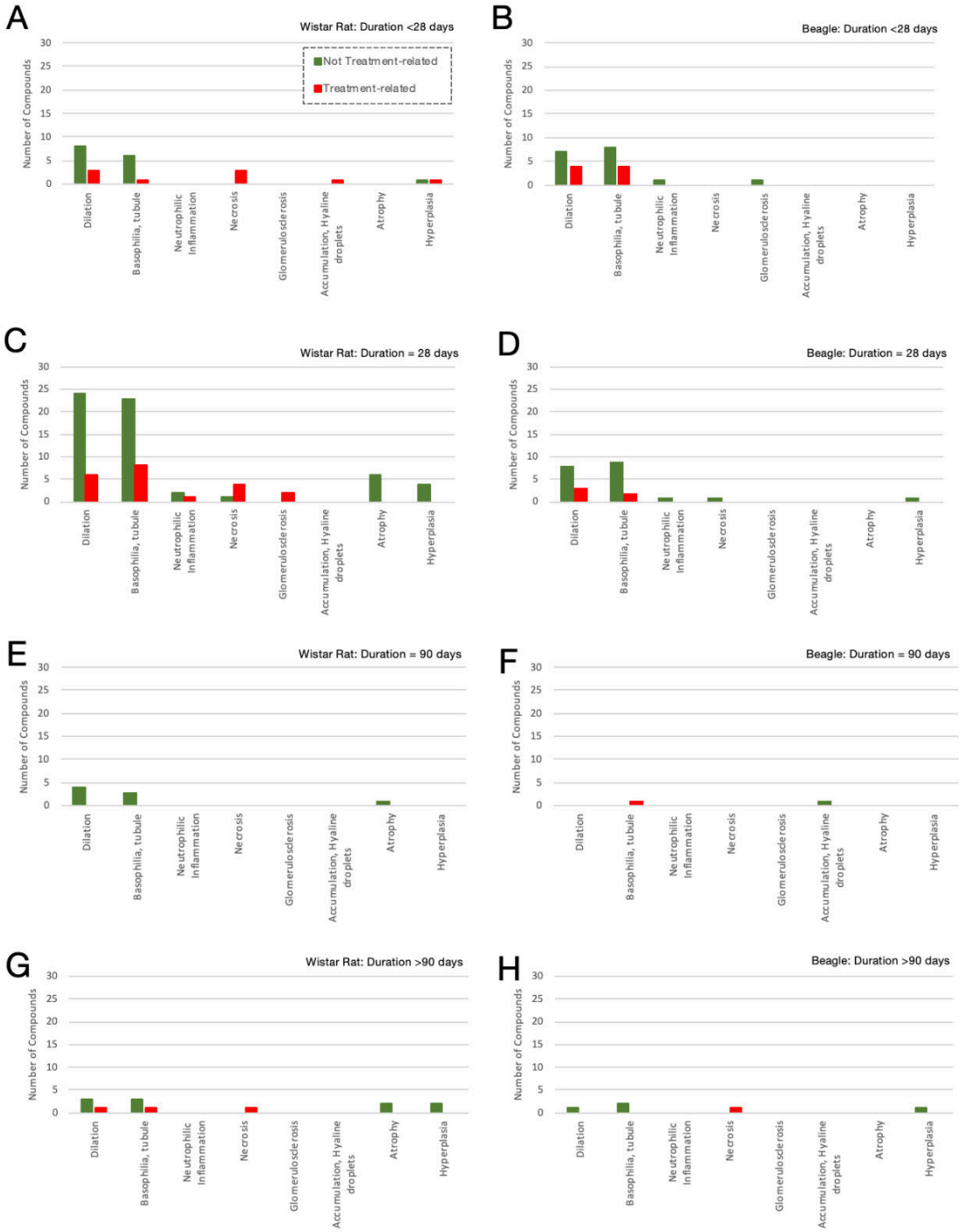


Figure 8 – Number of compounds with renal histopathological endpoints in < 28-day, 28-day, 90-day and > 90-day oral gavage studies in Wistar rats and Beagles. A Number of compounds with renal findings in Wistar rat studies with a duration of less than 28 days. **B** Number of compounds with renal findings in Beagle dog studies with a duration of less than 28 days. **C** Number of compounds with renal findings in Wistar rat 28-day studies. **D** Number of compounds with renal findings in Beagle dog 28-day studies. **E** Number of compounds with renal findings in Wistar rat 90-day studies. **F** Number of compounds with renal findings in Beagle dogs 90-day studies. **G** Number of compounds with renal findings in Wistar rat studies with a duration of more than 90 days. **H** Number of compounds with renal findings in Beagle dog studies with a duration of more than 90 days. *Green bars in the chart represent compounds with not treatment-related findings, while red bars display substances with treatment-related findings.*

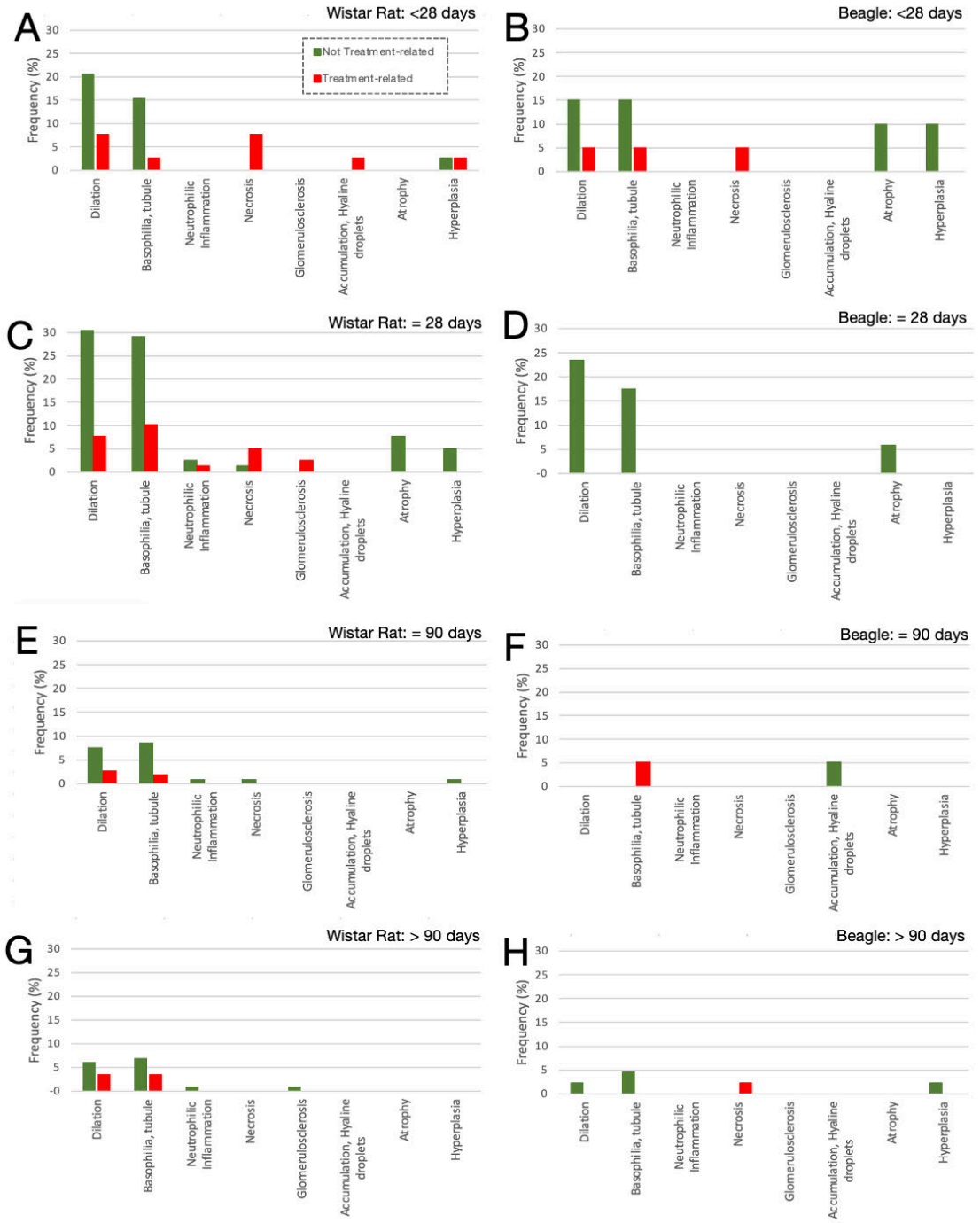


Figure 9 - Frequency of renal histopathological endpoints in < 28-day, 28-day, 90-day and > 90-day studies in Wistar rats and Beagles. A Frequency of compounds with renal findings in Wistar rat studies with a duration of less than 28 days. **B** Frequency of compounds with renal findings in Beagle dog studies with a duration of less than 28 days. **C** Frequency of compounds with renal findings in Wistar rat 28-day studies. **D** Frequency of compounds with renal findings in Beagle dog 28-day studies. **E** Frequency of compounds with renal findings in Wistar rat 90-day studies. **F** Frequency of compounds with renal findings in Beagle dogs 90-day studies. **G** Frequency of compounds with renal findings in Wistar rat studies with a duration of more than 90 days. **H** Frequency of compounds with renal findings in Beagle dog studies with a duration of more than 90 days. *Green bars in the chart represent compounds with not treatment-related findings, while red bars display substances with treatment-related findings.*

3.5 Analysis of the consistency of renal toxicity findings across study duration and species

As described in chapter 3.4.2, dilation and tubular basophilia were the most frequent histopathological endpoints observed in 28-day oral gavage studies in Wistar rat, irrespective of whether they were denoted as treatment-related or not treatment-related. Both endpoints also exhibited the highest background incidences within 28-day oral gavage studies in Wistar rat. In contrast, accumulation of hyaline droplets, atrophy, hyperplasia, neutrophilic inflammation, necrosis and glomerulosclerosis all displayed significantly lower spontaneous incidences. After determination of the frequency and consistency of renal toxicity findings across species, the following section will further analyse the eight selected renal findings across study duration and species. This information is of vital importance already in preclinical trials as the specific objective in determination of the consistency across time is to assess whether the selected findings are transient and therefore potentially reversible or whether they are rather permanent effects suggesting an irreversible effect. The aim within comparing the renal effects across species is to obtain data about the cross-species consistency, which is pivotal for the application and transferability of the occurrence of renal effects in preclinical studies to human beings.

3.5.1 Co-occurrence of different treatment-related renal histopathological endpoints in 28-day oral gavage studies in Wistar and Wistar Han rats

Simultaneous occurrence of different renal histopathological endpoints must be considered, specifically with regard to the ability of a particular clinical chemistry parameter to indicate the presence of a certain histological lesion. Histopathological findings such as dilation and tubular basophilia are more common in normal, healthy kidneys. Due to the fairly high background incidence, distinction between treatment and non-treatment related dilation and tubular basophilia is not always straightforward. Consequently, the diagnosis of nephrotoxicity based on these histopathological endpoints alone is problematic. In

contrast, histopathological findings such as necrosis and glomerulosclerosis, which rarely occur spontaneously in control animals, are relatively clear signs of nephrotoxicity. These endpoints are often accompanied by dilation and basophilia (Table 22). This can bias evaluation of consistency between histopathological endpoints and clinical chemistry parameters, more specifically the capability of a clinical chemistry value to signal the emergence of a particular histopathological finding. There are no 28-day oral gavage Wistar rat studies in the eTOX database, in which necrosis and glomerulosclerosis co-occurred.

Table 22 - Co-occurrence of treatment-related renal histopathological endpoints in 28-day studies in Wistar and Wistar Han rats. The numbers in the respective fields show and the number of affected animals relative to the total number of animals within all treatment groups (i.e. all male and female animals within all available dose groups with the exception of the control groups. (e.g.: For the compound *GGA_BI_001* three (male and female) animals were identified with treatment-related necrosis, nine with treatment-related dilation, whereas in total there were 100 male and female animals in which kidney histopathology was conducted after treatment with this compound.) Based on the data contained in the eTOX database, it was not possible to discern if different types of lesions were found in the same animal.

	Compound Name	Necrosis	Glomerulo-sclerosis	Dilation	Tubule Basophilia	Neutrophilic Inflammation
WISTAR	GGA_BI_001	3/100		9/100		
	AZ_GGA_200002321	7/60		3/60		
	AZ_GGA_200009505	11/59				
	AZ_GGA_203287734	11/56		6/56		
	AZD9935		22/96		55/96	
	AZ_GGA_205981792		14/36		16/36	
	AZ_GGA_200010598			6/36	21/36	5/36
	AZ_GGA_206269903			3/36	9/36	
	AZD4580			14/72	24/72	
	AZ_GGA_200010232				8/100	
	PLS_JJ_0052				4/40	
	PLS_JJ_0003				5/30	
WISTAR HAN	AZ_GGA_203287646		6/12			
	AZ_GGA_203287759	2/60				
	AZ_GGA_203287693	5/80		4/80	6/80	
	Deferasirox	5/13			9/13	
	AZ_GGA_203287757	2/120		1/120	13/120	
	AZ_GGA_203287755	7/60			26/60	

3.5.2 Accumulation, hyaline droplets

Searching the eTOX database yielded no 28-day oral gavage studies in Wistar and Wistar Han rats in which hyaline droplet accumulation was recorded.

Therefore, no conclusions on the consistency between hyaline droplet accumulation and clinical chemistry parameters as well as the consistency of hyaline droplet accumulation across time and species can be drawn.

3.5.3 Atrophy

Searching the eTOX database yielded no 28-day oral gavage studies in Wistar and Wistar Han rats in which renal atrophy was recorded. Consequently, evaluation of renal atrophy in relation to clinical chemistry parameters and consistency across time and species cannot be made.

3.5.4 Basophilia, tubule

3.5.4.1 Compounds associated with treatment-related tubular basophilia

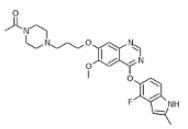
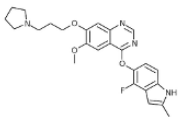
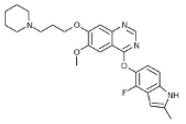
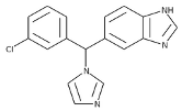
While high background incidences of tubular basophilia were observed in Wistar rats, tubular basophilia was also the most frequent treatment-related renal histopathological finding in 28-day oral gavage studies in Wistar and Wistar Han rat. Table 23 summarizes the compounds in which treatment-related tubular basophilia was identified in 28-day oral gavage studies in Wistar and Wistar Han rats, including their chemical structure and pharmacological mechanism.

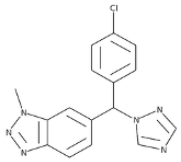
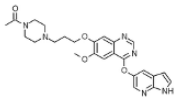
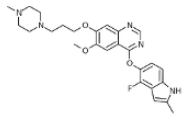
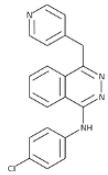
Table 23 - Search criteria for identification of compounds causing treatment-related tubular basophilia in a 28-day oral gavage study in Wistar or Wistar Han rats.

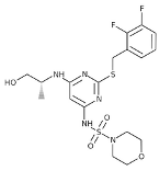
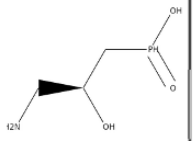
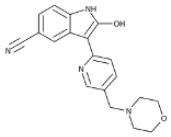
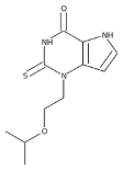
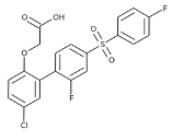
	Chosen Search Criteria	Wistar	Wistar Han	Total Hits
Species	Wistar; Wistar Han	408	430	838
Route of administration	Oral gavage	199	298	497
Duration	28-32 days	104	154	258
Result	Histopathology	92	147	239
Site	Kidney	79	129	208
Effect	Basophilia, tubule	34	56	90
Relevance	Treatment-related	8	10	18

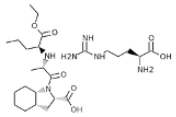
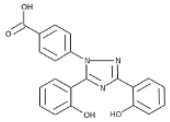
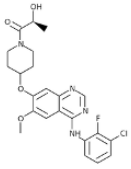
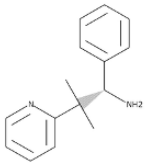
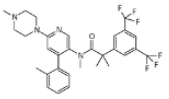
Six out of the eight compounds that caused tubular basophilia in a 28-day oral gavage study in Wistar rats are tyrosine kinase inhibitors. In contrast, the nine compounds identified as causing tubular basophilia in a 28-day oral gavage study in Wistar Han rats all belong to different pharmacological classes.

Table 24 - Compounds associated with treatment-related tubular basophilia and their pharmacology.

Compound Name	AZ_GGA_203287552	AZ_GGA_200010232	AZ_GGA_203287683	PLS_JJ_0003
Chemical Structure				
Pharmacology	Vascular endothelial growth factor receptor inhibitor (TKI)	Vascular endothelial growth factor receptor inhibitor (TKI)	Vascular endothelial growth factor receptor inhibitor (TKI)	Retinoic acid metabolism blocker
Rat Strain	Wistar			

Compound Name	PLS_JJ_0052	M596380	AZ_GGA_206269903	AZ_GGA_200010598
Chemical Structure				
Pharmacology	Not available	Tyrosine kinase inhibitor	Vascular endothelial growth factor receptor inhibitor (TKI)	Vascular endothelial growth factor receptor inhibitor (TKI)
Rat Strain	Wistar			

Compound Name	AZ_GGA_203287567	AZ_GGA_203287693	AZ_GGA_203287668	AZ_GGA_203287558	AZ_GGA_203287626
Chemical Structure					
Pharmacology	C-X-C chemokine receptor type 2 antagonist	GABA B receptor agonist	Glycogen synthase kinase 3 inhibitor	Myeloperoxidase inhibitor	CRTh2 receptor antagonist
Rat Strain	Wistar Han				

Compound Name	Perindopril	Deferasirox	AZ_GGA_203287757	AZ_GGA_206242732	Netupitant
Chemical Structure					
Pharmacology	Anti-Hypertensive	Iron chelation of bis-hydroxy-phenyl triazole type	Not available	Not available	Neurokinin 1 Antagonist, P-Glycoprotein Inhibitor
Rat Strain	Wistar Han				

In the 28-day oral gavage studies conducted with the compounds “AZ_GGA_203287552” and “AZ_GGA_205981792” the co-occurrence of glomerulosclerosis in animals may severely bias outcomes of consistency of tubular basophilia in relation to clinical chemistry, more specifically the ability of particular clinical chemistry parameters to indicate the presence of tubular basophilia.

3.5.4.2 Analysis of the consistency of tubular basophilia and clinical chemistry findings

To analyze the relationship between tubular basophilia and clinical chemistry parameters relevant to nephrotoxicity testing, treatment-related findings of tubular basophilia in 28-day studies in rats were plotted against the respective clinical chemistry data from the same study (Figure 10).

As is evident from Figure 10A, no statistically significant changes in serum creatinine or creatinine values outside of the calculated reference range were recorded in any of the 28-day oral gavage Wistar rat studies associated with treatment-related tubular basophilia, in any animal group.

Similarly, the concentration of serum urea was within the reference range in all treatment groups, apart from a statistically significantly increase in serum urea in a high-dose male treatment group administered “Vatalanib” for 28 days (Figure 10B).

A statistically significant decrease in serum phosphate together with treatment-related findings of tubular basophilia was only observed in the high-dose male group in response to “M596380” (Figure 10C). In human patients a number of drugs (e.g.: deferasirox, cisplatinum, imatinib, tenofovir, adenovir etc.) have been associated with the Fanconi syndrome, which is characterized by renal malabsorption of the proximal tubule of inter alia phosphate. As a consequence, the Fanconi syndrome causes hypophosphatemia and can have devastating consequences for the patient through manifestation of osteomalacia in adults and rickets in children (144).

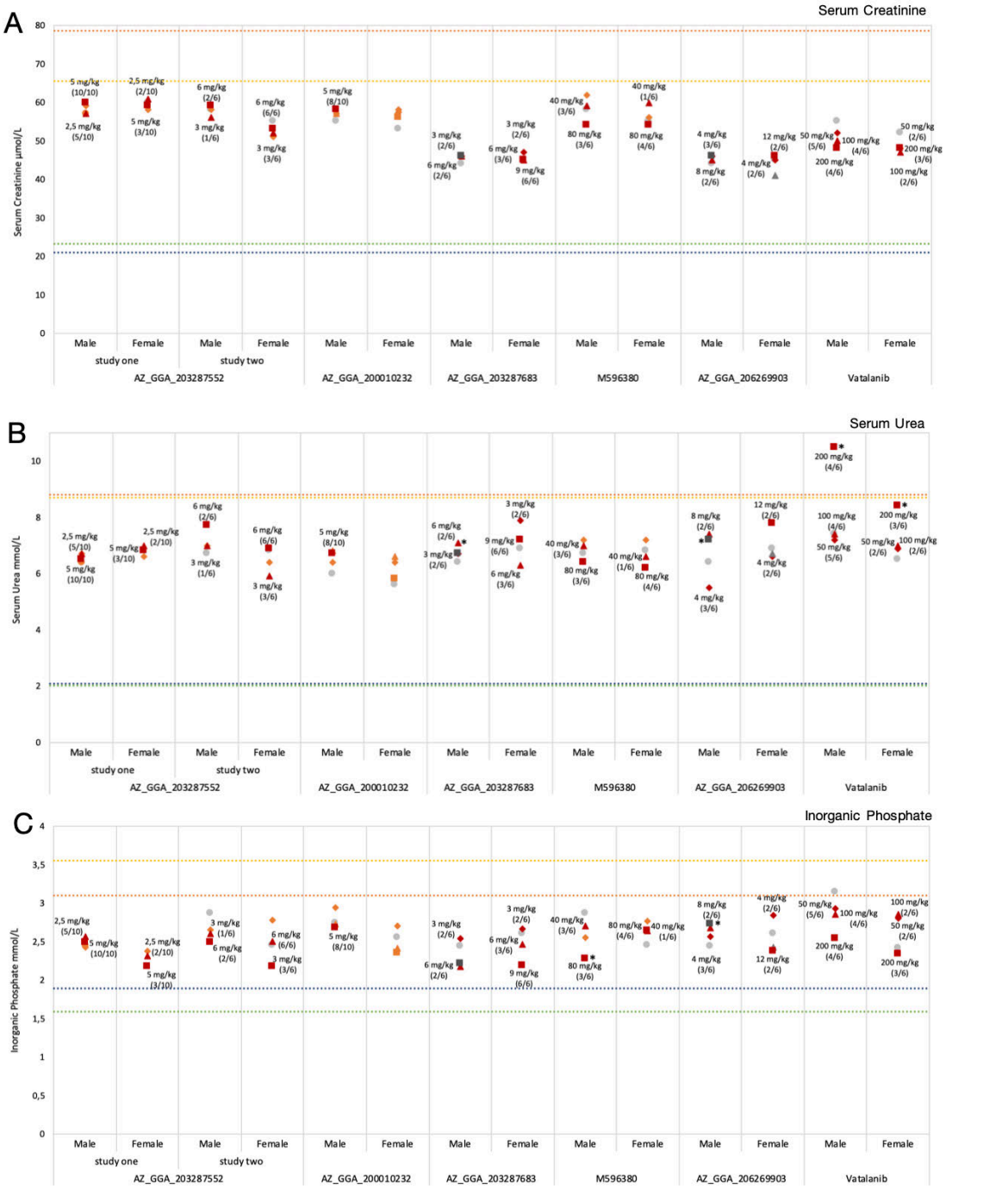


Figure 10 - Clinical chemistry data obtained from 28-day oral gavage studies in Wistar rats in which treatment-related tubular basophilia was observed. A Serum creatinine. **B** Serum urea. **C** Inorganic phosphate. For the compound "AZ_GGA_203287552" two matching studies were available. *The different shapes (circle, tilted square, triangle and square) mark the respective control groups as well as low- (LD), medium- (MD) and high-dose (HD) groups. Forms colored in red represent that treatment-related tubular basophilia was identified at least once in the dose-group, whereas orange color shows the cases in which tubular basophilia was identified, but not labelled as treatment-related. The boxes next to the red shapes indicate the dose and the frequency of tubular basophilia per dose group. Stars denote values which were reported as statistically significantly changed. The dotted lines indicate the calculated reference ranges for male and female clinical chemistry values (see chapter 3.3).*

3.5.4.3 Analysis of the consistency of tubular basophilia in relation to treatment duration, including consideration of dose

The toxicity finding of tubular basophilia and its relation to treatment duration are important for an improved understanding of this histopathologic endpoint, because the behavior of occurrence of a histopathological effect in preclinical studies is vital for final evaluation in the context of transience of an effect (i.e., a histopathologic lesion can possibly occur for only a certain period of time). Determination of the consistency across time of a histopathological finding can help to assess whether the selected finding is potentially reversible or a rather permanent effect detectable across different treatment durations, therefore suggesting an irreversibility. Moreover, toxicologic effects in preclinical studies after a certain amount of time might already indicate a possible maximum duration of administration of a compound or (if the substance is accumulating) a potential hint towards a maximum total dose, also regarding to the transferability to clinical trials. Furthermore, it is important to know how much time has to pass before a histopathological effect occurs. This knowledge for example is helpful in order to identify a possible mechanism on a cellular or biochemical level.

For the fewest compounds study durations other than 28 days are available. But while there is a number of corresponding studies over a comparable treatment duration in the eTOX database performed with compounds identified as causing tubular basophilia within a 28-day study in rats, only in few of those studies treatment-related tubular basophilia was recorded (Figure 11): The compounds exhibiting treatment-related tubular basophilia in 28-day oral gavage studies do not show consistent findings of renal tubular basophilia across time with the exception of the substance “Netupitant”. In addition to treatment-related tubular basophilia observed in the 30 mg/kg high-dose group (male: 4/6; female: 6/6 animals) in the 28-day study on “Netupitant”, treatment-related tubular basophilia was also reported to occur in the male mid-dose (100 mg/kg) and the male and female high-dose groups (300 mg/kg) within a corresponding fifteen-day study. While this suggest consistency of treatment-related tubular basophilia across time, tubular basophilia was not evident in a 91-day study in Wistar rats

with the same compound and identical dose groups as compared to the corresponding 28-day study (3, 10 and 30 mg/kg). Furthermore, cases of tubular basophilia were spotted in a 182-day study of compound “AZ_GGA_200010232” in both genders at a dose of 2 mg/kg. This compares to doses of 1.25, 2.5 and 5 mg/kg in the corresponding 28-day-study, in which treatment-related tubular basophilia was registered in the male high-dose group in eight out of ten animals. A 15-day study was conducted with the same compound with a dose of up to 1000 mg/kg without any histopathological evidence of treatment-related tubular basophilia. Therefore, a high consistency across time for treatment-related tubular basophilia can only partly be confirmed for this compound.

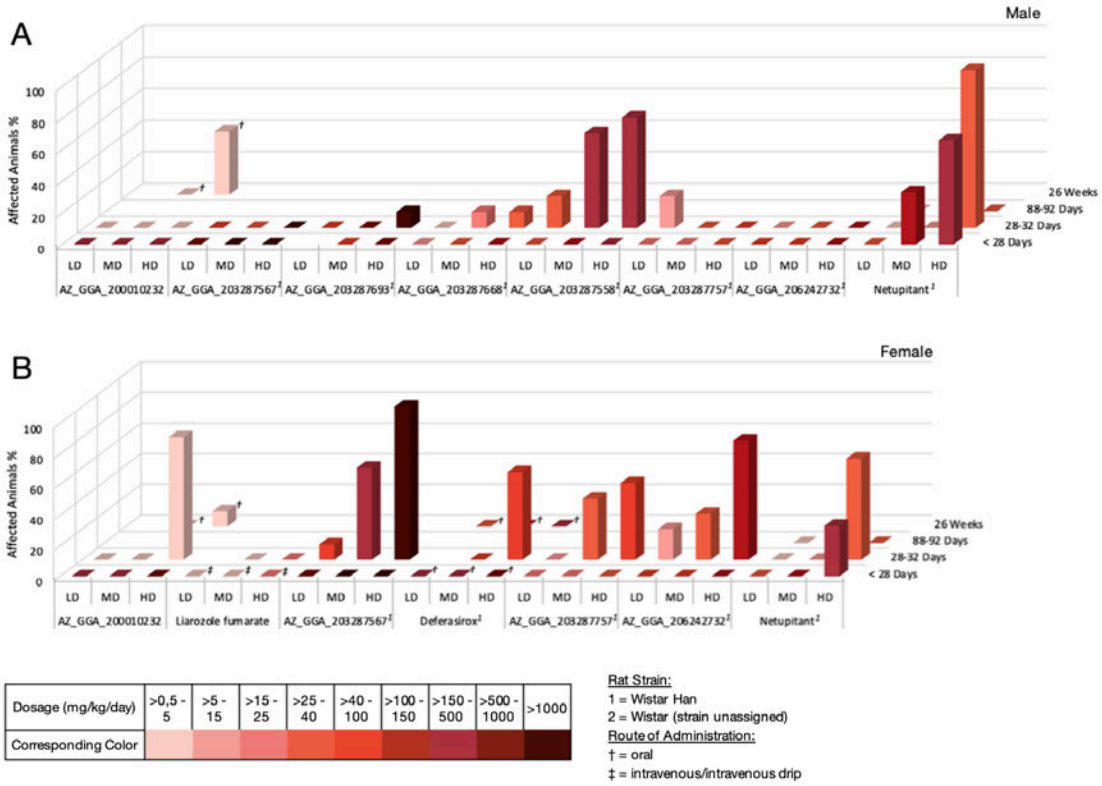


Figure 11 - Consistency of the occurrence of treatment-related tubular basophilia in relation to treatment duration in Wistar and Wistar Han rats. Comparison of the occurrence of treatment-related tubular basophilia across treatment duration in (**A**) male and (**B**) female rats. The comparison includes eight compounds with identification of treatment-related tubular basophilia in a 28-day oral gavage Wistar/Wistar Han rat study and at the same time with identification of at least one corresponding study with a different treatment duration. *A color code was introduced to indicate the dose-range (mg/kg bw). The numbers in superscript indicate substances conducted with another strain than Wistar - 1=Wistar Han; 2=Wistar (strain unassigned), whereas the cross and double cross indicate routes of administration other than “oral gavage”.*

3.5.4.4 Analysis of the consistency of tubular basophilia across species, including consideration of dose

An improved understanding of the consistency of tubular basophilia across species may contribute to a better understanding of the relevance of tubular basophilia as a renal adverse effect, in particular regarding the transferability of tubular basophilia observed in preclinical studies to humans and therefore clinical trials.

Even though six substances were identified with treatment-related tubular basophilia in 28-day studies in rats tubular basophilia was not observed as a compound-related finding in corresponding subacute studies in beagles after administration of the substances, which provoked tubular basophilia in a 28-day oral gavage study (Figure 12).

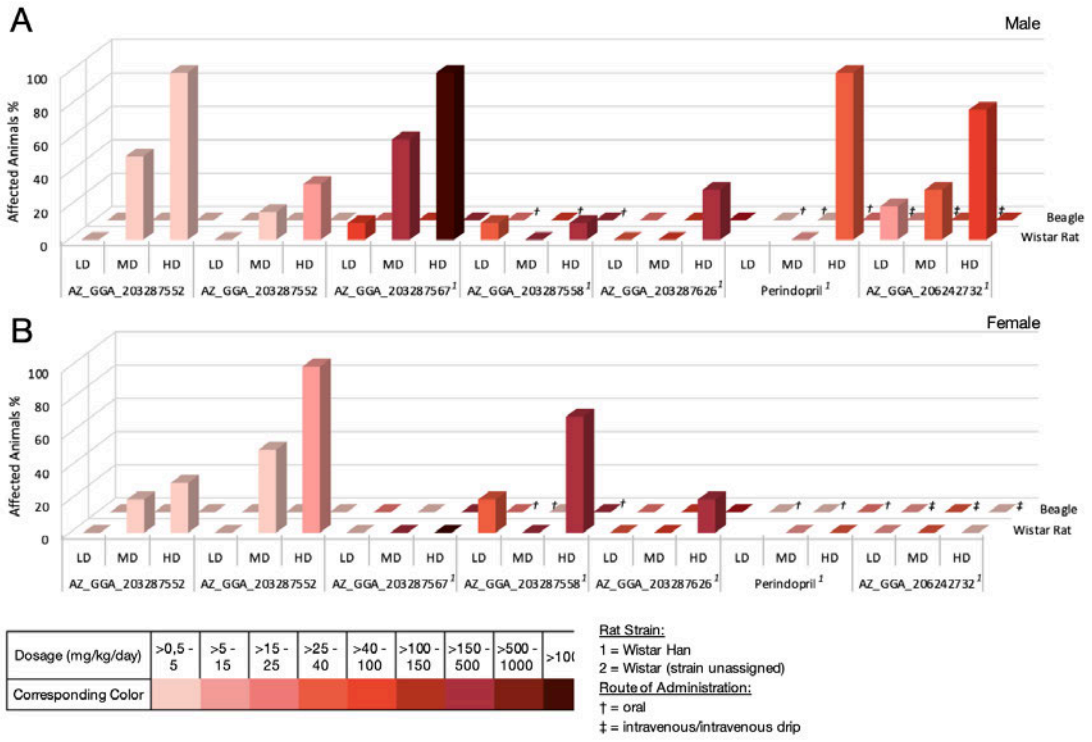


Figure 12 - Consistency of the occurrence of treatment-related tubular basophilia across species in 28-day oral gavage studies in rat and beagle. Comparison of the occurrence of treatment-related tubular basophilia across species in 28-day studies in (**A)** male and **(B)** female animals. The comparison includes compounds with identification of treatment-related tubular basophilia in a 28-day oral gavage Wistar/Wistar Han rat study and corresponding 28-day beagle dog studies. It is evident that in none of the seven corresponding 28-day beagle dog studies cases of treatment-related tubular basophilia were identified. *A color code was introduced to indicate the dose-range (mg/kg bw). The numbers in superscript indicate substances conducted in another strain than Wistar - 1=Wistar Han; 2=Wistar (strain unassigned) -, whereas the cross and double cross indicate routes of administration other than "oral gavage".*

3.5.5 Dilation

3.5.5.1 Compounds associated with treatment-related renal dilation

In total, eleven compounds were identified in the eTOX database with treatment-related renal dilation in 28-day oral gavage Wistar and Wistar Han rat studies (Table 25).

As evident from chapter 3.4.2 of this work, treatment-related findings of dilation in 28-day oral gavage Wistar rat studies contained in the eTOX database were always accompanied by the histopathological finding of either renal necrosis or tubular basophilia. In 28-day oral gavage Wistar rat studies, conducted with the compounds “AZD4580”, “AZ_GGA_206269903” and “AZ_GGA_200010598”, treatment-related tubular basophilia co-occurred with renal dilation after 28 days. These three compounds are all VEGF inhibitors (Table 26). Substances “GGA_BI_001”, “AZ_GGA_200002321” and “AZ_GGA_203287734” all exhibited findings of renal necrosis in the 28-day oral gavage Wistar rat studies in addition to the findings of treatment-related renal dilation.

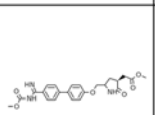
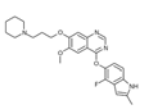
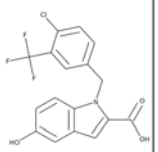
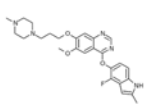
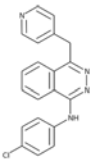
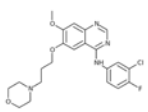
Amongst the range of compounds that induced treatment-related tubular basophilia there are, inter alia, four tyrosine kinase inhibitors and two chemokine receptor antagonists (Table 26).

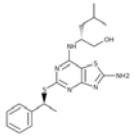
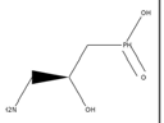
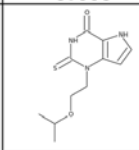
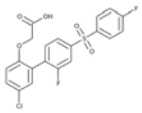
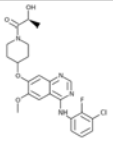
Based on identical dose groups, histopathological findings and clinical chemistry data, it is possible that in compounds “AZD4580” and “AZ_GGA_206269903” data may have been used twice: According to the eTOX database, six different dose groups with each sex were conducted with compound “AZD4580” (3, 6, 9, 4, 8, 12mg/kg bw). However, for the substance “AZ_GGA_206269903” three male and female dose groups (4, 8, 12mg/kg bw) were identified with identical histopathological and gross pathology results as well as with identical clinical chemistry findings. As a consequence, the three latter dose groups (4, 8, 12 mg/kg bw) of compound “AZD4580” were excluded from further analysis.

Table 25 - Search criteria for identification of compounds causing treatment-related renal dilation in a 28-day oral gavage study in Wistar or Wistar Han rats.

	Chosen Search Criteria	Wistar	Wistar Han	Total Hits
Species	Wistar; Wistar Han	408	430	838
Route of administration	Oral gavage	199	298	497
Duration	28-32 days	104	154	258
Result	Histopathology	92	147	239
Site	Kidney	79	129	208
Effect	Dilation; dilation, tubule	47	61	108
Relevance	Treatment-related	6	5	11

Table 26 - Compounds associated with treatment-related renal dilation and their pharmacology.

Compound Name	GGA_BI_001	AZD4580	AZ_GGA_2032 87734	AZ_GGA_2062 69903	AZ_GGA_2000 10598	AZ_GGA_2000 02321
Chemical Structure						
Pharmacology	Fibrinogen receptor antagonist	Vascular endothelial growth factor receptor inhibitor (TKI)	C-C chemokine receptor type 2 antagonist	Vascular endothelial growth factor receptor inhibitor (TKI)	Vascular endothelial growth factor receptor inhibitor (TKI)	Epidermal Growth factor receptor inhibitor (TKI)
Rat Strain	Wistar					

Compound Name	AZ_GGA_2032 87671	AZ_GGA_2032 87693	AZ_GGA_2032 87558	AZ_GGA_2032 87626	AZ_GGA_2032 87757
Chemical Structure					
Pharmacology	C-X3-C chemokine receptor 1 antagonist	GABAB receptor agonist	Myeloperoxidase inhibitor	CRTh2 receptor antagonist	Not available
Rat Strain	Wistar Han				

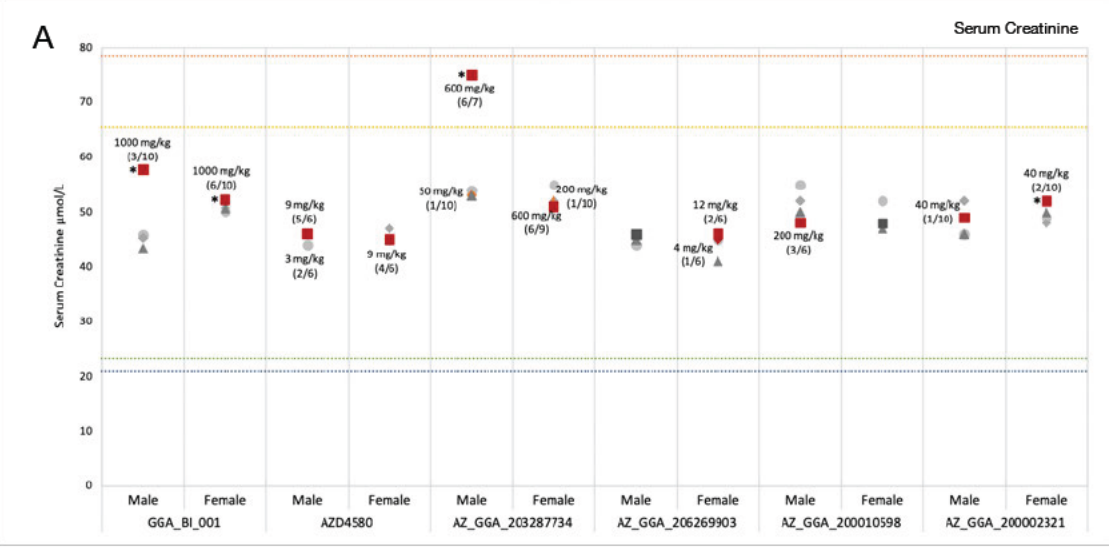
3.5.5.2 Analysis of the consistency of dilation and clinical chemistry findings

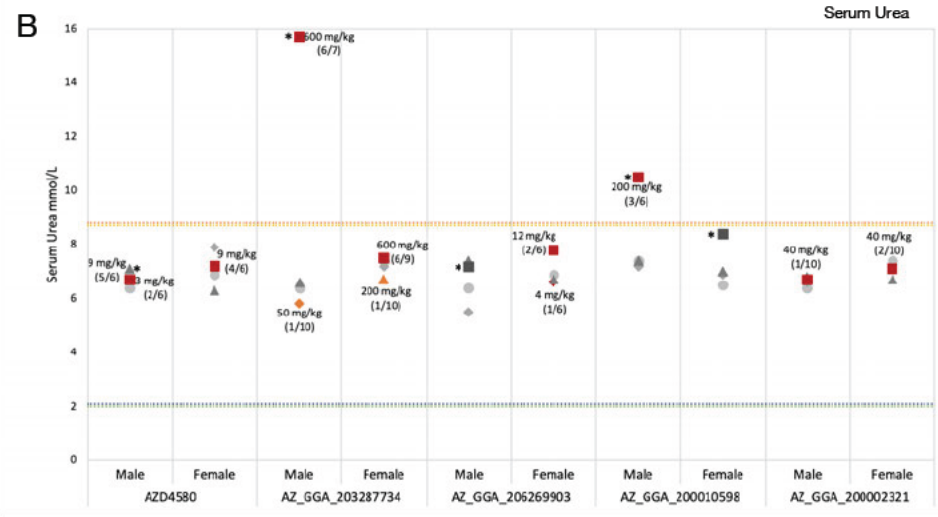
In order to enhance understanding of renal dilation as an adverse finding in preclinical development, relations between a histopathological effect – in this case renal dilation - and its corresponding clinical chemistry (Figure 13) is valuable. Through evaluation of this relationship, it can be possible to point out limitations of the examined clinical chemistry parameters in Wistar rats in regard to their ability to monitor toxic renal effects of compounds in Wistar rats.

As evident from Figure 13A, statistically significant increases in serum creatinine were observed in male and female high-dose groups following treatment with “GGA_BI_001”. Similarly, creatinine values were statistically significantly elevated in the female high-dose group treated with compound “AZ_GGA_200002321” as well as in the male high-dose group following treatment with “AZ_GGA_203287734”. However, it should be noted that necrosis was also recorded in these studies and thus it is likely that the increase in serum creatinine may reflect necrosis rather than tubule dilation.

Concentrations of serum urea can be located out of the reference range in two treatment groups: The male high-dose treatment groups treated with the high dose of substance “AZ_GGA_203287734” and “AZ_GGA_200010598”. Values of both treatment groups were marked as statistically significantly increased by the study pathologists. Both treatment groups exhibited findings of treatment-related dilation. (Figure 13B). Additionally, in the male mid-dose group treated with “AZD4580”, the male high-dose group administered “AZ_GGA_206269903” and the female high-dose group following treatment with substance “AZ_GGA_200010598” statistically significantly elevated urea levels were observed. However, for these three last-named treatment groups no consistent findings of treatment-related dilation were described.

In female mid- and high-dose animal groups of substance “AZ_GGA_203287734” statistically significantly increased serum levels of inorganic phosphate can be identified with simultaneous identification of treatment-related renal dilation (Figure 13C). These values are located within the calculated reference interval.


A
Serum Creatinine
Serum Creatinine µmol/L
1000 mg/kg (3/10)
1000 mg/kg (6/10)
9 mg/kg (5/6)
3 mg/kg (2/6)
9 mg/kg (4/6)
600 mg/kg (6/7)
50 mg/kg (1/10)
200 mg/kg (1/10)
600 mg/kg (6/9)
12 mg/kg (2/6)
4 mg/kg (1/6)
200 mg/kg (3/6)
40 mg/kg (1/10)
40 mg/kg (2/10)
Male
Female
GGA_BI_001
AZD4580
AZ_GGA_203287734
AZ_GGA_206269903
AZ_GGA_200010598
AZ_GGA_200002321


B
Serum Urea
Serum Urea mmol/L
9 mg/kg (5/6)
3 mg/kg (2/6)
9 mg/kg (4/6)
600 mg/kg (6/7)
50 mg/kg (1/10)
600 mg/kg (6/9)
200 mg/kg (1/10)
12 mg/kg (2/6)
4 mg/kg (1/6)
200 mg/kg (3/6)
40 mg/kg (1/10)
40 mg/kg (2/10)
Male
Female
AZD4580
AZ_GGA_203287734
AZ_GGA_206269903
AZ_GGA_200010598
AZ_GGA_200002321

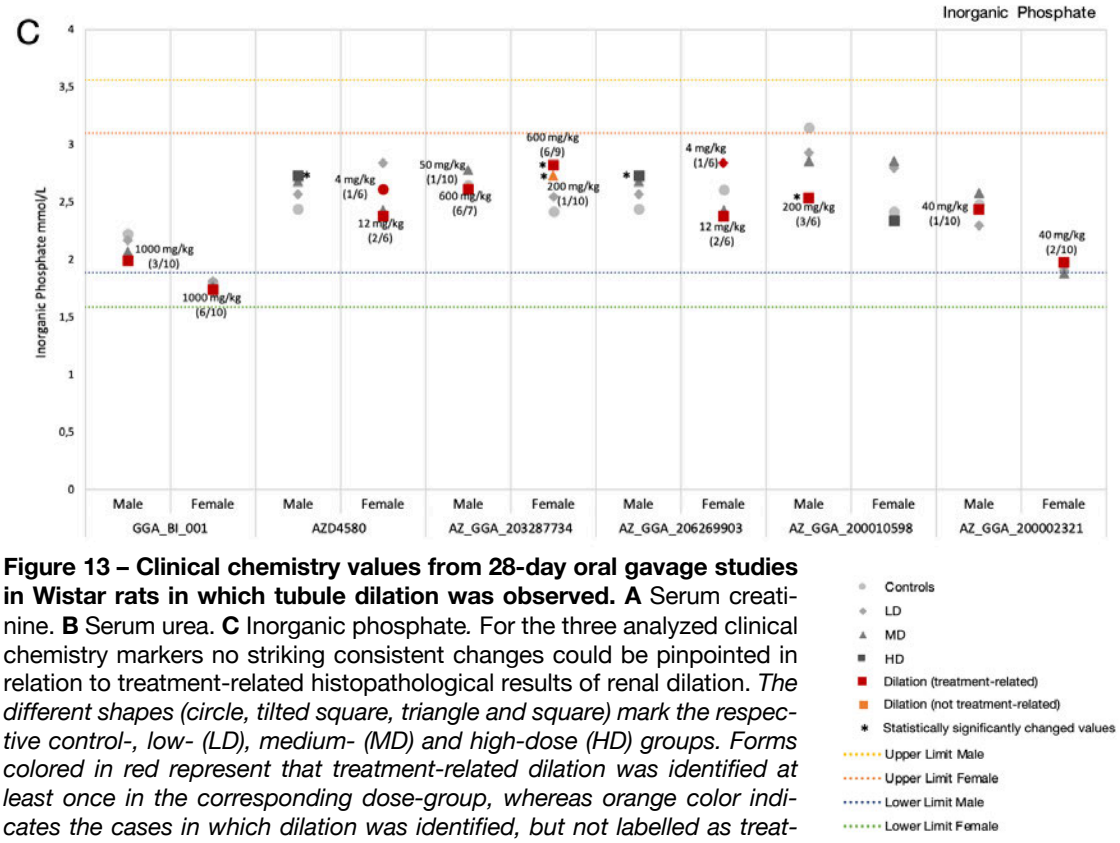


Figure 13 – Clinical chemistry values from 28-day oral gavage studies in Wistar rats in which tubule dilation was observed. A Serum creatinine. **B** Serum urea. **C** Inorganic phosphate. For the three analyzed clinical chemistry markers no striking consistent changes could be pinpointed in relation to treatment-related histopathological results of renal dilation. *The different shapes (circle, tilted square, triangle and square) mark the respective control-, low- (LD), medium- (MD) and high-dose (HD) groups. Forms colored in red represent that treatment-related dilation was identified at least once in the corresponding dose-group, whereas orange color indicates the cases in which dilation was identified, but not labelled as treatment-related. The boxes next to the red and orange shapes indicate the dosages, which were used in the respective groups as well as the number of identified results of renal dilation. Stars denote values which were statistically significantly changed according to the eTOX database. The dotted lines indicate the calculated clinical chemistry reference levels for male and female animals (see chapter 3.3).*

3.5.5.3 Analysis of the consistency of dilation in relation to treatment duration, including consideration of dose

Searching the eTOX database yielded in total seven substances with shorter- and longer term studies performed with the compounds identified as causing dilation within a 28-day study in rats (Figure 14).

In both male and female animals treated with “AZ_GGA_203287734”, treatment-related dilation was identified in an 8-day study in the mid- (1000 mg/kg bw) and high-dose (2000 mg/kg bw) groups. These data are consistent with treatment-related dilation observed in both male and female high-dose group (600 mg/kg bw) of the corresponding 28-day oral gavage Wistar rat study.

Treatment-related dilation was also consistently observed in rats treated with “AZ_GGA_200002321” for 8 and 28 days. Evidence of treatment-related dilation was detected in the female mid-dose group (1000 mg/kg bw) as well as

in the male and female high-dose group (2000 mg/kg bw) in an eight-day study, consistent with the occurrence of treatment-related tubular basophilia in the corresponding male and female high-dose groups (600 mg/kg bw) of the 28-day Wistar rat study. However, a 26-week study with a maximum dose of 25 mg/kg bw revealed no evidence of treatment-related renal dilation, therefore not confirming a high consistency of treatment-related dilation across treatment duration for this compound.

Even though similar dosages were used, no consistent findings of treatment-related dilation across different treatment durations were observable for compounds “AZ_GGA_203287671”, “AZ_GGA_203287626” and “AZ_GGA_203287693”. Thus, analysis of the selection of available compounds in the eTOX database implies a rather low consistency of treatment-related dilation across treatment-duration in rats.

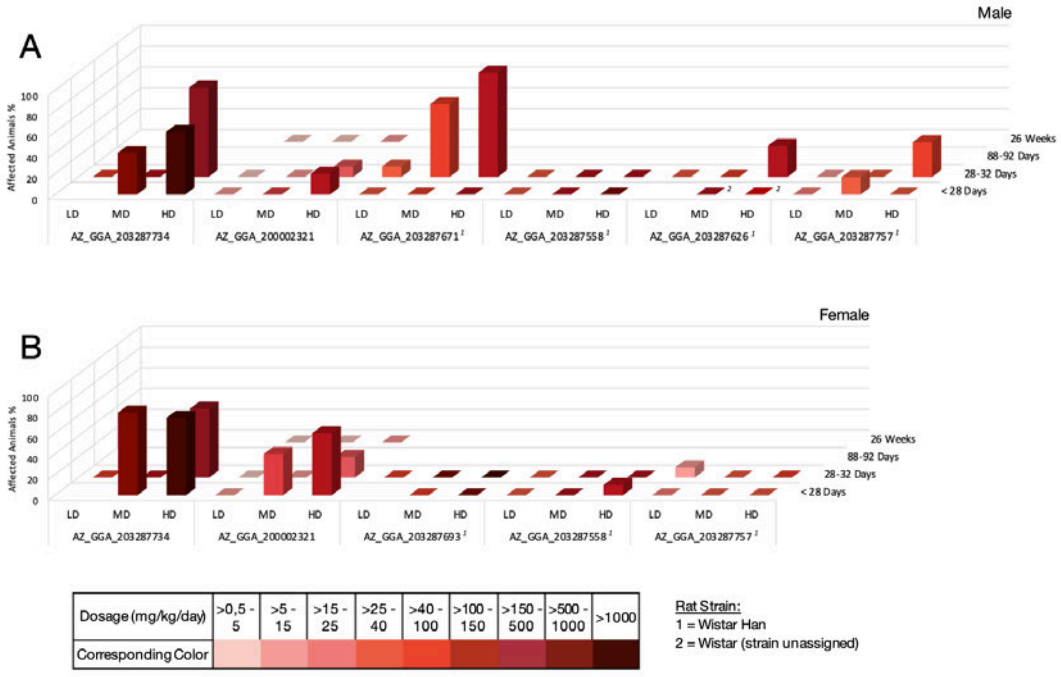


Figure 14 – Consistency of the occurrence of treatment-related renal dilation in relation to treatment duration in Wistar and Wistar Han rats. Comparison of the occurrence of treatment-related renal dilation across treatment duration in **(A)** male and **(B)** female rats. The comparison includes compounds with identification of treatment-related renal dilation in a 28-day oral gavage Wistar/Wistar Han rat study and at the same time with at least one available study over a different treatment duration. In total seven compounds with corresponding studies are available. *A color code was introduced to indicate the dose-range (mg/kg bw). The numbers in superscript indicate substances or studies conducted with another strain than Wistar - 1=Wistar Han; 2=Wistar (strain unassigned).*

3.5.5.4 Analysis of the consistency of dilation across species, including consideration of dose

Cross-species consistency between the frequently used species of rats and dogs is of high significance in preclinical development, particularly in view of possible species-differences. Due to the fact that renal dilation has already been demonstrated to exhibit high background incidence in both rats and dogs, consistency across species is of particular interest.

In total a comparison between 28-day oral gavage beagle dog studies and corresponding 28-day Wistar rat studies (conducted with the identical compound) is feasible for six substances. However, treatment-related renal dilation was recorded in these beagle dog studies for neither male, nor female animals (Figure 15). The dose levels of compounds administered to beagle dogs were equal or lower than doses in the respective rat studies.

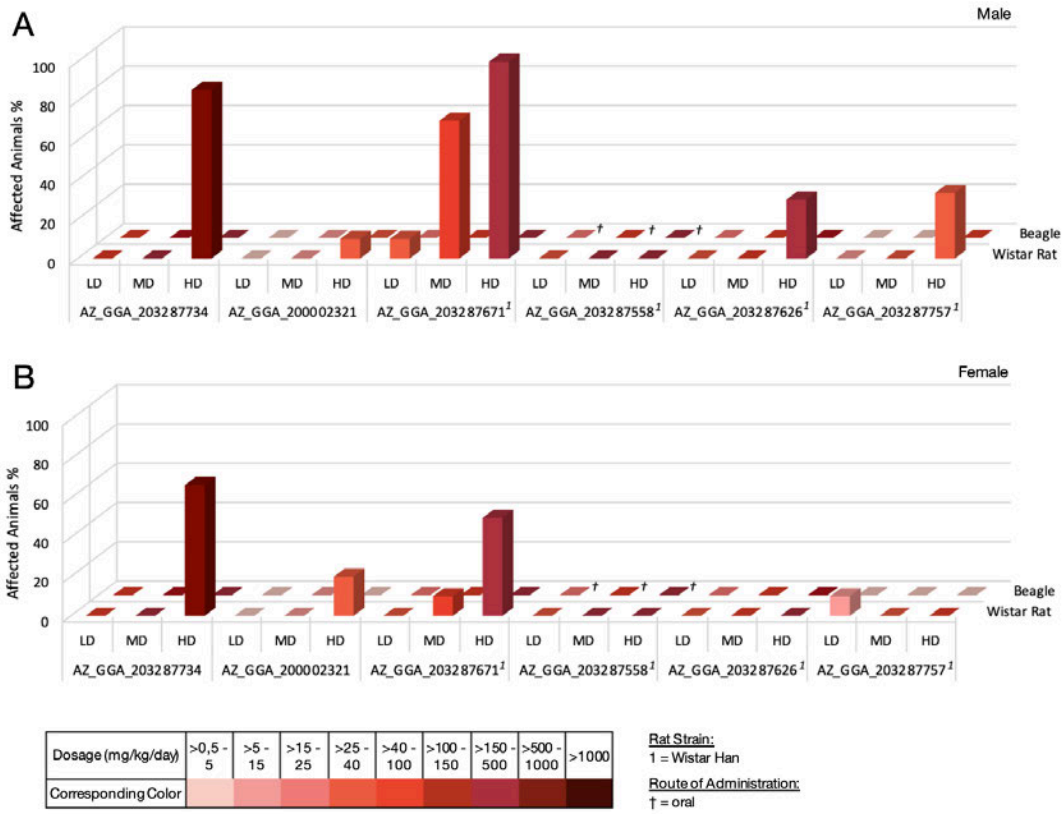


Figure 15 - Consistency of the occurrence of treatment-related renal dilation across species in 28-day oral gavage studies in rat and beagle. Comparison of the occurrence of treatment-related dilation across species in 28-day studies in **(A)** male animals and **(B)** female animals. The comparison includes compounds with identification of treatment-related dilation in a 28-day oral gavage Wistar/Wistar Han rat study and with available corresponding 28-day studies conducted in beagle dogs. There are seven comparable 28-day studies in beagle dogs, but no case of treatment-related dilation was recorded. However, the doses administered to the beagle dogs were consistently smaller or equal. *A color code was introduced to indicate the dose-range (mg/kg bw). The number one in superscript indicates that the rat strain was labelled as Wistar Han in the eTOX database and not as Wistar. Crosses denote treatment groups in which route of administration was labelled as "oral" instead of "oral gavage"*

3.5.6 Hyperplasia

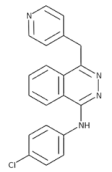
Searching the eTOX database yielded no 28-day oral gavage studies in Wistar and Wistar Han rats in which hyperplasia was recorded. Consequently, evaluation of renal hyperplasia in relation to clinical chemistry and consistency across time and species was not feasible.

3.5.7 Inflammation, neutrophilic

3.5.7.1 Compounds associated with treatment-related neutrophilic inflammation

The eTOX database includes only one 28-day oral gavage study that presents with histopathological evidence of neutrophilic inflammation in Wistar and Wistar Han rats (Table 27). Therefore, analysis of the frequency of neutrophilic inflammation, its relation to clinical chemistry parameters and consistency of neutrophilic inflammation across both time and species was not possible.

Table 27 - Compounds associated with neutrophilic inflammation and their pharmacology

Compound Name	AZ_GGA_200010598
Chemical Structure	
Pharmacology	Vascular endothelial growth factor receptor
Rat Strain	Wistar

3.5.8 Necrosis

3.5.8.1 Compounds associated with treatment-related renal necrosis

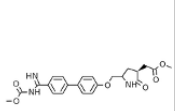
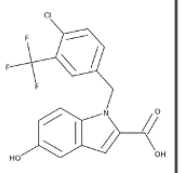
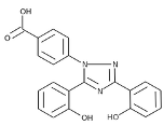
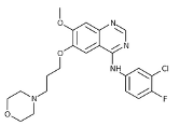
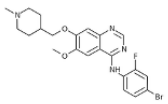
In order to assess occurrence of renal necrosis in 28-day Wistar and Wistar Han rat studies in relation to clinical chemistry parameters, study duration and species, the eTOX database was mined for 28-day oral gavage studies in Wistar and Wistar Han rats in which renal necrosis was observed (Table 28).

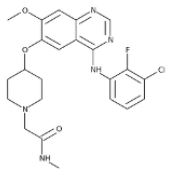
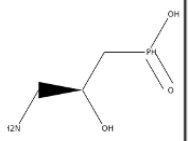
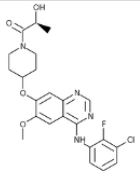
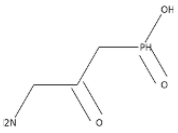
Table 28 - Search criteria for detection of compounds with 28-day oral gavage Wistar and Wistar Han rat studies with the histopathological effect of renal necrosis.

	Chosen Search Criteria	Wistar	Wistar Han	Total Hits
Species	Wistar; Wistar Han	408	430	838
Route of administration	Oral gavage	199	298	497
Duration	28-32 days	104	154	258
Result	Histopathology	92	147	239
Site	Kidney	79	129	208
Effect	Necrosis	4	5	9

In six of the nine identified compounds the type of necrosis is specified as papillary necrosis, while administration of the other three substances induced tubular necrosis (“AZ_GGA_203287734”), single cell necrosis (“Deferasirox”) and a type of necrosis designated as “not specified” (“GGA_BI_001”) respectively (Table 29).

Table 29 – Compounds associated with different types of renal necrosis and their pharmacology.

Compound Name	GGA_BI_001	AZ_GGA_203287734	Deferasirox	AZ_GGA_200002321	AZ_GGA_200009505
Chemical Structure					
Pharmacology	Fibrinogen receptor antagonist	CCR2b-Receptor antagonist	Iron chelation of bis-hydroxyphenyl triazole type	Epidermal growth factor receptor inhibitor (TKI)	Vascular endothelial growth factor receptor inhibitor (TKI)
Type of Necrosis	Not specified	Tubular Necrosis	Single Cell Necrosis	Papillary Necrosis	
Rat Strain	Wistar	Wistar Han	Wistar		

Compound Name	AZ_GGA_203287759	AZ_GGA_203287693	AZ_GGA_203287757	AZ_GGA_203287755
Chemical Structure				
Pharmacology	Inhibitor of the EGF and ErbB2 tyrosine kinases (TKI)	GABAB receptor agonist	Not available	Not available
Type of Necrosis	Papillary Necrosis			
Rat Strain	Wistar Han			

Three of the six compounds, “AZ_GGA_200002321”, “AZ_GGA_200009505” and “AZ_GGA_203287759”, in which papillary necrosis was identified, are pharmacologically described as tyrosine kinase inhibitors. In addition, even though the pharmacological mechanism of “AZ_GGA_203287757” was not stated, this compound may also be a tyrosine kinase inhibitor based on its structural similarity to “AZ_GGA_203287759”. One particularity of compound “GGA_BI_001” was the fact that in the 28-day oral gavage male high-dose rat study (1000 mg/kg bw) not only three cases of treatment-related necrosis were

described but also one case of papillary necrosis, which was marked as not treatment-related by the study pathologists.

3.5.8.2 Analysis of the consistency of necrosis and clinical chemistry findings

Clinical chemistry reference intervals describe the typical range of results seen in a healthy reference population. Values outside a reference range are not necessarily pathologic. Nonetheless, in the context of this work they can possibly indicate the occurrence of renal lesions. In order to further improve knowledge of the consistency between renal necrosis in 28-day oral gavage studies in Wistar rats and the biochemical parameters serum creatinine, serum album, serum urea and inorganic phosphate, a systematic evaluation was carried out by plotting the respective clinical chemistry values in the respective (low-, medium- and high-dose) group. In total concentrations of clinical chemistry parameters of eight compounds exhibiting necrosis in 28-day Wistar rat studies were plotted into the graph. Each 28-day study usually consisted of six different “treatment groups” – a male and female low-, medium and high-dose group.

Statistically significant increases in serum creatinine were observed in five out of 14 different “treatment groups” in which necrosis was identified (Figure 16A): The respective male high-dose group treated with compound “GGA_BI_001” and “AZ_GGA_203287734”, the female high-dose group treated with compound “AZ_GGA_200002321”, as well as the male and female high-dose group administered with compound “AZ_GGA_200009505”. In these five treatment groups necrosis was registered in at least 30 percent of the respective treatment group’s animals. However, despite the significant increase in these treatment groups, serum creatinine values exceeded the upper reference limit only in male rats administered the high-dose of “AZ_GGA_203287734”. In this specific treatment group, tubular necrosis was identified in five out of seven animals, which is the highest frequency amongst all compared treatment groups. The grade of necrosis in this particular treatment group (high-dosed males with

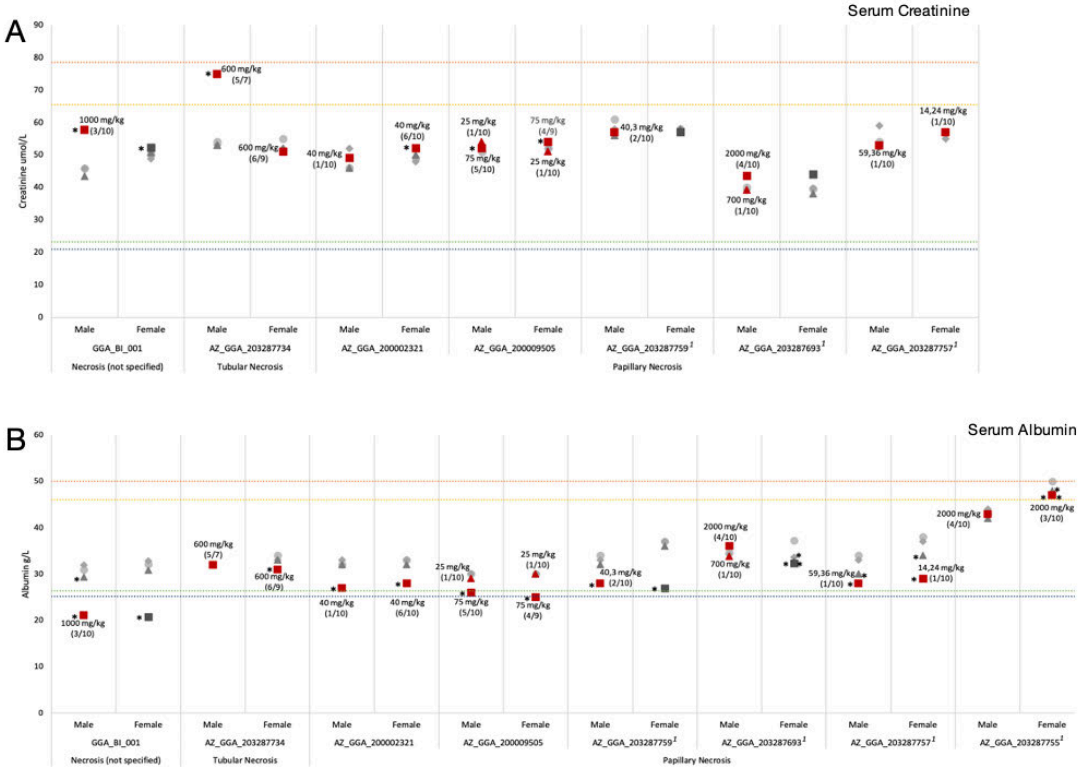
compound “AZ_GGA_203287734”) was described as low in two and as medium in three of the five male affected rats. In the corresponding female high-dose group treated with compound “AZ_GGA_203287734” five cases of low grade necrosis and a single case of medium-grade necrosis were described.

Levels of serum albumin were statistically significantly decreased in nine out of 16 treatment groups, in which the histopathological endpoint of renal necrosis occurred (Figure 16B). Two of these serum albumin values, identified in the male high-dose treated with compound “GGA_BI_001” and in the female high-dose group administered the substance “AZ_GGA_200009505”, were below the lower reference limit. In these groups three out of ten (grade of necrosis is not stated in substance “GGA_BI_001”) and four out of nine rats (three low grade and one medium grade in compound “AZ_GGA_200009505”) were affected by renal necrosis. The substance named “AZ_GGA_200009505” provoked one low grade case of necrosis in the male and female medium-dose group each and four low- and one medium grade case in the male high-dose animals. Two medium grade cases of necrosis were recorded after treatment with compound “AZ_GGA_203287759” (high-dose males), whereas after administration of “AZ_GGA_203287755“ only low grade necrosis occurred (four and three cases in the male and female high-dose group respectively). In compound “AZ_GGA_203287757” study pathologists observed one medium grade case of necrosis in the female mid-dose and the male high-dose group each. “AZ_GGA_203287693” provoked one case of low grade necrosis in its medium-dose male group and two low- and medium grade cases of necrosis each in its high-dose male group. The severity of necrosis was not specified for the compounds “GGA_BI_001” and “AZ_GGA_200002321”.

Concentrations of urea were statistically significantly increased in only three of the 15 groups that presented with findings of necrosis (Figure 16C). However, the concentration of serum urea in the male high-dose group treated with compound “AZ_GGA_203287734” (15,7 mmol/L) was considerably above the calculated reference level. This was also the case for the concentration of

serum creatinine in the same treatment group. Changes in both serum urea and creatinine may reflect kidney necrosis.

No statistically significantly increased serum phosphate levels were evident for the twelve treatment groups, in which renal necrosis was described with the exception of the female high-dose group given compound “AZ_GGA_203287734” (Figure 16D). In this particular group, phosphate levels were reported as statistically significantly increased, yet remained within the calculated reference range.



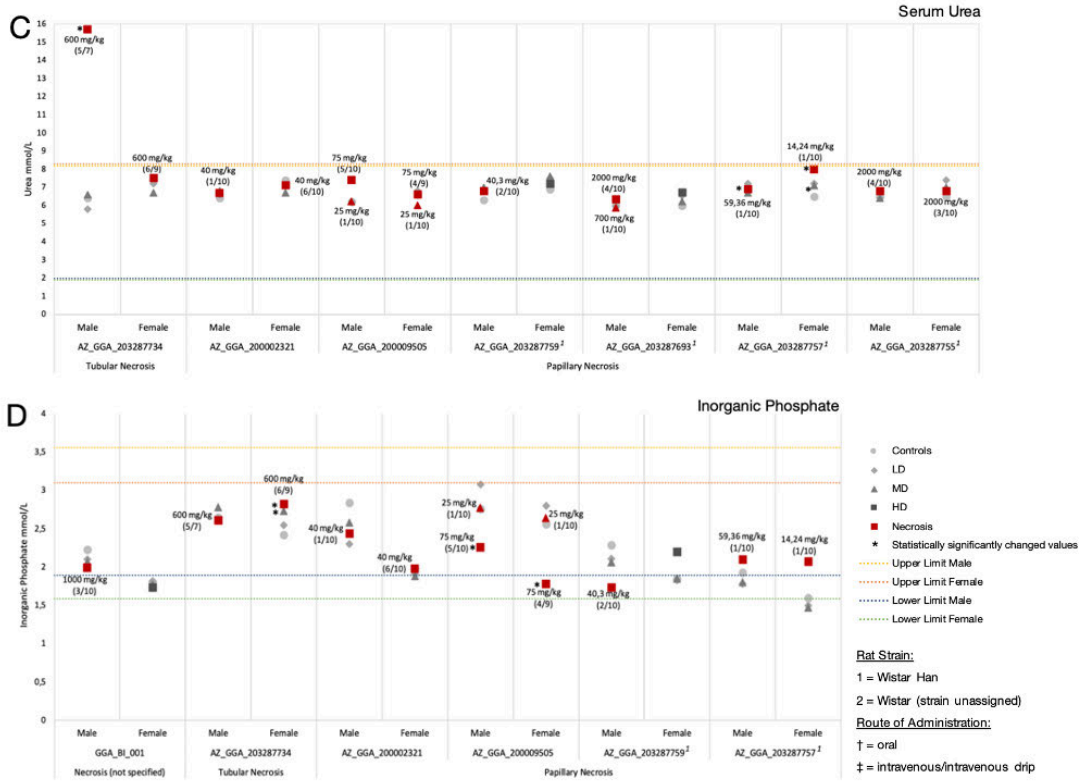


Figure 16 - Clinical chemistry data obtained from 28-day oral gavage studies in Wistar and Wistar Han rats in which renal necrosis was observed. A Serum creatinine. **B** Serum albumin. **C** Serum urea. **D** Inorganic phosphate. *The different shapes (circle, tilted square, triangle and square) mark the respective control groups as well as low- (LD), medium- (MD) and high-dose (HD) groups. Forms colored in red indicate that renal necrosis was identified at least once in the dose-group, whereas orange color shows the cases in which necrosis was identified, but not labelled as treatment-related. The boxes next to the red shapes indicate the dose and the frequency of renal necrosis per dose group. Stars denote values which were reported as statistically significantly changed. The dotted lines indicate the calculated reference ranges for male and female clinical chemistry values (see chapter 3.3).*

3.5.8.3 Analysis of the consistency of necrosis in relation to treatment duration, including consideration of dose

While renal necrosis was identified in 28-day oral gavage rat studies conducted with nine compounds, comparable rat studies with administration of the identical compound over a different study duration were available for seven of these nine compounds (Figure 17). However, renal necrosis was identified by study pathologists in only two of seven substances (“AZ_GGA_203287734” and “AZ_GGA_200002321”) in studies over a different treatment-duration other than 28 days after administration of the compounds which provoked necrosis in a 28-day study.

Treatment-related tubular necrosis was recorded in one out of five animals in the high-dose groups (2000 mg/kg bw) of both male and female rats treated with “AZ_GGA_203287734” for eight days. The administered dose was more than three times higher as compared to the corresponding 28-day study, in which rats were given 600 mg/kg/day bw in the high-dose group. In the corresponding 28-day study renal necrosis was evidenced five of seven male and six of nine female high-dose animals.

In rats treated with compound “AZ_GGA_200002321” renal papillary necrosis was identified consistently throughout different treatment durations. Renal papillary necrosis was reported in two out of five animals in both male and female rats treated with a high-dose (50 mg/kg bw) of “AZ_GGA_200002321” for 14 days. This compares to findings of renal necrosis in the 40 mg-high-dose groups (male: 1/10 animals; female: 6/10 animals) of the reference 28-day oral gavage rat study. Additionally, treatment-related papillary necrosis was recognized in a 26-week study in female high-dose animals (25 mg/kg bw respectively 15 mg from week nine on, 10/20 animals affected), as well as in three males and four females out of 50 animals each in a high-dose group (10 mg/kg bw) of a two-year rat study after treatment with compound “AZ_GGA_200002321”. This indicates a high consistency across treatment duration for this specific compound.

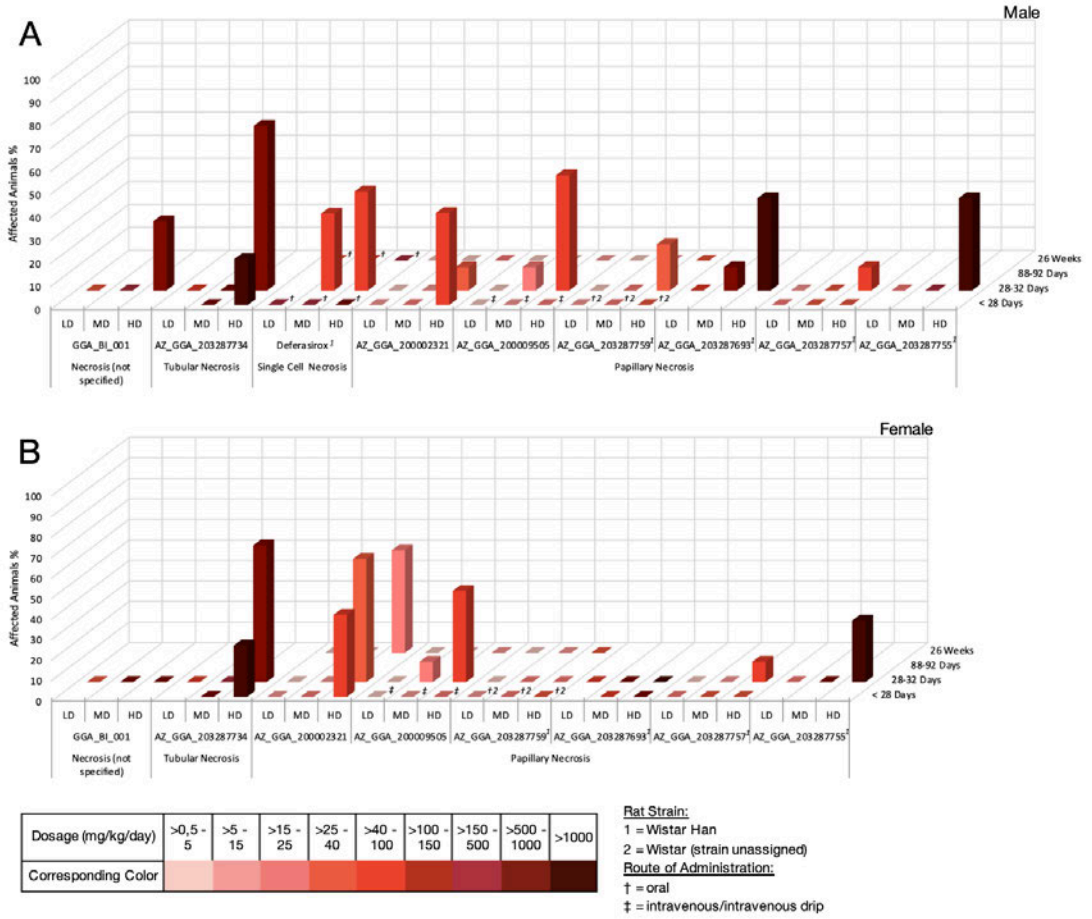


Figure 17 – Consistency of the occurrence of different types of renal necrosis in relation to treatment duration in Wistar and Wistar Han rats. Comparison of the occurrence of renal necrosis across treatment duration in **(A)** male and **(B)** female rats. The comparison includes compounds in which renal necrosis was identified in a 28-day oral gavage Wistar/Wistar Han rat study. At the same time at least one study over a different treatment duration had to be conducted with the identical compound. *A color code was introduced to indicate the dose-range (mg/kg bw). The numbers in superscript indicate substances conducted with another strain than Wistar - 1=Wistar Han; 2=Wistar (strain unassigned), whereas the cross and double cross indicate routes of administration other than "oral gavage".*

3.5.8.4 Analysis of the consistency of necrosis across species, including consideration of dose

With regard to the consistency of the histopathological effect of necrosis across species, no findings of renal necrosis were described in the corresponding 28-day oral gavage studies in the species beagle dogs with the only exception posed by compound "AZ_GGA_200002321" (Figure 18). In this particular 28-day oral gavage study, beagle dogs were dosed at 40 mg/kg bw in the high-dose group. This resulted in a single treatment-related low-grade case of papillary necrosis in the female high-dose treated with substance AZ_GGA_200002321 (1/3 animals). An identical dose of compound

"AZ_GGA_200002321" was administered in the reference 28-day Wistar rat high-dose group (40mg/kg bw), in which one (out of ten) and six (out of ten) rats were identified with renal necrosis in the male and female group respectively. No findings of renal necrosis were recorded in the 28-day oral gavage beagle dog studies of compounds "AZ_GGA_203287734", "AZ_GGA_200009505", "AZ_GGA_203287759" and "AZ_GGA_203287757". All of the corresponding 28-day oral gavage dog studies were carried out at lower dose levels in comparison to the 28-day reference rat studies.

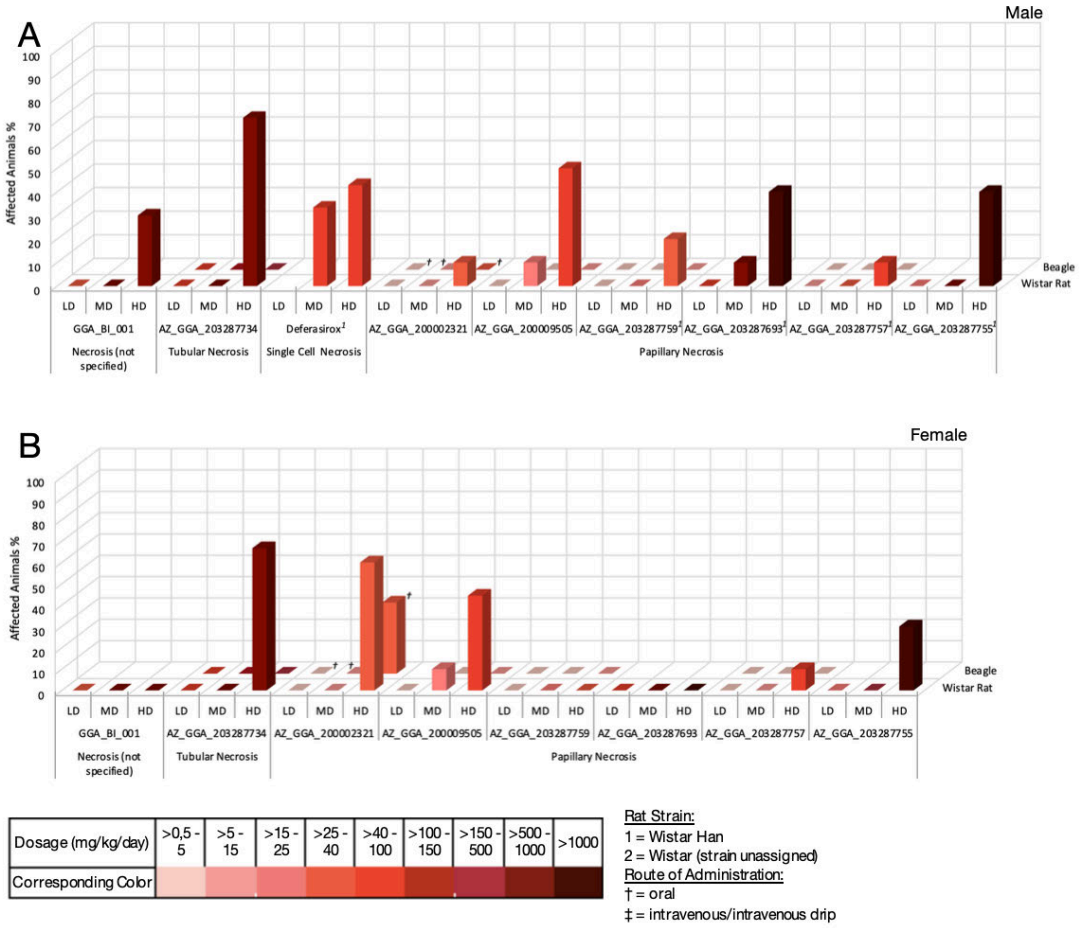


Figure 18 – Consistency of the occurrence of different types of renal necrosis across species in 28-day oral gavage studies in rat and beagle. Comparison of the occurrence of renal necrosis across species in 28-day studies in **(A)** male and **(B)** female animals. Included are compounds, in which renal necrosis was identified in a 28-day oral gavage Wistar/Wistar Han rat study. *A color code was introduced to indicate the dose-range (mg/kg bw). The numbers in superscript indicate substances conducted in another strain than Wistar - 1=Wistar Han; 2=Wistar (strain unassigned) -, whereas the cross and double cross indicate routes of administration other than "oral gavage".*

3.5.9 Glomerulosclerosis

3.5.9.1 Compounds associated with treatment-related renal glomerulosclerosis

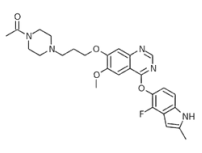
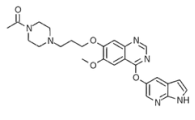
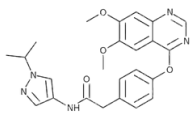
While no Wistar rat was identified with spontaneous occurrence of glomerulosclerosis in 28-day oral gavage studies (chapter 3.4.1), the eTOX database includes four 28-day oral gavage studies that presented with histopathological evidence of glomerulosclerosis in Wistar and Wistar Han rats. The search criteria are listed in Table 30.

Table 30 - Search criteria for identification of compounds causing glomerulosclerosis in a 28-day oral gavage study in Wistar or Wistar Han rats.

	Chosen Search Criteria	Wistar	Wistar Han	Total Hits
Species	Wistar; Wistar Han	408	430	838
Route of administration	Oral gavage	199	298	497
Duration	28-32 days	104	154	258
Result	Histopathology	92	147	239
Site	Kidney	79	129	208
Effect	Glomerulosclerosis	3	1	4

Compound “GGA-SAD-0127” was excluded from further analysis, since glomerulosclerosis was only detected in one mid-dose animal, and this was not considered to be treatment-related. Two of the other three compounds (“AZD9935” and “M596380”) were tyrosine kinase inhibitors, from which “AZD9935” was described as a VEGF-inhibiting tyrosine kinase inhibitor (Table 31).

Table 31 – Compounds associated with renal glomerulosclerosis and their pharmacology

Compound Name	AZD9935	M596380	AZ_GGA_203287646
Chemical Structure			
Pharmacology	VEGFR-Inhibitor	Tyrosine-Kinase-Inhibitor	Not available
Rat Strain	Wistar		Wistar Han

“AZD9935” and “M596380” both were examined in Wistar rat oral gavage studies, whereas for the analysis of the third substance with glomerulosclerosis

(“AZ_GGA_203287646”) Wistar Han rats were utilized. The pharmacological mechanism for “AZ_GGA_203287646” was not stated in the eTOX database.

For “AZD9935”, two 28-day oral gavage studies were identified in which treatment-related glomerulosclerosis was reported. For each of the other two compounds one matching study was identified.

3.5.9.2 Analysis of the consistency of glomerulosclerosis and clinical chemistry findings

In order to analyze the relationship between glomerulosclerosis and various clinical chemistry parameters relevant to nephrotoxicity testing, findings of glomerulosclerosis in 28-day studies in rats were plotted against the respective clinical chemistry data from the same study (Figure 19) - in total three compounds with three male and female dose groups each (plus one male and one female control group per compound).

No clinical chemistry levels were provided for compound “AZ_GGA_203287646”. Therefore Figure 19 gives a detailed graphical overview for the two substances “AZD9935” (study one and two) and “M596380”.

Consistent with histopathological evidence of glomerulosclerosis, a significant treatment-related decrease in serum albumin accompanied by a significant treatment-related increase in blood cholesterol was observed in high-dose male rats in response to “M596380” as well as in the mid- and high-dose females of study two and high-dose females of study one administered “AZD9935” (Figure 19C & E). Furthermore, protein levels were statistically significantly decreased in the three above-named female treatment groups. However, triglyceride levels were decreased rather than increased (Figure 19F). In contrast, no statistically significant effects were seen for the females treated with “M596380”, even though serum albumin was slightly decreased, and blood cholesterol increased. In male Wistar rats in study one dosed with “AZD9935”, no effects on serum albumin, but a statistically significant increase in blood cholesterol were observed. In six out of eight dose groups in which glomerulosclerosis was observed, cholesterol levels were above the calculated reference range, and in five of the eight cases denoted as statistically significantly elevated as well.

There was a statistically significant decrease in serum protein in both studies for high-dose female animals treated with “AZD9935” (Figure 19D). This effect was however not labelled as treatment-related in study two. In these particular high-dose females not only blood protein decreased statistically significantly but was also accompanied by a statistically significant decrease in serum albumin and a statistically significant increase in blood cholesterol. Neither of those effects was specified as treatment-related. In the corresponding high-dose male animals in study two conducted with compound “AZD9935”, concentrations of blood protein and serum albumin remain unchanged. However, both serum triglycerides and blood cholesterol increased in a statistically significant manner, but were denoted as not treatment-related. This shows that male Wistar rats indeed do seem to be affected by treatment with “AZD9935” even though no GS was reported.

Values of serum urea and creatinine were not significantly changed in any of the animal groups treated with “AZD9935” or “M596380” consistent with glomerulosclerosis (Figure 19A & B). The same applies for serum phosphate, for which no statistically significant changes were identified in any dose group of the 28-day oral gavage studies on compounds “AZD9935” (study one and two) and “M596380” (Figure 19G). This implies that glomerulosclerosis had no significant impact on these particular clinical chemistry concentrations.

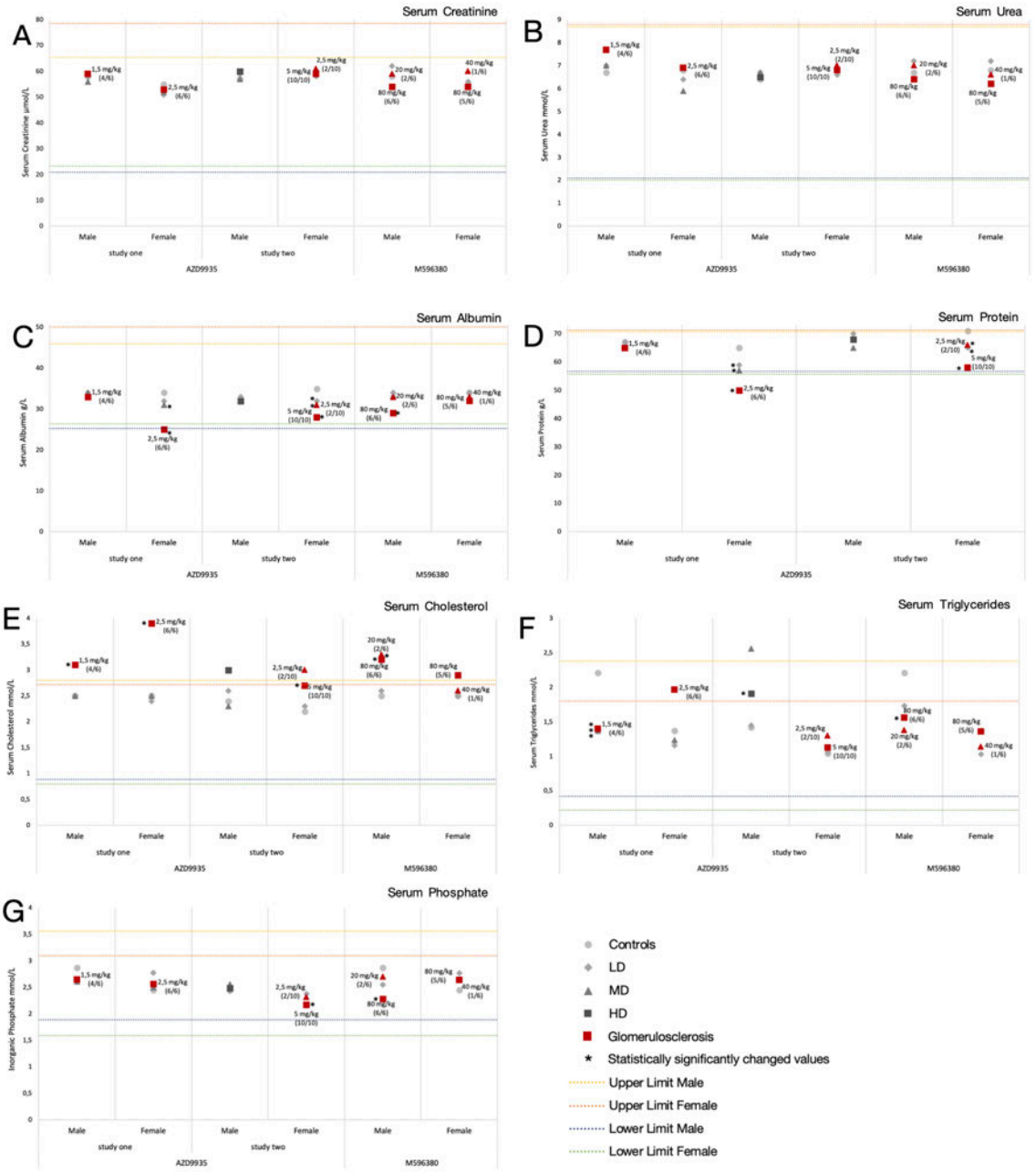


Figure 19 - Clinical chemistry data obtained from 28-day oral gavage studies in Wistar rats in which glomerulosclerosis was observed. A Serum creatinine. **B** Serum urea. **C** Serum albumin. **D** Serum protein. **E** Serum cholesterol. **F** Serum triglyceride. **G** Inorganic phosphate. *The different shapes (circle, tilted square, triangle and square) mark the respective control groups as well as low- (LD), medium- (MD) and high-dose (HD) groups. Forms colored in red indicate that glomerulosclerosis was identified in at least one animal per dose-group, whereas orange color shows the cases in which glomerulosclerosis was identified, but not labelled as treatment-related. The boxes next to the red shapes indicate the dose and the frequency of glomerulosclerosis per dose group. Stars denote values which were reported as statistically significantly changed. The dotted lines indicate the calculated reference ranges for male and female clinical chemistry values (see chapter 3.3).*

3.5.9.3 Analysis of the consistency of glomerulosclerosis in relation to treatment duration, including consideration of dose

Searching the eTOX database yielded no studies in Wistar and Wistar Han rats with a study duration other than 28 days for the substances of “AZD9935”, “M596380” and “AZ_GGA_203287646” (Figure 20). As a consequence, no conclusions can be drawn regarding the consistency of glomerulosclerosis across treatment duration.

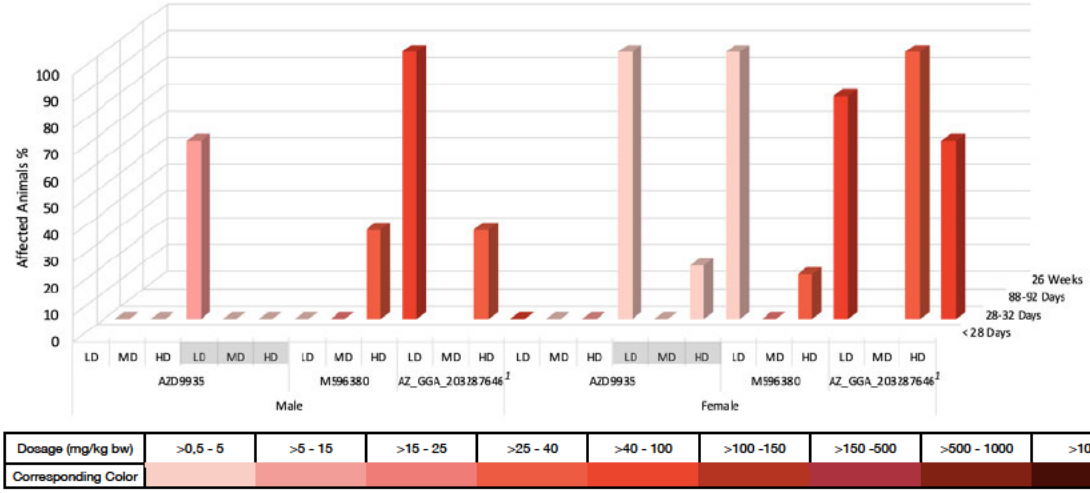


Figure 20 - Consistency of the occurrence of glomerulosclerosis in relation to treatment duration in Wistar and Wistar Han rats. As can be seen no rat studies over a different treatment duration other than 28 days are available for the above compounds. *A color code was introduced to indicate the dose-range. The number one in superscript denotes the use of Wistar Han rats instead of Wistar rat.*

3.5.9.4 Analysis of the consistency of glomerulosclerosis across species, including consideration of dose

Evaluation of cross-species consistency of glomerulosclerosis revealed no cases of glomerulosclerosis in any of the three 28-day oral gavage beagle dog studies after administration of “AZD9935” or “AZ_GGA_203287646” (Figure 21). Noteworthy is the fact that the maximum dose administered in these 28-day oral gavage dog studies was 0.5 mg/kg bw (“AZD9935”) and 2.2 mg/kg bw (“AZ_GGA_203287646”). Maximum doses in the corresponding rat studies were with 6 (“AZD9935”) and 49.2 mg/kg bw (“AZ_GGA_203287646”) considerably higher. No 28-day studies in beagle dogs are available for compound “M596380”.

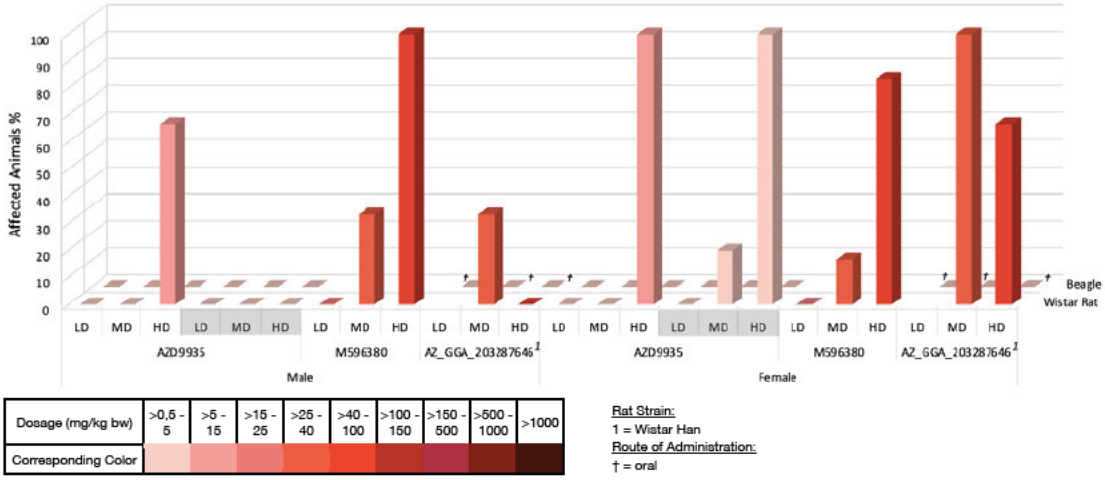


Figure 21 - Consistency of the occurrence of glomerulosclerosis across species in 28-day oral gavage studies in rat and beagle. Included are compounds, in which glomerulosclerosis was identified in a 28-day oral gavage Wistar/Wistar Han rat study. It is evident that in none of the corresponding 28-day beagle dog studies cases of glomerulosclerosis were identified. Doses used in the beagle dog studies were considerably lower in comparison to the corresponding 28-day rat studies. *A color code was introduced to indicate the dose-range. The number one in superscript indicate substances conducted in Wistar and the cross indicates an "oral" route of administration.*

4 DISCUSSION

Through the eTOX project a large, comprehensive database of high-quality in vivo preclinical toxicity data was established. This work took advantage of the extensive amount of raw data in the eTOX database in order to obtain reliable data on the frequency of different types of toxicity findings and consistency across study duration and species. This was accomplished through mining the eTOX database with regard to the frequency of renal lesions in preclinical 28-day oral gavage studies in rats, the relation between renal histopathological findings and routine clinical chemistry parameters indicative of kidney injury, as well as the consistency of renal histopathological findings across time and species. The overall objective of this work is to better understand and manage the development of certain toxicity findings and safety liabilities.

At the time of investigation, the eTOX platform contained 1395 compounds with 6501 associated preclinical safety studies. For 258 of these compounds, 28-day oral gavage studies conducted in Wistar or Wistar Han rat were available. Evidence of a treatment-related renal effect within 28-day oral gavage studies in rats were reported in response to 49 of these compounds. Tubular basophilia and dilation were the most frequent renal lesions in 28-day oral gavage rat studies amongst the eight histopathological endpoints investigated. These two histopathological effects also exhibited the highest background incidence. In contrast, glomerulosclerosis and renal necrosis were not or only rarely identified as a spontaneous lesion in control animals within 28-day oral gavage studies. Necrosis and glomerulosclerosis were also less frequently observed in 28-day oral gavage rat studies and were predominantly considered treatment related – as opposed to tubular basophilia and dilation, which were frequently denoted as non-treatment related by the study pathologist.

Changes in clinical chemistry consistent with the histopathological evidence of the analyzed renal toxicity findings were not observed with the exception of necrosis causing creatinine and urea to rise statistically significantly in approximately a third, and albumin to decrease statistically significantly in almost two thirds of the treatment groups consistent with the histopathological

evidence of necrosis. Furthermore, glomerulosclerosis occurred consistently with a pattern of statistically significantly lowered serum albumin and serum protein as well as a statistically significant increase in serum cholesterol in both female high-dose animal groups of study one and two of compound “AZD9935”.

A comparison of the examined histopathological findings over different study durations revealed a high consistency across treatment duration for the renal lesion of necrosis specifically for compound “AZ_GGA_200002321”. However, consistent findings across study duration were not observed for the other compared compounds exhibiting renal necrosis in 28-day oral gavage Wistar rat studies. Whereas a high consistency across study duration for the toxicity findings of tubular basophilia and renal dilation could only party be confirmed, search of the eTOX database yielded no studies in Wistar and Wistar Han rats with a study duration other than 28 days for the compounds that produced glomerulosclerosis in 28-day studies.

None of the analyzed renal lesions were observed consistently as a compound-related finding in corresponding subacute 28-day beagle dog studies.

4.1 Spontaneous renal findings in 28-day oral gavage studies in control Wistar rats and beagle dogs

The knowledge of spontaneous incidences of histopathological effects does not only contribute to a better understanding of renal adverse reactions to drugs in preclinical research but is also crucial for histopathologists at the bench in order to evaluate if a histopathological finding occurred spontaneously or whether it was provoked through administration of a (potentially toxic) compound in preclinical studies. For instance, does familiarity of the exact background incidence make a crucial difference for study pathologists when setting no observed effect levels (NOELs), where minor histological distinctions can have large implications for safety margin calculations (and therefore drug development). Determination of NOELs is particularly challenging if the difference between treated and control groups is small. Study pathologists then have to decide whether that difference is just a chance distribution of a naturally variable feature or a genuine treatment-related effect (145). Furthermore, the slightest

expression of nephrotoxicity simply might occur as a marginal exacerbation of the severity of a spontaneously occurring lesion. Spontaneous disease may also mask or mimic a toxicologic response (55).

The background incidence of spontaneous lesions varies by research laboratories and by the strain of rats used in preclinical toxicology studies. A majority of rats in chronic and carcinogenicity studies exhibit a range of age-related inflammatory, proliferative, and degenerative lesions in the kidney predominated by chronic progressive nephropathy (CPN) (55).

The calculated background incidences of renal findings in 28-day oral gavage studies in Wistar rats and beagle dogs were basically within expectations. As tubular basophilia is known to be a frequent finding in both rats and dogs (50, 52, 55), the high background incidence in both animal species is not particularly surprising. Specially in laboratory rats, tubular basophilia is a common microscopic change accompanying CPN (146). The spontaneous incidence of tubular basophilia in male rats has already been demonstrated to exceed background incidence in females (55), which matches the results of this work. According to literature, CPN also is associated with tubule dilation (55). This possibly explains the rather high background incidence of renal dilation particularly in male but also in female Wistar rats identified in the eTOX database in 28-day oral gavage studies. CPN is a rodent-specific entity (55). This may explain the lower incidence of renal dilation in beagle dogs in comparison to rats determined in this study. These findings are in line with *Khan et al*., who already described tubule dilation as a rare spontaneous finding in beagle dogs by (55). In literature, tubular atrophy has been reported to be frequently present in late stage CPNs (50). This can potentially be supported by this work with a background incidence of approximately two percent of renal tubular atrophy in 28-day oral gavage studies conducted in Wistar rat controls. Not a single finding of “hyaline droplet accumulation” was detected in Wistar rat control animals in 28-day oral gavage studies in the eTOX database. This is not in line with literature, in which “hyaline droplet accumulation” is characterized as a renal lesion with a spontaneous incidence of more than two percent in two- to thirteen-week studies in Wistar rats

(55). The histopathological effect of renal hyperplasia is one of the few analyzed findings actually occurring more commonly spontaneously in beagle dogs than in Wistar rats in the eTOX database, as demonstrated by the results of this work. However, literature reveals that this histopathological result occurs only rarely in beagle dogs spontaneously, but actually is considered as part of spontaneously occurring CPN in Wistar rats (55). Nonetheless renal hyperplasia was found in less than every 200th rat in the eTOX database in controls after 28 days. The very few or even no findings of glomerulosclerosis and necrosis not surprisingly are consistent with literature as they are not known to be spontaneous findings in neither Wistar rats nor beagle dogs. Total control animal number for the species of beagle dogs, with in total 549 control animals, was considerably lower in comparison to 1783 Wistar control rats. The bigger the total number of control animals, the higher the significance of results can be considered.

4.2 Evaluation of compounds linked with the histopathological result of tubular basophilia

Detailed knowledge of a histopathological finding is fundamental for pathologists in preclinical development. Tubular basophilia is a histopathological result, which has to be considered in parts as problematic to evaluate based on clinical chemistry biomarkers taking the fact into account that the co-occurrence of endpoints such as glomerulosclerosis and necrosis most certainly influence resulting clinical chemistry levels. Furthermore, tubular basophilia is known to be a common spontaneous finding as this work also confirmed, but at the same time it is considered as an initial and some of the earliest evidence of nephron injury potentially acting as a precursor lesion of more severe kidney injury as stated by literature (52). However, the fact, that tubular basophilia is more likely to occur spontaneously in comparison to other histopathological findings does not make it less important to discuss. This is because for example a minimal expression of nephrotoxicity simply might occur as a slight exacerbation of the severity of a spontaneously occurring lesion or the development of a spontaneous disease can also mask or mimic a toxicologic response.

Additionally, there is evidence that tubule basophilia is a histopathological finding, which can arise in very different cellular circumstances: Examples are tubular regeneration, renal degenerative conditions but also in kidney diseases in human patients like pyelonephritis and kidney nephroblastoma (50).

It is remarkable that five of the eight identified compounds consistent with the occurrence of treatment-related tubular basophilia in 28-day oral gavage Wistar rat studies pharmacologically are denoted as vascular endothelial growth factor-receptor-inhibitors (VEGFR): “AZ_GGA_203287552”, “AZ_GGA_200010232”, “AZ_GGA_203287683”, “AZ_GGA_206269903” and “AZ_GGA_200010598”. Besides those five compounds the eTOX database contains data on one further VEGFR-inhibitor, which however did not produce tubular basophilia in Wistar rats after 28-day administration via oral gavage. VEGFR-inhibitors are nowadays commonly used for cancer treatment due to their ability to inhibit angiogenesis. In human patients, VEGFR-inhibitors have been linked to a high risk of nephrotoxicity, specifically to the production of interstitial nephritis, glomerulonephritis, proteinuria, and hypertension. Generally, vascular endothelial growth factor (VEGF) is described as an endothelial-specific growth factor that induces endothelial cell proliferation and migration, differentiation and survival, mediates endothelium-dependent vasodilatation, induces microvascular hyperpermeability and is involved in interstitial matrix remodeling. In the kidney, VEGF expression is most prominent in glomerular podocytes and in tubular epithelial cells, whereas VEGF receptors can primarily be identified on preglomerular, glomerular, and peritubular endothelial cell (55, 147-149).

4.2.1 Consistency of treatment-related tubular basophilia and clinical chemistry findings

Delivery of clear statements concerning the interpretation of the clinical chemistry results in relation to the consistency of treatment-related tubular basophilia in 28-day oral gavage Wistar rat studies is as expected challenging for the analyzed parameters of serum creatinine, urea and inorganic phosphate. As traditional clinical biomarkers serum creatinine and urea in literature are described to be late indicators of renal damage and not to rise significantly until at least one-

half of the kidney mass has been compromised (55), no significant changes may be expected, which can be confirmed by this work. Also, serum phosphate concentrations are within the calculated reference intervals, which goes in line with assumptions that no significant effects on clinical chemistry concentrations result from the occurrence of treatment-related tubular basophilia.
With regard to the reference intervals, it has to be stated that the calculated reference ranges are probably relatively large due to the data from different companies and thus statistically significant changes in treatment groups relative to the respective controls of the same study are more meaningful.

4.2.2 Consistency of treatment-related tubular basophilia in relation to treatment duration, including consideration of dose

The occurrence of treatment-related tubular basophilia is not consistent throughout different study durations in Wistar rats in the eTOX database. In fact, there are seven corresponding studies conducted with the same compound with a study duration less than 28 days to compare against the 28-day oral gavage Wistar rat studies in the eTOX database. From these seven subacute studies treatment-related tubular basophilia was solely recorded in a 15-day study of compound “Neputitant”. It can potentially be deduced that brief administration of a substance - even with use of higher doses - is not enough to cause tubular basophilia consistently. Nonetheless it has to be mentioned at this point again that this research included only those findings, which were marked as treatment-related. Those designations (e.g. in which case a histopathological result is labelled as treatment related) are not transparent in the eTOX database, which will be discussed in chapter 4.7.

4.2.3 Consistency of treatment-related tubular basophilia across species, including consideration of dose

Consistency of treatment-related tubular basophilia across species is remarkably low as no case of this histopathological result can be identified in any of the six comparable 28-day oral gavage beagle dog studies after treatment with the identical six compounds, which provoked tubular basophilia in rats after

28-days. This is particularly surprising when looking at the fact that tubular basophilia is known to be a frequent finding in both rats and dogs (50, 52, 55). Additionally, a high background incidence in both animal species was observed in 28-day oral gavage studies of the eTOX database (12,4% male and 10,7% female rats vs. 5,6% male and 1,8% female beagle dogs).

4.3 Evaluation of compounds linked with the histopathological result of dilation

The toxicity result of renal dilation can partly be compared to the histopathological effect of tubular basophilia: Interpretation of results is challenging, because dilation also is a common spontaneous finding in rodents, already demonstrated in this research. A reason for the high spontaneous incidence is the fact that renal dilation is part of the renal disorder of chronic progressive nephropathy. Moreover renal dilation has been described to occur in the kidney through manifold causes: Examples are chronic hypokalemia, renal necrosis or regeneration, and the appearance of some dilated tubules even is histologically described as normal (55), (48, 57). Similar to the histopathological lesion of tubular basophilia, endpoints such as necrosis might well influence the results of the analysis, in particular the interpretation of clinical chemistry results.

The first interesting observation is that in half of the six compounds with findings of treatment-related dilation in 28-day oral gavage Wistar rat studies, parallel cases of treatment-related necrosis can be observed after 28 days, precisely in substances “GGA_BI_001”, “AZ_GGA_200002321” and “AZ_GGA_203287734”. For the five compounds examined in the rat species of Wistar Han, this is the event twice, in compounds “AZ_GGA_203287693” and “AZ_GGA_203287757”. This supports the fact that, as stated above, dilation is a frequent finding alongside renal necrosis. The fact that in total in five of the compounds with the finding of treatment-related dilation in 28-day oral gavage studies co-occurrence of treatment-related tubular basophilia is observable, might lead to the possible theory that in those studies chronic progressive nephropathy might have played a triggering role as both dilation and tubule basophilia are common findings in this age-related disease of the rodents’ kidney.

4.3.1 Consistency of treatment-related renal dilation and clinical chemistry findings

As expected, the results of the analysis of clinical chemistry in 28-day oral gavage Wistar rat studies do not allow to draw clear conclusions as values of serum creatinine, serum urea and inorganic phosphate mostly are not significantly changed. Thus, it can be deduced that renal dilation does not seem to consistently impact on either of the three tested clinical chemistry parameters serum creatinine, urea and phosphate in the tested 28-day oral gavage studies in Wistar rats. A possible example of the interference of a co-occurring endpoint (in this case renal necrosis) might be given by the comparison of serum creatinine values, where in three compounds with parallel findings of treatment-related dilation and necrosis, in four high-dose groups statistically significantly elevated creatinine values can be identified: In male and female high-dose groups following treatment with “GGA_BI_001”, in the female high-dose treated with compound “AZ_GGA_200002321” and in the male high-dose group treated with “AZ_GGA_203287734”.

4.3.2 Consistency of treatment-related renal dilation in relation to treatment duration, including consideration of dose

Consistency of the histopathological result of dilation in relation to different treatment duration can only partly be seen. In three out of seven compounds with 28-day oral gavage Wistar rat studies with observation of the histopathological result of treatment-related renal dilation, treatment-related dilation is detectable in corresponding subacute studies conducted with the same compound (in an eight-day study of “AZ_GGA_200002321”, an eight-day study of “AZ_GGA_203287734” and a 21-day study of “AZD_GGA_203287757”). Unsurprisingly, the used doses were consistently considerably higher in the subacute studies. Nevertheless, this demonstrates that renal dilation can be provoked over a short treatment duration as well.

4.3.3 Consistency of treatment-related renal dilation across species, including consideration of dose

Not a single observation of treatment-related renal dilation can be made in the six corresponding 28-day oral gavage beagle dog studies after administration of the same compounds, which induced dilation in Wistar rats after 28 days. Thus, consistency across species has to be denoted as low. This is remarkable when keeping the fact in mind that occurrence of some dilated tubules is considered as normal in the beagle dog's kidney according to literature (48, 57), which also was validated by this work with background incidence of renal dilation being at just below 2% in male as well as in female beagle dog 28-day studies.

4.4 Evaluation of compounds linked with the histopathological result of necrosis

Whereas tubular basophilia and dilation are more common in normal, healthy kidneys and exhibit a high background incidence, renal necrosis is a rather clear indication of nephrotoxicity and does not usually occur spontaneously.

Three compounds, "AZ_GGA_200002321", "AZ_GGA_200009505" and "AZ_GGA_203287759", in which papillary necrosis was identified, are tyrosine kinase inhibitors (TKI). This obviously provokes the question whether TKI's already have been linked to the occurrence of papillary necrosis, according to literature a relatively common form of toxicity observed in preclinical safety testing and initially known under the term analgesic nephropathy (55). In fact, literature reveals data of TKIs provoking tubular necrosis (150), but no evidence of TKI's causing papillary necrosis. As the eTOX database partly consists of drug candidates which were stopped during preclinical or clinical development, it is possible that the consistent occurrence of papillary necrosis was an abort criterion for those studies conducted with a TKI. This has to be stated as an assumption since no further explanation is provided in the eTOX database about the compounds and their history of development. However, *Briggs et al.* describe

the substances included in the eTOX database as compounds that “have become drugs and many more chemicals that have failed to reach the market. These compounds may have been dropped from further development for many reasons, including safety issues.”
It is already mentioned and discussed in the chapter 4.3 above that the consistent occurrence of treatment-related renal dilation with findings of renal necrosis in 28-day oral gavage rat studies in five out of nine compounds is potentially associated with the disease complex of chronic progressive nephropathy.

4.4.1 Consistency of renal necrosis and clinical chemistry findings

Clinical chemistry levels of treatment groups with cases of renal necrosis are considerably more altered in comparison to the clinical chemistry parameters in animals that presented with tubular basophilia and dilation. Statistically significantly increased concentrations of serum creatinine and urea can be detected approximately in a third of the treatment groups with treatment-related necrosis. It is remarkable that in the male high-dose group treated with the only compound causing tubular necrosis, “AZ_GGA_203287734”, levels of serum creatinine and serum urea were far above the calculated reference range, and therefore present a big exception. Renal tubular necrosis is known to be accompanied by a profound kidney dysfunction (151), which serves as an explanation for the extremely altered clinical chemistry values of creatinine and urea. Serum albumin levels in 28-day oral gavage rat treatment groups with treatment-related findings of papillary necrosis are statistically significantly decreased in more than 50 percent of the cases, thus showing high consistency. This possibly confirms a reduced kidney function in those animal groups, probably concurrent with the occurrence of albuminuria. Albuminuria has already been associated with the occurrence of renal papillary necrosis in rats (152) as well as in humans (153, 154).

No statistically significant increased serum phosphate levels can be recognized for the examined treatment groups except for the female high-dose treated with compound “AZ_GGA_203287734”, in which tubular necrosis was

identified. This increase in serum phosphate occurs simultaneously with a decrease in serum albumin. Looking at the four analyzed renal clinical chemistry parameters altogether, the results possibly show that decreased serum albumin is the most sensitive and consistent parameter exhibiting any type of kidney necrosis in 28-day oral gavage rat studies. Moreover, the results can lead to the assumption that tubular necrosis has a higher impact on clinical chemistry parameters in comparison to papillary necrosis.
However, at this point it also has to be stressed that the lack of individual animal clinical chemistry levels is a major limitation of the database since only the mean level is given in the eTOX database. For example, if only two out of five animals exhibit a histopathological effect, there may be a change in clinical chemistry in these two animals. The mean value of the group, on the other hand, may not show a significant effect, which may lead researchers to the conclusion that the correlation between histopathology and clinical chemistry in this case is not very strong. However, if values for histopathology and clinical chemistry were available for each animal individually, the picture might be quite different.

Analysis of the severity of necrosis in the 28-day studies consistent with findings of renal necrosis does not lead to any powerful conclusions. In fact, with a few exceptions, most findings of necrosis in these particular studies are described as low grade, if the severity of necrosis was described at all. An exception presents the only compound, in which tubular necrosis was identified in 28-day Wistar rat studies (“AZ_GGA_203287734”). The grade of necrosis in the high-dosed males after administration of “AZ_GGA_203287734” was described as low in two and as medium in three of the five male affected rats. In the corresponding female high-dose treated with the identical compound five cases of low-grade necrosis and a single case of medium-grade necrosis were identified. The only substances with a record of more than one case of medium grade papillary necrosis were compound “AZ_GGA_203287759” (high-dose males with two medium cases of necrosis) and compound “AZ_GGA_203287693” with one case of low-grade necrosis in its medium-dose male group and two low- and medium grade cases of necrosis each in its high-dose male group. In none of

the analyzed 28-day oral gavage Wistar rat studies cases of high-grade necrosis were identified. The low overall severity of necrosis in the analyzed 28-day oral gavage rat studies also explains the lack of consistent changes in the concentrations of serum creatine and serum urea, since elevations of these two parameters are linked particularly with serious kidney damage (55, 95, 101).

4.4.2 Consistency of renal necrosis in relation to treatment duration, including consideration of dose

In order to evaluate the consistency across time in oral gavage studies in Wistar rats, a distinction between tubular and papillary necrosis is important. Consistency for tubular necrosis is high as the only compound "AZ_GGA_203287734" with findings of tubular necrosis shows cases in both sexes in a corresponding 8-day study conducted with the same compound. Compared to that, consistency of papillary necrosis is lower as it was detected consistently across time in response to only one out of six compounds that produced papillary necrosis in a 28-day study in rats ("AZ_GGA_200002321"). In animals treated with substance "AZ_GGA_200002321" papillary necrosis was identified in the male and female high-dose group of a 14-day study. Furthermore, it was identified in a 26-week- and a 2-year study following treatment with the same compound in Wistar rats.

4.4.3 Consistency of renal necrosis across species, including consideration of dose

Consistency of renal necrosis across species is low as in total solely one case of papillary necrosis is observable in a 28-day oral gavage beagle study of compound "AZ_GGA_200002321" corresponding to the identification of necrosis in the 28-day Wistar rat reference study, in which rats were administered the same substance. A reason for the low consistency of renal necrosis across species could well be the utilization of lower doses of the trial substances in the 28-day beagle dog studies without any exceptions.

4.5 Evaluation of compounds linked with the histopathological result of glomerulosclerosis

Whereas the eTOX database contained four compounds that induced glomerulosclerosis in 28-day oral gavage rat studies, no cases of spontaneous occurrence of glomerulosclerosis were identified. The very low background incidence of glomerulosclerosis is not surprising, even though it is recognized as one of the histopathological hallmarks of the disease pattern of chronic progressive nephropathy (CPN). However, glomerulosclerosis is described not to appear as early as other histopathological characteristics (e.g. tubular basophilia) do in CPN (55).

In today's research, tyrosine kinase inhibitors (TKIs) have already been associated with the occurrence of glomerulosclerosis (155, 156), which can be supported by the fact that two out of three identified compounds in the eTOX database with treatment-related findings of glomerulosclerosis - "AZD9935" and "M596380" - are TKIs as well.

4.5.1 Consistency of glomerulosclerosis and clinical chemistry findings

Clinical chemistry values are only partly changed in Wistar rats which presented with histopathological evidence of glomerulosclerosis in 28-day oral gavage studies. No statistically significant changes associated with glomerulosclerosis were seen in serum creatinine, urea, triglyceride and inorganic phosphate levels. However, a pattern is apparent for the changes in serum albumin, protein and cholesterol in treatment groups in which glomerulosclerosis was recorded. In several treatment groups, i.e. in high-dose female rats in response to "AZD9935" in study one as well as in the mid- and high-dose females of study two of the same compound, statistically significantly decreased serum albumin and protein levels are accompanied by a significant increase in serum cholesterol. The same pattern of clinical chemistry changes was observed in high-doses male rats treated with compound "M596380", except that serum protein

levels were not recorded. A potential explanation for this is that the kidney damage through manifestation of glomerulosclerosis leads to a loss of proteins, and as a compensation mechanism cholesterol levels increase due to the shift in the ratio of blood lipids.

4.5.2 Consistency of glomerulosclerosis in relation to treatment duration, including consideration of dose

Analysis of glomerulosclerosis in consideration of treatment duration was not possible since no comparative rat studies conducted with the same compounds over different treatment periods were available in the eTOX database.

4.5.3 Consistency of glomerulosclerosis across species, including consideration of dose

28-day beagle dog studies were available for compounds “AZ_GGA_203287646” and “AZD9935” corresponding to the 28-day studies in which glomerulosclerosis was evident in Wistar rats after treatment with the identical compounds. Based on the limited data available, the consistency of glomerulosclerosis in 28-day studies across species appears to be low as no comparable cases of glomerulosclerosis were identified in any beagle dogs. However, doses administered to the beagle dogs were considerably lower in both compounds “AZ_GGA_203287646” and “AZD9935”.

4.6 Benefits and opportunities of the eTOX database

The creation of a large-scale in-vivo database, such as the eTOX database, presents tremendous opportunities for the research of preclinical development such as the potential *in silico* prediction of in vivo toxicological outcomes in order to increase the quality of drug candidates and to contribute to the reduction of animal use in preclinical development of medicines. At present, most preclinical drug development is still carried out through the implementation of various animal studies with the extensive use of laboratory animals. Alone in Europe, approximately 20.000 dogs are used yearly in scientific procedures

(131), while the total number of animals being used in Europe per year for pharmaceutical research has been described to exceed ten million (157). There is a lot of controversial discussion in regard to the efficiency of animal utilization in preclinical development. The effectiveness of non-rodent data combined with rodent-data in predicting human toxicities has been pointed out in a study by *Olsen et al.* (38), who showed a true positive human toxicity concordance rate of 71% for rodent and nonrodent species, with nonrodents alone being predictive for 63% of human toxicities and rodents alone for 43% in a study including 150 compounds from twelve pharmaceutical companies. However, there is also evidence describing the use of additional non-rodent animal data as not valuable concerning the predictive value (130, 158). *Bailey et al.* for example concluded after analysis of 2,366 drugs, for which both animal and human data were available, “that the absence of toxicity in the animal provides little or virtually no evidential weight that adverse drug reactions will also be absent in humans” (158).

With the help of the eTOX database an optimization of systemic toxicity studies or even their replacement through improved in silico models can potentially contribute largely to reduce the number of necessary animal studies. More specifically, the eTOX project inter alia aims at developing in silico tools to enable researchers to predict the toxicity of small molecules during early stages of the drug development pipeline by means of information that is already available before preclinical drug development even begins. The topic of in silico methods is of great current importance, and is not limited to the field of drug safety, but is also being used in other industries (e.g. industrial chemical and cosmetic sector) in a wide variety of scenarios (159). As toxicity in preclinical testing has long been identified as one of the major causes for drug attrition failures (27, 160), in silico techniques are increasingly being accepted and exploited for safety assessment with the aim of supporting the evaluation of hazard and risk following exposure to potential drugs.

Preclinical studies yield preliminary efficacy, toxicity, pharmacokinetic and safety information and may vary in length lasting up to several years. Before

progressing with clinical trials on human patients, toxicological and pharmacokinetics information from preclinical animal studies is analyzed and extrapolated to human. Detection of unpredicted toxicity and side effects can result in time-consuming setbacks or even total compound failures preventing the development of new medicines. Approximately a third of all drug development projects failures is caused through detection of toxicity during preclinical safety studies (28). In order to evade wasting years of research, vast amount of resources, time and money, it does not come as a surprise that the pharmaceutical industry craves for a more efficient use of resources and time. In its 2007 report “Toxicity Testing in the 21st Century: A Vision and a Strategy” the National Research Council already suggested transforming toxicology “from a system based on whole-animal testing to one founded primarily on in vitro methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin” (161). Without a doubt the wealth of preclinical toxicity data that the pharmaceutical industry has generated in recent decades is not capitalized on as exceedingly well as it could be. That is where the eTOX project broke ground through merging the poorly used, highly relevant preclinical drug safety data of compounds, that have become drugs and many more chemicals having failed to reach the market, into one large scale database, which can now be mined for further insight. The eTOX database can for example be used to compare potential drug lead substances with already tested compounds in order to raise hypotheses in terms of possible organ toxicities and side effects. Through reliable prediction the endeavor of this project also has the potential to accelerate the development of new drugs and ultimately to improve attrition rate. Furthermore, another promising hope is the identification of viable future biomarkers of toxicity through analysis of this enormous volume of data. Such biomarkers are significant in modern drug development as they greatly support the monitoring of side effects in both preclinical and clinical testing. This could potentially lead to the initiation of human studies even in the presence of animal toxicity, and to the possibility of quitting compound trials in advance of

the occurrence of unwanted serious health effects. Through the described improved safety surveillance by means of those specific biomarkers, even drugs which would have been considered as failed candidates, could be introduced to the market.

4.7 Challenges and limitations of working with the eTOX database, possibilities of improvements

While the chances of the establishment and research with the eTOX database are undeniable, also the difficulties of working with such a database, its limitations and possibilities of improving the database's downsides need to be highlighted and discussed.

It is undeniable that there are many difficulties to overcome for the development of such an enormous database: The presumably biggest hurdle for creating preclinical safety databases with pharmaceutical compounds is the pharmaceutical companies´ reluctance to share proprietary data as well as the shortage of published data sets. Another obstacle to overcome is the standardization of nomenclature in order for the eTOX database to be established (e.g. 181 terms for Phosphorous including: Inorganic phosphorous, Phospho-rous, Phosphate, Phos, PO4-IN, IN_PHOS, Phosporous (P3-), INP: Inorg. phosphate) (36).

As stated above, there is no doubt about the benefits and possibilities that such a database can provide. Nonetheless, analysis of data was partly challenging for the following reasons:

Literature denotes the pathology of chronic progressive nephropathy (CPN) as an age-related disease (55). Without a doubt CPN has a big influence on several histopathological endpoints discussed in this work (e.g. tubular basophilia, dilation). This example outlines the crucial importance of the detailed knowledge about the laboratory animal's age, which is not included in the eTOX database. The fact that age cannot be controlled for also might limit the significance of the incidence rates calculated in this work, which are obviously greatly influenced by the animal's age. Even though the animals' age is specified in preclinical safety testing guidelines, the exact age is not stated in the eTOX database for the respective compound studies.

Undoubtedly, it was the goal to keep the database as clearly designed as possible, while at the same time not to lose any valuable information. Nonetheless this work would have benefitted from additional information about compounds, for instance due to which cause and at which stage research and trials with the respective compound was stopped. Of course, it would have been very interesting and potentially valuable to know whether some of the analyzed compounds may have been dropped from further development because of kidney safety issues or perhaps for other reasons.

Furthermore, it has to be criticized that identical studies were mistakenly reported several times as different studies or even associated with different compounds in the eTOX database. Examples can be found for compounds AZD9935 (glomerulosclerosis, studies three and four) or AZ_GGA_20002321 (necrosis, studies five and ten). In the second case, the way of administration was even reported to differ (oral gavage/oral) and in one of the two studies clinical chemistry parameters were partly marked as statistically significantly changed, while they were not highlighted in the other study at all, even though clinical chemistry values were exactly the same.

The lack of individual animal clinical chemistry levels is a major limitation of the database and has already been discussed in chapter 4.4.1.

Moreover, the absence of individual animal data can provoke questions related to the designations of effects as treatment-related or not treatment-related. A specific example of a study, in which case it is not comprehensible, why the histopathological finding in one dose-group is labelled as treatment-related, whilst the ones in the other groups are specifically marked as not treatment-related, is given in Table 32. The designation of histopathological findings as treatment-related or not treatment-related of course had great impact on database search results and therefore on the selection of compounds for this work.

Table 32 – Overview over study one of compound AZ_GGA_200010232 (treatment duration: 29 days, species: Wistar rat, way of administration: oral gavage). *TR = Treatment-related; M = Male; F = Female.*

Dosage (mg/kg bw)	Sex	Number of Animals per Group	Frequency of Tubular Basophilia	Relevance	Grade of Tubular Basophilia		
					Low	Medium	High
0	M	10	8	-	8	-	-
0	F	10	6	-	6	-	-
1,25	M	10	7	Not TR	7	-	-
1,25	F	10	9	Not TR	7	2	-
2,5	M	10	7	Not TR	7	-	-
2,5	F	10	10	Not TR	7	3	-
5	M	10	8	TR	5	3	-
5	F	10	7	Not TR	7	-	-

Moreover, several studies had to be excluded from further analysis because of possible errors in data transfer. For instance, in study one of compound “AZD9935”, a 14-day oral gavage Wistar rat study, used dosages were more than 200 times higher than the ones in comparable 28-day Wistar rat studies conducted with the same compound. Furthermore, in that 14-day study two cases of tubular basophilia were identified even though only one animal was tested.

4.8 Outlook

At present the overwhelming majority of preclinical drug development is implemented through the straightforward performance of numerous animal studies with the extensive use of laboratory animals. However, alterations in the near future are not unlikely. Undoubtedly, statistical assessment of preclinical in vivo data within joint research ventures, such as the eTOX project has been, present an immense opportunity of aiding preclinical research towards a more efficient research in the preclinical stage of drug development. More specifically, this could be achieved through the deployment of methodological strategies and possibly novel software tools in order to predict in vivo toxicology of new molecular entities by means of information that is already available before early stages of the drug development pipeline even begin. With the help of the large sets of in-vivo data an optimization of systemic toxicity studies or even their replacement through improved in silico models have the potential to contribute

greatly to reduce the number of necessary animal studies and to improve predictive power of animal toxicities for humans in order enable researchers to launch safer drugs onto the market. It has recently been shown that machine learning and deep learning methodologies are capable of synergically complementing the experimental methods in toxicology (162, 163). Additionally, collaborations with the aim of increasing the availability of relevant data frameworks and developing the aforementioned integrative approaches on top of those data have been launched in recent years in order to establish and analyze sets of data. Examples of those projects are the “Intelligent Assessment of Pharmaceuticals in the Environment” program (iPiE) (164), the “Hazard Evaluation Support System” (HESS) (165), “EU-ToxRisk” (166) and of course the “eTOX” project itself.

While the past initiatives have mainly been focused on the development of databases with exclusively preclinical toxicity studies, another huge opportunity is represented by the dimension of the possible integration of human data in such projects. Again, inaccessible large sets of data are in the hands of pharmaceutical industry but have been started to be uncovered, the most contemporary example posed by the new IMI project “Enhancing TRANslational SAFEty Assessment through Integrative Knowledge Management” (eTRANSAFE) (167). “eTRANSAFE” is a continuation of the collaboration of many of the eTOX partners and will jointly evaluate preclinical data and clinical safety information for a better prediction of potential human safety liabilities in the future.

5 CONCLUSION

This research aimed to obtain reliable data on the frequency of different types of renal toxicity findings in 28-day oral gavage studies in Wistar rats, their consistency across species and study duration, as well as the correlation between histopathological endpoints and routinely used clinical chemistry parameters indicative of kidney injury. Analysis of renal histopathological findings was carried out through extraction of information from the IMI eTOX database.

Spontaneous renal histopathological findings in 28-day oral gavage studies in control Wistar rats and beagle dogs confirmed tubular basophilia and renal dilation as the most frequent incidental findings in controls, whereas necrosis and glomerulosclerosis were not identified at all or only rarely as a background lesion.

Histopathological evidence of necrosis and glomerulosclerosis was associated with changes in clinical chemistry parameters in 28-day oral gavage Wistar rat studies. Necrosis was frequently accompanied by a statistically significant rise in serum creatinine and serum urea, whereas serum albumin was frequently found to decrease statistically significantly in treatment groups in which necrosis was recorded. In contrast to necrosis, glomerulosclerosis was not associated with statistically significant changes in serum creatinine and urea in any of the 28-day oral gavage Wistar rat treatment groups, but appears to be best reflected by a pattern of statistically significantly lowered serum albumin and serum protein together with a statistically significant increase in serum cholesterol. As might have been expected based on the high background incidences of tubular basophilia and dilation, no consistent changes in any of the clinical chemistry parameters were evident in animals in which renal lesions were confined to renal tubular basophilia or dilation. In summary, the routinely provided clinical chemistry parameters are rather insensitive - novel kidney biomarkers such as Cystatin C, β-trace protein and Kidney injury molecule 1 should further be evaluated and integrated into routine preclinical and clinical practice. However, evaluation of clinical chemistry data was limited by the lack of individual animal data.

Even though an extensive amount of preclinical studies is accessible through the eTOX database, comparison of consistency across time was limited by the limited number of shorter- and longer term studies conducted with the compounds identified as causing renal histopathological changes within a 28-day study in rats. A high consistency across time for both treatment-related tubular basophilia and treatment-related dilation cannot be confirmed for either of the two effects as these two findings were both induced only rarely in studies over a different treatment-duration other than 28 days after administration of the compounds which provoked the respective effect in a 28-day study. For the finding of necrosis consistency across time was low with the exception of "AZ_GGA_200002321", in which renal papillary necrosis was identified consistently throughout different treatment durations (2, 4, 26, 104 weeks). No shorter- and longer-term studies were available for the compounds identified as causing glomerulosclerosis within a 28-day study in rats.

No consistent findings of the selected histopathological endpoints were identified in any of the corresponding 28-day oral gavage beagle dog studies after treatment with the identical compounds, which caused the respective effect after 28-day treatment in rats. However, in the overwhelming majority of cases, beagle dogs were administered lower doses in these studies in comparison to the corresponding 28-day Wistar rat studies.

Searching the eTOX database yielded no 28-day oral gavage studies in Wistar and Wistar Han rats in which accumulation of hyaline droplets, tubular atrophy or hyperplasia was recorded. Only one 28-day oral gavage Wistar rat study was identified with the histopathological result of neutrophilic inflammation. Consequently, evaluation of these four renal findings in relation to clinical chemistry parameters and consistency across time and species cannot be made.

In summary, this work contributes knowledge through mining and evaluating the eTOX database on a variety of specific renal endpoints that frequently occur after administration of trial substances in 28-day oral gavage studies in Wistar rats in the field of preclinical toxicity with specific focus on their frequency

in relation to background findings, as well as consistency across time and species. Targeted statistical evaluation of in vivo data within joint research ventures such as the eTOX project, presents an enormous opportunity for an innovative future way of aiding preclinical research towards a more efficient research in the preclinical stage of drug development. This could be achieved through the augmentation of methodological strategies and possibly novel software tools in order to predict in vivo toxicology of new molecular entities by means of information that is already available before early stages of the drug development pipeline begin.

6 ZUSAMMENFASSUNG

Diese Arbeit zielte darauf ab, verlässliche Daten über die Häufigkeit verschiedener Arten von Nierentoxizitätsbefunden in 28-tägigen oralen Sondenstudien an Wistar-Ratten zu erhalten. Untersucht wurde weiterhin die Konsistenz der Toxizitätsbefunde unterschiedlicher Spezies und Studiendauer sowie die Korrelation zwischen histopathologischen Endpunkten und routinemäßig verwendeten klinisch-chemischen Parametern, die auf eine Nierenschädigung hinweisen. Die Analyse der histopathologischen Nierenbefunde wurde durch Extraktion von Informationen aus der IMI eTOX-Datenbank durchgeführt.

Spontane renale histopathologische Befunde in 28-tägigen oralen Sondenstudien an Wistar-Ratten und Beagles bestätigten tubuläre Basophilie und renale Dilatation als häufigste Nebenbefunde bei den Kontrolltieren, während Nekrose und Glomerulosklerose gar nicht oder nur selten als Hintergrundläsion identifiziert wurden.

Der histopathologische Nachweis von Nekrose und Glomerulosklerose war mit Änderungen der klinisch-chemischen Parameter in 28-tägigen Wistar-Rattenstudien mit oraler Sonde verbunden. Nekrose ging häufig mit einem statistisch signifikanten Anstieg von Serumkreatinin und Serumharnstoff einher, während Serumalbumin in Behandlungsgruppen, in denen Nekrose aufgezeichnet wurde, häufig statistisch signifikant abnahm. Im Gegensatz zur Nekrose war Glomerulosklerose in keiner der 28-tägigen Wistar-Ratten-Behandlungsgruppen mit oraler Sonde mit statistisch signifikanten Veränderungen von Serumkreatinin und Harnstoff assoziiert, sondern scheint sich am besten in einem Muster von statistisch signifikant erniedrigtem Serumalbumin und Serumprotein zusammen mit einem statistisch signifikanten Anstieg des Serumcholesterins widerzuspiegeln. Wie aufgrund der hohen Hintergrundinzidenzen von tubulärer Basophilie und Dilatation zu erwarten war, waren bei Tieren, bei denen Nierenläsionen auf renale tubuläre Basophilie oder Dilatation beschränkt waren, keine konsistenten Änderungen der klinisch-chemischen Parameter erkennbar. Zusammenfassend sind die routinemäßig bereitgestellten klinisch-chemischen Parameter eher unempfindlich - neuartige Nieren-Biomarker wie „Cystatin C“, „β-trace protein“

und „Kidney injury molecule 1“ sollten weiter evaluiert und in die routinemäßige präklinische und klinische Praxis integriert werden. Die Auswertung der Daten zur klinischen Chemie war jedoch durch das Fehlen individueller Tierdaten begrenzt.

Trotz der umfangreichen Anzahl an präklinischen Studien in der eTOX-Datenbank wurde der zeitliche Vergleich der Konsistenz durch die begrenzte Anzahl von Kurz- und Langzeitstudien eingeschränkt, welche mit denselben Substanzen durchgeführt wurden, die innerhalb einer 28-Tage-Studie an Ratten als Verursacher von renalen histopathologischen Veränderungen identifiziert wurden. Eine hohe zeitliche Konsistenz sowohl für die behandlungsbedingte tubuläre Basophilie und Dilatation kann für keinen der beiden Effekte bestätigt werden, da diese beiden Befunde nur selten in Studien über eine andere Behandlungsdauer als 28 Tage nach Verabreichung derselben Substanzen, die den jeweiligen Effekt in einer 28-Tage-Studie hervorriefen, induziert wurden. Für den Befund der Nekrose war die zeitliche Konsistenz gering. Eine Ausnahme stellte Substanz "AZ_GGA_200002321" dar, bei der über verschiedene Behandlungsdauern (2, 4, 26, 104 Wochen) hinweg konstant renale papilläre Nekrose festgestellt wurde. Für die Substanzen, die in einer 28-Tage-Studie an Ratten als glomerulosklerose auslösend identifiziert wurden, waren keine Kurz- und Langzeitstudien verfügbar.

In keiner der korrespondierenden 28-Tage-Studien an Beagles mit oraler Sonde wurden konsistente Befunde der ausgewählten histopathologischen Endpunkte nach Behandlung mit den identischen Verbindungen, die den jeweiligen Effekt nach 28-tägiger Behandlung in Ratten verursachten, festgestellt. In der überwiegenden Mehrheit der Fälle wurden den Beagles in diesen Studien im Vergleich zu den entsprechenden 28-Tage-Wistar-Rattenstudien niedrigere Dosen verabreicht.

In der eTOX-Datenbank konnten keine 28-tägigen oralen Sondenstudien an Wistar- und Wistar-Han-Ratten gefunden werden, in denen eine Akkumulation von hyalinen Tröpfchen, tubuläre Atrophie oder Hyperplasie aufgezeichnet

wurde. Nur eine 28-tägige Wistar-Rattenstudie wurde mit dem histopathologischen Ergebnis einer neutrophilen Entzündung identifiziert. Folglich kann eine Bewertung dieser vier Nierenbefunde in Bezug auf klinische Chemie und Konsistenz über Zeit und Spezies nicht vorgenommen werden.

Insgesamt zeigt dieser Arbeit, dass eine gezielte statistische Auswertung von in vivo-Daten im Rahmen von Forschungsverbünden wie dem eTOX-Projekt eine enorme Chance bietet, die präklinische Forschung in Zukunft auf dem Weg zu einer effizienteren Forschung in der präklinischen Phase der Arzneimittelentwicklung zu unterstützen. Dies könnte außerdem durch die Erweiterung methodischer Strategien und möglicherweise neuartiger Software-Tools erreicht werden, um die In-vivo-Toxikologie neuer molekularer Entitäten mit Hilfe von Informationen vorherzusagen, die bereits vor Beginn der Arzneimittelentwicklungspipeline verfügbar sind.

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APPENDIX

I - List of Tables

II - List of Figures

III – Acknowledgment

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