Chapter 10
Nonclinical Strategies for Investigating Potential Tumor Signals Detected in Clinical Trials

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Abstract Potential signals of human carcinogenicity may arise in the course of clinical development or post marketing experience for a drug having shown a lack of evidence of a carcinogenic risk in nonclinical studies. It is always possible that, given the small numbers of patients in clinical trials, such signals may be due to chance or, for example, ascertainment bias; however, any signal of potential treatment-related malignancy must be evaluated and possible avenues of clinical and nonclinical investigation assessed. Investigations to characterize these signals should be considered thoughtfully, on a case-by-case basis, and grounded in scientific rationale. Given the relatively short time course of clinical development, tumor events are unlikely to have arisen de novo during the trial. Thus, potential mechanisms of tumor promotion and progression may also need to be considered. In this chapter, some nonclinical models to study tumor promotion and progression are discussed, and case studies are presented to illustrate various courses of follow-up investigations. Development and validation of innovative models for assessing tumor promotion and progression that are more human-based warrant further scientific investigation.

Keywords **N**onclinical models • Carcinogenicity • Clinical tumor • Tumor progression • Tumor promotion

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10.1 Introduction

Assessment of potential carcinogenic risk to humans is an important component of safety assessment in drug development. Such assessments are multi-factorial and include: considerations of "on- and off-" target pharmacology; the ability of a drug to interact directly or indirectly with genetic material (in vitro and in vivo assays of genotoxicity); cellular or tissue toxicity that might presage the evolution of a carcinogenic effect (e.g., chronic inflammatory states, hyperplastic or dysplastic alterations, immunosuppression); and tumorigenic effects in traditional lifetime and/or alternative short-term carcinogenicity bioassays. Though long-term safety is evaluated in people during clinical trials and in post-market settings, carcinogenicity studies, per se, are not conducted in human subjects. Rather, lifetime bioassays in rodents are often employed as the "gold standard" to assess the potential of a compound to be carcinogenic. The lifetime nature of these studies allows for realization of all the stages of tumor development: initiation, promotion, and progression [52]. However, there may be translational issues in rodent bioassays models: analysis of study outcomes show that ~50 % of molecules tested are tumorigenic in lifetime rodent bioassays [21], though relatively few of the compounds (e.g., 20 % for pharmaceutical drugs) that are carcinogenic in rodents have been confirmed as human carcinogens [1]. Still, results from the lifetime studies in rodents have been shown to identify virtually all known human carcinogens [22].

In general, scientists and physicians take comfort in the belief that drugs which have shown no genotoxic or carcinogenic effects in nonclinical studies imply a low risk of carcinogenic risk in humans. However, potential signals of carcinogenicity may arise in the course of clinical development or post marketing experience for a drug having shown no potential for carcinogenic risk in nonclinical studies. This chapter will describe how potential clinical signals of carcinogenicity are evaluated and what nonclinical and clinical courses of investigation (additional to traditional genotoxicity and rodent carcinogenicity assays) might be considered to further understand and characterize possible human risk. Finally, case studies illustrative of potential human carcinogenicity signals in the (relative) absence of nonclinical signals will be discussed.

10.2 Detection and Investigation of Potential Clinical Signals of Cancer

Clinical trials are carefully designed to test whether a treatment is effective for a given disease state. However, even well-designed studies (such as a randomized, double-blind, placebo-controlled study) will have limitations with regard to controlling for all possible types of bias and the ability to detect a "true" rare event. Statistical power – the ability to detect a treatment- emergent event of a particular size or larger – will be limited by the number of patients in the trial. Small numbers

of patients can lead to false signals due to chance or bias. "Detection" or "ascertainment" bias occurs when an event is more likely to be observed for a particular set of study subjects (e.g., prasugrel; see Sect. 10.3 below). For example, women taking an oral contraceptive will have more frequent cervical smears than women who are not on the pill and so are more likely to have any existing cervical cancer diagnosed. Not all potential signals raised in initial trials with limited numbers of patients are confirmed in larger trials or in post-marketing studies (e.g., orlistat; see Sect. 10.3 below) or with more extensive experience (e.g., angiotensin II AT-1 receptor blockers; [27]), while some potential signals are not detected until significant human post-marketing experience has accumulated (e.g., pioglitazone; see Sect. 10.3 below).

Monitoring for and interpreting adverse events in Phase 3 clinical trials is guided by what is known or reasonably anticipated about the therapeutic target and class, the biologic mechanism of action, previous (Phase 1 or 2) clinical experience, and the totality of the nonclinical safety assessment package. In the absence of evidence that a treatment may be associated with cancer – e.g., (depending on the circumstances of the product's development) previous experience with a related pharmacologic class having a known or suspected potential risk of human carcinogenicity and nonclinical signals – standard adverse event reporting would be performed in clinical trials. There would be no prospectively defined parameters for monitoring tumor events, and detection of a possible signal in the clinic would be unexpected. A report of cancer is a rare event in clinical trials and one for which there is a high degree of sensitivity. Minor numerical imbalances between control and drug treatment groups may represent a potential cause for concern. When one arm of a clinical trial suggests a possible increase in "all types" or a particular subtype of cancer, investigation of the potential for an identifiable cancer risk should be further evaluated.

10.2.1 Clinical Investigation

As mentioned, clinical investigators should be prepared to seek relevant clinical information for cancer events. There are many factors that might relate to carcinogenic risk including the baseline characteristics of the patient population (e.g., age, co-morbidities, smoking status, pre-existing conditions). As previously noted, while study groups are randomized for many baseline characteristics, one cannot account for every possible parameter; potential areas of bias need to be thoroughly investigated to rule out false positive signals. Link et al. [27] described the case of angiotensin II AT-1 receptor blockers (ARBs) in which a positive signal detected in a meta-analysis of five clinical trials was not recapitulated in a subsequent, much larger meta-analysis (31 clinical trials) which found no evidence of any excess site-specific cancer. (The larger meta-analysis results were supported by negative results in 19 mostly lifetime rodent bioassays for cancer when viewed en masse by FDA.)

10.2.2 Potential Relationship Between Pharmacologic Mechanisms and Tumorigenicity

Any assessment of carcinogenic potential begins with careful consideration of mechanism of action of an agent to hypothesize theoretical risks. There may be causes for concern for some pharmacological classes whether or not there is a signal in the nonclinical studies. An in-depth knowledge of the target biology will inform possible mechanisms by which a molecule may exert a carcinogenic effect. Such considerations form the basis for hypothesis generation that might be further tested in nonclinical studies.

There are several potential mechanisms of carcinogenesis. Genotoxic (DNA-reactive) agents interact directly and covalently with DNA to produce procarcinogenic mutations which lead to neoplastic transformation. Such agents are generally eliminated from consideration early in drug development. Epigenetic mechanisms of carcinogenesis (not associated with direct effects on the DNA sequence) can be quite complex in nature and may involve sustained adaptive effects and/or disruption of endocrine, paracrine, nervous, and immune systems; it is these types of pathways that are typically of interest for drugs in development [40].

The accumulated experience with rodent carcinogenicity studies makes evident that several pharmacological classes are associated with the development of rodent tumors at specific organs or tissues. Underlying mechanisms of rodent tumorigenicity may include the persistent stimulation of the involved target or of target-associated cell cascades or suppression of defense mechanisms against tumor cells. The potential relevance of these mechanisms for humans needs to be thoroughly understood in terms of whether tumorigenesis derives from the pharmacologic mode of action (e.g., is "target-related") and under which conditions (e.g., threshold doses, treatment schedules, exposure duration). The human relevance of animal data is dependent on multiple factors, including species specificities in: disposition and metabolic pathways; receptor levels, isoforms, polymorphisms; cross-talk between signal transduction pathways. Other sources of inter-species variability include regulatory pathways of cell repair, proliferation and death, and differences in patterns of cell/tissue adaptation or potentiation of responses during chronic exposures to chemicals. Differences in stem cell populations which are targets for neoplastic transformation across species also may influence receptor-mediated carcinogenic responses [2]. Therefore, extensive knowledge on the primary and secondary targets for any active substance, the associated cellular mechanisms, and their similarities and differences between rodents and humans is fundamental for understanding possible human risk posed by any rodent tumorigen. When the pharmacological mechanism responsible for the tumorigenesis in rodents is considered of possible/plausible relevance to humans, a potential concern with the pharmacological class may emerge even in the absence of positive results in rodents for some member(s) of such class.

Examples of rodent tumorigenesis triggered by certain pharmacological classes of drugs, several of which have been shown to have potential relevance to humans, are described below.

10.2.2.1 Receptor-Mediated Tumorigenesis

The pharmacologic activity of many drugs is mediated through binding to cellular receptors, which may result in proliferative responses at target tissues, and which, in turn, has been hypothesized to be associated with tumorigenesis. Hormones or hormone analogues may constitute the active substance of a medicinal product directly interacting with the respective receptor, or hormones may be indirectly modulated by an active substance. Agents acting at the parathyriod hormone, calcitonin, and dopamine receptors may be associated with a hormonally-mediated mechanism of tumorigenesis.

Calcitonin (CT) is a hormone produced by the C cells of the thyroid and participates in the regulation of blood calcium levels and calcium mobilization from the bone. Mainly, CT acts by decreasing calcium in the blood and inhibiting bone resorption. Salmon calcitonin (sCT) is a calcitonin analogue which was indicated for the chronic treatment of osteoporosis and reduction of fracture-associated pain. Unlike human calcitonin which acts specifically at the CT receptor, sCT has affinity to other receptors of the CT receptor family like calcitonin gene related peptide (CGRP) and amylin receptors. Levels of CT receptors are elevated in different tumors/tumor cells, and CT has been proposed to mediate tumor progression [42, 53]. In fact, a small but significant increase in post-marketing reports of tumors (different types) was reported in patients treated with sCT, and the use of injectable sCT-containing products has been restricted to shorter-term indications [16].

Dopamine antagonists (used as neuroleptics, for example) induce hyperprolactinemia through inhibition of the inhibitory action of dopamine on prolactin secretion in the hypothalamus. In line with this activity, mammary tumors are commonly observed in rats with this pharmacological class. However, those tumors have been considered non-relevant for humans based on the difference in prolactin physiology in rodents and humans. An increased incidence in breast tumors has not been established in patients receiving dopamine antagonists in clinical trials. Some relatively recent publications have raised the concern of a possible increase of breast cancer in patients treated with neuroleptics, however the available information remains inconclusive [51].

The pharmacologic actions of insulin analogues are mediated through insulin receptor (IR) stimulation, but the insulin hormone may also have proliferative effects which are triggered by its activation of insulin-like growth factor receptors (IGFR). These proliferative effects induced by insulin(s) in tissues expressing the IGFR may suggest a theoretical cause for concern even when no tumors (e.g., mammary tumors) are observed in chronic rodent studies.

10.2.2.2 Immunosuppressive Agents

There is compelling evidence that the immune system can identify and destroy nascent tumors and thereby function as a primary defense against cancer [41]. Particular immune cell types, effector molecules, and pathways can sometimes collectively

function as extrinsic tumor suppressor mechanisms. Therefore, chronic disturbance of the immune system may reduce immune surveillance and possibly allow tumor growth. Although immunosuppression is a recognized risk factor for human carcinogenesis, rat carcinogenicity testing results with immunosuppressive agents do not reliably reflect this human risk [5], thus potentially yielding "missed signals" for human carcinogenicity. The increase in cancer risk after transplantation has been widely documented for different immunosuppressive regimens with, for example, cyclosporin, azathyoprin, methotrexate, and tacrolimus. Immunomodulatory therapies against several chronic diseases with autoimmune etiology, like rheumatoid arthritis, multiple sclerosis, and psoriasis may potentially be associated with risk of tumorigenicity. Even in the absence of a positive rodent carcinogenicity study or evidence in clinical trials for an individual immunosuppressant, the tumorigenic risk posed by immunosuppression itself should be considered when establishing the benefit: risk ratio of a drug.

In conclusion, there are several pharmacological classes associated with a consistent pattern of tumorigenesis. For most of these classes, it may be plausible that the mechanism of tumorigenesis is associated to their primary or secondary target receptors and may also occur in humans, but the human relevance in the real life conditions of clinical use should be clarified. Agents that are growth factors, hormones or which cause hormonal stimulation, or suppress the immune system may be potentially associated with carcinogenic risk. In these cases, any potential safety risks need to be evaluated and identified. Additionally, for new molecular entities with innovative mechanisms of action, a deep and thorough knowledge of the target biology and associated pharmacologic cascades is needed regarding hypotheses of any carcinogenic risk.

10.2.3 Nonclinical Safety Assessment

Prior to Phase 3 clinical trials, the genotoxic potential of the drug has been characterized; however, typically, the nonclinical carcinogenicity studies have not been completed and in some cases, the results of chronic toxicology studies may not yet be available [10]. The results of subchronic (≤3 months) or chronic (≤9 months) repeated dose toxicology studies together with a thorough knowledge of the pharmacologic attributes of the product may help identify potential risk factors, e.g., hyperplasia, endocrine activity, immunotoxicity.

Given the relatively short course of time during clinical development – many trials are less than 6 months in duration – tumor events are unlikely to have arisen de novo during course of the trial. If tumors are observed in clinical trials, they are likely pre-existing, although the possibility of some drug-associated mechanism of tumor promotion or progression should also be considered. The rodent carcinogenicity studies may provide some evidence of tumor promotion or progression activity if a treatment-related increase in the incidence or onset of spontaneously developed tumors occurs. Other nonclinical models which have been used to investigate the promotion/progression are described below.

10.2.3.1 Investigative Nonclinical Models

Experimental animal models for examining tumor promotion are limited and have not been validated for predicting human risk. The most widely used model is the 2-stage tumor promotion model in which rodents are first dosed with a tumor initiator for a short period of time followed by the putative tumor promoter or a vehicle control. The promotion phase of the model usually involves several months of repeated dosing following initiation. After several months of promoter treatment, the incidence and severity of tumors are scored and compared between rodents receiving the hypothesized tumor promoter and those receiving the vehicle control. Compounds that significantly increase the incidence and severity of tumors present over those treated with the vehicle control are classified as tumor promoters.

The 2-stage tumor promotion model has been used to examine tumor promotion in several different tissues including skin, liver, stomach, urinary bladder, and pancreas [9]. In most cases, the initiating agent is a genotoxic agent, while the tumor promoter is usually non-genotoxic and acts by increasing cellular proliferation. Cell proliferation can occur in response to tissue injury or in response to a mitogenic stimulus.

Although widely used in the literature, several problems have been identified with this model. It has been demonstrated in many cases that promoters themselves can act as complete carcinogens and induce tumors without the need of prior exposure to an initiating agent. Therefore, it is difficult to definitively distinguish agents as tumor initiators or promoters [9]. There is also considerable variation in how the 2-stage tumor promotion models are conducted with regards to types of initiating agents used, timing of administration of initiators and putative promoters, lag time for the development of tumors, and differences in species, sex and strains used. These differences make standardization of the model for routine tumor promoter identification difficult. Finally, no effort has been made to assess the model with respect to its utility in identifying potential tumor promoters in humans. Sodium ascorbate for example is a urinary bladder tumor promoter in rats [9], but there is no indication that sodium ascorbate functions as a urinary bladder tumor promoter in humans [23]. Therefore the model has not been validated with regards to identifying human tumor promoters and is not currently required by health authorities for assessing the safety of drugs. Since an agent that increases cell proliferation will test positive as a potential tumor promoter, it has been argued that use of the 2-stage tumor promotion model is unnecessary, and that agents that induce cell proliferation by any mechanism (cell toxicity or mitogenic stimulus) can be identified by screening for the presence of hyperplasia in chronic toxicity studies [9].

Assays to characterize the potential association between a drug and tumor growth and progression in animals are commonly conducted in pharmaceutical biology laboratories to enable discovery of potential oncolytic therapies. Typically, in vitro systems with human tumor cell lines are employed to measure inhibition of cell proliferation. Subsequently, in vivo studies are conducted to evaluate tumor growth using mouse xenograft models. Although not standardly employed to evaluate drugs and enhancement of tumor cell proliferation and growth, studies of this type were

conducted with prasugrel and dapagliflozin; methodological details are described in Buckley et al. [4] and Reilly et al. [37]. In brief, the methods used were as follows:

For the in vitro cell proliferation experiments, human tumor cell lines of interest (related to potential concerns identified in the clinical trials) are plated in complete media containing 10 % Fetal Bovine Serum (FBS). Following an overnight incubation, the 10 % FBS-containing media is removed and replaced with serum-free media, thus "starving" the cells of growth factors. The drug is added after 24–48 h of starvation at concentrations approximating human plasma levels or some multiple thereof. Cell proliferation is then measured (the WST-1 cell proliferation assay was used in the prasugrel studies) and statistically analyzed. Both a negative (vehicle) control and a positive control (10 % FBS) are included.

For the mouse xenograft studies, the drug is repeatedly administered to nude mice harboring human tumor xenografts derived from subcutaneous implants of human tumor cell lines. Drug administration commences when tumors reached approximately 100 mm^3. The dosing period is based on the anticipated growth rates of the tumors; the study should be terminated when tumors reach a predetermined size considered to cause undue stress to the animals. Rates of growth (size and estimated volume) of the xenografts are statistically compared with those of the vehicle control treated animals throughout the study. Weights and volumes of excised tumors at study termination are also measured and analyzed. Excised tumor tissue can be preserved for possible histopathological examination.

As mentioned, these models are not well-validated for use in the evaluation of tumor promotion and progression for human risk assessment of drugs, and such studies should be considered on a case-by-case basis considering the biology of the molecule and other relevant factors. While good animal models suitable to assess carcinogenic risk associated with impaired immune function are lacking, the potential risk to humans for this class of agents is recognized and accepted, regardless of whether any tumor imbalances are observed in the clinical program. If the known biology or pharmacology of an agent is not sufficient to explain tumor findings in clinical trials, alternative models should be considered. Given that significant uncertainty exists with such systems based on limited knowledge of clinical relevance and inexperience with alternative assessments, it is hoped that technological and scientific advances in the areas of systems biology, computational biology, predictive in silico approaches, molecular biology and genomics centered in human-based systems will provide better tools for identifying true human carcinogens (e.g., [3]).

10.3 Case Studies

Drugs characterized by a lack of evidence of carcinogenic potential in nonclinical assessments have been associated with varying degrees of evidence of potential clinical tumor signals and consequent courses of signal evaluation. The case studies

below provide some examples and illustrate various pathways of investigation that may be considered in evaluating potential cancer risks.

10.3.1 Dapagliflozin

Dapagliflozin is a selective sodium-glucose co-transporter 2 (SGLT2) inhibitor that is currently marketed in the United States (Farxiga, AstraZeneca), EU (Forxiga, AstraZeneca), and several other countries for the treatment of Type 2 diabetes. SGLT2 inhibitors promote urinary glucose excretion thereby reducing hyperglycemia and lowering glycosylated hemoglobin (HbA_{1c}) levels. SGLT2 is selectively expressed in the proximal tubule of the kidney [7, 25, 39] and therefore SGLT2 inhibitors would be expected to have no direct pharmacologic effects on other tissues. Humans with functional mutations in SGLT2 exhibit familial renal glucosuria but are largely asymptomatic. SGLT2 inhibitors are insulin-independent and can therefore be used in conjunction with many existing antidiabetic therapies. Other benefits of the SGLT2 inhibitors include low risk of hypoglycemia, weight loss due to loss of calories in the urine, and decreases in blood pressure due to the diuretic effects of urinary glucose excretion.

Dapagliflozin was subjected to a standard battery of nonclinical toxicology testing as required by regulatory health authorities [43]. There was no evidence that dapagliflozin was genotoxic as assessed by in vitro bacterial reverse-mutation assays. There was also no evidence that dapagliflozin was clastogenic in vivo in rats after daily dosing of 200 mg/kg dapagliflozin for 1 month (Cmax exposures ≤544× maximum recommend human dose [MRHD]). Dapagliflozin did not induce an increase in unscheduled DNA synthesis in male rats or induce clastogenicity in bone marrow micronucleus studies when tested to the maximum tolerated dose (700 mg/kg) required by ICH guidelines. In the 2-year rodent carcinogenicity assays, there was no evidence that dapagliflozin induced tumors or shortened the latency period for tumor development. Dapagliflozin did not increase the incidence of spontaneous background tumors in either the mouse or rat carcinogenicity studies. Mammary gland tumors, for example, are a common background lesion in female rats. There was no evidence that dapagliflozin increased the incidence of mammary gland tumors over those observed in control rats. Therefore, based on the weight of the nonclinical evidence, there was no evidence suggesting that dapagliflozin was a carcinogen.

However, during the dapagliflozin global clinical program (2011 cutoff), a numerical imbalance was observed in the number of urinary bladder tumors detected in dapagliflozin treated patients (nine patients with tumors—incidence rate 0.15) vs. those receiving placebo (one patient with tumor—incidence rate 0.03) [24]. The overall incidence of malignancies and unspecified tumors was balanced between dapagliflozin and placebo groups. Both the sponsor and the FDA indicated that there was no nonclinical evidence for dapagliflozin inducing cancer. The FDA acknowledged that there was a possibility that glucuosuria (with increased urination) and related

genito-urinary infections in dapagliflozin-treated patients may contribute to a detection bias for cases of urinary bladder cancer. According to this hypothesis, patients treated with dapagliflozin may have a greater detection of hematuria compared to those treated with placebo requiring further work-up and a higher rate of cancer diagnosis. Despite the lack of any nonclinical signal, the imbalance in urinary bladder tumors still raised regulatory concerns. There was no evidence that dapagliflozin acted as tumor initiator, but it was suggested that dapagliflozin may act as a urinary bladder tumor promoter or enhance tumor progression. A reassessment of previously conducted toxicity studies and additional nonclinical studies were conducted to evaluate whether dapagliflozin may act as a tumor promoter or enhance tumor progression.

Metabolism: The primary human metabolite of dapagliflozin is dapagliflozin 3-O-glucuronide (D3OG) [43]. This metabolite is a stable non-reactive ether glucuronide that lacks pharmacologic activity. The 3-O-glucuronide is formed at a lower rate in preclinical species, but plasma concentrations comparable to or higher than human exposures were achieved at the doses of dapagliflozin used in the toxicological assessment of dapagliflozin. Non-human species also form a pharmacologically active O-deethylated metabolite, which is only a minor metabolite in humans [36]. The possibility that urinary metabolites of dapagliflozin may be involved in the induction of bladder cancer was also assessed. No unique human dapagliflozin metabolites were found in the urine. Bridging studies were used to extrapolate urinary exposures to dapagliflozin metabolites. Similar to plasma exposures, extrapolated urinary concentrations of dapagliflozin in mouse, rat, and dog toxicity studies were >700× relative to humans, and extrapolated urinary concentrations of the D3OG metabolite were 1–15× in rodents and 30× in dogs relative to humans.

Off-Target Pharmacology: Dapagliflozin and its primary human metabolite, D3OG were screened in more than 300 secondary pharmacology assays that include enzyme inhibition and receptor binding for potential off target activity. No significant off-target pharmacology was observed at pharmacologically relevant concentrations in any of these in vitro assays, suggesting that dapagliflozin and dapagliflozin-3-O-glucuronide do not exhibit off-target pharmacology.

Cell Proliferation: Since increased cellular proliferation may be associated with tumor promotion, the incidence of urinary bladder hyperplasia was assessed in the mouse and rat carcinogenicity studies and in the chronic dog toxicity studies. Dapagliflozin did not induce urinary bladder tumors in either the 2-year mouse or rat carcinogenicity studies at systemic exposures that were >70× human exposures at the maximum recommended human dose (MRHD) [37]. Dapagliflozin did not increase the incidence of hyperplasia in the urinary bladder in either the 2-year mouse or rat carcinogenicity studies. There was also no evidence that dapagliflozin directly increased the incidence of urinary bladder hyperplasia following daily administration for up to 12-months in dogs at systemic exposures that were >3000× exposure in humans at the MRHD. In vitro assessments indicate that dapagliflozin and 3ODG did not enhance cell proliferation when tested in six human bladder transitional cell lines [37]. Additionally, gene transcription analysis

conducted on rat pharmacology studies with dapagliflozin indicated that dapagliflozin administration had no effect on cell cycling, cell regulation, or cell contact gene expression in the liver, kidney, skeletal muscle, or adipose tissues [37].

SGLT2 Inhibition and Increases in Urinary Glucose: A series of experiments was conducted to test whether SGLT2 inhibition and resulting increase in urinary glucose could be associated with the tumor promotion or progression. SGLT2 knockout (KO) and wild type (WT) mice were maintained until they were 15 months of age. Despite a lifetime of glucosuria, 86 % of the KO mice survived compared to 85 % of the WT mice. There was also no evidence of any renal dysfunction in the KO mice. Microscopic evaluation of the urinary bladder, kidneys, liver, heart, pancreas, adrenal glands, thyroids, spleen, female reproductive tract, male sex glands, skin, brain, and skull did not reveal any adverse effect attributable to prolonged exposure to glucosuria. Of particular note, no hyperplasia or neoplasia was observed in the urinary bladder mucosa, urogenital tract, or kidneys of SGLT2 KO mice compared to WT controls. It should also be noted that increases in urinary glucose (up to 400–500 mM) was a common feature of the toxicity studies conducted with dapagliflozin [37]. In no case were increases in urinary glucose associated with urinary bladder hyperplasia or the development of tumors. To test for a potential association between increasing glucose concentrations and transitional cell bladder tumors, an in vitro experiment was conducted in which six human bladder transitional cell lines were exposed to increasing concentrations of glucose in the media. The growth of all cell lines was completely inhibited at 50 mM glucose, well below the concentrations of glucose measured in clinical studies with dapagliflozin (mean of 166 mM at 10 mg dose; [28]). Based on this work, it was concluded that increases in urinary glucose excretion observed in the clinic were unlikely to contribute to enhanced growth of bladder tumors in patients.

Tumor Progression: To assess dapagliflozin and tumor progression, dapagliflozin was administered to nude mice implanted with either EJ-1 or UM-UC-3 (human transitional cell carcinoma cell lines) tumor xenografts. Dapagliflozin was not associated with growth of either tumor implants at doses 75× human clinical exposures [37].

Dapagliflozin was approved in the EU on November 2012 and in the US on January 2014. As part of the approval in the US, the FDA required a nonclinical post-marketing requirement. The FDA required the sponsor to evaluate dapagliflozin in an orthotopic rodent bladder tumor promotion model.

10.3.2 Prasugrel

Prasugrel (Ef(f)ient™),a novel member of the thienopyridine class of antiplatelet agents that includes ticlopidine and clopidogrel, is indicated for the reduction of thrombotic cardiovascular events (including stent thrombosis) in patients with acute

coronary syndrome. Prasugrel is an orally administered prodrug requiring in vivo metabolism to form an active metabolite (R-138727) which specifically and irreversibly antagonizes the $P2Y_{12}$ class of adenosine diphosphate (ADP) receptors on platelets, thus inhibiting ADP- mediated platelet activation and aggregation. Following oral administration, prasugrel is rapidly metabolized to form the active metabolite (R-138727) which is further metabolized and inactivated to R-106583, the major circulating metabolite in humans. The metabolic pathways of prasugrel in mice, rats, and humans are generally similar.

A comprehensive nonclinical safety assessment including genotoxicity and carcinogenicity studies supported the chronic use of prasugrel in patients with atherothrombotic disease [13, 49]. Prasugrel was negative in a battery of genotoxicity studies: the Ames bacterial reverse mutation assay; an in vitro chromosomal aberration study in Chinese Hamster Lung cells; and an in vivo mouse bone marrow micronucleus test. When tested in traditional chronic rodent bioassays at exposures up to 74-fold (inactive metabolite) to 1081-fold (active metabolite) higher than the clinical exposure during 10-mg/day maintenance dosing, prasugrel was negative in a 2-year rat carcinogenicity study. Statistically significant increases in hepatocellular adenomas were seen in the 2-year mouse carcinogenicity study at a dose 250-fold the clinical exposure. The increase in liver tumors in prasugrel-treated mice was considered related to hepatic drug-metabolizing enzyme induction and not of significant relevance to human risk [4]. There were no increases (statistically significant compared to concurrent controls or compared with historical control data) in tumor incidence for either species at any site except for the increased incidence of tumors in the mouse liver.

However, in the Phase 3 clinical trial with prasugrel (and a comparator antiplatelet agent, clopidogrel), excess neoplasms (all types) were reported in the prasugrel group: the frequency of newly diagnosed cancers was 1.6 % in the prasugrel group versus 1.2 % in the clopidogrel group [46]. It must be noted that the Phase 3 study was not designed to capture baseline cancer information (e.g., cancer history, stage at diagnosis, and treatment), and the tumor observations were collected "ad hoc" through adverse event reporting. Due to regulatory concerns about the apparent increase in malignancies, additional nonclinical studies were undertaken to examine the possibility that prasugrel may accelerate tumor growth [4]. These studies included both in vitro evaluations of human tumor cell lines in culture and in vivo nude mouse xenograft models of lung, colon, and prostate origin, collectively allowing an evaluation of diverse human tissue types that may be relevant to human disease.

Tumor cell proliferation (in vitro): Prasugrel's active and inactive metabolites did not increase tumor cell proliferation in human lung, colon, or prostate tumor cells in vitro. The data also demonstrated that the assay conditions employed in these studies maintained the ability of the cells to respond to mitogenic stimuli as shown by the response to fetal bovine serum.

Tumor progression (in vivo): In the in vivo tumor xenograft studies, exposures (AUC) at 10-mg/kg doses in the tumor-bearing nude mice were approximately 34-fold higher than the exposure to R-138727 and 22-fold higher for the exposure

to R-106583 in humans administered 10-mg maintenance doses. There was no significant difference in mean terminal body weights for control versus treated animals for each tumor type. Prasugrel did not increase tumor cell proliferation (tumor volumes or weights and tumor growth rate) in human colon, lung, or prostate tumor cells in vivo.

Pharmacology: Prasugrel is highly specific for its target, the $P2Y_{12}$ class of adenosine diphosphate (ADP) receptors on platelets. Consistent with the experimental findings, there is little plausible biological basis for antithrombotic agents such as prasugrel to be carcinogenic; research has, in fact, suggested the opposite (described in [4]). It is generally accepted that prohemostatic or prothrombotic pathways, namely the coagulation cascade and platelet activation and aggregation are pro-carcinogenic. Several reports have concluded that the co-aggregation of platelets with tumor cells provide a means for tumor cells to travel to distal sites and metastasize and to avoid immune surveillance. Accordingly, preclinical studies have documented the tumor-inhibitory activity of both anticoagulants and antiplatelet agents (as referenced in [4]).

Ascertainment bias was suspected, e.g., that the higher incidence of bleeding in the prasugrel (vs clopidogrel) arm of the Phase 3 clinical study resulted in additional medical attention, during which more pre-existing cancers were discovered. In addition, several factors were weighed in the FDA's consideration as to whether prasugrel was causally related to the higher rate of tumors in prasugel treated patients in the Phase 3 study [46]:

- It was difficult to conceptualize a potential mechanism through which prasugrel could initiate or stimulate nonspecific tumor development.
- Given the relatively brief duration of the study (15 months) and the early emergence of many of the tumors, it was not thought that induction of new tumors could plausibly explain the increase.
- In silico structure activity assessment suggested that prasugrel is not carcinogenic. There were no proliferative signals (e.g., hyperplasia) in the rodent carcinogenicity studies or in chronic studies in rats or dogs. Moreover, animal carcinogenicity studies of prasugrel were negative (with the exception of the clinically irrelevant mouse liver tumors).
- Prasugrel was negative in tumor-progression studies to assess the potential effects of prasugrel and its metabolites in human colon, lung, and prostate tumor-cell lines grown in vitro and in congenitally immunodeficient "nude" mice in vivo. To FDA knowledge, the only products thought to stimulate tumor development are the erythropoietins, which, unlike prasugrel, are growth factors.
- Finally, given the observational nature of safety analyses, the fact that numerous comparisons were performed without statistical correction, and the lack of prespecified hypotheses, as well as the marginal statistical support for the finding, the possibility of a false positive finding seemed high.

The FDA and a Scientific Advisory Panel concluded that causality between prasugrel treatment and tumorigenicity or tumor promotion was unlikely. The Sponsors

were assigned a postmarketing requirement to collect baseline and subsequent data on cancer in a large, at-the-time ongoing clinical trial.

The results of another clinical investigation of Dual Antiplatelet Therapy (DAPT; [32]) highlight the complexity of analyzing a potential tumor signal in clinical trials. In the DAPT study, subjects received dual antiplatelet therapy (either clopidogrel or prasugrel) beyond 1 year in duration. This study clearly showed a reduction in both stent thrombosis and myocardial infarction when dual antiplatelet therapy is extended beyond 1 year after implantation of a drug-eluting stent; however, there was an observed increase in moderate or severe bleeding, as well as a possible increase in all-cause mortality. While the study might be considered of sufficient duration (12–33 months) to test for some treatment-related signal of cancer, limitations in the study design (e.g., inconsistent reporting of cancer and characterization of cancer history) rendered any relationship of rates of cancer deaths per treatment to study drug uncertain. Additional blinded adjudication initially revealed a statistical increase in cancer-related deaths; however, the apparent increase was subsequently determined to be related to an imbalance in patient entry criteria. The added adjudication process discovered there were patients who had entered the study with advanced cancer, and there was an imbalance at baseline of eight vs one in the two respective groups of 30- versus 12- months' thienopyridine treatment; when these patients are removed, the non-cardiovascular deaths were no longer statistically significant. This initial finding prompted a meta-analysis of more than 69,000 clopidogrel-treated patients with over 139,000 patient years which showed that extended duration dual antiplatelet therapy was not associated with a difference in the risk of all-cause, cardiovascular, or non-cardiovascular death compared with aspirin alone or short duration dual antiplatelet therapy [12]. Analyses of the DAPT study highlight the importance of duration (sufficient to examine treatment-emergent development of cancer), sufficient experience (the meta-analysis allowed assessment of significant numbers of patients), and understanding bias (in this case, enrollment bias) when assessing potential cancer signals in clinical trials.

10.3.3 Cladribrine

Clabridine (Litak) was approved in the EU in 2004 [17] for the treatment of hairy cell leukemia (HCL). It is an antimetabolite chemically derived from deoxyadenosine, where the hydrogen atom in the two-position of the purine ring has been replaced by a chlorine atom, thus rendering the molecule resistant to the deamination by adenosine deaminase. Intracellularly, clabridine is phosphorylated by deoxycytidine kinase (which is present in a high concentration particularly in normal and malignant lymphoid cells). Because lymphoid cells also have a low content of 5′-nucleotidase, there is accumulation of two- chlorodeoxyadenosine-5′-triphosphate (CdATP) which is incorporated into DNA strands, thereby blocking DNA chain elongation, inhibiting DNA repair and ribonucleotide reductase. Cell death then occurs from energy depletion and apoptosis.

Clabridine is a cytotoxic medicinal product shown to be mutagenic to cultured mammalian cells. In vitro studies in various cell lines have shown that clabridine induces dNTP imbalance, DNA strand breaks, depletion of NAD and ATP, and cell death. It also inhibits DNA repair. These properties support generally the proposed mechanism of action and the therapeutic effect of clabridine. The carcinogenic potential of clabridine was tested in a single 22-month study in mice and in a TgrasH2 transgenic mouse bioassay. In the 22-month study, a significant increase in Harderian gland tumors was observed. Except for three adenocarcinomas in the high dose group, tumors were mostly benign adenomas, and there were no histomorphologic signs of progression to adenocarcinomas. Harderian gland tumors were not considered clinically relevant, as humans do not have a comparable anatomical structure [6]. The TgrasH2 transgenic mouse bioassay was negative, and the absence of any sign of Harderian gland alteration was considered to further add to the conclusion regarding the clinical irrelevance of Harderian gland tumors. The EMA Committee for Medicinal Products for Human Use (CHMP) concluded that, overall, the mouse studies did not reveal evidence of clinically relevant carcinogenic potential of clabridine [18, 19].

In HCL patients, there appeared to be no evidence that clabridine-treated patients had a higher frequency of secondary malignancies than patients treated with alpha-interferon or deoxycoformycin. However, since the incidence of secondary malignancies was significantly higher compared to the general population, the CHMP recommended that patients treated with clabridine be regularly monitored and that an annual follow-up report on secondary malignancies be provided [18, 19]. Warnings about secondary malignancies and regular monitoring as a precaution were incorporated in the SPC.

In 2009, an oral tablet formulation of clabridine (Movectro) was developed for the treatment of Multiple Sclerosis (MS). As of August 2010, five malignancies in clabridine-treated patients vs. one in placebo patients were reported. Over the entire clinical program, 22 cases of malignancies were reported in clabridine-treated MS patients, while only two cases were reported in placebo-treated patients (one basal cell carcinoma and one ovarian cancer). The Relative Risk (RR) of malignancies based on patients from all studies suggested a five-fold increase in the risk of cancer but with a broad CI (95 % CI: 0.67–38.43). However, the sponsor considered that a more appropriate estimation of RR should be derived from analyses restricted to clabridine-treated MS patients in double-blind controlled trials, thus avoiding confounding by dissimilar follow-up periods of the treated and placebo cohorts. That analysis yielded an RR of 2.31 (95 % CI: 0.27–19.81), suggesting only a two-fold increase in the risk of cancer among clabridine exposed patients which was statistically not significant. The CHMP agreed that, while RR calculation based on all studies might be biased, the more conservative analysis based on all studies was more appropriate and suggested an increased risk of malignancy with increased exposure time. The concern for the disproportion of number of malignancies in the clabridine groups compared to placebo during the whole clinical trial program contributed to the negative opinion issued by CHMP in 2010, which was reiterated after

a reexamination in 2011 [18, 19]. In contrast to HCL indication, the benefit/risk for MS patients was considered negative.

The Movectro case is a good example of a situation where a potential cancer signal emerged through clinical experience in the absence of a relevant positive rodent carcinogenicity study. It must be said, however, that concern for possible human carcinogenic risk was prudent based on the positive genotoxicity and the pharmacological properties of clabridine, even though the mouse carcinogenicity bioassays results had been reassuring.

10.3.4 Avandia

Rosiglitazone (Avandia™) was approved to improve glycemic control in patients with Type 2 diabetes mellitus [14, 47]. Thiazolidinediones such as rosiglitazone produce their effects by activating peroxisomal proliferator-activated receptor gamma (PPARγ), altering gene expression associated with multiple molecular and cellular processes. The marketing application was supported by a comprehensive nonclinical safety assessment which included genotoxicity and carcinogenicity studies [47]. Genotoxicity tests of chromosomal aberration, unscheduled DNA synthesis, and the in vivo mouse micronucleus were negative, while the incidence of forward mutations at the TK locus of mouse lymphoma L5178Y cells was slightly increased (ca. 2×) in the presence of S-9. In the rodent carcinogenicity studies, there were no remarkable findings except an increase in the incidence of adipose hyperplasia in mice and significant increases in benign adipose tissue tumors (lipomas) in rats. The proliferative changes in both species were considered due to the persistent pharmacological overstimulation of adipose tissue.

Rosiglitazone is an example of a drug for which there was no signal of animal (excepting the target-related lipomas) or human tumorigenicity but which faced questions because, with piogliatozone, a related thiazolidinedione with PPARγ activity, tumors were observed in the urinary bladder of male rats in a 2-year carcinogenicity study [45] and there were reports of bladder cancer in some patients taking the drug [44]. Clinically, rosiglitazone has received little attention regarding bladder cancer risk in patients, in large part due to FDA-imposed stringent prescribing restrictions and the EMA suspension of the marketing authorization related to cardiovascular risks [15, 48]. In 2004, a number of PPARγ agonists were being screened for potential chemopreventive properties in a nonclinical model. Lubet et al. [29] reported that a relatively high dose of rosiglitazone appeared to promote bladder cancer formation in the hydroxybutyl(butyl)nitrosamine (OHBBN, a urinary bladder specific carcinogen) -induced rat bladder tumor model. At that time, the FDA was reporting that a number of recently synthesized PPARγ and PPARα/γ agonists were themselves inducing bladder tumors in rats or mice or in both species [11]. A subsequent 2-stage tumor promotion study expanded upon the initial data, suggesting that lower doses of rosiglitazone may also have significant tumor promoting activity in the OHBBN rat model [30]. The potential for rosiglitazone to be associated with later-stage promo-

tional activity was considered somewhat surprising in that the PPARγ receptor, although highly expressed in normal bladder urothelium and hyperplastic lesions, was expressed at lower levels in established bladder cancers.

This case study provides an example in which a rat model of initiation and promotion was employed to investigate an initial report of potential tumor promoting activity of rosiglitazone (in that same model) and a concern regarding the pharmacologic class [11]. However, unlike pioglitazone, there have been no reports of any theoretical association of rosiglitazone treatment and bladder hyperplasia or cancer in animals or patients. In a systematic review and meta-analysis of clinical trial and observational studies, Turner et al. [44] concluded that "no significant risk was seen with rosiglitazone" and that "the evidence for any relationship between bladder cancer risk and rosiglitazone cumulative duration is limited and inconsistent."

10.3.5 Orlistat

Orlistat is a specific and long-acting inhibitor of pancreatic and gastric lipases and is currently marketed as a treatment for obesity in the US and Europe as both a prescription (Xenical™, Roche) and over-the-counter (Alli, GlaxoSmithKline) drug. Orlistat is a partially hydrated derivative of lipstatin that functions by decreasing the breakdown and subsequent absorption of an estimated one-third of dietary ingested fats [33]. Pharmacokinetic studies indicate that orlistat has very low oral bioavailability and suggest that the effects of orlistat are restricted to the intestines [54]. In a 2-year efficacy study, obese patients receiving 120 mg orlistat three times a day lost significantly more weight (8.8 %) than those patients receiving placebo (5.8 %) after the first year of the study. During the second year, twice as many patients receiving placebo (63 %) regained their weight compared to those maintained on orlistat (35 %). The most common adverse events observed in patients receiving orlistat included abdominal pain, fatty/oily evacuation, and fecal incontinence [33].

During the Phase 3 clinical trials, nine cases of breast cancer were observed in women taking orlistat compared to one patient in the placebo group. During follow-up surveys, two more patients receiving orlistat (11 total) were diagnosed with breast cancer compared to three in the placebo group [31]. The FDA indicated that the data submitted supported the efficacy of orlistat but asked Roche to gather further information on the breast cancer cases observed in the clinical trials. The reason for the clinical imbalance in breast cancer was unknown but was speculated to be due to chance or detection bias. In August 1997, Roche withdrew its NDA and then resubmitted it in November 1997 [31]. At the Xenical™ FDA Advisory Committee meeting (March 1998), independent experts in the fields of oncology, histopathology, and mammography agreed that the majority of the breast cancers observed in the orlistat clinical trials were pre-existing and that 3 of the cases in the orlistat treatment group and two in the placebo group emerged after treatment initiation [38]. Therefore, with this new data, there was no difference in the incidence of breast cancer in patients treated with orlistat compared to placebo [33]. Nonclinical

studies were also supportive of a lack of a tumor risk with orlistat [38]. Orlistat did not induce tumors in the 2-year rodent carcinogenicity studies and was not genotoxic in nonclinical testing. Therefore, it was the opinion of the experts at the Advisory Committee meeting that there was no evidence that orlistat induced breast cancer. More data from open-label Phase 3b trials confirmed this conclusion. In these trials, three additional cases of breast cancer were observed (all in the placebo group) with no observed imbalance in breast cancer cases [50]. This additional data was provided to regulatory authorities in January 1999 and lead to the approval of orlistat (Xenical™, Roche) by the FDA in April of 1999.

Interestingly, orlistat has been shown in recent years to exhibit potent antitumor activity in vitro through its ability to block cellular fatty acid synthesis activity and induce apoptosis in colon and breast cancer cells [26, 34]. Although the low bioavailability of orlistat may prevent its utility in treating breast cancer, it has been suggested that the antitumor properties of orlistat may have beneficial effects for the treatment of tumors of the gastrointestinal tract [35].

10.4 Summary

In summary, any signal of potential treatment-related malignancy should be considered and appropriately evaluated. The case studies presented illustrate the various kinds of hypothesis-driven nonclinical investigations that may be conducted to evaluate potential cancer risk when a human tumor signal is identified. While these post-hoc or retrospective assessments should take into account the biology and pharmacology of the molecule, they may also include studies addressing tumor promotion and progression on a case-by-case basis. However, it is very important to recognize that these models are not standard or well-validated, and the development and validation of innovative models for assessing tumor promotion and progression that are more human-based warrants further scientific investigation.

In practice, risk evaluation and management of potential safety signals in the settings of late-phase clinical development and/or real world use may take several forms. Risk Management Plans (RMPs; [20]) include a set of pharmacovigilance activities and interventions designed to identify, characterize and manage risks related to a medicine. Pharmacovigilance actions to investigate specific safety concerns such as tumorigenicity may include targeted safety studies, postmarketing surveillance, observational and epidemiologic studies, and mechanistic or descriptive studies. Risk minimization activities may include treatment axations, patient restriction or exclusion and updated labeling requirements.

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