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Review

Successful Drug Development Despite Adverse Preclinical Findings
Part 1: Processes to Address Issues and Most Important Findings

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Abstract: Unexpected adverse preclinical findings (APFs) are not infrequently encountered during drug development. Such APFs can be functional disturbances such as QT prolongation, morphological toxicity or carcinogenicity. The latter is of particular concern in conjunction with equivocal genotoxicity results. The toxicologic pathologist plays an important role in recognizing these effects, in helping to characterize them, to evaluate their risk for man, and in proposing measures to mitigate the risk particularly in early clinical trials. A careful scientific evaluation is crucial while termination of the development of a potentially useful drug must be avoided. This first part of the review discusses processes to address unexpected APFs and provides an overview over typical APFs in particular classes of drugs. If the mode of action (MoA) by which a drug candidate produces an APF is known, this supports evaluation of its relevance for humans. Tailor-made mechanistic studies, when needed, must be planned carefully to test one or several hypotheses regarding the potential MoA and to provide further data for risk evaluation. Safety considerations are based on exposure at no-observed-adverse-effect levels (NOAEL) of the most sensitive and relevant animal species and guide dose escalation in clinical trials. The availability of early markers of toxicity for monitoring of humans adds further safety to clinical studies. Risk evaluation is concluded by a weight of evidence analysis (WoE) with an array of parameters including drug use, medical need and alternatives on the market. In the second part of this review relevant examples of APFs will be discussed in more detail. (J Toxicol Pathol 2010; **23**: 189–211)

Key words: adverse preclinical finding, hazard identification and characterization, risk evaluation and management, mode of action, safety ratio, weight of evidence

Introduction and Overview

Adverse preclinical findings (APFs) can arise anytime and often unexpectedly during the development of a drug candidate. They can fall in any of the following categories:

- Functional APFs, covered essentially by safety pharmacology testing[1, 2], including in particular
  - QT prolongation[3–10]
  - Immunotoxicity incl. immunostimulation[11–15]
  - CNS-related symptoms, such as seizures[a 16]
- Genotoxicity including mutagenicity[17–22]
- Morphological toxicity such as cardiotoxicity or nephrotoxicity[23–28]
- Carcinogenicity[17, 22, 29–31]
- Reproductive toxicity: Functional and gross pathological observations[32–36]

The above categories of APFs are essentially related to the methodology of investigation. An APF can belong to more than one category, e.g. have functional symptoms and result in morphological changes.

Figure 1 is an example of a subtle morphological APF. What do these vacuoles in the pancreatic β cells mean? Is this finding insignificant or the first sign of a major issue? A lot can go wrong at this point[37] including e.g. ignoring the finding as not significant or becoming hyperactive. There is no general answer to the question what these vacuoles mean for further development of the drug candidate, as will be explained below.

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[a] Not much published literature is available on this subject. Examples of drugs with functional adverse preclinical CNS findings are listed in the Physicians' Desk Reference PDR[16]. Some examples are also discussed below under "Evaluation of various types of adverse preclinical findings – Functional effects".

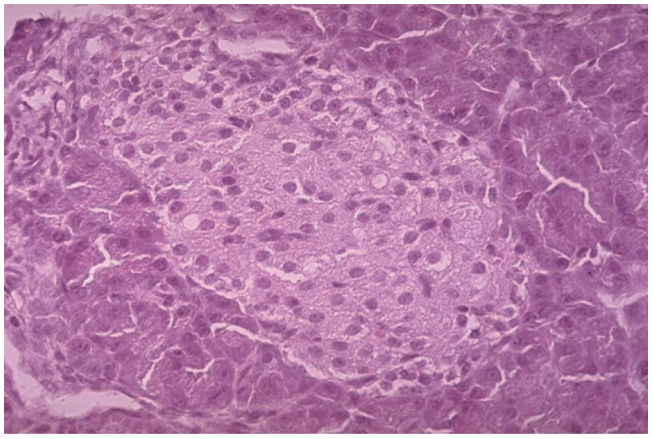


Fig. 1. Beta-cell vacuolization in the pancreas of a SIV 50 rat. H&E, lens 25×.

This review outlines proven processes for dealing with unexpected APFs in order to optimize the chances of successful development of drug candidates or—if necessary—to create a scientific basis for their early termination. This first part of the review addresses strategic aspects regarding the "troubleshooting" approach—others prefer to call it the "problemsolving" approach. In the second part some examples are discussed in more detail. Drug development is a complex process[38]. The accurate prediction of human drug toxicity remains a major challenge in drug development[39]. Therefore, toxicologists and toxicologic pathologists need to be prepared to address unexpected APFs. Toxicity studies are designed to produce toxicity at least at the high dose. Absence of APFs may mean for example that dose selection was wrong or that the drug candidate has no major therapeutic value, or that the model used to detect toxicity is not valid. The examples used in this review are mainly from drug development; however, the same approach is also meaningful for chemicals or food additives.

Processes in Case of Adverse Preclinical Findings—Overview

Guidance

The guiding principle of those involved in developing and administering drugs is still *"Primum non nocere"* (above all do not harm), as formulated by Hippocrates almost 2,500 years ago. Simultaneously the aim must be to bring value adding drugs to the market for the benefit of patients and the company. Over 90% of withdrawals of marketed drugs are due to clinical toxicity, particularly hepatotoxicity and cardiovascular toxicity[40–43], which underlines the importance of a careful and intelligent preclinical and clinical safety assessment before registration and marketing of new drugs. As rare APFs may only be noticed once drugs are widely used, post-marketing surveillance is important and provides also new insights for the development of further drug candidates[44].

Organization

The initiative to assess APFs must be kept in-house but consulting with external specialists is often necessary and very helpful.

Whenever possible, an experienced company-internal associate should take the lead and the responsibility for resolving issues in connection with an unexpected APF. This is primarily a scientific issue, but business aspects must also be taken into account e.g. regarding the financial resources a company is willing to invest in view of the potential benefits of the drug candidate for patients and the company. If internally no "troubleshooting" leader is available, a trusted external expert with the necessary business sense can be commissioned with the task. The company management, often at several levels depending on the issue and its anticipated impact, generally likes to be kept informed and to take major decisions e.g. regarding resources (manpower, money). However, the team leader and the team must be empowered with relevant decision making competence within company-defined limits and must have adequate resources.

Good management of the various steps for assessing the human relevance of APFs is a key success factor and involves various steps as listed below. These steps need not necessarily be taken in sequential order and a specific approach tailored to a particular problem is recommended. The various steps frequently involve:

- Assembling a team of in-house specialists and external experts, e.g. drug development consulting services. Experts from universities recognized for their achievements in the scientific field related to the APF in question can be helpful.
- Determining in a first step the potential relevance of the APF, e.g. by collecting information about similar drugs as available from literature, through the USA freedom of information acts, from the scientific community including consultants, etc.
- Examining the options at the current state of knowledge, in essence whether to
  - Abandon development, e.g. for the following reasons: The risk/benefit ratio of the drug most likely is too small, the investment at stake is still minor e.g. because the drug candidate is in an early phase of development, or follow-up drug candidates are available
  - License drug candidate out, as other companies might be interested in developing the drug candidate in different indications or are willing to take a higher development risk
  - Review the therapeutic indication of the drug candidate, since e.g. a harder indication for a more serious disease could justify a higher therapeutic risk (see also below under risk-benefit evaluation), or a different therapeutic indication might reduce the therapeutic dose

  - Try to resolve the issue, which is addressed below in more detail
- Setting up a plan for issue resolution including tentative objectives, a time frame, budget limits and potential exit points, among other aspects. If a back-up drug candidate is available, one may consider starting to develop it while still working on the first drug candidate. However, if the issue is due to a class effect (see below), similar problems are to be expected with a follow-up drug candidate of the same chemical and/or pharmacological class. In such as case it may be worthwhile to run short-term screening tests to determine the relative potential of further drug candidates to cause the APF in question, thereby supporting the selection of the optimal drug candidate
- Securing continuous support from the upper company management through reliable reporting
- Making sure that also members not belonging to the core team are regularly updated and kept interested in contributing their knowledge to resolve the issue
- Contacting authorities in the event that an application for clinical trials with an investigational new drug (IND) exists or that clinical trials are ongoing. Generally authorities need to be informed within 15 calendar days after the sponsor's receipt of the respective APF information (see respective guidelines of the various regulatory authorities). However, it is not always necessary to file an adverse event (safety) report; it may be sufficient to inform the authorities about the findings and currently planned measures. It is understood that the latter may have to be updated later following further insight into the issue
- Conducting the necessary activities including scientific evaluation and producing the necessary documents. The end product of "troubleshooting" activities ideally is a scientific story which explains the unexpected APF. It is advisable to consider publishing the results in a recognized peer-reviewed journal, as this will increase the credibility of the conclusions.
  Unfortunately, sometimes not all aspects of an APF can be fully explained by scientific data, as will be shown in the second part of this review. Also this must be discussed openly and addressed in the conclusions
- Obtaining agreement with authorities and continuing with or terminating development. Even if not all aspects of the APF can be explained, risk evaluation and precautions for risk management frequently allow proceeding with clinical development, until a final risk evaluation becomes possible based on good human data

The steps

Identification and resolution of unexpected APFs occur in various steps such as defined below. In short and general terms, after an APF is identified, it needs to be verified and characterized in detail. Consequently, a risk evaluation is conducted, which includes in particular an evaluation of the relevance of the finding for man and safety ratios, which then serve as a basis for risk management. This step-wise approach is well established for environmental agents[45–48]and useful also for drugs[28].

There are four major steps to deal with unexpected APFs
1. Hazard identification: Recognition of a suspected APF
2. Hazard characterization particularly regarding dose-response, severity, and reversibility of the APF
3. Risk evaluation: Essentially an intellectual process to determine if and under which conditions the drug may be used in man
4. Risk management: Implementation of precautions for the use of the drug in man

An example regarding the various steps in dealing with APF is the following:

1. Hazard identification: An increased incidence of thyroid follicular tumors is seen in a mice lifetime bioassay.
2. Hazard characterization: The working hypothesis is that, in the absence of genotoxicity, these tumors are likely to be of epigenetic origin and related to the hormonal control of the thyroid. Deep frozen sera of the 13 week dose-finding study are investigated, and decreased thyroxin (T4) and increased thyroid stimulating hormone (TSH) levels are found. Liver and thyroid weights are increased. An additional 4 week investigative study shows increased T(4) uridine 5'-diphosphoglucuronosyltransferase (UDP-GT) activity.
3. Risk evaluation: The drug candidate is an inducer of the microsomal enzyme UDP-GT. Increased glucuronidation of T4 increases T4 excretion and lowers T4 serum levels, thus leading to TSH elevation. TSH stimulates the proliferation of thyroid follicular cells, which in the lifetime bioassay is associated with an increased incidence of thyroid follicular tumors[49].
4. Risk management: Human beings are known to be less susceptible to hormonal imbalance, though high TSH levels e.g. in case of a Hashimoto goiter lead to benign thyroid hyperplasia or thyroid adenoma also in humans[50]. Therefore, thyroid hormones and TSH are monitored in clinical studies to make sure that at therapeutic doses no changes occur.

The above steps sometimes blend, that is they may not necessarily be conducted strictly sequentially. APFs considered minor, e.g. clinical chemistry findings without morphological correlate, need not to be investigated in a complex manner, and one can proceed directly to risk evaluation (e.g. calculation of safety ratios, see below), and risk management (e.g. limitation of the starting dose in new clinical trials and monitoring of the respective enzymes).

1. Hazard identification

Hazard identification means recognition and qualitative assessment of unexpected APFs keeping in mind that sensitivity of preclinical safety test systems is more important than specificity. In other words: The test systems are designed not to miss potential APFs, but in return may generate insignificant or false positive findings.

Some basic questions to answer during the hazard identification step are:
- Is there indeed an adverse effect?
- What else is known about the drug in question?
- Were there other relevant findings?
- Is the study technically valid?
- Is the model valid?
- Were there other modifying factors?

Is there indeed an adverse effect?

In preclinical safety studies essentially incidences, severity levels, and time of onset (as far as possible) of lesions in dosed and control animals are compared. Each finding must be examined regarding its relationship with treatment[28, 51, 52].

Distinction of treatment-related lesions vs. artifacts: The following findings are often histological artifacts: CNS vacuolation, terminal or post-mortem acute renal tubular necrosis and collapsed lungs particularly in dogs mimicking interstitial pneumonia. Many more morphological artifacts can be encountered and have to be recognized as such[53].

Examples of "artifacts" because of experimental conditions are: Carcinogenicity at cytotoxic doses[54], positive "genotoxic" findings in *in vitro* assays at high concentrations[55], and teratogenicity at doses toxic to pregnant test animals. In an effort to decrease the incidence of irrelevant positive results, the regulatory authorities, in consultation with the scientific community, have agreed e.g. on dose selection for carcinogenicity studies[56] and have started to revise the International Conference on Harmonization (ICH) guideline on genotoxicity testing currently available as draft guideline S2(R1)[21]. Another experimental artifact can be light-induced retinopathy in albino rodent[57]; this artifact may be difficult to distinguish from drug-induced retinopathy, if dosed animals were constantly closer to the light source than control animals.

Distinction of treatment-related lesions vs. spontaneous alterations and naturally occurring variations: Laboratory animals show spontaneous alterations, partly related to species, strain, age, housing condition, diet, infections, and other factors. Such alterations are seen in many different organs[58–63]. They can also be due to embryonic remnants and misplacement of tissue[64–66]. Examples are spontaneous seminiferous tubular atrophy/hypoplasia particularly in dogs[67], seasonal arrest of spermatogenesis in hamsters, immature sexual organs especially in dogs, mammary estrous cycle changes, or vascular alterations[68]. More rare examples are e.g. spongiosis hepatis, as described in the next section, or retinal gliosis, a lesion originally described in humans[69]. The latter can occur spontaneously e.g. in rats and be mistaken as an induced lesion. Historical control data are very important when evaluating lesion incidences of dosed and control animals. The best historical control data are in-house data of the last five years. If such data are not available or not sufficient, external data from the same strain and breeder can be used, while literature data can serve for confirmation[70–73].

Misdiagnosis: Also for an experienced toxicologic pathologist it can be quite challenging to distinguish e.g. an age-related lymphoid thymus hyperplasia in female mice from a malignant lymphoma. Another source of error may be related to changes of the definition of diagnostic terms over time, which can lead to differing interpretations: For example, cystic degeneration or spongiosis hepatis in rats was assumed to be (pre-)neoplastic in the past, but is now known to occur also as spontaneous or secondary/reparative non-neoplastic lesion[74]. To minimize misdiagnoses, a review of pathology data by a second experienced toxicologic pathologist and a discussion between the study pathologist and the review pathologist is beneficial to assure quality of the histopathological evaluation[75, 76].

Data handling: Statistical tests are necessary, but do not tell anything about the biological relevance of a lesion. Testing at the 5% limit means that of one hundred statistical tests on normally distributed homogenous data pairs such as liver weights of high dose and control animals, 5 tests turn out positive by chance reflecting only normal biological variance. Therefore, toxicologists and toxicologic pathologists must have some basic understanding of statistical tests, know the limits of their application, and be able to correctly interpret test results. False positive statistical results can be particularly awkward in the case of rare findings or a slightly increased incidence only in a (high) dose group. As already pointed out above, historical control data for any type of toxicity studies are indispensable to analyze such findings and to show that the statistically significant "deviation" is within the historical control range [77].

Another fallacy derives from dividing lesions into different subcategories, such as in the following example: The incidence of pulmonary squamous cell carcinomas in the control, low, mid, and high dose groups is 0, 0, 0, and 3, respectively. These data suggest a carcinogenic effect in the high dose group. However, there were also broncho-alveolar carcinomas with group incidences of 4, 3, 3, and 1, respectively. If both carcinoma types are pooled to give 4, 3, 3, and 4 respectively, then no treatment-related effect is present. For a guideline of combining neoplasms see [78].

Review of earlier studies: It is mandatory to review earlier, often shorter-term studies with the drug candidate or related drug candidates for subtle changes, which might have been missed or dismissed as not significant. With hindsight and knowing what one is looking for, it is always easier.

What else is known about the drug in question?

Exaggerated pharmacological action: Could the

observed APF be a consequence of an exaggerated pharmacological action at high doses of the drug candidate, e.g. neuropathy, if healthy animals are treated with hypoglycemic drugs[79]? Is the APF mediated by a receptor that is responsible for the pharmacodynamic effect? Is that receptor relevant to man, similarly distributed, and similarly responsive in man?

Drug class effect: Some classes of drugs are known to induce APFs (Table 1). Information can be obtained e.g. from the scientific literature, through personal contacts directly from the scientific community or through the US freedom of information acts. Some of these class effects are known to be relevant to man, such as cytotoxicity of most anticancer drugs in rapidly proliferating tissues. Others are known to be more or less species-specific, such as many endocrine effects seen in test animals. Still others are of somewhat uncertain relevance for man, such as vasculitis observed with phosphodiesterase (PDE) IV inhibitors, and need to be assessed on a case-by-case basis (see also second part of this review). Class effect information is crucial to assess the relevance of an APF to man, to decide about potential precaution measures to be taken in clinical trials and to anticipate how registration authorities might regulate the drug in question. Also if a class effect is known not to be relevant to man, a new drug candidate showing the same APF has to be assessed carefully. As will be discussed in the second part of this review, peroxisome proliferation was originally and correctly regarded as not being relevant to man, but new peroxisome proliferator-activated receptor (PPAR) agonists are much more potent. Therefore it can not be excluded that APFs observed with new PPAR agonists are relevant to man. Already at this early stage, it may be useful to calculate risk/benefit ratios (see below under risk evaluation) based on structural similarities and/or therapeutic class in case of a suspected class effect.

Were there other relevant findings?

Particularly in case of unexpected APFs it is important to correlate in-life observations, clinical pathology/chemistry data, pathology findings, and any other observation of relevance made during the safety study. Clinical chemistry data[80, 81] often come from earlier time-points and can provide information on the time course of a target organ lesion. Pathology findings to be correlated include organ weights[82], lesions of other organs with potentially systemic consequences such as in case of the hepato-renal syndrome[83], organs of the endocrine system (see second part of review), or early lesions including preneoplastic lesions[28, 84–86]. It is clear that also findings from other studies, such as results from pharmacological or mechanistic studies, need to be taken into account.

Is the study technically valid?

Important issues to be examined include dose selection especially in rodent bio-assays[56], purity of the test substance, especially if after a change of the substance batch new toxicities are observed, and study conduct including e.g. tissue sampling[62, 87–90]. It is of utmost importance that control and dosed groups are treated in the same way with the only exception of dosing. This also includes that control and dosed groups should be necropsied and processed by the same team. Slide evaluation should be done by the same study pathologist. If for time-constraints two pathologists share the task, then splitting by dose must be avoided by all means. If necessary, then splitting should be by sex and the two pathologists must take some time to also examine typical slides of the other sex.

Is the model valid?

Animals are not humans and some particularities of test species commonly used in preclinical safety studies are summarized below. The endocrine regulation of rodents is markedly different from that of human beings [91, 92], e.g.

- Rats lack high-affinity thyroxin-binding globulin
- The estrogen/progesterone ratio is 1:100–200 in rats, but 1:1 in women
- The sexual endocrine system of old rats is progesterone dominated, while that of menopausal women is just waning with a natural decrease of estradiol and progesterone production by the ovaries [93]
- Prolactin has a trophic effect on rat mammary gland, but induces lactation in women
- Increased luteinizing hormone (LH) leads to Leydig cell (LC) tumors in rats, while human LCs are much less sensitive [94]

Anatomical particularities of rodents include rodent-specific organs such as forestomach, Harderian gland (eye region), Zymbal's gland (ear), and preputial/clitoral gland. Tumors occurring exclusively in rodent-specific organs are often regarded as not relevant to man. However,

- Tumors in the rat forestomach might indicate, for example, a risk for esophageal tumors in man
- The similarity of Zymbal's and preputial/clitoral glands of rats to human sebaceous glands must be kept in mind.

Target organ concordance between test animals and human beings need not be a prerequisite when evaluating animal tumor findings with regard to their relevance to humans. Therefore, also the significance of tumors in unique rodent tissues must be addressed using the mode of action/human relevance framework approach (see risk evaluation below).

Mice are known to have a high incidence of spontaneous tumors particularly in lungs, the liver, Harderian and adrenal glands, the hematopoietic system, and ovaries. Rats show high incidences of mammary gland and pituitary tumors[70, 95–100]. Also, differences in absorption-distribution-metabolism-excretion (ADME) parameters between test animals and man regarding e.g. metabolite pattern, (organ) accumulation or distribution of drug-metabolizing enzymes, may play a role[101–104]. Differences are also found between different strains of the same species[105–111]. For example, the incidence of the following rodent tumors is strongly strain- and possibly partly also breeder-influenced:

Table 1. Examples of Effects Seen with Selected Drug Classes, Including Selected References

Organ	Lesion	Examples of involved drug classes
Vessels	Arteritis	Phosphodiesterase inhibitors[249–252] Some dopaminergic drugs[220] Some endothelial antagonists[253] Other drugs[254]
Heart	Direct cardiotoxicity	Anticancer drugs of the anthracyclic antibiotic type and others[157, 255, 256]
	Cardiopathy/myocardial infarction, particularly in dogs	Positive inotropic and vasoactive drugs[209, 210, 255, 257]
Muscles	Rhabdomyolysis	Lipid-lowering agents[258, 259]
Kidney	General nephrotoxicity	Aminoglycosides and other antibiotics [260–264] Cyclosporine[265, 266]
	Papillary necrosis particularly in dogs	Non-steroidal anti-inflammatory drugs (NSAID)[262–264, 267, 268]
Nervous system	Neuronal toxicity	Many substances, but in particular environmental chemicals such as organophosphates[269, 270]
Liver	Hepatomegaly because of organelle proliferation	Smooth endoplasmic reticulum (SER) proliferation e.g. with phenobarbital-like drugs[52, 146, 271] Peroxisome proliferation e.g. with certain hypolipemic drugs[214, 272–274]
Gastro-intestinal tract	Ulcers in the intestinal tract	Non-steroidal anti-inflammatory drugs (NSAID) [275, 276]
Endocrine organs	Endocrine-mediated APFs are frequent	Many drugs, for overviews see e.g.[277, 278]
	Seminiferous tubule atrophy	Drugs with estrogenic properties [279–282]
	Pseudopregnancy with persistence of corpora lutea	Progestational and dopaminergic compounds, e.g. neuroleptics[120, 277]
	Thyroid insufficiency	Sulfonamides[283, 284]
	Other hormonal effects, e.g. skeletal and other changes[277]	Recombinant human parathyroid hormone[285]
Various organs	Atrophy of rapidly proliferating tissues, e.g. bone marrow, lymphoid organs, epithelia of gastrointestinal tract, seminiferous tubules	Cytotoxic anticancer agents[286–288]
Metabolism	Phospholipidosis	Amphiphilic and other drugs[231, 289, 290]

For additional references see text and second part of the review.

- LC tumor incidence: 88–96 % in F344 rats, below 10% in Sprague Dawley (SD) rats, 1–2% in Long-Evans rats
- Mononuclear cell leukemia incidence: 20% in F344 rats, rare in SD rats
- Mammary gland tumors, the incidence of which varies widely between different strains, possibly due to endocrine differences and viral infections

Despite the above facts the value of *in vivo* toxicology studies to predict for many significant human toxicities is established[112], but the prediction of human risk based on animal data needs to take all necessary parameters into account[28, 47, 113].

What other modifying factors have to be considered?

Many factors can influence the outcome of a study[114, 115], including the following conditions:

- Quality of animals, particularly of non-rodents and among them especially of monkeys[116, 117]
- Age of the animals[118–120]. As age can have a potentially important effect on drug safety, new drug candidates, unless they can not be used for children, need to be tested also in juvenile animals[121]
- Husbandry[71], including e.g. light intensity[122] and diet[123–128]
- Feeding
  - Feeding ad libitum, which actually means

overfeeding[128, 129], significantly increases tumor incidences of the pituitary[130], the mammary gland, and the lung in rats and of the liver in mice, but may decrease the incidence of uterine tumors in rats[131]. Overfeeding also increases the incidence and severity of degenerative diseases, including nephropathy [132], which may impact on excretion of xenobiotics, and the incidence of myocarditis, polyarteritis, and prostatitis. Overfeeding has been shown to shorten the life span of test animals
    - On the other hand, decreased feed intake e.g. because of toxicity-associated decreased well-being of the test animals or poor palatability of the drug candidate in feed admixture results in the following changes: Retarded growth of young animals, weight loss, decreased organ weights particularly of the lymphatic system, decreased resistance e.g. to preexisting infections, which might be visible as multiple foci of acute to chronic infection. Decreased feeding might also reduce the incidence of background changes as described including references under feeding ad libitum above.
- Contaminations of feed, water or the air and impurities in the drug substance[133]

2. Hazard characterization

This step serves in particular to characterize and quantify the observed APF in more detail.

The most important objectives of the hazard characterization step are to obtain additional data, as far as needed and possible, on:
- Dose response, including exposure at the maximal tolerated dose (MTD) and at the no-observed-adverse-effect level (NOAEL) in the most sensitive species relevant to humans[134]
- Early markers for the APF, which can be used in early human trials of the translational medicine phase as well as in later trials to monitor humans (risk management, see below) for the occurrence of the respective adverse effect(s)
- Mode of action (MoA)[47, 135]

Hazard characterization often involves additional experimental work. The amount of efforts invested depends among other factors especially on the stage of development of the drug candidate (past investment at stake), the proposed indication (risk-benefit considerations, see also below), available alternatives internally (follow-up drug candidates) and on the market (available therapeutic alternatives), the nature of the APF, and the conviction within the company regarding viability of the drug candidate.

Additional investigations of available samples

Samples may be available from the study in question or previously conducted studies and allow e.g. the following additional investigations:
- Hormone measurements in blood/serum samples particularly in case of findings in endocrine organs
- Assessment of gene or mRNA expression and/or marker proteins or protein patterns in blood or tissue samples
- Morphological investigations e.g. by electron microscope (EM) or by immunohistochemistry (IHC) on tissue samples regarding
    - Proliferative cell type[136–138]
    - Measurement of cell proliferation, e.g. by proliferating cell nuclear antigen (PCNA)[138–140], and of apoptosis[141–143]
    - Subcellular details partly also visible on EM pictures of formalin fixed tissue, e.g. to prove or disprove the presence of phospholipidosis
    - Molecular epitopes, which are reasonably well preserved even after many years in wet and paraffin-embedded tissue
    - Morphometry for numerical or volume changes, in particular to establish a NOAEL[144 –149]

Tailor-made mechanistic studies

It is crucial that the additional studies are relevant to resolving the APF issue in question. Therefore, it is necessary to
- Keep the design simple and the objective including what to (dis)prove in mind
- Avoid experiments yielding results which may not be interpretable, e.g. because of lack of experience, lack of historical data, unproven method, etc.

Tailor-made mechanistic studies can have various purposes, namely to better characterize the lesion in question and/or to test one or several hypotheses about the pathogenesis of the observed APF. They serve to obtain properly sampled and prepared material for investigation by often more sophisticated methods, such as
- EM investigations on glutaraldehyde-fixed tissues[150]
- IHC investigations on fresh tissue for detailed cell kinetic studies including cell proliferation, single cell necrosis, and similar investigations[139, 145, 151]
- Flowcytometry for cell typing, e.g. in connection with immunotoxicity issues[152, 153]
- Laser scanning cytometry or confocal laser scanning micrososcopy for a detailed assessment of cellular structures[153, 154]
- Microdissection followed by special analyses, especially –omics investigations (see next bullet point)[155]
- -omics investigations for DNA, RNA, and protein expression[156–160]. These investigations can be useful to find biomarkers for testing of follow-up drug candidates and in clinical trials, but identifying and validating a biomarker can be resource intensive and

demanding[161, 162]

- More detailed investigation of ADME parameters including e.g. metabolites or organ accumulation. Additional analytical work is also necessary to support Phase III clinical trials, if in earlier clinical studies human metabolites are observed at exposures greater than 10% of the total drug-related exposure and at significantly higher levels than the maximum exposure in the toxicity studies. This does not apply for metabolites which are not of toxicological concern, such as most glutathione conjugates. For details see[163, 164]

Tailor-made mechanistic studies allow an investigation of early findings and of their development over time, e.g. by sequential necropsies in a time-course study or by using newer imaging techniques on live animals[165]. Investigation of early lesions is important, as early lesions are often characteristic for the toxicity observed, while later lesions represent a more general reaction of the biological system to injury.

Toxicity generally starts with functional impairment e.g. of selected cellular membranes of specific cells, which results in subtle structural changes such as microvesiculation (accumulation of water e.g. in mitochondria or lysosomes). If the toxic insult persists, water accumulation increases and becomes visible at the light microscopic level as vacuoles in the primarily affected cells. At this stage cell organelles might be disrupted and the target cells might eventually die resulting in non-specific inflammation dominated by leucocytes and phagocytic macrophages, which may also damage other organ cell types. Lymphocytes generally appear somewhat later, but stay longer. In subchronic studies the end stage of target organ toxicity may be replacement of specific cells by non-specific scar tissue, which may also impinge upon other organs, such as e.g. in the hepato-renal syndrome[83]. Tumors are often preceded by signs of increased cell proliferation (see below) or by preneoplastic lesions, particularly in livers[84 – 86, 166].

To investigate if an APF is reversible, selected animals are necropsied after a treatment-free period at the end of a repeat dose study generally on a routine basis. It is important to keep in mind that early toxic lesions may disappear under continuation of exposure to the offending drug candidate because of adaptation and regeneration. E.g. one or two weeks after acute tubular necrosis kidney tubules might appear normal with exception of tubular basophilia in hematoxylin&eosin (H&E) sections, a tinctorial change which is characteristic of regenerated tubular cells. With progressing severity of a lesion, the likelihood of complete repair decreases. However, regenerative capacity is high especially in liver and kidney. If the lesion might have regressed to some degree or if it appears useful to investigate the details of the recovery process, tailor-made studies may be conducted with a longer recovery period and possibly with investigations at various time points during the recovery period.

In vitro studies with cell or tissue cultures, organ slices or the perfused target organ can be useful to investigate metabolism, effects on subcellular organelles, receptors, or gene expression. Such studies also serve to obtain data on dose-effect relationship at subcellular or molecular levels. For this purpose human cell lines are available.

Increased cell proliferation

Increased cell proliferation is generally associated with tumors in lifetime bioassays[92, 167–169], but this is not always the case [170]. Increased cell proliferation is seen particularly in the following conditions:

- Subacute to chronic cytotoxicity with increased cell death, which must be avoided in lifetime bioassays by correct dose selection[56]
- Chronic tissue irritation e.g. in case of
  - Crystals or aggregates with proteins and lipids in the urinary system. A well-known example is saccharine, which at very high doses formed bladder calculi leading to urinary bladder carcinoma in male rats[171, 172]. A more recent example is the occurrence of urinary bladder carcinomas following treatment with certain PPAR agonists, as discussed in more detail in the second part of this review
  - Solid state carcinogenicity e.g. at subcutaneous injection sites especially in rats or at implantation sites of microchips[173] or transponders[174], particularly in mice
  - Age-related "spontaneous" lesions, e.g. chronic progressive nephropathy, particularly following exacerbation by xenobiotic treatment, which seems to slightly increase renal tubule cell neoplasms[175]
- Increased physiological stimulation of cells leads to cellular hypertrophy (increase in size, generally with proliferation of cellular organelles) and hyperplasia (increase in cell number). The best known examples are the hormonally-mediated tumors in rodents, which are frequent in bioassays and generally without significance to man[92].

For an overview of various types of epigenetic (not genotoxicity-related) carcinogenicity see Table 2.

3. Risk evaluation

The most important parameters for assessing the relevance of an observed unexpected APF for man are

a. Mode of action (MoA) of the drug candidate eliciting the APF
b. Safety ratio (also called safety factor or safety margin) between the highest non-toxic exposure in the most sensitive and relevant animal species and the therapeutic drug exposure of man
c. Weight of evidence (WoE) based on the above and further parameters as appropriate

a. Mode of action

Understanding the MoA as established during step 2 of

Table 2. Epigenetic Carcinogenicity is Generally without Relevance to Man Unless Mentioned in the Table, with Selected References

Pathogenesis		**Examples of compound(s) and, if appropriate, target organs and details regarding MoA**
Cytotoxicity	Cell proliferation for reparation of target organ	Many drugs at cytotoxic doses for prolonged periods of time[291] *Note*: Man is less sensitive to tumorigenicity associated with increased cell proliferation
		Chemicals binding to α2μ globulin in renal tubular cells leading to kidney tumors in male rats, which produce significant amounts of α2μ globulin under testosterone[92, 292]
Chronic irritation	Cell proliferation for reparation and adaptation of target organ/site	Drugs which - are irritating at subcutaneous injection site [167] - predispose for urolithiasis such as saccharine, PPAR agonists, etc.[172, 293] *Note:* Man is less sensitive to tumorigenicity caused by chronic irritation
Induction of drug metabolizing enzymes	Trophic effects on liver cells, particularly in rats	Phenobarbitone and similar inducers of drug metabolizing enzymes[52, 180, 227, 271] leading to adaptive hepatocyte hypertrophy, hyperplasia, and finally tumors
	Trophic effect on thyroid of rats	Microsomal enzyme inducers (see also above) increase T3 and T4 excretion leading to TSH elevation in rats which lack high-affinity thyroxin-binding globulin[50, 294, 295] *Note:* High TSH levels e.g. in case of a Hashimoto goiter lead also in humans to benign thyroid hyperplasia or thyroid adenoma
Induction of peroxisome proliferations	Increased oxidative stress (?)	Liver tumors with "early" hypolipemic drugs, such as clofibrates[273, 274]
Stimulation of adipocytes (?)	Cytokine and growth factor release (?)	Lipo- and fibrosarcomas in rats and hemangiosarcomas in mice with new PPAR agonists[296, 297]
Hormonal stimulation	Direct hormonal stimulation has a trophic effect	Drugs with hormonal action lead to hypertrophy, hyperplasia, and tumors in the target organ [92]
	Hormonal imbalance	Dopaminergics by lowering of prolactin lead to estrogen dominance in old rats and uterine tumors[93, 120] *Note:* As sex hormones are decreased in aging women, this MoA is not relevant to humans
	Decrease of hormone receptors	The dopaminergic mesulergine reduces the number of LH receptors in Leydig cells (LC) resulting in increased LH and LHRH levels *Note:* Not relevant to man tolerating large increases of LH, e.g. in case of the Klinefelter syndrome[94, 120]
	Block of hormone production results in increased stimulation of target organ	H2-blockers lower gastric HCl resulting in increased gastrin levels leading to stimulation of enterochromaffin-like cells (ELC) with hyperplasia and carcinoids particularly in rats[92, 298–302] *Note*: H2-blockers are basically not considered to be tumorigenic in man, but may lead to ELC hyperplasia[303]. Therefore, some residual risk can not be excluded[304, 305]
		Antithyroid agents lower T3 and T4 thus increasing TSH which leads to thryroid tumors particularly in rats being sensitive to inhibition of thyroid peroxidase[50, 92, 294] *Note:* High levels of TSH in Grave's disease (M. Basedow) are not associated with thyroid tumors in man
		Sulfonamides decrease iodine thyroid uptake and lead to thyroid tumors, particularly in rats[50, 294, 306]
Unknown		*β*2-agonists e.g., salbutamol or terbutaline induce hyperplasia of salivary glands and mesovarian leiomyomas in rats[92, 307]. Can be suppressed by *β*-blockers such as propranolol

For additional references see text.

addressing APF is the optimal basis for predicting the relevance of an unexpected APF for man and, if necessary, for managing and minimizing human risk. This has increasingly been emphasized again in recent years in a number of publications in particular regarding the relevance of tumors in rodent bioassays for man[47, 176–183], but also for other types of toxicities[176, 184]. Examples showing how the elucidation of the MoA helped in risk evaluation are given in the second part of the review.

b. Safety ratios

The safety ratio is defined as follows:

$$\text{Safety ratio}^{*} = \frac{\text{Animal NOAEL exposure}^{**}}{\text{Max (anticipated) human exposure}^{***}}$$

* The safety ratio is often called "safety factor" and in some regions "safety margin". However, the latter is sometimes also used for the ratio between exposure at NOAEL and exposure at LOAEL (low observed adverse effect level, within or between species). Therefore, it is advisable to clearly define these terms when using them in a report or publication.

** Exposure at NOAEL of the most sensitive species relevant to humans regarding the particular APF[134] .

*** If the anticipated human dose is not well known, as it is often the case during very early phases of development, also the calculation of the safety ratio between animal NOAEL exposure and exposure at pharmacodynamically effective doses in the same species is helpful. For clinical trials the safety ratios between animal NOAEL exposure and exposure at the human starting dose is relevant.

The safety ratio is an important quantitative means to set the first human doses to be tested in early clinical trials in man[185, 186]. As a basic rule and on practical grounds, a safety ratio is estimated by a safety (or uncertainty) factor of 10 for extrapolation from animal to man and an additional factor of 10 for inter-individual variation in man, resulting in a total safety ratio of 100. However, this comfortable safety ratio is frequently not achieved, and a safety ratio of 10–20 is often acceptable.

According to the FDA guidance for industry on estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers[185], usually the NOAEL from the most relevant animal studies should first be converted to a human equivalent dose (HED) using standard factors presented in Table 1 of the guidance and taking into account the body surface. Using sound scientific judgment, a safety ratio (called safety factor in the guidance) should be applied to the relevant HED to arrive at the maximum recommended starting dose (MRSD). This guidance says that in general a safety ratio of at least 10 should be considered.

High safety ratios are less important for life-saving indications and/or elderly target patient populations particularly in the following context:

- Marginal increase of tumor incidence
- No therapeutic alternatives on the market or significant therapeutic or safety advantages over available alternatives
- MoA is well understood and/or the issue is manageable e.g. by using early biomarkers in clinical and in outpatient institutions.

Conversely, safety ratios are important in case of APFs which may be relevant to man and irreversible, such as neuronal degeneration or toxicity to the reproductive organs leading to sterility.

The calculation of a safety ratio is only possible under the assumption that below a limit or threshold dose the drug candidate does not induce toxicity. Since many decades thresholds are accepted for general toxicity and epigenetic tumorigenicity, but in recent years also increasingly for genotoxicity[187–190]. However, others argue that covalent binding of genotoxic drugs or of parts/metabolites thereof to cellular macromolecules like proteins and DNA may not be repaired completely: Therefore some effects may persist and accumulate with repeated exposure[191]. The discussion is ongoing, but according to the 2006 European Medicines Agency (EMA, former EMEA) guideline on the limits of genotoxic impurities in drug substance a "threshold of toxicological concern" (TTC) value of 1.5 μg/day of a genotoxic impurity resulting in a mathematical excess cancer risk of <1 in 100,000 over lifetime exposure is considered to be acceptable for most pharmaceuticals[192]. This allows calculating an acceptable daily intake based on the expected daily dose. Higher limits may be justified under certain conditions such as short-term exposure.

Low safety ratios including safety ratios below 1, which means significant animal toxicity at dose levels below anticipated human doses, do not necessarily stop development, in particular in case of central nervous (CNS) drugs. CNS-related APFs such as sedation, ataxia, convulsions or death may actually require a slowly increasing dose regimen to allow the healthy test animals to adapt to the treatment. To proceed to early clinical trials in case of a low safety ratio, the APF should be relatively easy to monitor in man and be recognizable early, before permanent toxic damage is inflicted. In addition, an earlier than usual completion of the preclinical program is mandatory in most cases, as will be discussed for CNS drugs in more detail in the second part of this review.

c. Weight of evidence (WoE)

The WoE evaluation is the conclusion of the risk evaluation process, similar to evidence-based human medicine[193, 194]. The WoE as used in this paper is established on a case-by-case basis using a multifactorial, multidisciplinary approach[181, 195–198]. The WoE evaluation is a summary of the relative importance and causal relationships of various aspects of the particular issue, including dose/exposure-response, metabolism, tissue distribution, severity, reversibility, (anticipated) relevance to man, (anticipated) relative

sensitivity of man, and means to look for early signs of adverse findings in man. In addition, the WoE evaluation also takes into account the conditions of drug use and the overall therapeutic context, such as

- The severity of the disease condition and how the new treatment option impacts on quality of life and survival
- The novelty of the drug in question, i.e. whether there is a true medical need for this drug and/or whether it has a relevant new mode of action
- The availability of alternative therapies and their relative safety and efficacy as compared to the drug in question
- The target population, in particular its age
- The intended duration of use

A WoE analysis and conclusion is a process, which has to be continued throughout drug development. Typical milestones are first trials in humans, each time when testing larger populations, escalating doses, extending the duration of treatment in clinical trials, and finally before submitting registration documents to the approving authorities. In case of significant findings for a drug on the market, the WoE analysis and conclusions need to be repeated for the specific case.

For example: A WoE conclusion that a tumor response in rodent bioassays does not preclude administration of the drug candidate to humans, may be based on several of the following facts:

- In comparison with control animals only slight increase in tumor number, no shift to less-differentiated tumor type, no earlier occurrence of tumors, no reduction of the life span of test animals
  No clear dose-response (e.g. highest incidence of the questionable tumor finding in the mid dose)
  Or in other words: The tumors in dosed animals appear and behave similar to tumors of control animals
- A sufficient safety ratio
- Absence of genotoxicity allowing to adopt a threshold concept of the carcinogenic mechanism

On the other hand a real carcinogenic alert exists, if one or the other genotoxicity test is positive and/or one or several of the following conditions are observed: Marked dose-related increase in tumor incidence, early onset of tumor formation potentially associated with early pre-neoplastic lesions and/or shift to a less differentiated tumor type in comparison with control animals, potentially associated with decreasing longevity of the treated animals. The WoE approach should also take into account decreased incidences of certain tumor types and weigh them against increased tumor incidences[199].

Many drugs are on the market which are carcinogenic in laboratory animals, generally rodents, but are found to be safe in humans. For overviews see e.g.[181, 200, 201]. Often the situation is not as clear as given in the above examples and—as shown in the second part of this review—there are also new drugs on the market e.g. with animal tumors *and* positive genotoxicity tests.

Evaluation of various types of adverse preclinical findings

Functional effects: This section is limited to functional toxicity without morphological correlate as e.g. seen in safety pharmacology studies[202] or occurring in toxicity studies with certain drug candidates. Low safety ratios do not *a priori* preclude successful development, though additional safety data are generally needed and special precautions should be applied for first-in-man trials.

Human diseases caused by a regulatory imbalance are relatively frequent, affecting e.g. the endocrine system (over- or underproduction of hormones), the cardiovascular system, in particular the blood pressure, or the metabolic system (e.g. human metabolic syndrome). Drug candidates developed for such disorders actually disturb the corresponding regulatory system in healthy test animals and may therefore be associated with dose-limiting and marked APFs. E.g. anti-diabetes drugs can lead to hypoglycemic brain damage[203, 204] or drug candidates overstimulating neurons can result in neuronal degeneration[204], an effect also observed in humans at excessive doses[205].

As mentioned, test animals are often very sensitive to drug candidates intended for treatment of disorders of the central nervous system (CNS) and react with tremors, decreased activity, sedation, recumbency, loss of balance and ataxia, seizures, and also death already at relatively low doses, occasionally below the intended therapeutic dose. Such APFs are usually without histopathological correlates, dose-limiting, and reversible on cessation of dosing. Frequently the severity decreases with continuing dosing and an escalating dose regimen can help to achieve acceptable exposure in preclinical safety studies. Many drugs on the market show such CNS signs at relatively low doses in test animals, including clozapine, haloperidol, risperidone, buproprion, tricyclic antidepressants or acetylcholine esterase (AChE) inhibitors such as rivastigmine and benzodiazepines (see respective drug information in the PDR[16]).

Another important group of functional APFs is related to the cardiovascular system. Drug candidates that promote QT prolongation potentially associated with the much feared torsades de pointes, include the antiarrhythmics quinidine, disopyramide, procainamide, sotalol, dofetilide, and ibutilide as well as methadone, thioridazine, and haloperidol[3, 5–10, 206–208]. The relevance of QT prolongation found in preclinical safety tests needs to be established in specific clinical trials[7] and does per se not preclude further drug development. Drug candidates with positive inotropic activity increase heart rates in dogs, resulting over time in morphological alterations including myocardial necrosis[209, 210]. For more detail see second part of this review.

Adverse clinical chemistry findings in laboratory animals, such as moderate elevation of selected serum enzymes without significant morphological alterations, do not prevent progression of a drug candidate into clinical trials, as the corresponding parameter can be monitored in man. However, clinical chemistry investigations provide useful biomarkers, particularly to screen series of drug candidates before start-

ing development.

Morphologic effects: Morphologic toxicity[23–28] falls into one or several of the following basic pathological reaction patterns of biological systems.

Regressive alterations include in particular cytotoxicity which may progress to cell death. Almost any drug tested at high enough doses is cytotoxic. A sufficient safety ratio (a quantitative measure) is needed for continuing development, unless the MoA suggests that the APF is qualitatively not relevant to man. It depends upon the intended indication what constitutes a sufficient safety ratio (risk-benefit evaluation, see also Fig. 3 below).

Progressive alterations are hypertrophy, hyperplasia, and neoplasia. Hypertrophy and hyperplasia generally indicate adaptation to cope with increased workload e.g. following induction of metabolic enzymes especially in the liver, stimulation of target organs by hormones (see below), or glomerular[211] and renal tubular hypertrophy[212] following treatment with diuretics and other drugs. To assess the human relevance of a proliferative APF understanding of the responsible MoA is crucial, especially in case of potentially epigenetic tumors arising as a result of prolonged cellular stimulation in the absence of genotoxicity[27, 113, 213–216]. A sufficient safety ratio is needed, unless the MoA is clearly irrelevant to man. Human evidence to trace the emergence of proliferative changes in man can generally only be obtained by invasive techniques including biopsies and is impracticable.

Inflammation can result from different types of injury. For instance exacerbation of background infections needs to be taken into account when treating test animals with immunosuppressive agents or when toxicity decreases the well-being and therefore potentially the resistance against infections. Some drug candidates lead to inflammation of organs or organ systems by unknown MoA, e.g. PDE IV inhibitors to vasculitis, as discussed in more detail in the second part of this review. However, inflammatory foci can also be the consequence of toxic injury e.g. around hepatocellular or renal papillary necrosis. It is important to understand the pathogenesis of inflammation, which provides at least some understanding of the MoA. Safety ratio calculations can support risk evaluation.

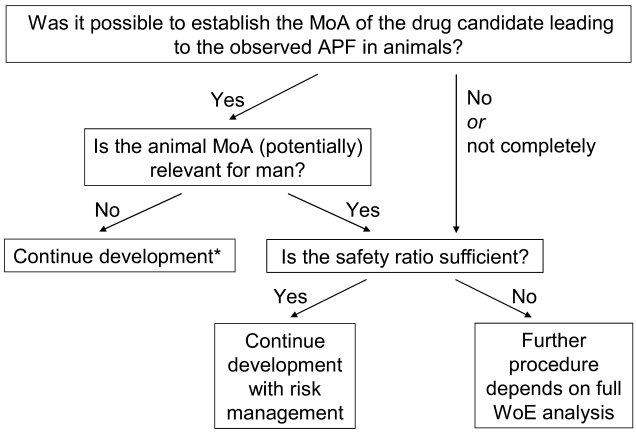
Metabolic changes manifest themselves often in form of storage of endogenous substances, e.g. fat, and may be the consequence of subtle toxicity. Storage diseases in the strict sense are e.g. phospholipidosis resulting from an impaired clearance of membranes (more detailed discussion in the second part of this review). Such changes may occasionally also be accompanied by functional impairment of the affected organ[217]. Accumulation of administered drugs or metabolites can sometimes be seen as pigment deposits in the respective organ, often without impairment of organ function[218].

Primary *circulatory and respiratory disturbances* are less frequent. The latter can occasionally be a consequence of phospholipidosis. Drug-induced arteritis in laboratory animals[219–221] is often without clinical dysfunction. Circulatory and respiratory disturbances including morphological changes can result from exaggerated pharmacological action of drug candidates as well: Particularly the aforementioned high sensitivity of dogs to drug candidates with positive inotropic activity is well-known[209, 210].

Malformations as toxicity endpoint are important in reproductive toxicity studies. Maternal toxicity needs to be taken into account to determine the relevance of potential developmental toxicity evidenced as fetal malformations and/or embryofetal toxicity. The pathogenesis of malformations is practically always unknown, unless due to cytotoxic drugs; however, for obvious reasons the latter need not to be tested in reproductive studies. Safety factors are of little help for the risk evaluation of potential teratogens: The drug candidate has to be labeled as potentially teratogen, if developmental toxicity is observed in the absence of maternal toxicity. Well controlled large human studies proving the absence of human teratogenicity can not be conducted for ethical reasons. Pharmaceuticals are classified according to their potential fetal risk according to animal and, where available, human data and the potential benefit of the drug for mothers. The categories range from "no fetal risk based on human data" to "proven human fetal risk outweighing the potential benefit for the pregnant mother". The definitions of these pregnancy categories differ between regulatory agencies[222–224]. Malformations will be discussed in more detail in the second part of this review.

Affected organelles: If unexpected morphological APFs are noted, EM investigations may be used to investigate early subcellular changes. EM investigations can also serve to select drug candidates with low potency for inducing a specific APF known to occur with a particular class of drugs, e.g. antidepressant drug candidates with a low potential for inducing phospholipidosis. Newer methods including gene expression and fluorescent microscopy can partly replace the more resource intensive EM investigations[225]. EM and other methods may support drug candidate selection and may help avoiding longer animal studies. However, fortuitously detected subcellular changes without functional consequences and/or progression to histopathological alterations are not relevant and therefore such sophisticated investigations are not needed in routine toxicity studies.

Not infrequently drug candidates have marked effects on specific cellular components. Best known is the effect of inducers of drug metabolizing enzymes associated with hyperplasia of the *smooth endoplasmic reticulum* (SER), the site of enzymes metabolizing xenobiotics, particularly in the liver. SER proliferation occurs in dose-dependent fashion and leads to cellular hypertrophy and hyperplasia with increased liver weight. It can be recognized histologically in H&E sections by a clearly eosinophilic cytoplasm[226]. In electromicrographs the proliferation of the SER is easily seen, also in formalin-fixed tissues from routine toxicity studies. SER hyperplasia and the associated phenomena are reversible upon cessation of treatment. However, in lifetime studies marked SER proliferation is generally associated with liver tumors in rodents because of sustained increased hepatocyte stimulation as explained above. Induction of



* Safety ratios may be calculated for providing additional evidence of safety of drug candidate in humans

Fig. 2. The main aspects of a simplified risk evaluation as part of the weight of evidence analysis (WoE, for details see text) for significant adverse preclinical findings (APFs) with drug candidates: Mode of action (MoA) assessment combined with calculation of safety ratios (NOAEL exposure in most sensitive and relevant animal species vs. maximal human therapeutic exposure). For the definition of a sufficient safety ratio see text.

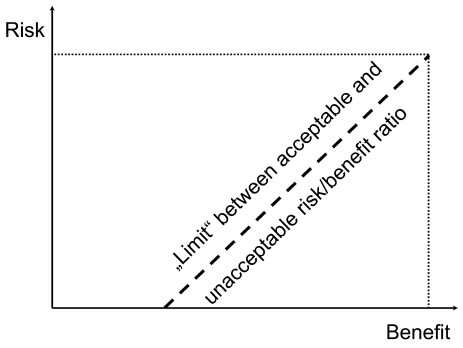


Fig. 3. Risk/benefit ratio. Greater benefit justifies higher risk.

metabolic enzymes occurs to a greater extent in rodents than in man and liver tumors due to SER proliferation are without much relevance to man[180, 213, 227]. However, enzyme induction may have significant consequences for the pharmacodynamics of drugs[228].

Lysosomes are particularly abundant in cells specialized in phagocytosis such as leukocytes and macrophages. If ingested material, often cellular membranes, can not be degraded, phagolysosomes accumulate in the cell or in the draining lymphatic organs, the spleen, the reticuloendothelial system of the liver or in other organs. This results in a storage-type disease[229, 230]. If the accumulation is mild, it can resolve[231]. Excessive accumulation of phagolysosomes can impair the physiological functioning of the cell and also lead to single cell necrosis. This type of storage disease is therefore of some concern to regulators and will be discussed in more detail in the second part of this review. Its significance to man needs to be evaluated on a case-by-case basis. A sufficient safety ratio is important.

Peroxisomes are particularly abundant in liver cells and are rich in peroxide reducing enzymes. Peroxisome proliferation is relatively easy to induce in rodent livers. At least one factor contributing to the resulting liver tumors is an increased production of hydrogen peroxides[232]. The older PPAR α agonists such as clofibrate were not considered to be carcinogenic in humans at therapeutic doses. However, today's PPAR agonists in development are much more potent than fibrates[233]. In rodent carcinogenicity studies PPAR α and dual α/γ agonists induce tumors at multiple sites, in multi-species and strains of both sexes. Though the known PPAR agonists are not genotoxic in the standard ICH genotoxicity battery, they have to be labeled as "probable human carcinogens" according to the USA Environmental Protection Agency (EPA) and the International Agency for Research on Cancer (IARC) criteria[234]. For a more detailed discussion of PPAR agonists see second part of this review.

In recent years *mitochondrial toxicity* has received some attention and is increasingly being implicated in drug-induced clinical idiosyncratic toxicity[235–243]. However, the study of chemical effects on mitochondrial respiration dates back many decades[244]. Mitochondrial toxicants are partly known to inhibit or uncouple oxidative phosphorylation, thus leading to oxidative stress and inhibition of DNA replication, transcription or translation. Mitochondrial alterations need to be assessed on a case-by-case basis.

Summary of the risk evaluation process

A decision chart for dealing with unexpected APFs is shown in Fig. 2.

The risk evaluation process includes both a qualitative and quantitative analysis: The *qualitative* evaluation answers the question if the observed APF can be relevant to man in principle. For this purpose, the establishment of the MoA is crucial. The *quantitative* analysis, that is the calculation of safety ratios, becomes important when the APF is not (entirely) test species-specific or when the MoA of the APF is not (entirely) clear.

Safety ratios in conjunction with other information have to be included into a complete WoE analysis. An important part of the latter is also an assessment of the risk-benefit ratio[245] as depicted in Fig. 3.

A greater benefit of drug treatment in case of a severe disease and/or missing therapeutic alternatives justifies a higher treatment risk. The limit between acceptable and unacceptable risk-benefit ratio is blurred. Therefore the package insert can only make the treating physician aware of the therapeutic risk. If the risk is considerable, the treating physician then has to make his/her own decision based on a case-by-case evaluation regarding the patient and the clinical conditions and then choose the optimal treatment solution under the given circumstances.

Additional evidence, if and to which extent an APF is relevant to man, often comes from translational medicine

with first-in-man clinical trials. The ultimate proof may only become available with long-term clinical follow-up studies and post-marketing drug monitoring, which is also addressed in the next chapter. However, clinical observations do generally not allow verifying in humans potential genotoxicity or teratogenicity observed in preclinical development: Genotoxic effects are generally too weak and cancer may, if at all, develop only after many years of exposure. Ethical barriers do not allow exposing pregnant women; if on rare occasions pregnant women were exposed to potentially teratogenic drugs, generally no final conclusions are possible.

4. Risk management

Risk management means to take precautions to minimize the risk for man[195], e.g. by

- More carefully monitoring patients with increased risk for adverse reactions e.g. because of chronic kidney disease in case of drugs mainly excreted by the kidney
- Excluding women in child bearing age in case of potentially teratogenic drugs, unless they are under contraceptive therapy and the expected benefit outweighs the potential risk
- Carefully selecting the initial doses for the first-in-man clinical trials[185]
- Escalating the dose in clinical studies with particular care
- Increasing clinical monitoring, e.g., serum and urinary biomarker measurements throughout treatment
- Adequately instructing health professionals
- Employing an effective post-marketing surveillance program[246]

Risk management is a task of the physicians responsible for clinical trials on behalf of the drug company and as actual trial leaders in hospitals. Once the drug is on the market, risk management is a task of the treating physicians in hospitals and in outpatient institutions. However, toxicologists and toxicologic pathologists are required to contribute their knowledge based on preclinical data of the drug, particularly for setting dose limitations and suggesting monitoring activities in early clinical trials, but also when it comes to increase doses in later human trials or when signs of potentially new toxicities (in the clinical environment often called "side-effects") are observed during the post-marketing phase.

Recently, the USA Food and Drug Administration (USA FDA) has published a draft guidance on risk evaluation and mitigation strategies (REMS)[247, 248]. This document requires e.g. a communication plan to health care providers, measures to assure safe use of the drug in question, an implementation plan for such measures, and a timetable for submission of follow-up assessments.

Conclusions

Drug development is complex and not infrequently the path to market is paved with obstacles. Toxicologists and toxicologic pathologists play an important role in drug development and have to contribute to the well-being of patients who will be treated with a new drug. They also have to avoid being overcautious and promoting premature termination of a potentially useful drug. Drug development is routine in many aspects, but becomes challenging when sound scientific judgment is needed. The toxicologic pathologist with training in medical sciences is well equipped to contribute significantly to hazard identification, hazard characterization, risk evaluation, and proposing measures for risk management. This paper has outlined possible processes to resolve unexpected APFs and has provided an overview over typical issues encountered, from functional toxicity to morphological toxicity, genotoxicity, carcinogenicity, and reproductive toxicity.

This first part of the review emphasizes that "troubleshooting" is a task which must be managed from within the company, though external experts might be able to contribute significantly to solutions. The clarification of the pathogenesis of an APF, that is the MoA of the drug candidate leading to this APF, is important in the process to evaluate the relevance of the finding to man. In addition, the calculation of safety ratios provides a quantitative measure for the potential risk to man particularly in early clinical trials. Identification of early markers of toxicity adds further safety to these studies in man. Additional tailor-made mechanistic studies need to be carefully planned, taking into account potential hypotheses regarding the MoA. The methods employed must be well established in the facility running investigative studies and historical control data must be available to assure that the results are interpretable. The conclusion of the risk evaluation process is an analysis of the WoE of the various parameters characterizing the APF, taking into account also the context of the drug use, medical need, and alternatives on the market.

In the second part of this review a number of APFs will be discussed in more detail, in particular with regard to their MoA and their relevance to man. Examples will cover toxicity, both of the functional and morphologic type, genotoxicity, tumorigenicity, and reproductive toxicity. The examples will also show that the MoA of drugs leading to APFs can sometimes not entirely be clarified, but that a well founded risk evaluation may still be possible.

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References

1. International Conference on Harmonization (ICH). ICH harmonized tripartite guideline. Safety pharmacology studies for human pharmaceuticals. S7A. 2000.
2. Kinter LB and Valentin JP. Safety pharmacology and risk assessment. Fundam Clin Pharmacol. **16**: 175–182. 2002.

3. Yap YG and Camm AJ. Drug induced QT prolongation and torsades de pointes. Heart. **89**: 1363–1372. 2003.
4. International Conference on Harmonization (ICH). ICH harmonized tripartite guideline. Nonclinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals. S7B. 2005.
5. De Bruin ML, Pettersson M, Meyboom RH, Hoes AW, and Leufkens HG. Anti-HERG activity and the risk of drug-induced arrhythmias and sudden death. Eur Heart J. **26**: 590–597. 2005.
6. Morganroth J and Gussak I. Cardiac Safety of Noncardiac Drugs: Practical Guidelines for Clinical Research and Drug Development. Humana Press, Totowa. 2005.
7. Ahmad K and Dorian P. Drug-induced QT prolongation and proarrhythmia: An inevitable link? Europace. **9 Suppl 4**: iv16–22. 2007.
8. Borer JS, Pouleur H, Abadie E, Follath F, Wittes J, Pfeffer MA, Pitt B, and Zannad F. Cardiovascular safety of drugs not intended for cardiovascular use: Need for a new conceptual basis for assessment and approval. Eur Heart J. **28**: 1904–1909. 2007.
9. Sager PT. Key clinical considerations for demonstrating the utility of preclinical models to predict clinical drug-induced torsades de pointes. Br J Pharmacol. **154**: 1544–1549. 2008.
10. Pugsley MK, Authier S, Towart R, Gallacher DJ, and Curtis MJ. Beyond the safety assessment of drug-mediated changes in the QT interval... what's next? J Pharmacol Toxicol Methods. **60**: 24–27. 2009.
11. Yabu K, Warty VS, Gorelik E, and Shinozuka H. Cyclosporine promotes the induction of thymic lymphomas in C57BL/6 mice initiated by a single dose of gamma-radiation. Carcinogenesis. **12**: 43–46. 1991.
12. Kuper CF, Harleman JH, Richter-Reichelm HB, and Vos JG. Histopathologic approaches to detect changes indicative of immunotoxicity. Toxicol Pathol. **28**: 454–466. 2000.
13. Penn I. Post-transplant malignancy: The role of immunosuppression. Drug Saf. **23**: 101–113. 2000.
14. Bustami RT, Ojo AO, Wolfe RA, Merion RM, Bennett WM, McDiarmid SV, Leichtman AB, Held PJ, and Port FK. Immunosuppression and the risk of post-transplant malignancy among cadaveric first kidney transplant recipients. Am J Transplant. **4**: 87–93. 2004.
15. Vos JG and Kuper CF. Chemically-induced immunopathology and immune functional changes. J Toxicol Pathol. **17**: 137–146. 2004.
16. Physicians' Desk Reference. Thomson PDR, 64th ed. Montvale. 2010.
17. Milman HA and Weisburger EK. Handbook of Carcinogen Testing, 2nd ed. Noyes Publications, Park Ridge NJ. 1994.
18. Thybaud V, Aardema M, Clements J, Dearfield K, Galloway S, Hayashi M, Jacobson-Kram D, Kirkland D, MacGregor JT, Marzin D, Ohyama W, Schuler M, Suzuki H, and Zeiger E. Strategy for genotoxicity testing: Hazard identification and risk assessment in relation to in vitro testing. Mutat Res. **627**: 41–58. 2007.
19. Ku WW, Bigger A, Brambilla G, Glatt H, Gocke E, Guzzie PJ, Hakura A, Honma M, Martus HJ, Obach RS, and Roberts S. Strategy for genotoxicity testing—metabolic considerations. Mutat Res. **627**: 59–77. 2007.
20. Lorge E, Gervais V, Becourt-Lhote N, Maisonneuve C, Delongeas JL, and Claude N. Genetic toxicity assessment: Employing the best science for human safety evaluation part IV: A strategy in genotoxicity testing in drug development: Some examples. Toxicol Sci. **98**: 39–42. 2007.
21. International Conference on Harmonization (ICH). ICH harmonized tripartite step 2 draft consensus guideline. Guidance on genotoxicity testing and data interpretation for parmaceuticals intended for human use. S2(R1). 2008.
22. Brambilla G, Mattioli F, Robbiano L, and Martelli A. Genotoxicity and carcinogenicity testing of pharmaceuticals: Correlations between induction of DNA lesions and carcinogenic activity. Mutat Res. **705**: 20–39. 2010.
23. Riddell RH. Pathology of Drug-Induced and Toxic Diseases. Churchill Livingstone, New York. 1982.
24. Glaister JR. Principles of Toxicological Pathology. Taylor & Francis, London, Philadelphia. 1986.
25. Mottet NK. Environmental Pathology. Oxford University Press, New York. 1985.
26. Gopinath C, Prentice DE, and Lewis DJ. Atlas of Experimental Toxicological Pathology. MTP Press, Lancaster, Boston. 1987.
27. Haschek WM, Rousseaux CG, and Wallig MA. Handbook of Toxicologic Pathology, 2nd ed. Academic Press, San Diego. 2002.
28. Ettlin RA and Prentice DE. Application of pathology in safety assessment. In: Preclinical Drug Development, 2nd ed. MC Rogge and DR Taft (eds). Informa Healthcare. 271–335. 2009.
29. International Conference on Harmonization (ICH). ICH harmonized tripartite guideline. Testing for carcinogenicity of pharmaceuticals. 1997.
30. Kitchin KT. Carcinogenicity: Testing, Predicting, and Interpreting Chemical Effects. M. Dekker, New York. 1999.
31. Ettlin RA and Prentice DE. Unexpected tumour findings in lifetime rodent bioassay studies—what to do? Toxicol Lett. **128**: 17–33. 2002.
32. Palmer AK. Incidence of Sporadic Malformations, Anomalies and Variations in Random Bred Laboratory Animals. Thieme, Stuttgart. 1977.
33. Brent RL. Utilization of animal studies to determine the effects and human risks of environmental toxicants (drugs, chemicals, and physical agents). Pediatrics. **113**: 984–995. 2004.
34. Bremer S, Balduzzi D, Cortvrindt R, Daston G, Eletti B, Galli A, Huhtaniemi I, Laws S, Lazzari G, Liminga U, Smitz J, Spano M, Themmen A, Tilloy A, and Waalkens-Behrends I. The effects of chemicals on mammalian fertility. The report and recommendations of ECVAM Workshop 53—the first strategic workshop of the EU ReProTect Project. Altern Lab Anim. **33**: 391–416. 2005.
35. Bremer S, Brittebo E, Dencker L, Knudsen LE, Mathisien L, Olovsson M, Pazos P, Pellizzer C, Paulesu LR, Schaefer W, Schwarz M, Staud F, Stavreus-Evers A, and Vahankangas K. In vitro tests for detecting chemicals affecting the embryo implantation process. The report and recommendations of ECVAM workshop 62—a strategic workshop of the EU ReProTect project. Altern Lab Anim. **35**: 421–439. 2007.
36. Brent RL and Fawcett LB. Developmental toxicology, drugs, and fetal teratogenicity. In: Clinical Obstetrics: The Fetus & Mother, 3rd ed. EA Reece and JC Hobbins (eds). Blackwell Pub, Malden. 217–235. 2007.
37. Williams GM. Safety assessment of pharmaceuticals: Examples of inadequate assessments and a mechanistic

approach to assuring adequate assessment. Toxicol Pathol. **25**: 32–38. 1997.
38. Pray LA. Understanding the Benefits and Risks of Pharmaceuticals: Workshop Summary. National Academies Press, Washington. 2007.
39. Li AP. Accurate prediction of human drug toxicity: A major challenge in drug development. Chem Biol Interact. **150**: 3–7. 2004.
40. Fung M, Thornton A, Mybeck K, Wu H-HJ, Hornbuckle K, and Edumundo M. Evaluation of the characteristics of safety withdrawal of prescription drugs from worldwide pharmaceutical markets-1960 to 1999. Drug Info J. **35**: 293–317. 2001.
41. Lasser KE, Allen PD, Woolhandler SJ, Himmelstein DU, Wolfe SM, and Bor DH. Timing of new black box warnings and withdrawals for prescription medications. JAMA. **287**: 2215–2220. 2002.
42. Lexchin J. Drug withdrawals from the Canadian market for safety reasons, 1963–2004. CMAJ. **172**: 765–767. 2005.
43. Schuster D, Laggner C, and Langer T. Why drugs fail—a study on side effects in new chemical entities. Curr Pharm Des. **11**: 3545–3559. 2005.
44. Wysowski DK and Swartz L. Adverse drug event surveillance and drug withdrawals in the United States, 1969–2002: The importance of reporting suspected reactions. Arch Intern Med. **165**: 1363–1369. 2005.
45. Copley MP. Environmental Protection Agency risk assessment—process and toxicologic pathology. Toxicol Pathol. **25**: 68–71. 1997.
46. Kleinjans JC. Principles in toxicological risk analysis. Toxicol Lett. **140–141**: 311–315. 2003.
47. Cohen SM, Klaunig J, Meek ME, Hill RN, Pastoor T, Lehman-McKeeman L, Bucher J, Longfellow DG, Seed J, Dellarco V, Fenner-Crisp P, and Patton D. Evaluating the human relevance of chemically induced animal tumors. Toxicol Sci. **78**: 181–186. 2004.
48. Hasegawa R, Koizumi M, and Hirose A. Principles of risk assessment for determining the safety of chemicals: Recent assessment of residual solvents in drugs and di(2-ethylhexyl) phthalate. Congenit Anom (Kyoto). **44**: 51–59. 2004.
49. Hood A, Allen ML, Liu Y, Liu J, and Klaassen CD. Induction of T(4) UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers. Toxicol Appl Pharmacol. **188**: 6–13. 2003.
50. McClain RM. Mechanistic considerations for the relevance of animal data on thyroid neoplasia to human risk assessment. Mutat Res. **333**: 131–142. 1995.
51. Kimber I and Dearman RJ. Immune responses: Adverse versus non-adverse effects. Toxicol Pathol. **30**: 54–58. 2002.
52. Williams GM and Iatropoulos MJ. Alteration of liver cell function and proliferation: Differentiation between adaptation and toxicity. Toxicol Pathol. **30**: 41–53. 2002.
53. Luna LG. Histopathologic Methods and Color Atlas of Special Stains and Tissue Artifacts. American Histolabs, Gaitheresburg. 1992.
54. Butterworth BE, Popp JA, Conolly RB, and Goldsworthy TL. Chemically induced cell proliferation in carcinogenesis. IARC Sci Publ. 279–305. 1992.
55. Kirkland DJ, Henderson L, Marzin D, Muller L, Parry JM, Speit G, Tweats DJ, and Williams GM. Testing strategies in mutagenicity and genetic toxicology: An appraisal of the guidelines of the European Scientific Committee for Cosmetics and Non-Food Products for the evaluation of hair dyes. Mutat Res. **588**: 88–105. 2005.
56. International Conference on Harmonization (ICH). ICH harmonized tripartite guideline. Dose selection for carcinogenicity dtudies of pharmaceuticals for human use. S1C(R2). 2008.
57. Perez J and Perentes E. Light-induced retinopathy in the albino rat in long-term studies. An immunohistochemical and quantitative approach. Exp Toxicol Pathol. **46**: 229–235. 1994.
58. Peter CP, Burek JD, and van Zwieten MJ. Spontaneous nephropathies in rats. Toxicol Pathol. **14**: 91–100. 1986.
59. Robertson JL. Spontaneous renal disease in dogs. Toxicol Pathol. **14**: 101–108. 1986.
60. Holland T. A survey of discriminant methods used in toxicological histopathology. Toxicol Pathol. **29**: 269–273. 2001.
61. Karbe E, Williams GM, Lewis RW, Kimber I, and Foster PM. Distinguishing between adverse and non-adverse effects. Session summary. Exp Toxicol Pathol. **54**: 51–55. 2002.
62. Suwa T, Nyska A, Haseman JK, Mahler JF, and Maronpot RR. Spontaneous lesions in control B6C3F1 mice and recommended sectioning of male accessory sex organs. Toxicol Pathol. **30**: 228–234. 2002.
63. Clemo FA, Evering WE, Snyder PW, and Albassam MA. Differentiating spontaneous from drug-induced vascular injury in the dog. Toxicol Pathol. **31 Suppl**: 25–31. 2003.
64. Leininger JR, McDonald MM, and Abbott DP. Hepatocytes in the mouse stomach. Toxicol Pathol. **18**: 678–686. 1990.
65. Iwata H, Hosoi M, Miyajima R, Yamamoto S, Mikami S, Yamakawa S, Enomoto M, Imazawa T, and Mitsumori K. Morphogenesis of craniopharyngeal derivatives in the neurohypophysis of Fisher 344 rats: Abnormally developed epithelial tissues including parotid glands derived from the stomatodeum. Toxicol Pathol. **28**: 568–574. 2000.
66. Takeuchi Y, Yoshida T, Chiba Y, Kuwahara M, Maita K, and Harada T. Pulmonary sequestration with ectopic pancreatic tissue in a Wistar Hannover GALAS rat. Toxicol Pathol. **30**: 288–291. 2002.
67. Goedken MJ, Kerlin RL, and Morton D. Spontaneous and age-related testicular findings in beagle dogs. Toxicol Pathol. **36**: 465–471. 2008.
68. Lilbert J and Burnett R. Main vascular changes seen in the saline controls of continuous infusion studies in the cynomolgus monkey over an eight-year period. Toxicol Pathol. **31**: 273–280. 2003.
69. Yanoff M, Zimmerman LE, and Davis RL. Massive gliosis of the retina. Int Ophthalmol Clin. **11**: 211–229. 1971.
70. Eiben R. Frequency and time trends of spontaneous tumors found in B6C3F1 mice oncogenicity studies over 10 years. Exp Toxicol Pathol. **53**: 399–408. 2001.
71. Morawietz G and Rittinghausen S. Variations in prevalence of endocrine tumors among different colonies of rats? A retrospective study in the Hannover registry data base. Arch Toxicol Suppl. **15**: 205–214. 1992.
72. Deschl U, Kittel B, Rittinghausen S, Morawietz G, Kohler M, Mohr U, and Keenan C. The value of historical control data-scientific advantages for pathologists, industry and agencies. Toxicol Pathol. **30**: 80–87. 2002.
73. Keenan C, Hughes-Earle A, Case M, Stuart B, Lake S,

Mahrt C, Halliwell W, Westhouse R, Elweee M, Morton D, Morawietz G, Rittinghausen S, Deschl U, and Mohr U. The north american control animal database: A resource based on standardized nomenclature and diagnostic criteria. Toxicol Pathol. **30**: 75–79. 2002.
74. Karbe E and Kerlin RL. Cystic degeneration/Spongiosis hepatis in rats. Toxicol Pathol. **30**: 216–227. 2002.
75. Long G. Perspectives on pathology peer review. Toxicol Pathol. **24**: 645–646. 1996.
76. McKay JS, Barale-Thomas E, Bolon B, George C, Hardisty J, Manabe S, Schorsch F, Teranishi M, and Weber K. A commentary on the process of peer review and pathology data locking. Toxicol Pathol. **38**: 508–510. 2010.
77. Hayashi M, Hashimoto S, Sakamoto Y, Hamada C, Sofuni T, and Yoshimura I. Statistical analysis of data in mutagenicity assays: Rodent micronucleus assay. Environ Health Perspect. **102 Suppl 1**: 49–52. 1994.
78. McConnell EE, Solleveld HA, Swenberg JA, and Boorman GA. Guidelines for combining neoplasms for evaluation of rodent carcinogenesis studies. J Natl Cancer Inst. **76**: 283–289. 1986.
79. Mohseni S. Hypoglycemic neuropathy. Acta Neuropathol. **102**: 413–421. 2001.
80. Proceedings of the International Workshop on Clinical Pathology Testing in Preclinical Safety Assessment. Washington DC, July 27, 1991. Toxicol Pathol. **20**: 469–543. 1992.
81. Weingand K, Brown G, Hall R, Davies D, Gossett K, Neptun D, Waner T, Matsuzawa T, Salemink P, Froelke W, Provost JP, Dal Negro G, Batchelor J, Nomura M, Groetsch H, Boink A, Kimball J, Woodman D, York M, Fabianson-Johnson E, Lupart M, and Melloni E. Harmonization of animal clinical pathology testing in toxicity and safety studies. The Joint Scientific Committee for International Harmonization of Clinical Pathology Testing. Fundam Appl Toxicol. **29**: 198–201. 1996.
82. Carr GJ and Maurer JK. Precision of organ and body weight data: Additional perspective. Toxicol Pathol. **26**: 321–323. 1998.
83. Fleck C and Engelbert K. The hepato-renal syndrome: Renal amino acid transport in bile duct ligated rats (DL)—influence of treatment with triiodothyronine or dexamethasone on renal amino acid handling in amino acid loaded rats. Exp Toxicol Pathol. **50**: 356–364. 1998.
84. Bannasch P, Haertel T, and Su Q. Significance of hepatic preneoplasia in risk identification and early detection of neoplasia. Toxicol Pathol. **31**: 134–139. 2003.
85. Su Q and Bannasch P. Relevance of hepatic preneoplasia for human hepatocarcinogenesis. Toxicol Pathol. **31**: 126–133. 2003.
86. Tsuda H, Fukushima S, Wanibuchi H, Morimura K, Nakae D, Imaida K, Tatematsu M, Hirose M, Wakabayashi K, and Moore MA. Value of GST-P positive preneoplastic hepatic foci in dose-response studies of hepatocarcinogenesis: Evidence for practical thresholds with both genotoxic and nongenotoxic carcinogens. A review of recent work. Toxicol Pathol. **31**: 80–86. 2003.
87. Ruehl-Fehlert C, Kittel B, Morawietz G, Deslex P, Keenan C, Mahrt CR, Nolte T, Robinson M, Stuart BP, and Deschl U. Revised guides for organ sampling and trimming in rats and mice - part 1. Exp Toxicol Pathol. **55**: 91–106. 2003.
88. Kittel B, Ruehl-Fehlert C, Morawietz G, Klapwijk J, Elwell MR, Lenz B, O'Sullivan MG, Roth DR, and Wadsworth PF. Revised guides for organ sampling and trimming in rats and mice—Part 2. A joint publication of the RITA and NACAD groups. Exp Toxicol Pathol. **55**: 413–431. 2004.
89. Morawietz G, Ruehl-Fehlert C, Kittel B, Bube A, Keane K, Halm S, Heuser A, and Hellmann J. Revised guides for organ sampling and trimming in rats and mice—Part 3. A joint publication of the RITA and NACAD groups. Exp Toxicol Pathol. **55**: 433–449. 2004.
90. Melnick RL, Thayer KA, and Bucher JR. Conflicting views on chemical carcinogenesis arising from the design and evaluation of rodent carcinogenicity studies. Environ Health Perspect. **116**: 130–135. 2008.
91. Hamolsky MW. The role of plasma transport proteins in hormone homeostasis, with particular reference to thyroid hormone. Surg Clin North Am. **49**: 541–546. 1969.
92. Alison RH, Capen CC, and Prentice DE. Neoplastic lesions of questionable significance to humans. Toxicol Pathol. **22**: 179–186. 1994.
93. Richardson I, Turkalji I, and Flueckiger E. Bromocriptine. In: Safety Testing of New Drugs: Laboratory Predictions and Clinical Performance. DR Laurence, AEM McLean, and M Weatherall (eds). Academic Press, London, Orlando. 19–63. 1984.
94. Prentice DE and Meikle AW. A review of drug-induced Leydig cell hyperplasia and neoplasia in the rat and some comparisons with man. Hum Exp Toxicol. **14**: 562–572. 1995.
95. Rittinghausen S, Kaspareit J, and Mohr U. Incidence and spectrum of spontaneous neoplasms in Han: NMRI mice of both sexes. Exp Toxicol Pathol. **49**: 347–349. 1997.
96. Kaspareit J and Rittinghausen S. Spontaneous neoplastic lesions in Harlan Sprague-Dawley rats. Exp Toxicol Pathol. **51**: 105–107. 1999.
97. Tillmann T, Kamino K, and Mohr U. Incidence and spectrum of spontaneous neoplasms in male and female CBA/J mice. Exp Toxicol Pathol. **52**: 221–225. 2000.
98. Gold LS, Manley NB, Slone TH, and Ward JM. Compendium of chemical carcinogens by target organ: Results of chronic bioassays in rats, mice, hamsters, dogs, and monkeys. Toxicol Pathol. **29**: 639–652. 2001.
99. Kamino K, Tillmann T, and Mohr U. Spectrum and age-related incidence of spontaneous tumours in a colony of Han: AURA hamsters. Exp Toxicol Pathol. **52**: 539–544. 2001.
100. Nakazawa M, Tawaratani T, Uchimoto H, Kawaminami A, Ueda M, Ueda A, Shinoda Y, Iwakura K, Kura K, and Sumi N. Spontaneous neoplastic lesions in aged Sprague-Dawley rats. Exp Anim. **50**: 99–103. 2001.
101. Green T. Species differences in carcinogenicity: The role of metabolism and pharmacokinetics in risk assessment. Ann Ist Super Sanita. **27**: 595–599. 1991.
102. Lin JH. Species similarities and differences in pharmacokinetics. Drug Metab Dispos. **23**: 1008–1021. 1995.
103. Ulrich AB, Standop J, Schmied BM, Schneider MB, Lawson TA, and Pour PM. Species differences in the distribution of drug-metabolizing enzymes in the pancreas. Toxicol Pathol. **30**: 247–253. 2002.
104. Deshmukh R and Blomme EA. A dog is not a rat: Importance of understanding species differences in drug absorption, distribution, metabolism and excretion (ADME).

Vet J. **179**: 8–9. 2009.
105. Owen RA and Heywood R. Strain-related susceptibility to nephrotoxicity induced by aspirin and phenylbutazone in rats. Toxicol Pathol. **14**: 242–246. 1986.
106. Solleveld HA and Boorman GA. Spontaneous renal lesions in five rat strains. Toxicol Pathol. **14**: 168–174. 1986.
107. Wolff GL. Variability in gene expression and tumor formation within genetically homogeneous animal populations in bioassays. Fundam Appl Toxicol. **29**: 176–184. 1996.
108. Kacew S, Ruben Z, and McConnell RF. Strain as a determinant factor in the differential responsiveness of rats to chemicals. Toxicol Pathol. **23**: 701–714. 1995.
109. Harvell DM, Strecker TE, Tochacek M, Xie B, Pennington KL, McComb RD, Roy SK, and Shull JD. Rat strain-specific actions of 17beta-estradiol in the mammary gland: Correlation between estrogen-induced lobuloalveolar hyperplasia and susceptibility to estrogen-induced mammary cancers. Proc Natl Acad Sci USA. **97**: 2779–2784. 2000.
110. Son WC, Kamino K, Lee YS, and Kang KS. Strain-specific mammary proliferative lesion development following lifetime oral administration of ochratoxin A in DA and Lewis rats. Int J Cancer. **105**: 305–311. 2003.
111. Aupperlee MD, Drolet AA, Durairaj S, Wang W, Schwartz RC, and Haslam SZ. Strain-specific differences in the mechanisms of progesterone regulation of murine mammary gland development. Endocrinology. **150**: 1485–1494. 2009.
112. Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, Lilly P, Sanders J, Sipes G, Bracken W, Dorato M, Van Deun K, Smith P, Berger B, and Heller A. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol. **32**: 56–67. 2000.
113. Cohen SM. Human relevance of animal carcinogenicity studies. Regul Toxicol Pharmacol. **21**: 75–80; discussion 81–76. 1995.
114. Hardisty JF. Factors influencing laboratory animal spontaneous tumor profiles. Toxicol Pathol. **13**: 95–104. 1985.
115. Vater ST, McGinnis PM, Schoeny RS, and Velazquez SF. Biological considerations for combining carcinogenicity data for quantitative risk assessment. Regul Toxicol Pharmacol. **18**: 403–418. 1993.
116. Maurer JK, Cheng MC, Boysen BG, Squire RA, Strandberg JD, Weisbrode SE, Seymour JL, and Anderson RL. Confounded carcinogenicity study of sodium fluoride in CD-1 mice. Regul Toxicol Pharmacol. **18**: 154–168. 1993.
117. Lerche NW and Osborn KG. Simian retrovirus infections: Potential confounding variables in primate toxicology studies. Toxicol Pathol. **31 Suppl**: 103–110. 2003.
118. Owen RA and Heywood R. Age-related variations in renal structure and function in Sprague-Dawley rats. Toxicol Pathol. **14**: 158–167. 1986.
119. Davis BJ, Travlos G, and McShane T. Reproductive endocrinology and toxicological pathology over the life span of the female rodent. Toxicol Pathol. **29**: 77–83. 2001.
120. Ettlin RA, Junker U, and Prentice DE. Dopamine agonists. In: Classic Examples in Toxicologic Pathology, 3rd ed. E Karbe, W Drommer, PG Germann, G Morawietz, and R Kellner (eds). European Society of Toxicologic Pathology, Hannover. CD-ROM. 2009.
121. Anderson T, Khan NK, Tassinari MS, and Hurtt ME. Comparative juvenile safety testing of new therapeutic candidates: Relevance of laboratory animal data to children. J Toxicol Sci. **34 Suppl 2**: SP209–215. 2009.
122. Rao GN. Light intensity-associated eye lesions of Fischer 344 rats in long-term studies. Toxicol Pathol. **19**: 148–155. 1991.
123. Christian MS, Hoberman AM, Johnson MD, Brown WR, and Bucci TJ. Effect of dietary optimization on growth, survival, tumor incidences and clinical pathology parameters in CD Sprague-Dawley and Fischer-344 rats: A 104-week study. Drug Chem Toxicol. **21**: 97–117. 1998.
124. Proceedings of the Symposium on the Role of Diet and Caloric Intake in Aging, Obesity, and Cancer. Reston VA, October 26–28, 1998. Toxicol Sci. **52**: 1–146. 1999.
125. Nold JB, Keenan KP, Nyska A, and Cartwright ME. Society of toxicologic pathology position paper: Diet as a variable in rodent toxicology and carcinogenicity studies. Toxicol Pathol. **29**: 585–586. 2001.
126. Rao GN, Morris RW, and Seely JC. Beneficial effects of NTP-2000 diet on growth, survival, and kidney and heart diseases of Fischer 344 rats in chronic studies. Toxicol Sci. **63**: 245–255. 2001.
127. Rao GN. Diet and kidney diseases in rats. Toxicol Pathol. **30**: 651–656. 2002.
128. Rao GN and Crockett PW. Effect of diet and housing on growth, body weight, survival and tumor incidences of B6C3F1 mice in chronic studies. Toxicol Pathol. **31**: 243–250. 2003.
129. Keenan KP, Laroque P, Ballam GC, Soper KA, Dixit R, Mattson BA, Adams SP, and Coleman JB. The effects of diet, ad libitum overfeeding, and moderate dietary restriction on the rodent bioassay: The uncontrolled variable in safety assessment. Toxicol Pathol. **24**: 757–768. 1996.
130. Molon-Noblot S, Laroque P, Coleman JB, Hoe CM, and Keenan KP. The effects of ad libitum overfeeding and moderate and marked dietary restriction on age-related spontaneous pituitary gland pathology in Sprague-Dawley rats. Toxicol Pathol. **31**: 310–320. 2003.
131. Roe FJ, Lee PN, Conybeare G, Kelly D, Matter B, Prentice D, and Tobin G. The Biosure Study: Influence of composition of diet and food consumption on longevity, degenerative diseases and neoplasia in Wistar rats studied for up to 30 months post weaning. Food Chem Toxicol. **33 Suppl 1**: 1S–100S. 1995.
132. Keenan KP, Coleman JB, McCoy CL, Hoe CM, Soper KA, and Laroque P. Chronic nephropathy in ad libitum overfed Sprague-Dawley rats and its early attenuation by increasing degrees of dietary (caloric) restriction to control growth. Toxicol Pathol. **28**: 788–798. 2000.
133. Bercu JP, Hoffman WP, Lee C, and Ness DK. Quantitative assessment of cumulative carcinogenic risk for multiple genotoxic impurities in a new drug substance. Regul Toxicol Pharmacol. **51**: 270–277. 2008.
134. Dorato MA and Engelhardt JA. The no-observed-adverse-effect-level in drug safety evaluations: Use, issues, and definition(s). Regul Toxicol Pharmacol. **42**: 265–274. 2005.
135. Butterworth BE, Conolly RB, and Morgan KT. A strategy for establishing mode of action of chemical carcinogens as a guide for approaches to risk assessments. Cancer Lett. **93**: 129–146. 1995.
136. Qureshi SR, Perentes E, Ettlin RA, Kolopp M, Prentice DE, and Frankfurter A. Morphologic and immunohistochemical characterization of Leydig cell tumor variants in Wistar rats.

Toxicol Pathol. **19**: 280–286. 1991.
137. Dietrich DR. Toxicological and pathological applications of proliferating cell nuclear antigen (PCNA), a novel endogenous marker for cell proliferation. Crit Rev Toxicol. **23**: 77–109. 1993.
138. Kellner R. RITA CEPA: Cell proliferation and apoptosis. 2010, from RITA CEPA website: http://reni.item.fraunhofer.de/reni/public/cepa
139. Lardelli P, Perentes E, Meier G, Navarro N, and Ettlin RA. Quantification of hepatocytic proliferation in the laboratory mouse. A comparative study using immunohistochemical detection of bromodeoxyuridine (BrdU) incorporation and proliferating cell nuclear antigen (PCNA) expression. Exp Toxicol Pathol. **46**: 95–100. 1994.
140. Goldsworthy TL, Morgan KT, Popp JA, and Butterworth BE. Guidelines for measuring chemically induced cell proliferation in specific rodent target organs. Prog Clin Biol Res. **369**: 253–284. 1991.
141. Schulte-Hermann R, Bursch W, Grasl-Kraupp B, Torok L, Ellinger A, and Mullauer L. Role of active cell death (apoptosis) in multi-stage carcinogenesis. Toxicol Lett. **82-83**: 143–148. 1995.
142. Schulte-Hermann R, Bursch W, and Grasl-Kraupp B. Active cell death (apoptosis) in liver biology and disease. Prog Liver Dis. **13**: 1–35. 1995.
143. Schulte-Hermann R, Bursch W, Marian B, and Grasl-Kraupp B. Active cell death (apoptosis) and cellular proliferation as indicators of exposure to carcinogens. IARC Sci Publ. 273–285. 1999.
144. Ettlin RA, Qureshi SR, Perentes E, Christen H, Gschwind R, Buser MW, and Oberholzer M. Morphological, immunohistochemical, stereological and nuclear shape characteristics of proliferative Leydig cell alterations in rats. Pathol Res Pract. **188**: 643–648. 1992.
145. Ettlin RA, Perentes E, Kolopp M, Lardelli P, Arnold J, Karamitopoulou E, and Oberholzer M. Overview of quantitative methods in toxicologic pathology. In Vivo. **7**: 315–324. 1993.
146. Kanai K, Watanabe J, Fujimoto S, and Kanamura S. Quantitative analysis of smooth endoplasmic reticulum proliferation in periportal, midzonal and perivenular hepatocytes of mice after administration of phenobarbital. Exp Toxicol Pathol. **45**: 199–203. 1993.
147. Mandalunis P, Gibaja F, and Ubios AM. Experimental renal failure and iron overload: A histomorphometric study in the alveolar bone of rats. Exp Toxicol Pathol. **54**: 85–90. 2002.
148. Wanke R. Stereology—benefits and pitfalls. Exp Toxicol Pathol. **54**: 163–164. 2002.
149. Mandalunis P and Ubios A. Experimental renal failure and iron overload: A histomorphometric study in rat tibia. Toxicol Pathol. **33**: 398–403. 2005.
150. Dykstra MJ, Mann PC, Elwell MR, and Ching SV. Suggested standard operating procedures (SOPs) for the preparation of electron microscopy samples for toxicology/pathology studies in a GLP environment. Toxicol Pathol. **30**: 735–743. 2002.
151. Gillett NA and Chan C. Applications of immunohistochemistry in the evaluation of immunosuppressive agents. Hum Exp Toxicol. **19**: 251–254. 2000.
152. Burchiel SW, Lauer FT, Gurule D, Mounho BJ, and Salas VM. Uses and future applications of flow cytometry in immunotoxicity testing. Methods. **19**: 28–35. 1999.
153. Roman D, Greiner B, Ibrahim M, Pralet D, and Germann PG. Laser technologies in toxicopathology. Toxicol Pathol. **30**: 11–14. 2002.
154. Peterson RA, Krull DL, and Butler L. Applications of laser scanning cytometry in immunohistochemistry and routine histopathology. Toxicol Pathol. **36**: 117–132. 2008.
155. Eltoum IA, Siegal GP, and Frost AR. Microdissection of histologic sections: Past, present, and future. Adv Anat Pathol. **9**: 316–322. 2002.
156. Boorman GA, Anderson SP, Casey WM, Brown RH, Crosby LM, Gottschalk K, Easton M, Ni H, and Morgan KT. Toxicogenomics, drug discovery, and the pathologist. Toxicol Pathol. **30**: 15–27. 2002.
157. Brown HR, Ni H, Benavides G, Yoon L, Hyder K, Giridhar J, Gardner G, Tyler RD, and Morgan KT. Correlation of simultaneous differential gene expression in the blood and heart with known mechanisms of adriamycin-induced cardiomyopathy in the rat. Toxicol Pathol. **30**: 452–469. 2002.
158. Orphanides G. Toxicogenomics: Challenges and opportunities. Toxicol Lett. **140–141**: 145–148. 2003.
159. Yim SH, Ward JM, Dragan Y, Yamada A, Scacheri PC, Kimura S, and Gonzalez FJ. Microarray analysis using amplified mRNA from laser capture microdissection of microscopic hepatocellular precancerous lesions and frozen hepatocellular carcinomas reveals unique and consistent gene expression profiles. Toxicol Pathol. **31**: 295–303. 2003.
160. Blomme EA, Yang Y, and Waring JF. Use of toxicogenomics to understand mechanisms of drug-induced hepatotoxicity during drug discovery and development. Toxicol Lett. **186**: 22–31. 2009.
161. Amacher DE, Adler R, Herath A, and Townsend RR. Use of proteomic methods to identify serum biomarkers associated with rat liver toxicity or hypertrophy. Clin Chem. **51**: 1796–1803. 2005.
162. Kilty CG, Keenan J, and Shaw M. Histologically defined biomarkers in toxicology. Expert Opin Drug Saf. **6**: 207–215. 2007.
163. USA FDA. Guidance for industry. Safety testing of drug metabolites. 2008.
164. International Conference on Harmonization (ICH). ICH harmonized tripartite guideline. Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. M3(R2). 2009.
165. Hockings PD, Roberts T, Campbell SP, Reid DG, Greenhill RW, Polley SR, Nelson P, Bertram TA, and Kramer K. Longitudinal magnetic resonance imaging quantitation of rat liver regeneration after partial hepatectomy. Toxicol Pathol. **30**: 606–610. 2002.
166. Cohen SM and Arnold LL. Cell proliferation and carcinogenesis. J Toxicol Pathol. **21**: 1–7. 2008.
167. Grasso P, Sharratt M, and Cohen AJ. Role of persistent, non-genotoxic tissue damage in rodent cancer and relevance to humans. Annu Rev Pharmacol Toxicol. **31**: 253–287. 1991.
168. Cohen SM. Role of cell proliferation in regenerative and neoplastic disease. Toxicol Lett. **82–83**: 15–21. 1995.
169. Cohen SM. Comparative pathology of proliferative lesions of the urinary bladder. Toxicol Pathol. **30**: 663–671. 2002.
170. Ward JM, Uno H, Kurata Y, Weghorst CM, and Jang JJ. Cell proliferation not associated with carcinogenesis in rodents and humans. Environ Health Perspect. **101 Suppl 5**: 125–135. 1993.

171. Cohen SM and Lawson TA. Rodent bladder tumors do not always predict for humans. Cancer Lett. **93**: 9–16. 1995.
172. Whysner J and Williams GM. Saccharin mechanistic data and risk assessment: Urine composition, enhanced cell proliferation, and tumor promotion. Pharmacol Ther. **71**: 225–252. 1996.
173. Tillmann T, Kamino K, Dasenbrock C, Ernst H, Kohler M, Morawietz G, Campo E, Cardesa A, Tomatis L, and Mohr U. Subcutaneous soft tissue tumours at the site of implanted microchips in mice. Exp Toxicol Pathol. **49**: 197–200. 1997.
174. Blanchard KT, Barthel C, French JE, Holden HE, Moretz R, Pack FD, Tennant RW, and Stoll RE. Transponder-induced sarcoma in the heterozygous p53+/– mouse. Toxicol Pathol. **27**: 519–527. 1999.
175. Seely JC, Haseman JK, Nyska A, Wolf DC, Everitt JI, and Hailey JR. The effect of chronic progressive nephropathy on the incidence of renal tubule cell neoplasms in control male F344 rats. Toxicol Pathol. **30**: 681–686. 2002.
176. Lock EA and Smith LL. The role of mode of action studies in extrapolating to human risks in toxicology. Toxicol Lett. **140–141**: 317–322. 2003.
177. Meek ME, Bucher JR, Cohen SM, Dellarco V, Hill RN, Lehman–McKeeman LD, Longfellow DG, Pastoor T, Seed J, and Patton DE. A framework for human relevance analysis of information on carcinogenic modes of action. Crit Rev Toxicol. **33**: 591–653. 2003.
178. Dellarco VL and Baetcke K. A risk assessment perspective: Application of mode of action and human relevance frameworks to the analysis of rodent tumor data. Toxicol Sci. **86**: 1–3. 2005.
179. Seed J, Carney EW, Corley RA, Crofton KM, DeSesso JM, Foster PM, Kavlock R, Kimmel G, Klaunig J, Meek ME, Preston RJ, Slikker W Jr., Tabacova S, Williams GM, Wiltse J, Zoeller RT, Fenner-Crisp P, and Patton DE. Overview: Using mode of action and life stage information to evaluate the human relevance of animal toxicity data. Crit Rev Toxicol. **35**: 664–672. 2005.
180. Holsapple MP, Pitot HC, Cohen SM, Boobis AR, Klaunig JE, Pastoor T, Dellarco VL, and Dragan YP. Mode of action in relevance of rodent liver tumors to human cancer risk. Toxicol Sci. **89**: 51–56. 2006.
181. The International Programme on Chemical Safety (ICPS). IPCS Mode of Action Framework. World Health Organization. Geneva, Switzerland. 2008.
182. Proctor DM, Gatto NM, Hong SJ, and Allamneni KP. Mode-of-action framework for evaluating the relevance of rodent forestomach tumors in cancer risk assessment. Toxicol Sci. **98**: 313–326. 2007.
183. Peters JM. Mechanistic evaluation of PPAR{alpha}-mediated hepatocarcinogenesis: Are we there yet? Toxicol. Sci. **101**: 1–3. 2008.
184. Julien E, Boobis AR, and Olin SS. The Key Events Dose-Response Framework: A cross-disciplinary mode-of-action based approach to examining dose-response and thresholds. Crit Rev Food Sci Nutr. **49**: 682–689. 2009.
185. USA FDA. Guidance for industry. Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. 2005.
186. EMA. Draft. Guideline on requirements for first-in-man clinical trials for potentially high risk medicinal products. 2007.
187. Waddell WJ. Thresholds in chemical carcinogenesis: What are animal experiments telling us? Toxicol Pathol. **31**: 260–262. 2003.
188. Fukushima S, Wanibuchi H, Morimura K, Iwai S, Nakae D, Kishida H, Tsuda H, Uehara N, Imaida K, Shirai T, Tatematsu M, Tsukamoto T, Hirose M, and Furukawa F. Existence of a threshold for induction of aberrant crypt foci in the rat colon with low doses of 2-amino-1-methyl-6-phenolimidazo[4,5-b]pyridine. Toxicol Sci. **80**: 109–114. 2004.
189. Asano N, Torous DK, Tometsko CR, Dertinger SD, Morita T, and Hayashi M. Practical threshold for micronucleated reticulocyte induction observed for low doses of mitomycin C, Ara-C and colchicine. Mutagenesis. **21**: 15–20. 2006.
190. Humfrey CD. Recent developments in the risk assessment of potentially genotoxic impurities in pharmaceutical drug substances. Toxicol Sci. **100**: 24–28. 2007.
191. Greim H. Mechanistic and toxicokinetic data reducing uncertainty in risk assessment. Toxicol Lett. **138**: 1–8. 2003.
192. EMA. Guideline on the limits of genotoxic impurities. 2006.
193. Guzelian PS, Victoroff MS, Halmes NC, James RC, and Guzelian CP. Evidence-based toxicology: A comprehensive framework for causation. Hum Exp Toxicol. **24**: 161–201. 2005.
194. Hoffmann S and Hartung T. Toward an evidence-based toxicology. Hum Exp Toxicol. **25**: 497–513. 2006.
195. USA FDA. Guidance for industry. Premarketing risk assessment. 2005.
196. Weed DL. Weight of evidence: A review of concept and methods. Risk Anal. **25**: 1545–1557. 2005.
197. Boobis AR, Cohen SM, Dellarco V, McGregor D, Meek ME, Vickers C, Willcocks D, and Farland W. IPCS framework for analyzing the relevance of a cancer mode of action for humans. Crit Rev Toxicol. **36**: 781–792. 2006.
198. Boobis AR, Doe JE, Heinrich-Hirsch B, Meek ME, Munn S, Ruchirawat M, Schlatter J, Seed J, and Vickers C. IPCS framework for analyzing the relevance of a noncancer mode of action for humans. Crit Rev Toxicol. **38**: 87–96. 2008.
199. Davies TS and Monro A. The rodent carcinogenicity bioassay produces a similar frequency of tumor increases and decreases: Implications for risk assessment. Regul Toxicol Pharmacol. **20**: 281–301. 1994.
200. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. VJ Cogliano (ed). WHO IARC, Lyon. 1987–2008.
201. Torok G, Csik M, Pinter A, and Surjan A. Carcinogenicity of medicinal drugs: A survey of IARC monographs. Cent Eur J Public Health. **8 Suppl**: 83–85. 2000.
202. Claude JR and Claude N. Safety pharmacology: An essential interface of pharmacology and toxicology in the non-clinical assessment of new pharmaceuticals. Toxicol Lett. **151**: 25–28. 2004.
203. Auer RN. Hypoglycemic brain damage. Metab Brain Dis. **19**: 169–175. 2004.
204. Gazit V, Ben-Abraham R, Coleman R, Weizman A, and Katz Y. Cysteine-induced hypoglycemic brain damage: An alternative mechanism to excitotoxicity. Amino Acids. **26**: 163–168. 2004.
205. Fujikawa DG. Prolonged seizures and cellular injury: Understanding the connection. Epilepsy Behav. **7 Suppl 3**: S3–11. 2005.
206. The Academy of Medical Sciences. Safer Medicines. A report from the Academy's Forum with Industry. London.

2005.
207. Guth BD. Preclinical cardiovascular risk assessment in modern drug development. Toxicol Sci. **97**: 4–20. 2007.
208. Drew BJ, Ackerman MJ, Funk M, Gibler WB, Kligfield P, Menon V, Philippides GJ, Roden DM, and Zareba W. Prevention of torsade de pointes in hospital settings. A scientific statement from the American Heart Association and the American College of Cardiology Foundation. Circulation. **121**: 1047–1060. 2010.
209. Sandusky GE, Means JR, and Todd GC. Comparative cardiovascular toxicity in dogs given inotropic agents by continuous intravenous infusion. Toxicol Pathol. **18**: 268–278. 1990.
210. Dogterom P, Zbinden G, and Reznik GK. Cardiotoxicity of vasodilators and positive inotropic/vasodilating drugs in dogs: An overview. Crit Rev Toxicol. **22**: 203–241. 1992.
211. Lane PH. Long-term furosemide treatment in the normal rat: Dissociation of glomerular hypertrophy and glomerulosclerosis. Am J Kidney Dis. **33**: 1058–1063. 1999.
212. Kobayashi S, Clemmons DR, Nogami H, Roy AK, and Venkatachalam MA. Tubular hypertrophy due to work load induced by furosemide is associated with increases of IGF-1 and IGFBP-1. Kidney Int. **47**: 818–828. 1995.
213. Williams GM. Epigenetic mechanisms of liver tumor promotion. Prog Clin Biol Res. **331**: 131–145; discussion 138–147. 1990.
214. Melnick RL, Kohn MC, and Portier CJ. Implications for risk assessment of suggested nongenotoxic mechanisms of chemical carcinogenesis. Environ Health Perspect. **104 Suppl 1**: 123–134. 1996.
215. Silva Lima B and Van der Laan JW. Mechanisms of nongenotoxic carcinogenesis and assessment of the human hazard. Regul Toxicol Pharmacol. **32**: 135–143. 2000.
216. Roberts RA, Goodman JI, Shertzer HG, Dalton TP, and Farland WH. Rodent toxicity and nongenotoxic carcinogenesis: Knowledge-based human risk assessment based on molecular mechanisms. Toxicol Mech Methods. **13**: 21–29. 2003.
217. Bredehorn T, Clausen M, Duncker G, and Lullmann-Rauch R. Morphological and functional changes due to drug-induced lysosomal storage of sulphated glycosaminoglycans in the rat retina. Graefes Arch Clin Exp Ophthalmol. **239**: 788–793. 2001.
218. Gordon G, Sparano BM, Kramer AW, Kelly RG, and Iatropoulos MJ. Thyroid gland pigmentation and minocycline therapy. Am J Pathol. **117**: 98–109. 1984.
219. Isaacs KR, Joseph EC, and Betton GR. Coronary vascular lesions in dogs treated with phosphodiesterase III inhibitors. Toxicol Pathol. **17**: 153–163. 1989.
220. Kerns WD, Arena E, Macia RA, Bugelski PJ, Matthews WD, and Morgan DG. Pathogenesis of arterial lesions induced by dopaminergic compounds in the rat. Toxicol Pathol. **17**: 203–213. 1989.
221. Kerns WD, Arena E, and Morgan DG. Role of dopaminergic and adrenergic receptors in the pathogenesis of arterial lesions induced by fenoldopam mesylate and dopamine in the rat. Am J Pathol. **135**: 339–349. 1989.
222. Australian Therapeutic Goods Administration (TGA). Prescribing medicines in pregnancy. An Australian categorisation of risk of drug use in pregnancy. 1999. Last amendment: 2007.
223. Meadows M. Pregnancy and the drug dilemma. FDA Consum. **35**: 16–20. 2001.
224. USA FDA. CFR—Code of Federal Regulations Title 21, Part 201 - Labeling - Subpart B - Labeling requirements for prescription drugs and/or Insulin. Sec. 201.57 Specific requirements on content and format of labeling for human prescription drug and biological products described in 201.56(b)(1). 2009. From USA FDA website: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=201.57
225. Nioi P, Perry BK, Wang EJ, Gu YZ, and Snyder RD. In vitro detection of drug-induced phospholipidosis using gene expression and fluorescent phospholipid based methodologies. Toxicol Sci. **99**: 162–173. 2007.
226. Amacher DE, Schomaker SJ, Boldt SE, and Mirsky M. The relationship among microsomal enzyme induction, liver weight, and histological change in cynomolgus monkey toxicology studies. Food Chem Toxicol. **44**: 528–537. 2006.
227. Mirsalis JC and Steinmetz KL. The role of hyperplasia in liver carcinogenesis. Prog Clin Biol Res. **331**: 149–161. 1990.
228. Worboys PD and Carlile DJ. Implications and consequences of enzyme induction on preclinical and clinical drug development. Xenobiotica. **31**: 539–556. 2001.
229. Nyska A, Nold JB, Johnson JD, and Abdo K. Lysosomal-storage disorder induced by elmiron following 90-days gavage administration in rats and mice. Toxicol Pathol. **30**: 178–187. 2002.
230. Anderson N and Borlak J. Drug-induced phospholipidosis. FEBS Lett. **580**: 5533–5540. 2006.
231. Chatman LA, Morton D, Johnson TO, and Anway SD. A strategy for risk management of drug-induced phospholipidosis. Toxicol Pathol. **37**: 997–1005. 2009.
232. Reddy JK and Qureshi SA. Tumorigenicity of the hypolipidaemic peroxisome proliferator ethyl-alpha-p-chlorophenoxyisobutyrate (clofibrate) in rats. Br J Cancer. **40**: 476–482. 1979.
233. El Hage J. Peroxisome proliferation-activated receptor agonists: Carcinogenicity findings and regulatory recommendations. International Atherosclerosis Society Symposium on PPAR. Monte Carlo. 2005.
234. El Hage J. Preclinical and clinical safety assessments for PPAR agonists. DIA meeting, Washington DC. 2004.
235. Amacher DE. Drug-associated mitochondrial toxicity and its detection. Curr Med Chem. **12**: 1829–1839. 2005.
236. Forner F, Foster LJ, Campanaro S, Valle G, and Mann M. Quantitative proteomic comparison of rat mitochondria from muscle, heart, and liver. Mol Cell Proteomics. **5**: 608–619. 2006.
237. Dykens JA and Will Y. The significance of mitochondrial toxicity testing in drug development. Drug Discov Today. **12**: 777–785. 2007.
238. Nadanaciva S, Dykens JA, Bernal A, Capaldi RA, and Will Y. Mitochondrial impairment by PPAR agonists and statins identified via immunocaptured OXPHOS complex activities and respiration. Toxicol Appl Pharmacol. **223**: 277–287. 2007.
239. Dykens JA, Jamieson J, Marroquin L, Nadanaciva S, Billis PA, and Will Y. Biguanide-induced mitochondrial dysfunction yields increased lactate production and cytotoxicity of aerobically-poised HepG2 cells and human hepatocytes in vitro. Toxicol Appl Pharmacol. **233**: 203–210. 2008.

240. Dykens JA, Jamieson JD, Marroquin LD, Nadanaciva S, Xu JJ, Dunn MC, Smith AR, and Will Y. In vitro assessment of mitochondrial dysfunction and cytotoxicity of nefazodone, trazodone, and buspirone. Toxicol Sci. **103**: 335–345. 2008.
241. Nadanaciva S and Will Y. The role of mitochondrial dysfunction and drug safety. Drugs. **12**: 706–710. 2009.
242. Russmann S, Kullak-Ublick GA, and Grattagliano I. Current concepts of mechanisms in drug-induced hepatotoxicity. Curr Med Chem. **16**: 3041–3053. 2009.
243. Vickers AE. Characterization of hepatic mitochondrial injury induced by fatty acid oxidation inhibitors. Toxicol Pathol. **37**: 78–88. 2009.
244. Bachmann E and Zbinden G. Effect of antidepressant and neuroleptic drugs on respiratory function of rat heart mitochondria. Biochem Pharmacol. **28**: 3519–3524. 1979.
245. EMA. Reflection paper on benefit risk assessment methods in the context of the evaluation of marketing authorisation applications of medîcinal products for humane use. 2008, from EMA website: http://www.ema.europa.eu/pdfs/human/brmethods/1540407enfin.pdf
246. USA FDA. Guidance for industry. Good pharmacovigilance practices and pharmacoepidemiologic assessment. 2005.
247. USA FDA. Guidance for industry. Format and content of proposed risk evaluation and mitigation strategies (REMS), REMS assessments, and proposed REMS modifications. Draft guidance. 2009.
248. American Pharmacists Association. White paper on designing a risk evaluation and mitigation strategies (REMS) system to optimize the balance of patient access, medication safety, and impact on the health care system. J Am Pharm Assoc (2003). **49**: 729–743. 2009.
249. Joseph EC. Arterial lesions induced by phosphodiesterase III (PDE III) inhibitors and DA(1) agonists. Toxicol Lett. **112–113**: 537–546. 2000.
250. Zhang J, Herman EH, Knapton A, Chadwick DP, Whitehurst VE, Koerner JE, Papoian T, Ferrans VJ, and Sistare FD. SK&F 95654-induced acute cardiovascular toxicity in Sprague-Dawley rats—histopathologic, electron microscopic, and immunohistochemical studies. Toxicol Pathol. **30**: 28–40. 2002.
251. Weaver JL, Snyder R, Knapton A, Herman EH, Honchel R, Miller T, Espandiari P, Smith R, Gu YZ, Goodsaid FM, Rosenblum IY, Sistare FD, Zhang J, and Hanig J. Biomarkers in peripheral blood associated with vascular injury in Sprague-Dawley rats treated with the phosphodiesterase IV inhibitors SCH 351591 or SCH 534385. Toxicol Pathol. **36**: 840–849. 2008.
252. Sobry C and George C. PDE4 inhibitors I: Vasculopathies. In: Classic Examples in Toxicologic Pathology, 3rd ed. E Karbe, W Drommer, PG Germann, G Morawietz, and R Kellner (eds). European Society of Toxicologic Pathology, Hannover. CD-ROM. 2009.
253. Jones HB, Macpherson A, Betton GR, Davis AS, Siddall R, and Greaves P. Endothelin antagonist-induced coronary and systemic arteritis in the beagle dog. Toxicol Pathol. **31**: 263–272. 2003.
254. Proceedings of the Toxicology Forum, Aspen CO, July 9–11, 1987. Arteritis and arterial drug toxicity in the safety assessment of drugs. Toxicol Pathol. **17**: 65–231. 1989.
255. Proceedings of the Ninth International Symposium of the Society of Toxicologic Pathologists, Ottawa ON, Canada, June 24–28, 1990. Toxicologic Pathology of the Cardiovascular System. Toxicol Pathol. **18**: 437–635. 1990.
256. Robert J. Preclinical assessment of anthracycline cardiotoxicity in laboratory animals: Predictiveness and pitfalls. Cell Biol Toxicol. **23**: 27–37. 2007.
257. Greaves P. Patterns of drug-induced cardiovascular pathology in the beagle dog: Relevance for humans. Exp Toxicol Pathol. **50**: 283–293. 1998.
258. Hodel C. Myopathy and rhabdomyolysis with lipid-lowering drugs. Toxicol Lett. **128**: 159–168. 2002.
259. Lemarchand TX, Newton RK, and Searfoss GHI. Peroxisome proliferators II: Muscle lesions. In: Classic Examples in Toxicologic Pathology, 3rd ed. E Karbe, W Drommer, PG Germann, G Morawietz, and R Kellner (eds). European Society of Toxicologic Pathology, Hannover. CD-ROM. 2009.
260. Hottendorf GH and Williams PD. Aminoglycoside nephrotoxicity. Toxicol Pathol. **14**: 66–72. 1986.
261. Reinhard MK, Hottendorf GH, and Powell ED. Differences in the sensitivity of Fischer and Sprague-Dawley rats to aminoglycoside nephrotoxicity. Toxicol Pathol. **19**: 66–71. 1991.
262. Proceedings of the Fourth International Symposium of the Society of Toxicologic Pathologists, Washington DC, June 5–7, 1985. Part 1. Renal Pathology and Toxicity. Toxicol Pathol. **14**: 11–128. 1986.
263. Proceedings of the Fourth International Symposium of the Society of Toxicologic Pathologists, Washington DC, June 5–7, 1985. Part 2. Renal Pathology and Toxicity. Toxicol Pathol. **14**: 158–262. 1986.
264. Proceedings of the Sixteenth International Symposium of the Society of Toxicologic Pathologists, Beaver Creek CO, June 22–26, 1997. Toxicologic Pathology of the Kidney. Toxicol Pathol. **26**: 1–176. 1998.
265. Ryffel B, Weber E, and Mihatsch MJ. Nephrotoxicity of immunosuppressants in rats: Comparison of macrolides with cyclosporin. Exp Nephrol. **2**: 324–333. 1994.
266. Su Q, Weber L, Le Hir M, Zenke G, and Ryffel B. Nephrotoxicity of cyclosporin A and FK506: Inhibition of calcineurin phosphatase. Ren Physiol Biochem. **18**: 128–139. 1995.
267. Black HE. Renal toxicity of non-steroidal anti-inflammatory drugs. Toxicol Pathol. **14**: 83–90. 1986.
268. Bach PH and Nguyen TK. Renal papillary necrosis—40 years on. Toxicol Pathol. **26**: 73–91. 1998.
269. Proceedings of the Eighth International Symposium of the Society of Toxicologic Pathologists, Cincinnati OH, May 21–25, 1989. A Modern Approach to Toxicologic Pathology of the Nervous System. Toxicol Pathol. **18**: 81–223. 1990.
270. Proceedings of the Eighteenth International Symposium of the Society of Toxicologic Pathologists, Washington DC, June 13–17, 1999. Toxicologic Pathology of the Nervous System. Toxicol Pathol. **28**: 3–214. 2000.
271. Whysner J, Ross PM, and Williams GM. Phenobarbital mechanistic data and risk assessment: Enzyme induction, enhanced cell proliferation, and tumor promotion. Pharmacol Ther. **71**: 153–191. 1996.
272. Ashby J, Brady A, Elcombe CR, Elliott BM, Ishmael J, Odum J, Tugwood JD, Kettle S, and Purchase IF. Mechanistically-based human hazard assessment of peroxisome proliferator-induced hepatocarcinogenesis. Hum Exp Toxicol. **13 Suppl 2**: S1–117. 1994.
273. Lake BG. Mechanisms of hepatocarcinogenicity of

peroxisome-proliferating drugs and chemicals. Annu Rev Pharmacol Toxicol. **35**: 483–507. 1995.
274. Cattley RC, DeLuca J, Elcombe C, Fenner-Crisp P, Lake BG, Marsman DS, Pastoor TA, Popp JA, Robinson DE, Schwetz B, Tugwood J, and Wahli W. Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans? Regul Toxicol Pharmacol. **27**: 47–60. 1998.
275. Black HE. The effects of steroids upon the gastrointestinal tract. Toxicol Pathol. **16**: 213–222. 1988.
276. Rainsford KD. Gastrointestinal damage from nonsteroidal anti-inflammatory drugs. Toxicol Pathol. **16**: 251–259. 1988.
277. Proceedings of the Seventh International Symposium of the Society of Toxicologic Pathologists, Bosten MA, June 5–8, 1988. Endocrine Toxicologic Pathology. Toxicol Pathol. **17**: 233–456. 1989.
278. Capen CC. Overview of structural and functional lesions in endocrine organs of animals. Toxicol Pathol. **29**: 8–33. 2001.
279. Ettlin RA and Dixon RL. Reproductive toxicity. In: Environmental Pathology. NK Mottet (ed). Oxford University Press, New York. 129–180. 1985.
280. Russel LD, Ettlin RA, Hikim APS, and Clegg ED. Histological and Histopathological Evaluation of the Testis. Cache River Press, Clearwater. 1990.
281. Creasy DM. Pathogenesis of male reproductive toxicity. Toxicol Pathol. **29**: 64–76. 2001.
282. Foley GL. Overview of male reproductive pathology. Toxicol Pathol. **29**: 49–63. 2001.
283. Nishikawa S. Effects of sulfonamide on the pituitary-thyroid gland. 1. Morphological changes of thyroid gland and variation in plasma thyroxine and triiodothyronine. J Toxicol Sci. **8**: 47–59. 1983.
284. Nishikawa S. Effects of sulfonamide on the pituitary-thyroid gland. 2. Morphological changes of thyrotrophs in anterior pituitary gland. J Toxicol Sci. **8**: 61–70. 1983.
285. Vahle JL, Sato M, Long GG, Young JK, Francis PC, Engelhardt JA, Westmore MS, Linda Y, and Nold JB. Skeletal changes in rats given daily subcutaneous injections of recombinant human parathyroid hormone (1–34) for 2 years and relevance to human safety. Toxicol Pathol. **30**: 312–321. 2002.
286. Proceedings of the Elventh International Symposium of the Society of Toxicologic Pathologists, Phoenix AZ, May 31–June 5, 1992. Toxicologic Pathology of the Hematopoietic System. Toxicol Pathol. **21**: 113–257. 1993.
287. Nakae D, Onodera H, Fueki O, Urano T, Komiyama N, Sagami F, Kai S, Nishimura C, and Inoue T. Points to consider on the non-clinical safety evaluation of anticancer drugs. J Toxicol Sci. **33**: 123–126. 2008.
288. Double J, Barrass N, Barnard ND, and Navaratnam V. Toxicity testing in the development of anticancer drugs. Lancet Oncol. **3**: 438–442. 2002.
289. Lullmann-Rauch R. Drug-induced lysosomal storage disorders. Front Biol. **48**: 49–130. 1979.
290. Berg A-L and Ciaccio P. Compounds causing phospholipidosis. In: Classic Examples in Toxicologic Pathology, 3rd ed. E Karbe, W Drommer, PG Germann, G Morawietz, and R Kellner (eds). European Society of Toxicologic Pathology, Hannover. CD-ROM. 2009.
291. Klaunig JE, Kamendulis LM, and Xu Y. Epigenetic mechanisms of chemical carcinogenesis. Hum Exp Toxicol. **19**: 543–555. 2000.
292. Swenberg JA. Alpha 2u-globulin nephropathy: Review of the cellular and molecular mechanisms involved and their implications for human risk assessment. Environ Health Perspect. **101 Suppl 6**: 39–44. 1993.
293. Cohen SM, Ohnishi T, Clark NM, He J, and Arnold LL. Investigations of rodent urinary bladder carcinogens: Collection, processing, and evaluation of urine and bladders. Toxicol Pathol. **35**: 337–347. 2007.
294. McClain RM. Thyroid gland neoplasia: Non-genotoxic mechanisms. Toxicol Lett. **64–65**: 397–408. 1992.
295. Klaassen CD and Hood AM. Effects of microsomal enzyme inducers on thyroid follicular cell proliferation and thyroid hormone metabolism. Toxicol Pathol. **29**: 34–40. 2001.
296. Hardisty JF, Elwell MR, Ernst H, Greaves P, Kolenda-Roberts H, Malarkey DE, Mann PC, and Tellier PA. Histopathology of hemangiosarcomas in mice and hamsters and liposarcomas/fibrosarcomas in rats associated with PPAR agonists. Toxicol Pathol. **35**: 928–941. 2007.
297. Cohen SM, Storer RD, Criswell KA, Doerrer NG, Dellarco VL, Pegg DG, Wojcinski ZW, Malarkey DE, Jacobs AC, Klaunig JE, Swenberg JA, and Cook JC. Hemangiosarcoma in rodents: Mode-of-action evaluation and human relevance. Toxicol Sci. **111**: 4–18. 2009.
298. Betton GR, Dormer CS, Wells T, Pert P, Price CA, and Buckley P. Gastric ECL-cell hyperplasia and carcinoids in rodents following chronic administration of H2-antagonists SK&F 93479 and oxmetidine and omeprazole. Toxicol Pathol. **16**: 288–298. 1988.
299. Hirth RS, Evans LD, Buroker RA, and Oleson FB. Gastric enterochromaffin-like cell hyperplasia and neoplasia in the rat: An indirect effect of the histamine H2-receptor antagonist, BL-6341. Toxicol Pathol. **16**: 273–287. 1988.
300. Larsson H, Hakanson R, Mattsson H, Ryberg B, Sundler F, and Carlsson E. Omeprazole: Its influence on gastric acid secretion, gastrin and ECL cells. Toxicol Pathol. **16**: 267–272. 1988.
301. LePard KJ, Mohammed JR, and Stephens RL Jr. Gastric ECL-cell hyperplasia produces enhanced basal and stimulated gastric acid output but not gastric erosion formation in the rat. Gen Pharmacol. **28**: 415–420. 1997.
302. Havu N. Enterochromaffin-like cell carcinoids of gastric mucosa in rats after life-long inhibition of gastric secretion. Digestion. **35 Suppl 1**: 42–55. 1986.
303. Sanduleanu S, De Bruine A, Stridsberg M, Jonkers D, Biemond I, Hameeteman W, Lundqvist G, and Stockbrugger RW. Serum chromogranin A as a screening test for gastric enterochromaffin-like cell hyperplasia during acid-suppressive therapy. Eur J Clin Invest. **31**: 802–811. 2001.
304. Waldum HL, Brenna E, and Sandvik AK. Long-term safety of proton pump inhibitors: Risks of gastric neoplasia and infections. Expert Opin Drug Saf. **1**: 29–38. 2002.
305. Ali T, Roberts DN, and Tierney WM. Long-term safety concerns with proton pump inhibitors. Am J Med. **122**: 896–903. 2009.
306. Swenberg JA, Dietrich DR, McClain RM, and Cohen SM. Species-specific mechanisms of carcinogenesis. IARC Sci Publ. 477–500. 1992.
307. Colbert WE, Wilson BF, Williams PD, and Williams GD. Relationship between in vitro relaxation of the costo-uterine smooth muscle and mesovarial leiomyoma formation in vivo by beta-receptor agonists. Arch Toxicol. **65**: 575–579. 1991.