Module 2.4
Nonclinical Overview

XXX
(oral and parenteral formulations)

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Date: **Month XX, YYYY**

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2.4 Nonclinical Overview

Appendix I shows the results of computer-assisted investigations for publications on the subject of pharmacology, pharmacokinetics, and toxicology following administration of XXX, that appeared in the period of 01/01/1964 to 01/02/2016.

2.4.1 Overview of the Nonclinical Testing Strategy

XXX is a sulfonamide loop diuretic and cardiovascular drug. Preclinical data of XXX have been documented in studies for several decades. The mechanism and site of action, the pharmacological effects and the possible role of XXX as a diuretic in the treatment of edema have been investigated. Edemas are caused by abnormal accumulation of fluid in the interstitial tissue spaces of the body which may result from various disease conditions. The treatment of edema focuses on correcting the underlying cause and diuretics are administered to promote the excretion of sodium and water. XXX enhances the excretion of sodium, potassium, chloride and hydrogen.

Pharmacological and toxicological effects of XXX have been investigated in various species *in vivo*, following oral, intravenous and intraperitoneal routes of application, as well as *in vitro* studies. The applied doses were far above the doses of XXX recommended in clinical use. The renal and extra-renal pharmacodynamic effects of XXX have been studied (focus is given to oral administration) and were confirmed in dogs, hens, rats, mice, and rabbits. Investigation of XXX-drug interactions has contributed to the understanding of its mechanism of diuretic action and recent studies focus on the risks and benefits of XXX interaction with other clinically relevant drugs. XXX effects are evaluated in animal models of cardiac diseases and its pharmacodynamic actions are compared with those of the long-acting loop diuretic torasemide. Pharmacokinetic parameters are described and XXX metabolism is characterized. XXX interferes with the water and electrolyte balance within the organism and possible pharmacological consequences beyond fluid excretion have been evaluated as well.

Toxicity studies are performed to identify key target organs for toxicity and to define dose-response relationships for the effects observed. Toxicological data of XXX were determined with different methods of application in various species. Teratology studies of the drug have been undertaken in mice and rats. Nephrolithiasis and nephrocalcinosis associated with the use of XXX were seen in premature infants. Therefore, especially nephrocalcinosis was intensively studied in animal tests (rat). Potential ototoxic effects have been studied in cats, beagles, guinea pigs and rat pups.

2.4.2 Pharmacology

Renal Effects

Effects on Urinary Salt and Water Excretion

XXX acts at the luminal surface of the ascending limb of the loop of Henle by inhibiting the active cotransport of sodium, potassium and chloride. Inhibitors of the sodium/potassium-chloride cotransport also inhibit calcium and magnesium reabsorption by abolishing the transepithelial potential difference that is the dominant driving force for re-absorption of these cations. A reduced activity of the Na^+-K^+-ATPase is seen in parallel to the above

mentioned effects (*XXX et al. 1992*).

The diuretic effects of XXX were shown in several species. In experiments investigating the dose-effect relationship after oral administration of XXX to dogs, a dose-related increase in urine volume at dose levels between 1 and 12.5 mg/kg bodyweight was determined. Urine volume increased from 23.3 m/kg/4 hours in controls to 56.7 ml/kg/4 hours at 12.5 mg XXX/kg bodyweight. Sodium and chloride excretion was increased approximately by the factor five and potassium excretion by the factor three (*XXX and YYY 1964*). Studies after intravenous bolus doses of 10-50 µg/kg bodyweight to hens showed that XXX inhibits chloride absorption from the luminal side of the nephron, and this effect depends directly on the renal XXX secretion into the proximal tubule and the luminal XXX concentration at the loop of Henle (*XXX 1979*). After intravenous administration of 1 mg/kg bodyweight to dogs XXX significantly enhanced urine flow and sodium and potassium excretion as shown in the table below (*XXX et al. 1976*).

TABLE 2.4.2.1: Urine flow and sodium/potassium excretion in dogs

	Renal function	Electrolyte excretion		% proportion of reabsorbed sodium
	Urine volume ml/min	Urine Na^+ concentration µEq/min.	Urine K^+ concentration µEq/min	
Control	2.9	485	40	92
XXX	8.7	1156	81	80

Oral doses of 75 mg/kg bodyweight XXX applied to rats produced a significant increase in renal calcium excretion to 15.6 mg/5 days, compared with the control group (4.1 mg/5 days) (*XXX et al. 1986*). *XXX and XXX (1974)* obtained similar results in rabbits and the effect on calcium excretion was confirmed by *XXX et al. (1982) in vitro* on isolated cortical thick ascending limbs of the loop of Henle of rabbits. Results in mice showed that XXX enhanced urinary calcium excretion after XXX single dose (15 mg/kg bodyweight) administration or after chronic dosing (twice daily injection for 3 days) and increased calcium transport molecules. Coadministration of chlorothiazide decreased XXX-induced calciuria, either acutely or chronically, although still accompanied by upregulation of these transport molecules. It was concluded that increased abundance of calcium transport molecules in the distal convoluted tubule represents a solute load-dependent effect in response to increased calcium delivery and serves as a compensatory adaptation in the downstream segment (*XXX et al. 2007*).

In rabbits it has been shown that XXX pharmacodynamics depends on the mode of application. Natriuretic and diuretic efficiencies were greater with the infusion than with the bolus of XXX (5 mg/kg bodyweight). In addition, XXX net effect did not increase proportionally to fluid replacement, and the infusion of XXX prevented the hypoalbuminemia-induced decrease in response of XXX given as a bolus (*XXX - XXX et al. 2000*).

In dogs, oral doses of 40 mg XXX for 4 days led to an increase in renal excreted hydrogen ions of 63.6 mEq (*XXX et al. 1977).* This effect may result in hypochloremic metabolic alkalosis. In rats, after intraperitoneal administration of 100 mg/kg bodyweight/day for 7 days Na^+-K^+-ATPase activity was markedly enhanced in the distal portions of the nephron without changes in the proximal nephron (*XXX et al. 1987).* The site specific influence of XXX on the

Na^+-K^+-ATPase activity was also found by *XXX and XXX (1984)* in rats. Recent results (rats) suggest that chronic administration of XXX enhances distal acidification by increasing the abundance of H^+-ATPase irrespective of electrogenic Na^+ reabsorption. This upregulation of H^+-ATPase in the intercalated cells may be the result of tubular hypertrophy by diuretics (*XXX et al. 2007*).

XXX, even at very high dosage (rats, 10 mg/kg intravenously), does not act on the proximal tubule. By allowing the delivery of isotonic tubular fluid to the distal tubule, it causes a fall in volume absorption along the early distal convolution in proportion to its baseline water permeability. It increases urine flow by abolishing the interstitial hypertonicity and, consequently, the osmotic driven solvent flow across the distal epithelium and collecting ducts. Therefore the urine flow rate during XXX closely approximates volume delivery out of the proximal tubule (*XXX et al. 1998*). Short-term effects of intravenous administration of XXX on key functions in the kidney cortex and the outer and inner medulla of rats (by using magnetic resonance imaging) include an increased renal water content, an increase in the intracellular to extracellular volume fraction of water, an increased oxygen tension, and a decrease in the renal blood flow (*XXX et al. 2007*).

The diuresis profiles of phase inversion micronized XXX and XXX co-precipitated with Eudragit L100, as well as their blends with stock XXX, targeted at reducing the rapid spike in diuresis associated with immediate release formulations while maintaining cumulative urine output have been investigated systematically. Of the formulations tested, an equal parts blend of micronized XXX and stock XXX demonstrated optimal diuretic bioactivity profiles in a rat model (*XXX et al. 2015*).

Diuretic Resistance
In healthy dogs, diuretic resistance developed after 14 days of XXX (2 mg/kg bodyweight twice daily), but not torsemide (0.2 mg/kg bodyweight twice daily), administration; however, both loop diuretics were associated with increased blood urea nitrogen and plasma creatinine concentrations, compared with values before treatment. Compared with the baseline value, plasma aldosterone concentration was significantly increased after administration of either drug and was significantly higher after torsemide than after XXX treatment (*XXX et al. 2007*).

Effects on Renal Hemodynamics
XXX increases renal blood flow without increasing filtration rate. The increase in blood flow is accompanied by an increase in prostaglandin concentration in the renal venous blood and can be inhibited by indomethacin, a specific inhibitor of prostaglandin synthesis by reduction of cyclooxygenase activity. XXX may promote the synthesis of prostaglandins or inhibit the prostaglandin degradative enzymes. Prostaglandins A and E have natriuretic and diuretic activity (*XXX and XXX 1990a, XXX and XXX 1990b*). An increase of the glomerular filtration rate is prevented partly by an increase in hydrostatic pressure in the tubules. In rats, the hydrostatic pressure after intravenous administration of XXX increased from 10 to 40 mmHg (*XXX and XXX 1980*). Additionally, the reduction in the volume of extracellular fluid by the diuretic effect may attenuate the increase in renal blood flow and glomerular filtration rate (*Forth et al. 1992*, *XXX and XXX 1985*). In rats, after intraperitoneal administration of 100 mg/kg bodyweight for 7 days, marked diuresis with an increase of urine volume from 9.7 ml/24 hours to 34.6 ml/24 hours was accompanied by a relatively modest increase in glomerular filtration rate from 0.61 to 0.83 ml/minute (*XXX et al. 1987*). In dogs, XXX (2 mg/kg bodyweight intravenously) induced a significant increase in total renal blood flow, which was prevented by a pretreatment with indomethacin (*Data et al. 1978*) suggesting that prostaglandins play a role in the diuretic/natriuretic response of XXX and in the alteration of renal hemodynamics (*XXX and XXX 1990a, XXX and XXX 1990b*).

Data in mice indicate that XXX (2 mg/kg bodyweight intravenously) causes vasodilation of afferent arterioles and paradoxical increase of renal vascular resistance of the intact mouse kidney. Decreases in total renal blood flow and superficial blood flow and increases of tubular pressure by XXX were ameliorated by renal decapsulation. In addition, pretreatment with candesartan (2 mg/kg) or indomethacin (5 mg/kg) attenuated the reduction of renal blood flow and peak urine flows caused by XXX. It has been suggested that generation of angiotensin II and/or a vasoconstrictor prostaglandin combined with compression of peritubular capillaries by the expanding tubular compartment are responsible for the reduction of renal blood flow *in vivo* (*XXX et al. 2007*).

Effect on the Renin Angiotensin Aldosterone System
XXX leads to an increase in plasma renin activity with the plasma aldosterone concentration remaining unchanged. Immediately after administration of XXX there is a stimulation of renin secretion (rebound effect) which is further potentiated by the reduction in volume due to diuresis. The immediate effect is explained by a direct influence of XXX on the renal baroreceptors (*XXX and XXX 1980).* In lambs, infusions of 2 mg XXX/kg bodyweight for 1-2 minutes led to an increase in plasma renin activity from 5.37 ng/ml to 8.56 ng/ml after 8 minutes and to 20.36 ng/ml after 35 minutes. There was no change in serum aldosterone observed (*XXX et al. 1977, 1980). XXX et al. (1977)* obtained similar results in dogs. In a recent controlled study, however, plasma aldosterone increased. Following oral administration of a single dose of XXX (2 mg/kg bodyweight) or azosemide (1, 5, or 10 mg/kg bodyweight) in healthy dogs, compared with the effect of placebo, plasma aldosterone concentration was significantly increased by XXX and the 10 mg/kg dose of azosemide. The diuretic effects were similar for XXX and azosemide (5 mg/kg bodyweight) (*XXX et al. 2008*). Activation of the circulating renin-angiotensin-aldosterone system in clinically healthy dogs appeared to plateau at day 5 of XXX administration (*XXX et al. 2015*).

Other Renal Effects
Accumulation of methylguanidine and changes in guanidine compound levels in plasma, urine and kidneys were observed in XXX-treated rats. XXX (5 mg intraperitoneally) provoked a significant increase in plasma and urine levels of guanidino compounds compared with those of the controls. The renal distribution and content of guanidino compounds were weakly modified by XXX except for methylguanidine. The level of methylguanidine was enhanced by 10 to 16 times in all renal zones. The methylguanidine level was 60% higher in renal zones of rats treated with 10 rather than 5 mg XXX. The fractional excretion of methylguanidine was decreased by XXX (*XXX et al. 2008*).

Effects in Renal Failure
Data obtained in a rat model of surgical ischemic acute renal failure suggest that a low dose XXX infusion may attenuate ischemia/reperfusion-induced apoptosis and associated gene transcription. Compared to controls, treatment with XXX (30 µg/kg/h) significantly ($p<0.001$) reduced ischemia/reperfusion-induced apoptosis in both the cortex and medulla and attenuated the expression of 72 ischemia/reperfusion-induced apoptosis-related genes. Results imply a possible novel molecular basis for the mechanism of action of XXX in acute renal failure (*XXX et al. 2007*).

Extrarenal Effects
General Cardiovascular effects
The blood pressure lowering effect of the saluretics is partly attributable to a lowering of peripheral vascular resistance, probably due to intra-extracellular ion displacement in the vascular musculature, or mediated by the attenuation of the noradrenaline effect (XXX *1975,* XXX *and* XXX *1980).* In cats and dogs a reduction in blood pressure was induced by

intravenous administration of 25 or 50 mg/kg bodyweight (*XXX and XXX 1964).*

The direct effect of XXX on the vascular tonus of the smooth musculature was studied using the thoracic aorta and vena porta of rats. It was shown that only very high concentrations (> 500 mg/l) induce a reduction in tension of the arterial preparation, but that there is a dose-dependent reduction in tension in the portal vein. By lowering the extracellular sodium concentration to 120 mmol the venodilator effect could be significantly increased. This effect is supposed to be due to a XXX-induced change of the electromechanical behaviour of the portal vein caused by a reduction in sodium transport (*XXX et al. 1975*). The dilatation of capacity vessels leads to a reduction in cardiac preload after administration of XXX. In hypervolemic dogs made anuric by ureteral ligation the increased pulmonary artery pressure decreased 5-60 minutes after intravenous administration of XXX (2 mg/kg bodyweight) in the absence of any diuretic effect. The response was significantly inhibited by pretreatment with indomethacin and it was abolished by nephrectomy indicating that this is an effect mediated by renal prostaglandin synthesis (*XXX et al. 1977*). After treatment of dogs with 3 mg/kg bodyweight intravenously for 3 weeks there was a decrease in blood volume of 18% with a transient lowering of serum sodium values. No changes in serum potassium or calcium concentrations were observed (*XXX and XXX 1978*).

In rats, compared to untreated animals, XXX (20 mg/kg bodyweight orally twice daily for 5 weeks), either alone or in combination with captopril (0.05 mg/kg bodyweight), is capable to prevent myocardial calcification, cardiac hypertrophy and hypertension, maintaining blood Ca^{2+} and phosphate levels by slowing chronic renal failure (*XXX et al. 2010*).

Human G protein-coupled receptor 35 (GPR35) is a target of XXX and bumetanide. Both loop diuretics are agonists of human GPR35, but are not active against mouse or rat GPR35 (*XXX et al. 2016*).

Cardiovascular effects in Experimental Heart Failure
In a controlled porcine model of heart failure the progression of left ventricular systolic dysfunction was significantly accelerated by XXX (1 mg/kg bodyweight intramuscularly daily). In tachycardic pigs XXX shortened the time to left ventricular dysfunction (21.4 days XXX vs. 35.1 days placebo; p=0.038) and increased aldosterone levels (at day 14, 43.0 ng/dl vs. 17.6 ng/dl, respectively; p<0.05). Serum sodium was reduced (133.0 mmol/l vs. 135.7 mmol/l; p<0.05), but no difference in norepinephrine, potassium, magnesium, creatinine, or urea nitrogen was present. Basal sodium-calcium exchanger currents were significantly increased and isoproterenol responsiveness depressed by XXX (*XXX et al. 2014*).

In dogs with mitral valve regurgitation (n=5) XXX decreased left atrial pressure in proportion to its dosage. A placebo-controlled cross-over study found no significant difference between intravenous (i.v.) and oral treatment outcome of the same dosages. Left atrial pressure was significantly decreased with all administrations of XXX but not after placebo (p<0.05, respectively). The maximal reduction was observed 1 hour (1 mg/kg bodyweight i.v. 15.04 mmHg), 3 hours (2, 4 mg/kg i.v. 13.28, 9.23 mmHg), 4 hours (1 mg/kg orally, 14.68 mmHg), and 5 hours (2, 4 mg/kg orally, 13.19, 10.70 mmHg). E wave and E/Ea were significantly decreased corresponding to the reduction of left atrial pressure after administration of 2 and 4 mg/kg (p<0.05, respectively) (*XXX et al. 2015*).

Results obtained from dogs with heart failure (n=7) and treated twice daily orally with either XXX or torasemide (equivalent dose for 7 days) in a double-blind, crossover study indicate an equivalent control of clinical signs of congestive heart failure. Mean XXX dose on day 0 was 5.13 mg/kg bodyweight/day (range 2.8-9.6). However, torasemide appears to achieve greater diuresis as compared with XXX (*XXX et al. 2016*).

A comparison of the therapeutic effects of torasemide versus XXX in rats with heart failure resulted partly in favour of torasemide. Diuresis was increased dose dependently by both

diuretics. Torasemide and XXX showed an equipotent natriuretic effect. Moreover, both drugs significantly elevated plasma angiotensin II and decreased atrial natriuretic peptide in a dose-dependent manner. The urinary potassium excretion was significantly increased with XXX versus torasemide. Myocardial functional parameters were significantly improved by torasemide. Conversely, these parameters did not change in rats receiving XXX. Torasemide suppressed left ventricular fibrosis, myocardial protein levels of transforming growth factor-beta1, collagen III, and aldosterone synthase and improved survival rate to the control level, but XXX did not (*XXX et al. 2008*).

Effect on the inner ear
XXX inhibits adenylate cyclase and sodium-potassium-ATPase in the inner ear leading to a disturbance of the electrolyte secretion, resorption and balance in the inner ear. The disturbance is mostly reversible, but damage to the Corti organ may result after prolonged disturbance. Concomitant treatment with aminoglycosides potentiates the risk of ototoxic effects (*XXX et al. 1979*).

Effect on the cerebrospinal fluid (CSF)
XXX, as an inhibitor of the sodium and chloride cotransport mechanism in the kidneys, also displayed an effect on the electrolyte concentrations in the CSF. Studies in normal dogs after intravenous administration of 2.5 mg XXX/kg bodyweight revealed no changes in potassium, chloride and bicarbonate concentrations, and in pH and pCO_2, in the CSF, while the sodium concentration fell by 1.7 mmol, indicating that sodium coupled chloride transport is a major mechanism of chloride entry into the CSF. In dogs with hypercapnia intraventricular injections of XXX led to an increase of the chloride concentration. A dose of 20 mg/kg bodyweight (intravenously) in cats reduced the CSF and liquor production of the plexus choroideus by 80%. A reduction by 50% in rabbits was achieved at a dose of 50 mg/kg bodyweight, and a 22% reduction resulted in nephrectomised rabbits at a dose of 50 mg/kg bodyweight (*XXX et al. 1984, 1987*, *XXX 1969*). Similar results were found by *XXX et al. (1981)*, *XXX et al. (1986)* in dogs and by *XXX et al. (1986)* in rabbits.

Anticonvulsant Effect
Results of an experimental model of limbic status epilepticus in rats demonstrate anticonvulsant properties of XXX. In 7 of 10 animals, XXX (100 mg/kg bodyweight intraperitoneally) terminated status epilepticus after 68 minutes without sedative side effects (*XXX et al. 2003*). Optical imaging studies on adult hippocampal slices show that the blockade of epileptiform activity by loop diuretic (XXX and bumetanide) treatments is concomitant with their blockade of activity-driven changes of the extracellular space. In addition it is suggested that XXX and bumetanide mediate antiepileptic effects through their blockade of cell swelling, dependent on its antagonism of the glial Na^+-K^+-$2Cl^-$ cotransporter (*XXX 2016*).

Antinociceptive Effect
Intrathecal administration of XXX had antinociceptive effects in rats with incisional pain (model of postoperative pain). XXX (100 µg/20 µl) decreased the pain threshold (versus solvent) in intact rats for 2 hours after administration and caused excitatory behavior. However, XXX increased the pain threshold in incision model rats (at the incision point: at 20 minutes, 2 hours, 3 hours, 4 hours, and on the 2^{nd} to 5^{th} days after incision; at the remote point: at 20 minutes, 3-5 hours, and from the 3^{rd} to the 7^{th} day after incision) and improved wound recovery (*XXX et al. 2015*).

Other Pharmacological Effects

XXX has been shown to inhibit glucose utilization of sceletal muscle and other tissues independent of insulin. Representative of other tissues, the glucose transport across erythrocyte membranes was concentration dependent inhibited by XXX *in vitro* (*XXX and XXX 1976*). *In vitro*, XXX decreases the sensitivity of glucose utilization to insulin in skeletal muscle by directly inhibiting the glucose transport process (*XXX et al. 1998*).

Antioxidant properties of XXX have been demonstrated on Wistar rat red blood cells and plasma. *In vitro* results (oxidative challenge with AAPH) showed that oxidative stress was decreased due to a potent free radical scavenging effect of XXX. *In vivo* -studies confirmed the antioxidant capacity of XXX (0.1 mg/kg bodyweight daily) (*XXX et al. 2003*).

In neonatal rats XXX delays ductus arteriosus closure and dilates the constricted ductus arteriosus. If XXX has similar effects in human preterm neonates, caution may be warranted in its use in the treatment of infants with patent ductus arteriosus (*XXX et al. 2010*).

Drug Interactions

Cardiac Glycosides

Concomitant administration of glycosides and XXX led to a potentiation of both the positive inotropic and arrhythmic effect of the glycosides. In guinea pigs the toxic dose of the cardiac glycoside ouabain producing arrhythmia (0.152 mg/kg bodyweight) and the lethal dose (0.3 mg/kg bodyweight) was lowered by concurrent XXX application (dose not indicated). This effect was inhibited by a potassium-sparing diuretic (prorenoate) effect (*XXX et al. 1982*). In dogs a potassium or magnesium deficiency which potentiated the toxicity of ouabain was induced by XXX administration (*XXX and XXX 1981*). An increase in the plasma concentrations of the glycoside was also observed by *XXX and XXX (1979)* after concomitant administration of digoxin and XXX to dogs. *XXX et al. (1978)* demonstrated that the XXX-associated potentiation of glycoside toxicity is not caused by increased glycoside uptake into the myocardium, but by increased sensitivity.

Antibiotics

A synergistic nephrotoxic property of XXX (2.2 mg/kg bodyweight twice daily) and gentamicin (3 mg/kg bodyweight twice daily) administrated for a period of 8 days to a dog was described by *XXX et al. (1983)*. Acute tubular necrosis in the form of epithelial changes and hyperchromic nucleoli were observed. Studies of the nephrotoxicity of XXX and the concomitant administration of antibiotics were carried out in mice with intraperitoneal administration of 20 mg XXX/kg bodyweight 15 minutes before intraperitoneal administration of the antibiotics at the level of the LD_{50}. Damage to the renal tubules was observed only in the group pretreated with XXX after administration of gentamicin and tobramycin in dosages of 160 and 120 mg/kg bodyweight. The incidence and severity of tubular damage on concomitant administration of XXX and cephaloridine (600 and 1200 mg/kg bodyweight) or kanamycin (156 and 312 mg/kg bodyweight) was also increased. Cefazolin, cephalothin, cefamandole, cephapirin and cephacetrile displayed no nephrotoxicity on concomitant administration with XXX. Additionally the renal cortical antibiotic concentrations were clearly increased after concomitant XXX administration (*XXX et al. 1975*).

Anticonvulsants

XXX (100 mg/kg bodyweight intraperitoneal) potentiated the anticonvulsant action of valproate in the mouse maximal electroshock seizure model. In contrast, XXX at 100 mg/kg had no significant effect on the antielectroshock action of the other antiepileptic drugs tested (including carbamazepine, lamotrigine, oxcarbazepine, phenobarbital, topiramate) in mice (*XXX et al. 2007*).

COX-Inhibitors

Renal physiology is a partially cyclooxygenase (COX)-dependent system. Kidneys express both COX-1 and COX-2 enzymes. In rats, diclofenac-induced acute renal failure was prevented with a combination of diclofenac and XXX. The diuretic effect of XXX was neutralized by diclofenac. Whereas COX-1 expression was reduced by diclofenac and by the combination of diclofenac and XXX, renal COX-2 immunoreactivity was increased by XXX and diclofenac and the combination of both. Although creatinine clearance was lower in rats that were given diclofenac alone compared with the combination, COX-1 and COX-2 expression were similar in these groups (*XXX et al. 2009*). The selective COX-2 inhibitor rofecoxib neutralized the diuretic effect of XXX in rats treated with a combination of XXX and rofecoxib. Renal cortical COX-2 protein expressions due to XXX and rofecoxib with or without XXX were similar and significantly increased compared with controls. Renal failure due to rofecoxib did not developed in any rat, but selective COX-2 inhibitor, rofecoxib, might have similar renal effects as nonselective nonsteroidal drugs for blunting the diuretic effect of XXX (*XXX et al. 2010*).

Other Drugs

Ascorbic acid enhanced the diuretic effect of oral XXX in dogs, probably as the result of increases in the reabsorption of XXX from renal tubules and increases in the un-ionized fraction of XXX at the renal tubular receptor sites (*XXX and XXX 1998*).

Coadministration of XXX (5 mg/kg bodyweight) further augmented tacrolimus (1 mg/kg bodyweight)-induced impairment in kidney function in rats (*XXX et al. 2000*).

The concomitant intravenous administration of warfarin (1.2 mg/kg bodyweight) and XXX to rats at 5-10 mg/kg bodyweight led to a significant increase in the plasma elimination half-life of the anticoagulant and increased prothrombin activity. *In vitro* studies showed a competitive displacement of both substances from the plasma protein binding (*XXX et al. 1982*).

Co-administration of cisplatin and XXX (200 mg/kg) causes rapid and massive loss of cochlear hair cells in mice. Combined treatment with 0.5 mg/kg of cisplatin and XXX resulted in only moderate loss of outer hair cells in the basal 20% of the cochlea, only mild threshold shifts and minimal loss of distortion product otoacustic emission (DPOAE). In contrast, 1 mg/kg of cisplatin plus XXX resulted in a permanent 40-50 dB elevation of auditory brainstem response thresholds, almost complete elimination of DPOAE, and nearly total loss of outer hair cells (*XXX et al. 2015*).

In clinically healthy dogs, the mean urinary aldosterone-to-creatinine ratio increased significantly after administration of XXX (baseline, 0.37 µg/g; day 5, 0.89 µg/g) or the combination of XXX and standard dosage pimobendan (baseline, 0.36 µg/g; day 5, 0.88 µg/g). At day 10, the mean urinary aldosterone-to-creatinine ratio was 0.95 µg/g for XXX alone versus 0.85 µg/g for the combination of XXX and pimobendan. These results indicate that XXX-induced renin-angiotensin-aldosterone system (RAAS) activation appears to plateau by day 5 and pimobendan standard dosage does not enhance or suppress XXX-induced RAAS activation (*XXX et al. 2015*).

2.4.3 Pharmacokinetics

Absorption

XXX et al. (1979) investigated the bioavailability of XXX in dogs and monkeys after single dose application of 5 mg/kg bodyweight or after multiple application of the same dose for 20 days. In dogs the bioavailability was 41-52% with no significant difference between single and repeated dosing. Peak plasma levels of 2.29 µg/ml were reached within one hour and declined thereafter with a half-life of about 7 hours. In monkeys, at the same dose level peak plasma concentrations of only 0.277 µg/ml were also achieved within one hour and declined with a half-life of approximately 11 hours. The values found after repeated applications were not significantly different from those obtained after single application indicating the absence of an accumulation process. In investigations, based on the comparison of urinary excretion after oral and intravenous application of 5 mg/kg bodyweight to dogs, *XXX et al. (1976)* showed that about 50-60% of the dose was resorbed. In rats the main site of absorption was in the stomach at a pH of 3.0 ($T_{50\%}$ 2.5 hours), absorption was slower in the duodenum at a pH of 5.0 ($T_{50\%}$ 3.1 hours), and slowest from the jejunum at a pH of 5.0 ($T_{50\%}$ 11.7 hours) (*XXX et al. 1979*). Analyses of the plasma concentrations after intravenous bolus injection to rats at low dosages (10 mg/kg bodyweight) revealed a course of the x^3 form; with high dosages (30 mg/kg bodyweight) the form was x^2. There was no linear relationship between the plasma concentration under the curve (AUC) and the dose applied in the dose range of 10-30 mg/kg bodyweight (*XXX et al. 1983).*

Ascorbic acid significantly increases bioavailability of oral XXX in dogs. This increase appears to result from reduced gastric first-pass metabolism of XXX and not from enhanced gastrointestinal absorption of XXX. This might be supported by rat studies; the percentages of the oral doses of XXX recovered from the gastrointestinal tract at 8 hours after administration were similar without (39.5%) and with (44.7%) coadministration of ascorbic acid ($p<0.583$), and the amounts of XXX remaining per gram of stomach after 30-minutes incubations of XXX (50 µg) with 9000g supernatant fractions of stomach homogenates were increased significantly (48.5 µg vs. 42.4 µg) by the addition of ascorbic acid (100 µg) (*XXX and XXX 1998*).
Bile juice increased the gastrointestinal absorption of XXX in rats. With bile juice administration, the plasma concentrations of XXX were considerably higher and $AUC_{(0\text{-}8)\ h}$ was significantly greater (2570 vs. 658 µg x min/ml) (*XXX et al. 1999*).

Distribution

At 8 hours after intravenous application of 50 mg/kg bodyweight XXX to rats (*XXX et al. 1988)* determined the highest tissue concentration in the intestine (118 mg/kg) with only minor concentrations between 2 and 3 mg/kg in liver, muscle and lung and a slightly higher concentration of 19 mg/kg in kidney. The highest tissue levels were found by *XXX et al. (1969)* in kidneys of rats after oral application of XXX (approx. 60 mg/kg bodyweight) with nearly three times the plasma level. The concentration in liver tissue nearly reached the plasma level but concentrations in spleen, brain and muscle were very low and far below the plasma concentration. As determined *in vitro* using isolated slices of kidney tissue, XXX is absorbed by kidney tissue via active transport mechanisms (*XXX et al. 1975).* The distribution of XXX after intravenous application of 1 mg/kg bodyweight to dogs was determined by *XXX et al. (1976).* The highest concentrations were far above plasma concentrations (1.63 mg/kg), and were found in the liver (6.53 mg/kg) and kidney (8.45 mg/kg). Only minor amounts were detected in heart, lung, muscle or fat.
The first-pass effects of XXX by lung, heart, and liver seemed to be negligible in rats. The absolute bioavailability of XXX was 28.9% and 48.3% after oral and intraduodenal administration, respectively. Gastrointestinal and intestinal first-pass effects of XXX were

approximately 40% and 20% of the dose, respectively (*XXX et al. 2000*).

Protein Binding

A high plasma protein binding of 85.7-90.7% was found in dogs by *XXX et al. (1976). XXX and XXX (1982)* determined the binding to plasma proteins in rats after intravenous application. The protein binding decreased with increasing substance concentrations from nearly 100% at low concentrations to 90% at a concentration of 400 µg/ml. A high plasma protein binding of more than 97.5% was observed by *XXX et al. (1988)* in rats. *XXX et al. (1994)* found a comparable value in rats with 94.6% after intravenous application of 10 mg/kg bodyweight. Results of kinetic studies in the presence of a protein binding displacer, warfarin, and in hypoalbuminemic rabbits suggest that binding of XXX to plasma proteins, and not albumin per se, facilitates its renal secretion and pharmacological response in hypoalbuminemic rabbits. The decrease in XXX binding, secondary to drug displacement and/or hypoalbuminemia, can be a cause of resistance to the diuretic; and when XXX binding is decreased, the administration of XXX mixed with albumin enhances its renal secretion and diuretic effect (*XXX et al. 1999*).

Metabolism

In *in vitro* tests using homogenates of various tissues, *XXX and XXX (1983)* detected the metabolite 4-chloro-5-sulphamoyl-anthranilic acid and a further unknown metabolite of XXX in stomach, liver and small intestine homogenates of rats, rabbits and dogs. This metabolite was also found in urine of rats after intravenous or oral application of XXX at about 10-15% of the total urinary excretion. *XXX et al. (1976)* determined XXX after intravenous application of the radiolabeled drug to dogs in plasma, urine, liver and kidney. They identified 80-99% of the radioactivity in these samples 30 minutes after application as unchanged XXX, only minimal amounts were metabolized.

Three novel metabolites have been characterized recently. Administration of [^{14}C] XXX to bile duct-cannulated rats or mice demonstrated turnover to glutathione conjugate (8.8%), γ-ketocarboxylic acid metabolite (22.1%), N-dealkylated metabolite (21.1%), and XXX glucuronide (12.8%). XXX-glutathione conjugate was not observed in bile from mice. The novel γ-ketocarboxylic acid indicates bioactivation of the furan ring. Formation of γ-ketocarboxylic acid was cytochrome P450-dependent. In mouse liver microsomes, a γ-ketoenal XXX metabolite was trapped, forming an N-acetylcysteine/N-acetyl lysine XXX adduct. XXX (1 mM, 6 h) became irreversibly bound to primary mouse and rat hepatocytes, 0.73 and 2.44 nmol equivalent bound/mg protein, respectively, which was significantly reduced in the presence of 1-aminobenzotriazole (to 0.11 and 0.21 nmol equivalent bound/mg protein, respectively). Furan rings are part of new chemical entities, and bioactivation of the furan ring represent the mechanism of metabolic activation and hepatotoxicity of XXX. Mechanisms underlying species differences in toxicity are important to understand to decrease the drug attrition rate (*XXX et al. 2007*).

Elimination

According to *XXX et al. (1976)* dogs excreted 25% and monkeys 24% of a single oral dose of 5 mg/kg bodyweight within 24 hours in urine. Following intravenous application of the same dose to dogs, about 50% of the dose applied was excreted in urine and about 50% in feces indicating that half of the excretion takes place via the bile. *XXX et al. (1981)* found, after intravenous application of 2 mg/kg bodyweight to dogs, a renal excretion of 43% unchanged drug and 4.3% glucuronide. When XXX was applied to rats intraperitoneally at dose levels of 5, 10 or 20 mg/kg bodyweight urinary excretion was between 32 and 40% of the dose (*XXX et al. 1982*). *XXX and XXX (1982)* determined that the renal elimination of XXX in rats at dose levels between 1 and 100 mg/kg bodyweight applied intravenously increased linearly with dose from 27 to 38% of the dose applied. After intraperitoneal

administration of 6 mg XXX/kg bodyweight, adult rats excreted up to 78% of the dose administered unchanged and (in minor amounts) as a not defined metabolite renally within 120 minutes (*XXX and XXX 1976*). In investigations performed by *XXX et al. (1969)* rats received ca. 60 mg/kg bodyweight orally as a single dose or by repeated daily application for 10 days. After single application nearly equal parts of the substance were excreted in urine (47.5%) and feces (52.5% of total excretion). Results after repeated application indicated an accumulation to some degree.

2.4.4 Toxicology

Toxicity after Single Dose Administration

In studies for acute toxicity XXX showed a low toxicity after oral application and a moderate toxicity when applied parenterally. The LD_{50} values are shown below (see *TABLE 2.4.4.1*):

TABLE 2.4.4.1: Acute toxicity (LD_{50} [mg/kg bodyweight]) of XXX in various species and various forms of application

Species	Administration method				Source
	p.o.	i.p.	s.c.	i.v.	
Mouse	2000	430 900*	-	308	RTECS 2007
Rat	2600	800	4600	800	RTECS 2007
Rabbit	800	-	-	400	RTECS 2007
Dog	2000	-	-	> 400	RTECS 2007

Explanations: p.o. = oral; s.c. = subcutan; i.v. = intravenous; i.p. = intraperiteneal; LD_{50} = letal dose; * route not reported

Studies of the toxicity of XXX as a function of age in Charles River CD rats produced the following results: after oral administration the LD_{50} in 3-4 day old animals was 330-437 mg/kg bodyweight, in animals 60 days old, 1860-3640 mg/kg bodyweight (female) and 2070-3860 mg/kg bodyweight (male). The toxic ratio of young animals to adults was 7.1:1 (*XXX 1971).* According to *XXX (1980)* the symptoms of intoxication in acute toxicity studies were characterized essentially by water and electrolyte loss, the extent of which depends on the water and electrolyte supply. If the animals were given enough water and electrolytes to balance the loss, the toxicity could be considerably reduced.

Nephrotoxicity

Single intraperitoneal administrations of 50-200 mg XXX/kg bodyweight led in rat kidneys to epithelial damage to the pars recta of the proximal tubules. This damage was not observed after insertion of a vesico-venous shunt and prevention of XXX-induced water and electrolyte loss. These tubular lesions appear to develop as a result of XXX accumulation when intratubular urinary flow is reduced (*XXX et al. 1971*).

Hepatotoxicity

When XXX was applied to mice intraperitoneally at a single dose of 400 mg/kg bodyweight, cell necroses were detected within 12 hours especially in the centrilobular region. The extent of the damage was greatest after 24-48 hours (*XXX et al. 1974*). The hepatotoxic potential of XXX was dose dependent and occurred at dose levels between 200 and 400 mg/kg bodyweight with increasing incidence (*XXX et al. 1976*). *In vitro* studies on mouse hepatocytes showed a reduction in sulphohydrylic acid of 20-30% at XXX concentrations of 0.5-1.0 mmol/l, resulting in a reduction of cell vitality. In addition, cytoplasmic changes in the

form of a reduction in glycogen, deaggregation of polyribosomes, vesicle formation in the endoplasmic reticulum and the occurrence of lamellar structure, and changes to the surface structure of the cells in the form of a loss of microvilli and shape changes were observed. These changes could be prevented by N-acetylcysteine (6 mmol/l) (*XXX et al. 1987*). *XXX et al. (1976)* and *XXX and XXX (1981)* made similar observations after intraperitoneal doses of 400 mg XXX/kg bodyweight to mice.

XXX-induced hepatotoxicity is not initiated by mitochondrial injury. In the mouse, XXX administration (400 mg/kg bodyweight intraperitoneally) did not alter mitochondrial or cytosolic glutathione, and had no effect on respiration supported by complex I or II for up to 5 hours following dosing. However, alanine aminotransferase activity was significantly increased 5 hours following XXX administration (*XXX et al. 2000*).

TABLE 2.4.4.2: Lowest toxic dose (TDLo [mg/kg bodyweight]) of XXX in various species and various forms of application (from *RTECS 2007*)

Species	Administration method				Toxic effects
	p.o.	i.p.	s.c.	i.v.	
Mouse	1				Urine volume increased
	24				Other changes in urine composition
		500*			Changes in serum composition; transaminases
Rat	10				Urine volume increased; other changes in urine composition
	40				Changes in serum composition
	100				Changes in serum composition
Rat		500			Impaired liver function tests
			2		Changes in blood vessels or in circulation of kidney; urine volume increased; other changes in urine composition
			20		Behavioral effect (fluid intake); Changes in Na
				30	Urine volume increased; other changes in urine composition
				7.5	Blood pressure lowering not characterized in autonomic section; urine volume increased; changes in Na
Dog				0.1	Other changes in urine composition

Explanations: p.o. = oral; s.c. = subcutan; i.v. = intravenous; i.p. = intraperiteneal; * unreported route; TDLo = lowest toxic dose

Ototoxicity

A dose-response study in mice identified 200 mg/kg of XXX as the dose for disrupting the stria vascularis and opening the blood-ear barrier (*XXX et al. 2015*). Studies of acute ototoxicity were carried out in cats (n=76) and Beagle dogs (n=54) with intravenous doses of 0.5-100 mg XXX/kg bodyweight. In general, for the inhibition of afferent auditory nerve

activity (N1) (100% with 100 mg/kg bodyweight), lower doses are determined than for inhibition of the activity of the hair cells of the cochlea. Minimal doses for the inhibition of N1 in cats were 9-12 mg/kg bodyweight over a period of 30 minutes. In cats XXX reduced acoustically evoked cortical potentials and evoked brainstem excitation in guinea-pigs. This damage was reversible, and affected hearing capacity only temporarily. A change in sodium/potassium concentrations in the endolympha in guinea-pigs after intravenous doses of 50 mg/kg bodyweight was discussed as a pathophysiological correlate. Histological examinations revealed no changes in the stria vascularis of inner and outer hair cells, afferent and efferent peripheral nerves, and also central ganglia and nerve fibres (*XXX 1974, 1975, 1981, XXX et al. 1979, XXX and XXX 1982). XXX et al. (1991)* investigated the ototoxicity of XXX in developing rat pups 1-80 days old. He found a distinct functional disturbance correlated with a histologically detectable edema of the stria vascularis of the inner ear. The effect was much stronger in pups younger than 30 days than in pups older than 30 days. In guinea pigs XXX injected intraperitoneally at 40 mg/kg bodyweight or infused into the cochlea of the inner ear at 0.5 mM led to a functional disturbance and to morphological changes in the cochlear hair bundles (*XXX et al. 1990).*

Toxicity After Repeated Administration

In studies for toxicity after multiple dose XXX showed a low toxicity after various forms of application. The TDLo values are shown below (see also *TABLE 2.4.4.3*):

TABLE 2.4.4.3: Toxicity after multiple doses (TDLo [mg/kg bodyweight]) of XXX in various species and oral or intraperitoneal application (from *RTECS 2007*)

Species	Administration method			Toxic effects
	p.o./continous	p.o./intermittent	i.p.	
Mouse	77280/14D-C			Changes in tubules, death in the “U” date type field
		54600/13W-I		Changes in liver weight
Rat		1400/5W-I		Changes in tubules and in liver weight, increased urine volume
	32200/14D-C			Changes in tubules, death in the “U” date type field
		27300/13W-I		Changes in tubules and in liver weight
		15288/26W-I		Urine volume increased, changes in bladder weight
	4200/4W-C			Changes in heart weight, weight loss or decreased weight gain, changes in Na
		72/6D-I		Other changes in urine composition, changes in Na
		400/20D-I		Changes in calcium and dehydrogenases
		2000/20D-I		Changes in serum compostion, changes in Na
		1960/28D-I		Weight loss or decreased weight gain
			0.7/7D-I	Changes in K
Dog		210/4D-I		EKG changes not diagnostic of above; change in resting or action potential; changes in K

Explanations: p.o. = oral; i.p. = intraperiteneal; TDLo = lowest toxic dose; D = day; W = week; C = continous; I = intermittent; Na = sodium; K = potassium;

In the course of the US National Toxicology Program (US NTP) subacute and subchronic toxicity studies with rats and mice were performed: Rats received XXX at concentrations of 570, 1700, 5100, 15300 or 46000 ppm in food for 14 days. The treatment led to a dose dependent decrease in bodyweight and bodyweight gain beginning at 1700 ppm. Increased mortality was seen at the highest dose level and clinical signs of systemic intoxication occurred at the two highest dose levels. Histological investigations revealed nephrosis at a very high incidence in the two highest dose groups and in a single animal of the 5100 ppm group. XXX was administered to mice at the same concentrations in food for 14 days. In high-dose animals mortality was highly increased. The bodyweights and bodyweight gains were dose dependently decreased in all treated groups beginning at 1700 ppm. Clinical signs of systemic toxicity were restricted to the highest dose group. Nephrosis was seen in mice histologically in a dose dependent manner in the three higher dose groups *(NTP 1989).*

When XXX at dose levels of 10, 25 or 50 mg/kg bodyweight was orally applied to dogs and rats for 28 days, no mortality, no clinical signs and no histologically detectable effects on liver, spleen, kidneys, heart, brain and reproductive organs were seen in both species. Bodyweights were reduced only in high-dose dogs, not in rats. Intravenous application of 5, 10 or 50 mg/kg bodyweight to dogs led also to bodyweight reduction at the highest dose level but not to mortality and not to any histologically detectable organ damage in 12 examined organs (*XXX and XXX 1964).*

After oral and intravenous administration of 5 mg XXX/kg bodyweight to dogs and monkeys for 15-20 days, no treatment related histopathological changes to the liver, kidneys or testes were observed, compared to controls (*XXX et al. 1979).* After daily subcutaneous administration of 1 mg XXX/kg bodyweight for 4 weeks to rats with or without arteriosclerosis, treated rats had increased weights of adrenals and kidneys, increased blood pressure and increased aldosterone levels in blood. The hyperaldosteronism was postulated as the cause of the increase in blood pressure. Preexisting arteriosclerosis was exacerbated in treated animals. Focal myocardial necroses were observed in treated animals (*XXX 1981).* XXX was given to rats at concentrations of 625/938, 1250/875, 2500/3750, 5000/7500 or 10000/15000 ppm (males/females) in food. The treatment did not affect mortality. Bodyweight was dose dependently decreased in the three higher dose groups and liver weight was dose dependently increased in the four higher dose groups. Clinical signs of systemic toxicity were restricted to the two higher dose groups but diuresis, as pharmacodynamic effect, was seen in all treated groups. A treatment related and histologically visible nephrosis occurred only in the two highest dose groups. Mice received the substance at concentrations of 938/1250, 1875/2500, 3750/5000, 7500/10000 or 15000/20000 ppm (males/females) in food. There was no effect on mortality but bodyweight was depressed and liver weight was increased dose dependently in the three higher dose groups. Histologically detectable nephrosis was seen treatment related in the two highest dose groups *(NTP 1989).*

On the basis of these results, the dose levels in the two-year toxicity and carcinogenicity study were set at 0,350 and 750 ppm for rats and 0,700 and 1400 ppm/day for mice. In rats no significant differences in survival rate, bodyweight and clinical signs compared with the control group were found. The average dose consumed was 14/16 or 29/31 mg/kg bodyweight for males and females respectively. Histologically visible nephropathy occurred not at increased incidence but at increased severity in high-dose animals. The no effect level was 14 and 16 mg/kg bodyweight for males and females respectively. In mice the treatment led to increased mortality at the higher dose level and to dose dependently decreased bodyweight in all treated groups. The average dose consumed was 90/100 or 190/215 mg/kg bodyweight for males and females, respectively. The incidence and severity of histologically detectable nephropathy was severely increased in all treated groups. In high-dose animals an increased incidence of suppurative inflammation of uterus/ovaries and prostata was seen (*NTP 1989).*

Cardiotoxicity

XXX (10 mg/kg bodyweight/day) induced mortality in a rat model of chronic heart failure. The survival rate in the XXX group was lower than in the placebo group (hazard ratio 3.39; p=0.028). The XXX group had a lower body weight (-6%; p=0.028) at the end of the study and a higher sclerosis index of the glomeruli (+9%; p=0.026) than the placebo group. Wet lung weight, infarct size, and cardiac function were similar between the groups. This increased mortality could be prevented by additional administration of the ACE-inhibitor ramipril (1 mg/kg bodyweight/day) in a second study. The XXX group had a higher mortality rate than the XXX plus ramipril group (hazard ratio: 4.55; p=0.0003) (*XXX et al. 2016*).

Results after chronic administration of XXX to rats on thiamine standard food or thiamine-

deficient food indicate that XXX aggravates thiamine deficiency only in situations associated with insufficient thiamine intake, causing cardiac structural alterations, such as myocardial fiber hypotrophy, poor micro-vascularization, and mitochondrial degeneration (*da XXX et al. 2007*).

Nephrotoxicity
Nephrolithiasis and nephrocalcinosis was seen in premature infants (*BfArM 1996).* Therefore especially nephrocalcinosis was intensively studied in animal tests by different investigators. The reversibility of XXX induced nephrocalcinosis was investigated by *XXX ef al. (1996).* Weanling rats (age, 4 weeks) received XXX at a dose level of 40 mg/kg bodyweight intraperitoneally for 8 weeks, for 2 weeks or for 2 weeks with a 6 week recovery period. By histological determination nephrocalcinosis was found to be almost equal after 2 or 8 weeks of treatment but was partly, not fully, reversed within a 6 week recovery period. *XXX et al. (1997)* studied the effect of salt supplementation on the development of nephrocalcinosis in rats. Weanling rats received XXX at 40 mg/kg bodyweight intraperitoneally for 28 days with or without sodium enriched diet. Treated animals developed nephrocalcinosis at a significant incidence without any effect of the sodium supplementation. While treated animals without sodium supplementation showed growth retardation, sodium supplemented animals grew normally. There was no correlation between calcium excretion in urine and nephrocalcinosis indicating a pathogenic mechanism which cannot be easily explained by increased calcium excretion. In a further investigation weanling rats were given 40 mg/day (approx. 120 mg/kg bodyweight) in food for 6 weeks. Bodyweight was not decreased but absolute and relative kidney weight was increased in treated animals. Both glomerular volume and cortical tubular volume were highly increased when compared to controls. The effect was only partly prevented by the concurrent treatment with an angiotensin II receptor antagonist indicating that the effect on kidneys was partly mediated by angiotensin II (*XXX 1995).*

Long-term study results on Wistar rats fed with or without XXX (40 mg daily, from the time of weaning through 10 months of age) provide no evidence of detrimental glomerular effects of XXX in normal animals. Renal cortical hypertrophy and glomerular hypertrophy were sustained throughout the 9 months of treatment in the group receiving XXX. The cortical interstitial area was increased in the XXX group, but this did not appear to be the result of fibrosis. Proximal and distal tubule diameter were unaffected by treatment. No differences in glomerulosclerosis or glomerular ultrastructure were shown (*XXX 1999*).

Hepatotoxicity
Doses of 200 and 400 mg XXX (intraperitoneally) in mice for 7 days led to dose dependent cellular and focal necroses within the first two days. In the course of treatment the damage was restored indicating the development of tolerance to the test substance (*XXX et al. 1986).*

Species-specific differences in hepatotoxicity of XXX exist. XXX (1.21 mmol/kg) was shown to cause toxicity in mice, but not rats, at 24 hours, without resulting in glutathione depletion. *In vivo* covalent binding to hepatic protein was 6-fold higher in the mouse than rat (1.57 vs. 0.26 nmol equivalent bound/mg protein, respectively). *In vivo* covalent binding to mouse hepatic protein was reduced 14-fold by a predose of the cytochrome P450 inhibitor, 1-aminobenzotriazole, which also reduced hepatotoxicity. Three novel metabolites have been characterized (*see 2.4.3 Pharmacokinetics – Subtitle Metabolism*). Furan rings are part of new metabolites. It has been demonstrated that bioactivation of furan ring represent the mechanism of metabolic activation and hepatotoxicity of XXX (*XXX et al. 2007*).

Ototoxicity
After intravenous administration of 15, 30 or 60 mg XXX/kg bodyweight daily for a period of 30 or 40 days in guinea pigs, neither lasting damage to the cochlea nor changes in the histocochleogram, nor histopathological changes to the auditory organ were seen (*XXX and XXX 1973, XXX et al. 2015*).

In general, the literature to date contains no further reports on chronic toxicity of XXX (*RTECS 2007*, see also Appendix I *Literature investigation 2007/2016*).

Reproduction Toxicity
In reproductive studies lowest toxic doses (TDLo[mg/kg bodyweight]) of XXX mainly affect the musculoskeletal system and produce specific developmental abnormalities (see also *TABLE 2.4.4.4*

TABLE 2.4.4.4: Lowest toxic dose (TDLo [mg/kg bodyweight]) of XXX in mouse and rat after oral or intraperitoneal application (from *RTECS 2007*)

Species	Administration method		Toxic effects
	p.o.	**i.p.**	
Mouse	12500 (6-15D preg)		Postimplantation mortality; Fetotoxicity
	5000 (6-15D preg)		Specific developmental abnormalities, Musculoskeletal system
		1560 (1D male)	Spermatogenesis
Rat	150 (12-16D preg)		Specific developmental abnormalities, Musculoskeletal system
	300 (16D preg)		Specific developmental abnormalities, Musculoskeletal system
	22 (6W male)		Paternal Effects (spermatogenesis; testes; epididymis, sperm duct)
	Unreported route*:	1800 (6-17D preg)	Specific developmental abnormalities Musculoskeletal system

Explanations: p.o. = oral; i.p. = intraperitoneal; * unreported route; TDLo = lowest toxic dose; D = day; W = week; preg = pregnancy

Embryotoxic/Teratogenic Effects
Rabbits received injections of 12 mg XXX/kg bodyweight (intravenously) from day 1-10 of gestation. The treatment led to a slightly increased incidence of resorptions and fetal mortality. Skeletal malformations were seen in treated and control animals to a variable extent not interpretable with respect to the treatment (*XXX and XXX 1975).* A dose level of 75 mg/kg bodyweight (intraperitoneally) from days 7-11 and 14-18 of gestation to Wistar rats did not result in any significant changes in litter size, pup weight, renal weights or concentrations of sodium, potassium urea, creatinine or proteins in the navel arterial blood as compared to controls. However, XXX induced a lower formation and differentiation of the renal glomeruli (*XXX et al. 1985).* Further investigations showed that the treatment of pregnant rats according to the above mentioned scheme led to postnatal diuresis and disturbances in the plasma sodium and potassium concentrations in the pups as well as reduced pup weights. A delay of renal maturation in affected pups was supposed (*XXX and*

XXX 1996). XXX was administered to rats at dose levels of 37.5, 75, 150 or 300 mg/kg bodyweight from day 6 to day 17 of gestation. Embryo/ fetotoxicity was seen at 150 and 300 mg/kg bodyweight as increased resorption rate and decreased fetal weights. Skeletal variations indicating a substance related effect on bone mineralization were seen in fetuses of all treated groups (*XXX et al. 1981).* In a small number of rats the treatment with 50 mg/kg bodyweight XXX on days 12-14 of gestation led to the formation of ,,wavy ribs" at a high incidence in fetuses as a sign of impaired bone mineralization (*XXX et al. 1985). XXX and XXX (1988)* found corresponding results after oral administration of 80 mg XXX to rabbits from day 16 to 19 of gestation. Maternal metabolic alkalosis caused by XXX may be at least partially responsible for the effect on skeletal development and mineralization (*XXX et al. 1993).*

Perinatal/Postnatal Toxicity
Injections of XXX (5 mg/kg bodyweight daily) to rats from day 4-28 post-partum resulted in retardation of growth with a simultaneous fall in magnesium and calcium concentrations in the bone and an increase in magnesium and calcium concentrations in the urine (*XXX et al. 1986).*

Prenatally (pregnant rats 4 hours before delivery) subcutaneously administered XXX delayed postnatal ductus arteriosus closure (0.36 mm at 60 min after birth). XXX injection in 60-min-old rats dilated the constricted ductus arteriosus at 60 min (0.25 versus 0.02 mm in the controls) (*XXX et al. 2010*).

There have so far been no further studies on perinatal and postnatal toxicity (*RTECS 2007*, see also Appendix I *Literature investigation 2007/2016*).

Genotoxicity
The available investigations of the mutagenic potential of XXX were completely and competently reviewed by the German Health Office (*XXX et al. 1991)* and are summarized below: In a total of 6 mutagenicity tests in bacteria (Ames tests) XXX did not lead to any mutagenic effect with or without metabolic activation. Tests for mutagenicity or chromosome/DNA damage led to conflicting results. In a mouse lymphoma test for gene mutation in mammalian cells no mutagenic effect of XXX was seen without metabolic activation but with metabolic activation an increased incidence of mutations was seen at a very high cytotoxic concentration. In a range of tests for chromosome aberration or sister chromatid exchange, all of which were not adequately performed, positive as well as negative results were seen. Studies on chromosome aberrations and on sister chromatid exchange *in vitro* performed in the course of the US National Toxicology Program revealed minimal positive effects with and without metabolic activation but only at highly cytotoxic concentrations. In a test for DNA damage (unscheduled DNA synthesis, UDS) XXX did not induce any DNA damage. Confirming the results of *in vitro* tests, *in vivo* testing of XXX also led to conflicting results. In a micronucleus test in mice no clastogenic effect was found but the dose levels used were not high enough to exclude a possible clastogenicity. In investigations performed by an Indian group of scientists mutagenic effects on germ cells, possibly heritable, were seen. According to the German Health Office, the performance of these tests was generally not acceptable, with too low animal numbers and other serious shortcomings (*XXX and XXX 1977*).

In vivo genotoxicity of XXX at the hepatotoxic equivalent doses has been investigated in mice treated with intraperitoneal doses of 2.5, 5, 10, 20, 40, and 80 mg/kg bodyweight for both single (24 h) and repeated dose (7 consecutive days) toxicity studies. The results demonstrated toxic responses in the hepatocytes as evident from increased ALT/AST level, DNA damage, TUNEL positive cells and increased DNA fragmentation in mice *in vivo*. However, in bone marrow cells, XXX did not induce structural chromosomal aberrations, but

produced mild DNA strand breaks as observed by the comet assay (*XXX et al. 2016*).

The literature to date contains no further reports on genotoxicity studies, and shows no evidence of any mutagenic potential of XXX (*RTECS 2007*, see also Appendix I *Literature investigation 2007/2016*).

Carcinogenicity

Carcinogenicity studies with XXX were performed in the course of the National Toxicology Program 1989 in rats and mice (*NTP 1989).* Rats received XXX at dose levels of 15 or 30 mg/kg bodyweight, mice at dose levels of 95 and 200 mg/kg bodyweight. Non-neoplastic lesions were restricted to the kidney in rats and mice; these forms of nephropathy were seen in high-dose male rats with greater severity than in controls and in treated mice of all groups at a greater incidence as compared to controls. According to the author there was equivocal evidence of carcinogenic activity of XXX for male rats as shown by marginal increases in uncommon tubular cell neoplasms of the kidney and meningiomas of the brain. There was some evidence of carcinogenic activity of XXX for female mice as shown by an increase in malignant tumors of the mammary gland. In rats this increase was small and not dose dependent for both kinds of tumors. In mice it was also small but dose dependent in both treatment groups. In a tumor promotion study rats were pretreated (initiated) with N-nitrosobutyl-N-(4-hydroxybutyl)-amine for four weeks and afterwards received XXX at a total dose of 250 mg/kg bodyweight for 32 weeks. No increase in proliferative bladder lesions was seen in XXX treated groups compared to controls (*XXX et al. 1989).* No clear evidence of carcinogenic effects in humans is known from epidemiological studies or medical records despite the longterm use of XXX (*NN 1990).* According to *BfArM Mustertext 1996* it is the official opinion of the relevant authority that the longterm studies in rats and mice did not reveal a toxicologically relevant carcinogenic potential of XXX.

The literature to date contains no further studies of the tumorigenic potential of XXX, and shows no relevant evidence based on experimental clinical observations (*RTECS 2007*, see also Appendix I *Literature investigation 2007/2016*).

Local Tolerance

Available literature provides no evidence to suggest any particular local toxicity potential of XXX. For drug formulations intended only for oral intake generally no investigations regarding the local tolerance are necessary. There are no reports in the literature concerning the local toxicity of XXX after application to animals at present (*RTECS 2007*, see also Appendix I *Literature investigation 2007/2016*).

Immunotoxicity

There is no evidence to suggest any immunotoxic properties of XXX. XXX is considered to be a non-immunosuppressant drug (*XXX et al. 1994, 1995).*

There are no further reports in the literature concerning the immunotoxicity of XXX after application to animals at present (*RTECS 2007*, see also Appendix I *Literature investigation 2007/2016*).

Other Information

Single oral doses of 2000 mg XXX/kg bodyweight in mice led, after 2 hours, to hyperglycemia lasting 24 hours. This suggests an acute and prolonged effect of XXX on carbohydrate metabolism, possibly as a result of a reduction in insulin secretion (*XXX and XXX 1988a*, *XXX and XXX 1988b*).

2.4.5 Integrated Overview and Conclusions

XXX is a short-acting diuretic and selectively inhibits the carrier-mediated sodium, potassium and chloride co-transport at the luminal tubular membrane of the ascending loop of Henle, both in the medullary and cortical areas of the nephron leading to a strong diuresis. The renal effect is characterized by a marked excretion of water, chloride and sodium. XXX also enhances the excretion of potassium, hydrogen, calcium and magnesium. The enhanced excretion of hydrogen ions may lead to a subsequent hypochloremic metabolic alkalosis. Short-term oral XXX immediately increased urine volume significantly, but diuretic resistance developed after 14 days of XXX treatment (dogs).

XXX initially increases the renal blood flow without increasing filtration rate and concomitantly, prostaglandin concentration increases. Stimulation of renin secretion and increase in plasma aldosterone following oral administration of XXX has been shown. The activation of the renin-angiotensin-aldosterone system (RAAS) appears to plateau at day 5 of oral administration (dog). XXX causes renal vasodilatation. In mice vasodilatation of afferent arterioles and paradoxical increase of renal vascular resistance is observed. Data suggest that generation of angiotensin II and/or a vasoconstrictor prostaglandin combined with compression of peritubular capillaries by the expanding tubular compartment are responsible for the reduction of renal blood flow *in vivo*. The XXX-associated reduction of blood pressure is partly attributable to a reduction of peripheral vascular resistance. Beyond it, accumulation of methylguanidine and changes in guanidine compound levels in plasma, urine and kidneys is caused by XXX.

A novel molecular basis for the mechanism of action of XXX in renal failure has been suggested. In experimental postoperative acute renal failure XXX improved renal hemodynamics and attenuated ischemia/reperfusion-associated apoptosis and induced changes in angiogenesis-related gene expression. XXX is able to prevent metastatic myocardial calcification and hypertension following chronic renal failure (rat).

Diuretic therapy reduces preload and relieves congestion secondary to cardiac dysfunction. Myocardial functional parameters in experimental cardiovascular disease may improve with administration of loop diuretics. However, data are partly conflicting. In dogs with mitral valve regurgitation XXX dose-dependently decreased left atrial pressure. In a porcine model of heart failure XXX accelerated left ventricular systolic dysfunction. In rats, no myocardial functional improvement is seen with XXX as opposed to torasemide. But, both diuretics appear to provide an equivalent control of clinical signs of congestive heart failure in dogs.

Inhibition of the cation chloride cotransporter by XXX may account for various extrarenal effects. Antinociceptive and anticonvulsant effects are demonstrated in the rat model of postoperative pain and rat model of status epilepticus, respectively. Moreover, antioxidant properties of XXX have been shown. Beyond it, XXX induces disturbances of the electrolyte balance in the inner ear and in the cerebrospinal fluid. *In vitro*, XXX decreases glucose utilization of sceletal muscle by inhibiting the glucose transport process.

The pharmacokinetic profile of XXX resulting from studies in dogs and rats correlated well with the profile known in human pharmacology. After oral administration, XXX was rapidly absorbed from the gastrointestinal tract. In rats, absorption occurred most rapidly in the stomach of rats. The bioavailability after oral application ranges from 40-60% in dogs but was lower in rats (29%). Maximum plasma concentrations following oral administration were reached within one hour. Several studies showed that XXX is strongly bound to serum proteins (> 90%). According to findings in hypoalbuminemic rabbits, serum protein binding facilitates its renal secretion and the pharmacological response. The decrease in XXX

binding, secondary to drug displacement and/or hypoalbuminemia, can be a cause of resistance to the diuretic.

In rats, gastrointestinal and intestinal first-pass effects of XXX were approximately 40% and 20% of the dose, respectively. XXX is metabolized only in minor amounts. Three novel metabolites have been characterized recently in mouse and rat. Bioactivation of furan ring of metabolites represents the mechanism of XXX metabolic activation and hepatotoxicity. XXX is mainly excreted in its unchanged form in approximately equal amounts via urine and bile. The renal secretion is dependent on the extent of the unbound fraction.

Investigations of XXX-drug interaction have provided insight to its mechanism of diuretic action, but also reveal a great number of clinically relevant effects. Co-administration of hydrochlorothiazide decreased XXX-induced calciuria (mice). Pharmacokinetic interactions of XXX and warfarin result in elongation of the half-life of the anticoagulant (rat). Ascorbic acid increases the bioavailability of oral XXX and enhances the diuretic effect (dog). Diclofenac and also the selective COX-2 inhibitor rofecoxib neutralize the diuretic effect of XXX (rat), whereas XXX prevents the development of diclofenac-induced acute renal failure. XXX potentiated anticonvulsant action of valproate and had no effect towards other antiepileptic drugs (mice). This result may be of importance for epileptic patients treated with valproate and receive XXX for non-epilepsy reasons.

XXX increases effects of cardiac glycosides and potentiates the glycoside toxicity. A synergistic nephrotoxicity of XXX and certain antibiotics (gentamycin, tobramycin, kanamycin) are observed whereas with cefazolin, cephalothin, cefamandole, cephapirin and cephacetrile there is no toxic interaction. Tacrolimus-induced renal impairment is further increased by XXX. Moreover, XXX promotes cisplatin-induced ototoxicity (mice).

The acute toxicity of XXX was generally low and was reported as being dependent on the water and electrolyte supply. The *oral* LD_{50} was determined with 2000 mg/kg bodyweight in mice and in dogs, 2600 mg/kg bodyweight in rats, and 800 mg/kg bodyweight in rabbits. After *intravenous* administration the LD_{50} was between 308 mg/kg bodyweight (mouse) and 800 mg/kg bodyweight (rat). Following *intraperitoneal* administration, mean LD_{50} values of 430 mg/kg bodyweight (mouse) and 800 mg/kg bodyweight (rat) were obtained. After *subcutaneous* application, LD_{50} values of $>$ 4600 mg/kg bodyweight (mouse) were found. In studies for subacute, subchronic and chronic toxicity XXX induced bodyweight depression and increased mortality only at high-dose levels. Lowest toxic dose (TDLo) values after repeated oral administration of XXX in mice were $\leq$ 77280 mg/kg bodyweight or $\leq$ 54600 mg/kg bodyweight after 14 days continuous (producing changes in tubules) or 13 weeks intermittent application (producing changes in liver weight), respectively. In rats, TDLo values were obtained within a range from 72 mg/kg bodyweight and 6 days intermittent use (producing changes in urine composition) and 27300 mg/kg bodyweight and 13 week intermittent use (producing tubular and liver weight changes).

A substance related specific organ toxicity was seen in the kidneys as nephropathy. The overall no effect level was determined at 15 mg/kg bodyweight in a chronic study in the rat.

Hepatotoxicity of XXX (1.21 mmol/kg) was shown in mice, but was not found in rats. XXX-caused hepatic necrosis in mice may arise from metabolic bioactivation to a chemically reactive/toxic metabolite that binds to hepatic proteins. When compared to its genotoxicity on heptatocytes XXX is weakly genotoxic toward the bone marrow cells.

Studies of acute ototoxicity revealed a substance-related decrease of endocochlear potential with histological or ultrastructural changes of the stria vascularis and the hair cells and resulting in a functional impairment of the inner ear. The effects observed are probably

based on changes in the ion balance in the cochlea.

In a rat model of heart failure standard dose XXX was associated with increased mortality. However, co-administration of an ACE-inhibitor could prevent the XXX-associated mortality. In pigs with experimental heart failure XXX increased aldosterone and accelerated the progression of left ventricular dysfunction. Results suggest that activation of the renin-angiotensin-aldosterone system (RAAS) may contribute to progression of heart failure and mortality and XXX should be accompanied by RAAS-suppressive treatment.

XXX delays ductus arteriosus closure and dilates the constricted ductus arteriosus in neonatal rats. If XXX has similar effects in human preterm neonates, caution may be warranted in its use in the treatment of infants with patent ductus arteriosus.

Possible harm of XXX on reproduction includes embryotoxicity (increased frequency of resorption) and fetotoxicity (decrease of fetal weight). In specific investigations an impairment of the kidney development was determined. In reproductive studies lowest toxic doses (5000-12500 mg/kg bodyweight (mice) and 150-300 mg/kg bodyweight (rats)) of XXX mainly affect the musculoskeletal system and produce specific developmental abnormalities in female animals during pregnancy (day 6-16), and spermatogenesis in male animals (≈ 1500 mg/kg bodyweight (mice) and 22 mg/kg bodyweight (rats)). XXX should therefore be used in pregnancy only when strictly indicated. If it is to be used during lactation, it is recommended that breast-feeding be first discontinued.

Studies of mutagenicity revealed partly contradictory results. To date the question of whether a mutagenic potential should be attributed to XXX has been left undecided. Available data is generally not sufficient to draw a definite conclusion about the mutagenic potential of XXX. Positive effects were only seen in mammalian cells at high and cytotoxic concentrations. Often this indicates that possibly osmotic effects, effects on the pH-value or other physicochemical changes, or the cytotoxicity may play a role in such test results. No thoroughly performed *in vivo* test is available to clear up the toxicological relevance of the available data. Based on available data it seems possible that there may be no risk with regard to use in human therapy. Carcinogenicity studies in rats and mice revealed no clear carcinogenic potential but some evidence of carcinogenic effects in mice, based on an increase in mammary tumor incidence, and equivocal evidence of carcinogenic activity in rats, based on an increase in the incidence of tubular cell neoplasms of the kidney and meningiomas of the brain. The relevance of these findings to human therapy remains unclear.

On the basis of its defined pharmacodynamic profile of effects at the proposed dosage, which is well below the toxic level, XXX may be rated as an effective and safe compound. Overall, there has been no clear evidence of other possible, as yet unrecognized, risks. In view of the comprehensive scientific documentation currently available, it can be stated that any additional preclinical data significant for the assessment of the benefit/risk ratio of XXX containing preparations are available which results in a new benefit-risk evaluation, and the risk-benefit ratio of XXX is positive and has been unchanged over time in the period evaluated. According to present knowledge, no as yet unknown risks should be expected in relation to its use in human medicine.

2.4.6 List of Literature Citations

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Enclosures

Appendix I: Results from international literature investigation for publications on pharmacology, pharmacokinetics, toxicology, therapeutical effectiveness and adverse drug reactions following administration of XXX