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Original Article

Survey of tumorigenic sensitivity in 6-month rasH2-Tg mice studies compared with 2-year rodent assays

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Abstract: The pharmacokinetic endpoint of a 25-fold increase in human exposure is one of the specified criteria for high-dose selection for 2-year carcinogenicity studies in rodents according to ICH S1C(R2). However, this criterion is not universally accepted for 6-month carcinogenicity tests in rasH2-Tg mice. To evaluate an appropriate multiple for rasH2-Tg mice, we evaluated data for 53 compounds across five categories of rasH2-Tg mouse-positive [(1) genotoxic and (2) non-genotoxic] carcinogens and rasH2-Tg mouse-negative [(3) non-genotoxic carcinogens with clear or uncertain human relevance; (4) non-genotoxic rodent-specific carcinogens; and (5) non-carcinogens], and surveyed their tumorigenic activities and high doses in rasH2-Tg mice and 2-year rodent models. Our survey indicated that area under the curve (AUC) margins (AMs) or body surface area-adjusted dose ratios (DRs) of tumorigenesis in rasH2-Tg mice to the maximum recommended human dose (MRHD) were 0.05- to 5.2-fold in 6 category (1) compounds with small differences between models and 0.2- to 47-fold in 7 category (2) including three 2-year rat study-negative compounds. Among all 53 compounds, including 40 compounds of the rasH2-Tg mouse-negative category (3), (4), and (5), no histopathologic risk factors for rodent neoplasia were induced only at doses above 50-fold AM or DR in rasH2-Tg mice except for two compounds, which induced hyperplasia and had no relationship with the tumors observed in the rasH2-Tg mouse or 2-year rodent studies. From the results of these surveys, we confirmed that exceeding a high dose level of 50-fold AM in rasH2-Tg mouse carcinogenicity studies does not appear to be of value. (DOI: 10.1293/tox.2021-0031; J Toxicol Pathol 2022; 35: 53–73)

Key words: rasH2-Tg mouse, carcinogenicity, ICH S1C guideline, high dose selection, pharmacokinetic parameters, maximum recommended human dose

Introduction

Carcinogenicity assessments of small-molecule pharmaceuticals are generally conducted in a 2-year carcinogenicity study in one rodent species (usually rat) and either a short- or medium-term carcinogenicity study in an alternative model or a 2-year carcinogenicity study in another rodent species (generally mice). The CByB6F1-Tg(HRAS)2Jic (rasH2-Tg) mouse is a genetically modified mouse model[1] recommended for use in the ICH S1B carcinogenicity testing guideline. These mice are an F1 hybrid of genetically modified animals, in which three copies of a proto-oncogene of HRAS(c-Ha-ras) are inserted into chromosome 15[2]. The rasH2-Tg mouse has been used for most short-term carcinogenicity studies of pharmaceuticals in recent years[3–5] due to the low incidence of spontaneous tumors[6, 7] and the positive response to both genotoxic and non-genotoxic carcinogens[8].

Guidance for dose selection in rodent carcinogenicity studies of pharmaceuticals was presented in the ICH S1C guideline in 1994. This guideline, which was subsequently revised twice, provides six parameters for high-dose selection in a 2-year rodent carcinogenicity study. One of the accepted high-dose selection parameters is the 25-fold clinical

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exposure ratio.

A retrospective study conducted by the U.S. Food and Drug Administration (FDA) during the revision of the ICH-S1C guideline examined the association between rodent and human systemic exposure ratios (AUC margin, AM), kg body weight-based dose ratios (DR) [rat: mg/kg, human: mg/kg maximum recommended human dose (MRHD)], and body surface area-based DR (rat: mg/m^2, human: mg/m^2 MRHD) in 2-year rat carcinogenicity studies[9]. Based on the results, the body surface area-based DR was more appropriate than the body weight-based DR for estimating AM in the absence of exposure data, and most tumors occurred at AM or body surface area-based DR of less than 10-fold. Owing to these results, 25 times the maximum clinical exposure was proposed as an acceptable pharmacokinetic (PK) parameter for high-dose selection in 2-year rodent carcinogenicity studies.

Although more data have been accumulated for carcinogenicity studies using rasH2-Tg mice than those for other Tg mouse models (p53$^{+/-}$ deficient, Tg.AC, and XPA deficient models), the relationship between exposure and carcinogenicity in rasH2-Tg mice has not been fully investigated until now. Therefore, clinical exposure ratios are not commonly used as criteria for high-dose selection in rasH2-Tg mouse short-term carcinogenicity studies, and high doses in rasH2-Tg mouse studies are often determined by either the maximum tolerated dose (MTD), the limit dose, or the maximum feasible dose. A survey by U.S. pharmaceutical companies reported the use of rasH2-Tg mice by 64% of the mouse carcinogenicity studies conducted during the 8.5-year period up to July 2018. High doses were selected on the basis of MTD in more than 80% of the studies, and higher than 25-fold clinical exposure ratios in 45% of the studies[5]. For low toxicity compounds, high dose selection based on MTD often results in markedly higher exposures than clinical exposures. These conditions can significantly alter the physiology of animals, and the findings from these animals are considered irrelevant for human risk assessment.

To investigate the applicability of PK parameters to high dose selection in rasH2-Tg mouse carcinogenicity studies, we examined published study data for 53 compounds across five categories of rasH2-Tg mouse-positive [(1) genotoxic and (2) non-genotoxic] carcinogens and rasH2-Tg mouse-negative [(3) non-genotoxic carcinogens with clear or uncertain human relevance; (4) non-genotoxic rodent-specific carcinogens; and (5) non-carcinogens], and surveyed their tumorigenic activities and high doses in rasH2-Tg mice and 2-year rodent models.

Materials and Methods

Data sources

The 53 compounds across five categories were selected for the present survey from the international validation study of the ILSI/HESI Alternative Methods for Carcinogenicity Testing Project[10, 11], validation studies on rasH2-Tg mice carried out by the Central Institute for Experimental Animals (Kawasaki, Japan), and from available application materials for approval of pharmaceuticals. The five categories include (1) rasH2-Tg mouse-positive genotoxic carcinogens (6 compounds, Table 1), (2) rasH2-Tg mouse-positive non-genotoxic carcinogens (7 compounds including three 2-year rat study-negatives, Table 2), (3) rasH2-Tg mouse-negative non-genotoxic carcinogens with clear or uncertain human relevance (8 compounds, Table 3), (4) rasH2-Tg mouse-negative non-genotoxic rodent-specific carcinogens (14 compounds, Table 4), and (5) rasH2-Tg mouse-negative non-carcinogens (18 compounds, Table 5).

Calculation of the clinical exposure margin

When AUC values in clinical use and carcinogenicity studies are obtained from available application data, AMs were calculated using the steady-state AUC at repeated doses of MRHD (bazedoxifene, ozanimod, sunitinib, troglitazone, vascepa, dulaglutide, enzalutamide, raloxifene, tofacitinib, abiraterone, aliskiren, beclabuvir, cabozantinib, filgotinib, indacaterol, maraviroc, suvorexant, asunaprevir, baricitinib, bictegravir, daclatasvir, doravirine, etelcalcetide, evocalcet, glycopyrronium, tafamidis, telbivudine, teneligliptin, vadadustat, velpatasvir). The MRHD and clinical exposures used to calculate AM are shown in Supplementary Tables 1–5. If only AM values were available and no toxicokinetic (TK) data were found, the AM values presented in the application data were used (nilotinib).

In the calculation of AM, the AUC was essentially for the parent compound; however, AM was calculated using the sum of the AUC of the parent compound and the active major metabolites when these active metabolites exhibited the same level of pharmacological activity as the parent compound, and when the proportion of parent compound and major active metabolites differed largely in humans and rodents (sunitinib and ozanimod). The AM of ozanimod was calculated as the total AUC of the parent compound and the two major active metabolites (CC112273 and CC1084037). The total AUC value estimated from the AUC and/or its ratios of ozanimod and the two active metabolites, as shown in the CDER Clinical Pharmacology Review[12] and the Package Insert[13], was used as the clinical exposure value.

When the dosing intervals differed between clinical and rodent studies, AUC values were compared between clinical and non-clinical dosing in terms of exposure over the same time period based on the respective AUCtau (beclabuvir, dulaglutide, etelcalcetide, maraviroc, tofacitinib).

Calculation of the ratio of the human equivalent dose to the maximum clinical dose

For compounds for which TK data in carcinogenicity studies were not available to calculate AM, dose ratios to MRHD (DR), as shown in Supplementary Tables 1–5, were calculated based on body surface area (cyclophosphamide, diethylstilbestrol (DES), melphalan, phenacetin, procarbazine, thiotepa, clofibrate, ampicillin, cyclosporine, 17β-estradiol, methapyrilene, chlorpromazine, haloperidol, metaproterenol, phenobarbital, reserpine, sulfamethoxa-

Table 1. Tumorigenesis and HPRF of Category (1) rasH2-Tg Mouse-positive Genotoxic Carcinogens

Compound (Profile)	Model	6M rasH2-Tg mouse study				2Y mouse study				2Y rat study			
	Dose	VL	LD	MD	HD	VL	LD	MD	HD	VL	LD	MD	HD
Cyclophosphamide[26–30] (Alkylating agent)	DR		0.1	0.2	0.3		0.03	0.06	0.1	0.05	0.1	0.2	0.4
	Tumor		+	+	+		+	+	+	+	+	+	+
	HPRF		+	+	+			NA				NA	
Diethylstilbestrol[18, 19, 31–34] (Synthetic estrogen)	DR		0.07	0.2	0.7	0.03[c]	0.1[c]	0.3[c,d]	0.5[c,d,e]			0.2	1.9
	Tumor		−	−	+ (M)	+	+	+	+			−	+
	HPRF		+	+	+	NA	NA	NA	+			NA	
Melphalan[6, 35, 36] (Alkylating agent)	DR			0.1	0. 6			0.9	1.7			2.0	4.0
	Tumor			−	+[a]			+[b]	+[b]			+[b]	+[b]
	HPRF			NA				NA				NA	
Phenacetin[6, 37–41] (Analgesic)	DR			2.6	5.2			2.5	5.3		1.9	4.4	8.9
	Tumor			−	+ (M)			+ (M)	+ (M)		± (F)	+	+
	HPRF			NA				−	+		+ (F)	NA	NA
Procarbazine[42–44] (Alkylating agent)	DR			0.05	0.1			0.05	0.1			0.3	0.5
	Tumor			+ (M)[a]	+			+	+			+	+
	HPRF			NA				−	+ (F)			+	+
Thiotepa[42, 43, 45, 46] (Alkylating agent)	DR			0.3	0.6			0.4	0.7			0.4	0.9
	Tumor			+ (F)[a]	+			+	+			+	+
	HPRF			NA				−	−			−	−

+: positive; −: negative; ±: equivocal; DR: dose ratios of body-surface-based human equivalent doses to maximum recommended human dose (MRHD); F: Female; HD: high dose; HPRF: histopathologic risk factor; LD: low dose; M: male; MD: mid dose; NA: no data available or not done; VL: very low dose.

a, In house data summarized in Yamamoto *et al*[42].

b, Statistically significant difference when MD and HD were combined and compared to the control.

c, C57BL/6 mice were used.

d, Female C3H/HeN-MMTV$^+$ (high titer to mouse mammary tumor virus) mice were used.

e, Female C3H/HeN-MMTV$^-$ (low titer to murine mammary tumor virus) mice were used.

zole, bixalomer, cholestyramine, pasireotide, rifaximin, and sulfisoxazole). Specifically, the dose per kg body weight in the carcinogenicity study was divided by body surface area-converting factor (BSA-CF) to obtain the body surface area-adjusted human equivalent dose (HED), and the ratio of the obtained HED to MRHD (HED/MRHD) was calculated as DR[14]. The BSA-CF values of 12.3 for mice and 6.2 for rats were used in accordance with the FDA Guidance of Safety Starting Dose (FDA, 2005). If no compound intake based on measured food or water consumption was demonstrated in studies that administered feed or drinking water, doses per kg body weight were calculated using mean body weight, mean food consumption, and mean water consumption of SD, F344, and Wistar rats and B6C3F1 mice in long-term studies (Supplementary Table 6), as indicated by Blackburn[15]. For each rasH2-Tg mouse feeding study where food intake-based compound intake was not demonstrated, the dosage was calculated using the mean body weight at the start and end of the 26-week treatment and the mean weekly food consumption (Supplementary Table 6) as indicated by Paranjpe *et al*[16].

When the frequency of administration in carcinogenicity studies differed from that of clinical application, weekly accumulated HEDs and MRHD were compared (cyclophosphamide, procarbazine, melphalan, and thiotepa). The carcinogenicity study of pasireotide, which is administered intramuscularly once every 4 weeks in the clinic, was conducted with once-daily subcutaneous dosing; therefore, a single clinical dose was compared with 28-fold daily HEDs in carcinogenicity studies as exposure data were not available. When the routes of administration differed between carcinogenicity studies and clinical applications, the doses in the carcinogenicity studies were converted to BSA-based HEDs according to Nair *et al.*[14] and compared to MRHD (procarbazine, melphalan, thiotepa, pasireotide).

Analysis

For the 53 selected compounds, the highest doses tested, dose levels that caused tumor development and histopathologic risk factors (HPRFs) for rodent neoplasia were compared between rasH2-Tg mouse and 2-year rodent models in terms of AM or DR (Tables 1–5). Tumors, HPRFs including hyperplasia, hypertrophy, foci of cellular alteration, and preneoplastic lesions[17] are shown in Tables 8–12. MRHD, clinical exposure, doses of carcinogenicity studies, and AM or DR are shown in Supplementary Tables 1–5.

Table 2. Tumorigenesis and HPRF of Category (2) rasH2-Tg Mouse-positive Non-genotoxic Carcinogens

Compound (Profile)	Model	6M rasH2-Tg mouse study				2Y mouse study			2Y rat study			
	Dose	VL	LD	MD	HD	LD	MD	HD	VL	LD	MD	HD
Bazedoxifene[47–49] (SERM)	AM		18	33	54				0.1	0.6	1.9	4.8
	Tumor		−	+ (F)	+ (F)		NA		+ (M)	+ (M)	+	+
	HPRF		+ (F)	+ (F)	+ (F)				+ (M)	+ (M)	+ (M)	+
Clofibrate[50, 51] (PPARα agonist)	DR (M/F)		0.1	0.2/0.4	0.5/0.6	0.4	0.6	0.9			1.2	1.9
	Tumor		−	+ (M)	+ (M)	−	−	−			+	+
	HPRF		−	+ (F)	+	−	+	+			+	−
Nilotinib[52] (Bcr-Abl TK inhibitor)	AM		NA	15	35					NA	NA	2.5
	Tumor		−	−	+		NA			−	−	± (F)
	HPRF		NA	NA	+					NA	NA	+ (F)
Ozanimod[12, 13, 53] (S1P receptor modulator)	AM[a]		47[b]	144	450					0.2	1.0	3.6
	Tumor (M/F)		+/±	+	+		NA			−	−	−
	HPRF		−	−	+					−	−	−
Sunitinib[47, 54–56] (Receptor TK inhibitor)	AM (M/F)[c]		0.6	5.0	NC/7.7					0.2	0.7	5.9
	Tumor		−	+ (F)	+		NA			−	+ (F)	+
	HPRF		−	+	+					−	−	+ (M)
Troglitazone[57–60] (PPARγ agonist)	AM (M/F)			9.9	12	1.9	9.9	12		1.0/2.8	3.5/8.7	7.3/29
	Tumor			−	+ (F)	−	+ (F)	+		−	−	−
	HPRF			+	+	+	+	+		+	+	+
Vascepa[61] (EPA-mediated lipid reduction)	AM	0.7	1.2[d]	2.4	3.7					NA	3.0	6.8
	Tumor	−	−	−	+ (M)		NA			−	+	+
	HPRF	−	+ (M)	+	+					−	+	+

+: positive; −: negative; ±: equivocal; AM: AUC margin to MRHD based on AUC; DR: dose ratios of body-surface-based human equivalent doses to MRHD; F: Female; HD: high dose; HPRF: histopathologic risk factor; LD: low dose; M: male; MD: mid dose; NA: no data available or not done; VL: very low dose.

a, Margins of total AUC of ozanimod and two major active metabolites in the carcinogenicity studies to that in clinical use shown in the CDER clinical pharmacology review[12] and the Package Insert[13].

b, Average of 54-fold in males and 38-fold in females, both of which were dosed with ozanimod at 8 mg/kg/day.

c, Margins of total AUC of sunitinib and its major active metabolite to that at MRHD.

d, Not provided in the source data, but estimated on assumption that exposure increased dose-proportionally.

Results and Discussion

Category (1): rasH2-Tg mouse-positive genotoxic carcinogens (6 compounds, Tables 1, 6, 8)

The administration of any of these 6 compounds increased tumorigenesis in rasH2-Tg mice at 0.05 to 5.2-fold DR. In the 2-year carcinogenicity studies of these compounds, tumorigenesis was also increased at 0.03 to 2.5-fold DR in mice and 0.05 to 4.4-fold DR in rats. The ranges of DR in the 2-year studies were similar to those in the rasH2-Tg mouse studies; however, most of the tumors differed between the testing models. The carcinogenic DR for each of these compounds in the rasH2-Tg mouse studies ranged from 0.7- to 23-fold, and 0.2- to 2.0-fold to those in the 2-year mouse and rat studies, respectively. Of note, in the 2-year studies in mice, DES was an outlier with a 23-fold difference in DR compared with rasH2-Tg mice. All other compounds had 0.7- to 3.3-fold differences in the DR between the two models. DES tumorigenesis is generally accepted as a result of both genotoxic (epoxide or quinone intermediates have been shown to form DNA adducts) as well as mitogenic (estrogenic) activity[18]. In fact, in a 2-year study with C57BL/6 mice, thyroid tumors developed at very low doses (0.03-fold DR and higher). In contrast, rasH2-Tg mice were less susceptible to carcinogenesis via endocrine alterations caused by 17β-estradiol (estrogen), reserpine (catecholamine depletion), sulfamethoxazole (goitrogen), and suvorexant (CYP inducer), which were positive in the 2-year rodent studies (Tables 3, 4, 10, 11). As testicular Leydig cell tumors and hyperplasia were induced in the high-dose groups (0.7-fold DR) of DES-treated rasH2-Tg mice and non-Tg littermates[19], the difference in DES-induced carcinogenesis in the rasH2-Tg and 2-year mouse models may be due to inter-model differences in sensitivity to hormonal carcinogenesis with genotoxic and non-genotoxic mechanisms.

Category (2): rasH2-Tg mouse-positive non-genotoxic carcinogens (7 compounds, Tables 2, 6, 9)

Tumors induced by these compounds were observed in rasH2-Tg mice at AM/DR of 0.2- to 47-fold or more. Of these, bazedoxifene (33- to 54-fold AM), nilotinib (35-fold AM), and ozanimod (47- to 450-fold AM) increased tumors only at AM >25-fold in rasH2-Tg mice.

Four of the seven compounds were positive in 2-year rat studies. Of these compounds, the tumorigenic doses of clofibrate, sunitinib, and vascepa were within 5-fold AM/

Table 3. Tumorigenesis and HPRF of Category (3) rasH2-Tg Mouse-negative Non-genotoxic Carcinogens with Clear or Uncertain Human Relevance

Compound (Profile)	Model	6M rasH2-Tg mouse study				2Y mouse study				2Y rat study			
	Dose	VL	LD	MD	HD	VL	LD	MD	HD	VL	LD	MD	HD
Ampicillin[62, 63] (Antibiotic)	DR		0.9	2.4	7.3			2.6	5.2			2.6	5.2
	Tumor		−	−	−			−	−			+ (M)	+ (M)
	HPRF		−	−	−			+	+			+ (M)	+
Cyclosporin[42, 43, 64–66] (Immunosuppressant)	DR			0.04	0.1	0.001	0.004	0.02	0.1		0.005	0.02	0.09
	Tumor			−	±	−	−	−	+ (M)		−	−	−
	HPRF		NA			−	−	−	NA		NA		
Dulaglutide[67] (GLP-1 agonist)	AM		1.2	3.3	5.3					0.6	8.5	23	66
	Tumor		−	−	−		NA			−	+	+	+
	HPRF		+	+	+					−	+	+	+
Enzalutamide[68, 69] (Antiandrogen)	AM		0.1[a]	0.3[a]	1.0						0.3	NA	1.4
	Tumor		−	−	−		NA				+	+	+
	HPRF		−	−	−						+	+	+
17β-estradiol[23, 70–73] (Estrogen)	DR	0.5	2.4	9.8	24		0.1[b]	0.8[b]	4.2[b]				NA[c]
	Tumor	−	−	−	−		−	−	+ (F)				+
	HPRF	−	−	+	+		−	+ (F)	+ (F)				NA
Methapyrilene[74–76] (Antihistamine)	DR		26	52	103							2.0	4.1
	Tumor		−	−	−		NA					+	+
	HPRF		+	+	+							NA	NA
Raloxifene[49, 77, 78] (SERM)	AM				211		0.4	4.3	21	1.0 (M)	11	54	306 (F)
	Tumor				−		+ (F)	+	+	−	−	−	+
	HPRF				+		+	+	+	−	+	+	+
Tofacitinib[79, 80] (Immunosuppressant)	AM		2.3	8.9	20						7.0	25	67
	Tumor		−	−	−		NA				+ (M)	+	+
	HPRF		−	−	−						−	+ (M)	+ (M)

+: positive; −: negative; ±: equivocal; AM: AUC margin to MRHD based on AUC; DR: dose ratios of body-surface-based human equivalent doses to MRHD; F: Female; HD: high dose; HPRF: histopathologic risk factor; LD: low dose; M: male; MD: mid dose; NA: no data available or not done; VL: very low dose.

a, Not provided in the source data, but estimated on assumption that exposure increased dose-proportionally.

b, Female C3/HeJ-MMTV+ (high titer to murine mammary tumor virus) mice were used.

c, Three strains of rats were implanted with pellets of 5–6 mg 17β-estradiol twice at 4 weeks of age and 1 to 3 months later and kept until death.

DR in rasH2-Tg mouse and 2-year rat models, while those of bazedoxifene were 33-fold AM in rasH2-Tg mice for ovarian granulosa cell tumors, 0.1-fold AM for male rat and human irrelevant renal tumors, and 1.9-fold AM for female rat ovarian granulosa cell tumors. Thus, a 17-fold difference was found between rasH2-Tg mouse and rat models in susceptibility to ovarian carcinogenicity with bazedoxifene; however, small differences (0.2- to 7-fold) in carcinogenesis were found for the other three compounds.

Nilotinib, ozanimod, and troglitazone increased tumor incidence in rasH2-Tg mice at 12- to 47-fold AM and above; however, 2-year rat carcinogenicity studies revealed negative findings at the maximum tested doses. For nilotinib and ozanimod, no 2-year mouse studies were conducted, and the highest doses in the 2-year rat studies were lower (2.5-fold AM in nilotinib, 3.6-fold AM in ozanimod) than the tumorigenic AM in the rasH2-Tg mice studies. One possible factor that may have led to the negative rat carcinogenicity of these compounds is the lower MTD than the potential carcinogenic dose in rats owing to dose-limiting toxicity. Troglitazone was positive at 9.9- to 12-fold AM in the 2-year mouse study, but negative at 1.0- to 29-fold AM in the 2-year rat study. However, the high doses of AM were similar to the carcinogenic doses of rasH2-Tg mice. Ozanimod and troglitazone induced hemangiomas and hemangiosarcomas in rasH2-Tg or wild-type mice, but not in rats. Both classes of compounds are reported to induce the proliferation of vascular endothelial cells, specifically in mice via the sphingosine 1 (S1P) receptor (ozanimod) and hypoxia (troglitazone)[20, 21].

In the rasH2-Tg mouse study of ozanimod, the increase in the combined incidence of hemangioma and hemangiosarcoma was not statistically significant in females at the the low dose. However, biological significance in females at the low dose cannot be ruled out based on the drug class and dose-dependent increase. Tumorigenic AMs of ozanimod in males, females, and sex-combined were 54-, 38-, and 47-folds, respectively. Considering these factors, no compounds were found to increase tumor incidence only at doses greater than 50-fold AM.

Table 4. Tumorigenesis and HPRF of Category (4) rasH2-Tg Mouse-negative Non-genotoxic Rodent-specific Carcinogens

Compound (Profile)	Model	6M rasH2-Tg mouse study				2Y mouse study			2Y rat study			
	Dose	VL	LD	MD	HD	LD	MD	HD	VL	LD	MD	HD
Abiraterone[81, 82] (Androgen synthesis inhibitor; CYP17 inhibition)	AM		1.0	3.1	7.1				0.08[a] (M)	NA	0.8	1.5 (F)
	Tumor		−	−	−		NA		+	+	+	NA
	HPRF		+	+	+				+	+	+	NA
Aliskiren[83, 84] (Renin inhibitor; G.I. irritant)	AM		0.04 (F)	0.3	1.5					0.4	2.4	4.2
	Tumor		−	−	−		NA			−	−	+ (M)
	HPRF		−	+	+					−	+	+
Beclabuvir[25] (Antiviral; CYP inducer)	AM		0.1	1.0	7.2				2.0 (M)	3.5	14	36 (F)
	Tumor		−	−	−		NA		−	−	−	+
	HPRF		−	−	−				−	−	−	+
Cabozantinib[85] (Tyrosine kinase inhibitor)	AM		NA	0.8	2.5					0.1	0.2	0.7
	Tumor		−	−	−		NA			+ (M)	+	+
	HPRF		−	−	+					+ (F)	+ (F)	+ (F)
Chlorpromazine[86–89] (D2-R antagonist)	DR		1.0	1.9	3.8		NA			NA		
	Tumor		−	−	−		−			+		
	HPRF		+	+	+		NA			NA		
Filgotinib[90] (Immunosuppressant)	DR		1.0	3.0	13					0.9	1.7	6.0
	Tumor		−	−	−		NA			−	−	+ (M)
	HPRF		−	−	−					−	−	−
Haloperidol[91, 92] (D2-R antagonist)	DR	0.04 (M)	0.08 (F)	1.2 (M)	2.4 (F)		0.3	1.2				2.4
	Tumor	−	−	−	−		+ (F)	+ (F)				−
	HPRF	−	−	+	+		NA			NA		
Indacaterol[93, 94] (β2-stimulant)	AM		38	48	78					2.5	6.6	14
	Tumor		−	−	−		NA			−	−	±
	HPRF		+	+	+					−	−	+ (F)
Maraviroc[95, 96] (Antiviral; CYP inducer)	AM		7.2	15	46				0.8	2.8	11	15
	Tumor		−	−	−		NA		−	−	−	+
	HPRF		−	−	−				−	+ (M)	+	+
Metaproterenol[97, 98] (β2-stimulant)	DR		31	63	94		31	62				124
	Tumor		−	−	± (M)		+	+				± (F)
	HPRF		−	−	−		NA			NA		
Phenobarbital[99–102] (Anticonvulsant; CYP inducer)	DR		1.2	2.3	3.1	1.3	2.7	8.0				1.9
	Tumor		−	−	−	+	+	+				+
	HPRF		+	+	+	NA	NA	NA		NA		
Reserpine[103, 104] (Catecholamine depletion)	DR		3.1	6.3	11		4.3	8.6			4.1	8.2
	Tumor		−	−	−		+	+			+ (M)	+ (M)
	HPRF		−	+ (F)	+ (F)		−	−			−	−
Sulfamethoxazole[105, 106] (Antibiotic; Goitrogen)	DR		0.1	0.2	1.0				0.2[b]	0.7	1.5	2.9
	Tumor		−	−	−		NA		+	+	+	+
	HPRF		−	−	+					NA		
Suvorexant[107, 108] (Hypnotic; CYP inducer)	AM (M/F)	2.1	8.3	42	90				9.5 (F)	5.9/15	10 (M)	31/51
	Tumor	−	−	−	−		NA		−	−	+	+
	HPRF	−	+ (M)	+	+				−	+ (F)	+	+

+: positive; −: negative; ±: equivocal; AM: AUC margin to MRHD based on AUC; DR: dose ratios of body-surface-based human equivalent doses to MRHD; F: Female; HD: high dose; HPRF: histopathologic risk factor; LD: low dose; M: male; MD: mid dose; NA: no data available or not done; VL: very low dose.

a, Not provided in the source data, but estimated on assumption that exposure increased dose-proportionally.

b, In addition to these four doses, 25 mg/kg (0.1-fold) was selected for the lowest with tumorigenesis.

Category (3): rasH2-Tg mouse-negative non-genotoxic carcinogens with clear or uncertain human relevance (8 compounds, Tables 3, 7, 10)

Each of these compounds, with the exception of raloxifene in rats, increased tumor incidence at less than 25-fold AM/DR (0.1 to 8.5-fold AM/DR) in either rats or mice in a 2-year study. In the rasH2-Tg mouse studies, dulaglutide and methapyrilene induced HPRF at 1.2- and 26-fold AM/DR in C cells of the thyroid and the liver, respectively, in which organs tumors occurred in the 2-year studies.

Table 5. High Doses and HPRF of Category (5) rasH2-Tg Mouse-negative Non-carcinogens

Compound (Profile)	Model	6M rasH2-Tg mouse study				2Y mouse study			2Y rat study			
	Dose	VL	LD	MD	HD	LD	MD	HD	VL	LD	MD	HD
Asunaprevir[109] (Antiviral)	AM (M/F) HPRF		3.7 −	91 −	350 + (M)		NA			5.6/14 −	16/44 −	52/55 +
Baricitinib[110] (Immunosuppressant)	AM (M/F)		3.2/6.0	4.9/22	64/90		NA			1.0/2.9	2.8/9.2	6.9/31
Bictegravir[111] (Antiviral)	AM (M/F)		2.0/3.6	5.6/9.1	17/25		NA			NA		
Bixalomer[112] (Inhibition of phosphorus absorption)	DR[a]		0.7	2.2	8.5		NA			0.5	1.4	4.8
Cholestyramine[113–116] (Inhibition of cholesterol absorption)	DR[a]			1.5	3.1	0.6	1.2	2.9		0.5	1.0	2.2
Daclatasvir[117] (Antiviral)	AM		1.0	3.3	8.6		NA			0.2	0.8	4.7
Doravirine[118] (Antiviral)	AM HPRF		1.0 −	2.7 −	6.0 −		NA			0.5 −	2.3 −	7.4 + (M)
Etelcalcetide[119] (Ca receptor agonist)	AM (M/F)		0.04/0.03	0.1/0.09	0.2/0.3		NA		0.04	0.09	0.2	0.4
Evocalcet[120] (Ca receptor agonist)	AM (M/F) HPRF		1.1/0.9 −	4.4/3.0 −	14/12 +		NA			0.1/0.4 −	0.4/2.2 +	1.8/10 +
Glycopyrronium[121] (Muscarinic antagonist)	AM (M/F) HPRF		9.5/7.1[b] +	NA +	71/53 +		NA			18 +	48 +	79 +
Pasireotide[122] (Somatostatin receptor antagonist)	DR		1.1	2.3	5.7		NA			0.05	0.2	1.4
Rifaximin[123] (Antibiotic)	DR[a] (M/F)		0.6/1.0	2.0/3.1	6.1/8.1		NA			0.2	0.4	2.0
Sulfisoxazole[114, 124] (Antimicrobial)	DR HPRF		0.5 −	1.8 +	2.8 +		0.3 −	1.2 −			0.1 −	0.5 −
Tafamidis[125, 126] (Amyloidogenesis suppressant)	AM HPRF		1.1 −	3.3 + (M)	9.6 +		NA			3.4 +	9.5 +	18 +
Telbivudine[127] (Antiviral)	AM		3.8	6.4	14		NA			4.0	6.1	14[c]
Teneligliptin[128] (DPP-4 inhibitor)	AM (M/F) HPRF	1.1 −	5.0 −	25 +	123 +		NA			4.8/4.4 −	16/20 + (F)	65/77 + (F)
Vadadustat[129] (HIF-PH inhibitor)	AM		0.009	0.03	0.2		NA			0.01	0.09	0.3
Velpatasvir[130] (Antiviral)	AM		NA	25	74		NA			1.4	3.8	6.0

+: positive; −: negative; ±: equivocal; AM: AUC margin to MRHD based on AUC; DR: dose ratios of body-surface-based human equivalent doses to MRHD; F: Female; HD: high dose; HPRF: histopathologic risk factor; LD: low dose; M: male; MD: mid dose; NA: no data available or not done; VL: very low dose.

a, Non-absorbable drug.

b, Not provided in the source data, but estimated on assumption that exposure increased dose-proportionally.

c, Tumors increased but excluded from the analysis, since the high dose clearly exceeded the maximum tolerated dose.

In a 2-year rat carcinogenicity study of enzalutamide, many tumors, including Leydig cell tumors, were increased using the low dose of 0.3-fold AM; however, there was no increase in tumors or HPRF in the rasH2-Tg mice. Nevertheless, rasH2-Tg mice showed decreased vacuolation in Leydig cells at doses of 0.3-fold AM and higher, which may indicate alterations in the endocrine environment associated with tumorigenesis.

The rasH2-Tg mouse study of raloxifene only comprised one high dose (211-fold AM) with no tumors observed; however, ovarian interstitial cell hyperplasia as well as adrenal subcapsular hyperplasia were observed as HPRF. In the 2-year rat study, tumors, including granulosa and theca cell origin tumors of the ovary, were only observed at the high dose exceeding 300-fold AM and HPRFs were observed in the ovary and thymus at 11-fold AM and higher.

Table 6. Sensitivity Difference in Tumorigenesis between rasH2-Tg Mouse and 2-year Rodent Models (rasH2-Tg Mouse-positive Compounds)

Category	Compound	AM or DR					Tumor dose/tumor dose (rasH2-Tg mouse/2Y study)	
		6M rasH2-Tg mouse			2Y mouse	2Y rat		
		High dose	Tumor dose	HPRF dose	Tumor dose	Tumor dose	To mouse	To rat
(1) Genotoxic carcinogens	Cyclophosphamide	0.3	0.1	0.1	0.03	0.05	3.3	2.0
	Diethylstilbestrol	0.7	0.7	0.07	0.03	1.9	23	0.4
	Melphalan	0.6	0.6	NA	0.9	4.0	0.7	0.3
	Phenacetin	5.2	5.2	NA	2.5	4.4	2.1	1.2
	Procarbazine	0.1	0.05	NA	0.05	0.3	1.0	0.2
	Thiotepa	0.6	0.3	NA	0.4	0.4	0.8	0.8
(2) Non-genotoxic carcinogens	Bazedoxifene[a]	54	33	18	NA	1.9[b]	NA	17[b]
	Clofibrate	0.6	0.2	0.2	TN (0.9)[c]	1.2	NC	0.2
	Nilotinib[a]	35	35	35	NA	TN (2.5)[c]	NA	NC
	Ozanimod[a]	450	47	450	NA	TN (3.6)[c]	NA	NC
	Sunitinib[a]	7.7	5.0	5.0	NA	0.7	NA	7.1
	Troglitazone[a]	12	12	9.9	9.9	TN (29)[c]	1.2	NC
	Vascepa[a]	3.7	3.7	1.2	NA	3.0	NA	1.2

AM: AUC margin to MRHD based on AUC; DR: dose ratios of body-surface-based human equivalent doses to MRHD; NA: no available data; NC: not calculated; TN: tumor negative.
a, Margins are expressed based on AUC.
b, Comparison of ovarian tumors and HPRF.
c, AM or DR at the high dose.

On the other hand, in the 2-year mouse study, ovarian tumors including those of granulosa, theca cell and epithelial cell origin, cystic papillary and tubular hyperplasia of the ovary, and hyperplasia of other organs including those of males, developed at low doses (0.4-fold AM), which suggested that HPRF could be observed if lower doses had been tested in rasH2-Tg mice.

For 17β-estradiol, the frequency of cystic endometrial hyperplasia was decreased, although endometrial fibrosis and adrenal subcapsular cell hyperplasia (males) were increased at 9.8-fold DR or higher in rasH2-Tg mice. Compared with decreased proliferative lesions of the uterus in female rasH2-Tg mice, continued administration of 17β-estradiol was reported to increase its metabolism and decrease estrogen receptor-α expression in the uterus of rasH2-Tg mice, and downregulate the expression of various genes, including cell cycle genes[22]. In contrast, a 2-year mouse carcinogenicity study using mouse mammary tumor virus-high titer C3H/HeJ mice revealed increased adenocarcinoma of the uterus and mammary gland and mesothelioma of the uterus at 4.2-fold DR[23].

For the two immunosuppressants, no data for non-neoplastic changes were available (cyclosporin) or no HPRF developed (tofacitinib) in the rasH2-Tg mouse studies. Ampicillin, which increased mononuclear cell leukemia and pheochromocytoma development in F344 rats, also caused no increase in HPRF in rasH2-Tg mice, and no tumors were observed in the 2-year mouse study.

Category (4): rasH2-Tg mouse-negative non-genotoxic rodent-specific carcinogens (14 compounds, Tables 4, 7, 11)

In a 2-year rat study, abiraterone, aliskiren, cabozantinib, filgotinib, maraviroc, phenobarbital, reserpine, sulfamethoxazole, and suvorexant increased tumor incidence below 25-fold AM/DR (0.09- to 15-fold AM/DR), and indacaterol caused statistically insignificant increases in ovarian leiomyomas (14-fold AM). Beclabuvir (36-fold AM) and metaproterenol (124-fold DR, not statistically significant) increased the tumor incidence at dose levels above 25-fold AM/DR. Tumor incidences following the administration of haloperidol and metaproterenol increased at 0.3- and 31-fold DR, respectively, in the 2-year mouse studies, although the rat study was negative or did not show a statistically significant increase in tumor incidence. Although reported to have a positive outcome in the rat study, no information on carcinogenic doses was available for chlorpromazine in the 2-year rat study.

In the rasH2-Tg mouse study, abiraterone, aliskiren, haloperidol, phenobarbital, sulfamethoxazole, and suvorexant induced HPRF (0.3- to 8.3-fold AM/DR or higher) associated with carcinogenic target organs in the 2-year rat or mouse studies.

Cabozantinib, chlorpromazine, and indacaterol caused HPRF in the gastroduodenum (2.5-fold AM), liver (1.0-to 3.8-fold DR), and stomach (38- to 78-fold AM) of rasH2-Tg mice, respectively, but were not associated with tumor targets in the 2-year rat studies. In the 2-year carcinogenicity studies of reserpine, pheochromocytoma (male, 4.1-fold DR or more) in rats and mammary gland and seminal vesicle tumors were observed in mice (4.3-fold DR or more). In the rasH2-Tg mouse study of reserpine, increased ovarian

Table 7. HPRF Dose in rasH2-Tg Mouse Studies and Tumor Dose in 2-year Rodent Studies (rasH2-Tg Mouse-negative Compounds)

Category	Compound	AM or DR			
		6M rasH2-Tg mouse		2Y mouse	2Y rat
		High dose	HPRF dose[a]	Tumor dose	Tumor dose
(3) Non-genotoxic carcinogens with clear or uncertain human relevance	Ampicillin	7.3	HN	TN (5.2)[e]	2.6
	Cyclosporin	0.1	NA	0.1	TN (0.09)[e]
	Dulaglutide[b]	5.3	1.2	NA	8.5
	Enzalutamide[b]	1.0	HN	NA	0.3
	17β-estradiol	24	9.8	4.2	NA[h]
	Methapyrilene	103	26	NA	2.0
	Raloxifene[b]	211[c]	211[c]	0.4	306
	Tofacitinib[b]	20	HN	NA	7.0
(4) Non-genotoxic rodent-specific carcinogens	Abiraterone[b]	7.1	1.0	NA	0.09[d]
	Aliskiren[b]	1.5	0.3	NA	4.2
	Beclabuvir[b]	7.2	HN	NA	36
	Cabozantinib[b]	2.5	2.5	NA	0.1
	Chlorpromazine	3.8	1.0	NA[g]	NA[h]
	Filgotinib[b]	13	HN	NA	6.0
	Haloperidol	2.4	1.2	0.3	TN (2.4)[e]
	Indacaterol[b]	78	38	NA	14, equivocal
	Maraviroc[b]	46	HN	NA	15
	Metaproterenol	94	HN	31	124, equivocal
	Phenobarbital	3.1	1.2	1.3	1.9
	Reserpine	11	6.3	4.3	4.1
	Sulfamethoxazole	1.0	1.0	NA	0.1
	Suvorexant[b]	90	8.3	NA	10
(5) Non-genotoxic rodent non-carcinogens	Asunaprevir[b]	350	350	NA	TN (55)[e]
	Baricitinib[b]	90	HN	NA	TN (31)[e]
	Bictegravir[b]	25	HN	NA	NA
	Bixalomer	8.5	HN	NA	TN (4.8)[e]
	Cholestyramine	3.1	HN	TN (2.9)[e]	TN (2.2)[e]
	Daclatasvir[b]	8.6	HN	NA	TN (4.7)[e]
	Doravirine[b]	6.0	HN	NA	TN (7.4)[e]
	Etelcalcetide[b]	0.3	HN	NA	TN (0.4)[e]
	Evocalcet[b]	14	14	NA	TN (10)[e]
	Glycopyrronium[b]	71	9.5[d]	NA	TN (79)[e]
	Pasireotide	5.7	HN	NA	TN (1.4)[e]
	Rifaximin	8.1	HN	NA	TN (2.0)[e]
	Sulfisoxazole	2.8	1.8	TN (1.2)[e]	TN (0.5)[e]
	Tafamidis[b]	9.6	3.3	NA	TN (18)[e]
	Telbivudine[b]	6.1[f]	HN	NA	TN (14)[e]
	Teneligliptin[b]	123	25	NA	TN (71)[e]
	Vadadustat[b]	0.2	HN	NA	TN (0.3)[e]
	Velpatasvir[b]	91	HN	NA	TN (6.0)[e]

AM: AUC margin to MRHD based on AUC; DR: dose ratios of body-surface-based human equivalent doses to MRHD; NA: not available; HN: HPRF negative; HPRF: histopathologic risk factor; NA: no data; NC: not calculated; TN: tumor negative.
a, Dose level at which HPRF were found in the rasH2-Tg mouse studies.
b, Margins are expressed based on AUC.
c, Testing with one dose.
d, Estimated value from higher dose group.
e, AM or DR at the high dose.
f, 14-fold AM at the high dose was excluded from the analysis, since the high dose clearly exceeded the maximum tolerated dose with tumorigenesis.
g, 2-year mouse carcinogenicity study was negative, however detailed data was not available.
h, 2-year rat carcinogenicity study was positive, however detailed data was not available.

weight was observed as a change in endocrine environment that may be related to tumor development in the 2-year rodent studies at 6.3-fold AM or more.

In a 2-year rat study of filgotinib, Leydig cell tumors developed at 6-fold AM, and tumorigenesis was thought to be secondary to changes in the prolactin signaling pathway

Table 8. Tumor/HPRF of Category (1) rasH2-Tg Mouse-positive Genotoxic Carcinogens

Compound	Tumors/HPRF		
	6M rasH2-Tg mouse study	2Y mouse study	2Y rat study
Cyclophosphamide	**Urinary bladder**: transitional cell papilloma and carcinoma/ transitional cell hyperplasia **Harderian gland**: adenoma/ alveolar cell hyperplasia	**Hematopoietic system**: leukemia/NA	**Urinary bladder**: transitional cell papilloma and carcinoma/NA **Hematopoietic system**: leukemia/NA **Nervous system**: sarcoma/NA
Diethylstilbestrol	**Testis**: Leydig cell tumor/Leydig cell hyperplasia **Pituitary**: -/eosinophilic cell hyperplasia **Mammary gland**: -/ductal epithelium hyperplasia	**Pituitary**: adenoma and carcinoma[a,b]/ hyperplasia[a] **Mammary gland**: adenocarcinoma[a]/ hyperplasic alveolar nodules[a] **Thyroid**: follicular cell adenoma[b](M)/NA **Ovary**: tubular adenoma[a]/− **Uterine**: endometrial adenocarcinoma, mesothelioma[a]/adenosis[a]	**Pituitary**: pituitary tumor/enragement **Mammary gland**: fibroadenoma/− **Uterine**: −/hyperplasia and squamous metaplasia
Melphalan	**Stomach**: forestomach squamous cell papilloma and carcinoma/NA	**Lung**: tumor/NA **Hematopoietic system**: lymphosarcoma (M)/NA	**Peritoneum**: peritoneal sarcoma/NA
Phenacetin	**Lung**: adenoma/NA **Spleen**: hemangiosarcoma/NA	**Kidney**: cystic adenoma, cystpapillary adenoma, papillary adenoma, solid adenoma, tubulosolid carcinoma, tubulopapillary carcinoma and tubulopleomorphic carcinoma/ hyperplastic or dysplastic tubules **Urinary bladder**: −/papillary or nodular hyperplasia	**Kidney and urinary tract**: renal cell carcinoma, transitional cell carcinoma, papilloma/urothelial hyperplasia **Nasal cavity**: adenocarcinoma, transitional cell carcinoma, squamous cell carcinoma and adenoma/NA **Hematopoietic system**: leukemia/NA
Procarbazine	**Lung**: adenoma and adenocarcinoma/NA **Spleen**: hemangiosarcoma/NA	**Lung**: alveolar and bronchiolar adenoma/− **Nervous system**: olfactory neuroblastoma/− **Hematopoietic system**: malignant lymphoma, leukemia/− **Uterine**: adenocarcinoma/adenomatous hyperplasia	**Nervous system**: olfactory neuroblastoma/hyperplasia, squamous metaplasia **Hematopoietic system**: malignant lymphocytic lymphoma/lymphoreticular tissue hyperplasia **Mammary gland**: adenocarcinoma and cystadenocarcinoma/hyperplasia
Thiotepa	**Stomach**: forestomach squamous cell papilloma/NA	**Skin and associated glands**: squamous cell carcinoma (M)/− **Hematopoietic system**: lymphoma, lymphocytic leukemia/−	**Skin or ear canal**: squamous cell carcinoma/− **Hematopoietic system**: histiocytic malignant lymphoma, lymphocytic leukemia, granulocytic leukemia/− **Uterine**: adenocarcinoma/−

−: negative; HPRF: histopathologic risk factor; M: male; NA: no data available or not done.
a, Female C3H/HeN-MMTV- (low titer to murine mammary tumor virus) mice.
b, C57BL/6 mice.

caused by its pharmacological action, JAK inhibition[24]. In the rasH2-Tg mouse study of this compound, no hyperplasia of Leydig cells was observed; however, the degeneration of seminiferous tubules was observed at 13-fold AM. In the rasH2-Tg mouse studies of beclabuvir, maraviroc, and metaproterenol, no HPRF was observed at higher doses (7.2-fold, 46-fold, and 94-fold AM/DR, respectively).

Category (5): rasH2-Tg mouse-negative non-carcinogens (18 compounds, Tables 5, 7, 12)

The high doses of 5 of the 18 compounds in rasH2-Tg mouse studies were >50-fold AM/DR while those of four of these five compounds were >25-fold AM in rat carcinogenicity studies.

Among the 18 compounds, three are poorly absorbable: bixalomer (phosphorus absorption inhibition), cholestyramine (cholesterol absorption inhibition), and rifaximin (antibiotic). Clinical AUC values for these compounds were not obtained, and their high doses were <10-fold DR. In the rasH2-Tg mouse and 2-year rat studies of the three compounds, with the exception of the 2-year rat study of rifaximin, the high dose was administered as the limit dose or the maximum feasible dose (Supplementary Table 5). The development of HPRF was observed with six compounds, all of which showed HPRF at <50-fold AM (25-fold AM and lesser), except for one compound (asunaprevir), which showed centrilobular hypertrophy of hepatocytes at 350-fold AM.

Table 9. Tumor/HPRF of Category (2) rasH2-Tg Mouse-positive Non-genotoxic Carcinogens

Compound	Tumors/HPRF		
	6M rasH2-Tg mouse study	2Y mouse study	2Y rat study
Bazedoxifene	**Ovary**: granulosa cell tumor/interstitial cell hyperplasia **Adrenal**: −/subcapsular cell hyperplasia **Uterus**: −/− (stromal mucification, atrophy)[f]	NA	**Ovary**: granulosa cell tumor/granulosa cell hyperplasia **Kidney**: tubule adenoma, carcinoma (M)/−
Clofibrate	**Liver**: hepatocellular adenoma/increased weight	**Liver**: −/increased weight	**Liver**: hepatocellular carcinoma/hyperplastic nodule **Pancreas**: acinar cell carcinoma/−
Nilotinib	**Skin**: papilloma, carcinoma/epidermal hyperplasia	NA	**Uterus**: −(equivocal hemangiosarcoma)/endothelial and epithelial hyperplasia
Ozanimod	**Multiple organs**: hemangioma[a] and hemangiosarcoma[b]/− **Stomach**:−/glandular stomach mucosal hyperplasia	NA	−/−
Sunitinib	**Stomach**: carcinoma/foveolar hyperplasia **Duodenum**: carcinoma/− **Spleen**: hemangiosarcoma (F)/− **Uterus**: hemangiosarcoma/−	NA	**Duodenum**: carcinoma/− **Adrenal gland**: pheochromocytoma (M)/ hyperplasia of adrenal medulla (M) **Stomach**: −/glandular stomach mucosal cell hyperplasia (M)
Troglitazone	**Multiple organs**: hemangioma[c] and hemangiosarcoma[d]/− **Brown adipose tissue**: −/hypertrophy **Liver**: −/− (increased weight with centrilobular vacuolar degeneration and single cell necrosis)[e]	**Multiple organs**: hemangiosarcoma/− **Liver**: hepatocellular carcinoma (F)/− **Brown adipose tissue**: −/hypertrophy and hyperplasia	**Liver**: −/centrilobular hepatocellular hypertrophy **Brown adipose tissue**: −/hypertrophy and hyperplasia
Vascepa	**Skin and subcutis**: proximal tail squamous cell papilloma (M)/acanthosis, hyperkeratosis (erosion, ulceration, inflammation)[e] **Stomach**: −/ forestomach hyperplasia, hyperkeratosis (inflammation)[e]	NA	**Mesenteric lymph node**: hemangioma/− **Skin, subcutis**: fibroma, fibrosarcoma and sarcoma (NOS) (M)/− **Brain, spinal cord**: astrocytoma (M)/− **Thyroid**: −/C-cell hyperplasia (M) **Stomach**: −/forestomach mucosal hyperplasia **Larynx**: −/squamous epithelial cell hyperplasia (M)

−: negative; ±: equivocal; F: Female; HPRF: histopathologic risk factor; M: male; NA: no data available or not done; NOS: not otherwise specified.
a, Skin (M), mammary gland, ovary.
b, Spleen, femur bone marrow, ileum, jejunum (M), rectum (M), lung (M), skin, lumber (M), spinal cord (M), skeletal muscle.
c, Skin (F), jejunum (M), uterus.
d, Spleen, lung (F), skin (F).
e, Changes other than HPRF that may be factors in tumorigenesis (chronic inflammation and/or tissue damage).
f, Changes other than HPRF that may be factors in tumorigenesis (altered endocrine environment).

Relationship between high dose and tumor development (Tables 6, 7)

Among the 13 compounds positive in rasH2-Tg mice [Categories (1) and (2)], two compounds (bazedoxifene and ozanimod, both non-genotoxic) were administered at high doses of 50-fold AM or higher. Tumors occurred at doses <50-fold AM of these two compounds and occurred at doses >25-fold AM of three compounds (bazedoxifene, ozanimod, and nilotinib). In the rat studies, the high doses of all 13 of these compounds were <25-fold AM/DR (0.4 to 8.9-fold), except for troglitazone with 29-fold AM in males (Tables 1, 2), and 10 of these compounds increased tumorigenesis at 0.05- to 4.4-fold AM/DR.

For the 40 compounds that were negative in rasH2-Tg mice, high doses of 10 compounds exceeded 50-fold AM/DR. Of these, seven compounds had high doses exceeding 25-fold in the rat carcinogenicity studies (Tables 3–5): three compounds were positive for rat carcinogenicity, and seven compounds tested negative. In summary, among the 53 compounds tested in the rasH2-Tg mouse study, 12 (23%) had high doses of 50-fold AM/DR or higher, but none only caused tumor development at 50-fold AM/DR or higher.

HPRFs for rodent neoplasia (Tables 6, 7)

In the rasH2-Tg mouse studies of many of the compounds investigated in this study, hypertrophic/hyperplastic changes, altered cellular foci, and pre-neoplastic changes were found as HPRF[17], with or without carcinogenesis.

For the 6 rasH2-Tg mouse-positive genotoxic compounds [Category (1)], data on non-neoplastic lesions in the rasH2-Tg mouse studies for cyclophosphamide and DES were available, and both of these compounds induced tumor-related proliferative lesions at doses above 0.1 and 0.07-fold DR, respectively. In the rasH2-Tg mouse-positive

Table 10. Tumor/HPRF of Category (3) rasH2-Tg Mouse-negative Non-genotoxic Carcinogens with Clear or Uncertain Human Relevance

Compound	Tumors/HPRF		
	6M rasH2-Tg mouse study	2Y mouse study	2Y rat study
Ampicillin	−/−	**Stomach**: −/forestomach hyperkeratosis and acanthosis	**Hematopoietic system**: mononuclear cell leukemia/hematopoietic hyperplasia of bone marrow **Adrenal**: benign and malignant pheochromocytoma/− **Stomach**: −/forestomach hyperkeratosis and acanthosis **Thyroid**: −/C-cell hyperplasia
Cyclosporin	**Stomach**: equivocal forestomach papilloma/NA **Skin**: papilloma and squamous cell carcinoma/NA	**Thymus**: thymic lymphoma/−	−/NA
Dulaglutide	**Thyroid**: −/C-cell hypertrophy	NA	**Thyroid**: C-cell adenoma and carcinoma/C-cell hyperplasia
Enzalutamide	−/− (decreased vacuolation in Leydig cells)[a]	NA	**Testis**: Leydig cell tumor/Leydig cell hyperplasia **Thymus**: benign thymoma/− **Pituitary**: adenoma/pars distalis hyperplasia **Mammary gland**: fibroadenoma/− **Ovary**: benign granulosa cell tumor/granulosa cell hyperplasia **Urinary bladder**: urothelial adenoma and adenocarcinoma/urothelial hyperplasia (calculi)[c]
17β-estradiol	**Adrenal**: −/subcapsular cell hyperplasia (M) **Uterus**: −/− (endometrial fibrosis, decreased cystic endometrial hyperplasia)[a]	**Mammary gland**: adenocarcinoma/ hyperplastic alveolar nodules[b] **Uterus**: adenocarcinoma, mesothelioma/cervical adenosis[b]	**Pituitary**: adenoma/NA **Mammary gland**: adenocarcinoma, papillary carcinoma, anaplastic carcinoma/NA
Methapyrilene	**Salivary gland**: −/acinar cell hypertrophy **Liver**: −/hepatocytes hypertrophy, proliferation of small bile duct	NA	**Liver**: hepatocellular carcinoma, cholangiocarcinoma, hepatocellular neoplastic nodules/NA
Raloxifene	**Ovary**: −/interstitial cell hyperplasia **Adrenal**: −/subcapsular cell hyperplasia **Uterus**: −/− (stromal mucificaton, atrophy)[a]	**Ovary**: benign and malignant tumors of granulosa, theca cell and corpus luteum origin and benign tumors of epithelial cell origin/cystic papillary hyperplasia, tubular hyperplasia (cyst, persistent hemorrhagic follicular dilatation)[a] **Uterus**: −/deciduoma, diffuse papillary mucosal hyperplasia (atrophy, decreased cystic endometrial change)[a] **Testis**: benign and malignant Leydig cell tumor/Leydig cell hyperplasia **Prostate**: adenoma and adenocarcinoma/− **Mammary gland**: −/−(atrophy)[a]	**Ovary**: granulosa and theca cell origin tumor/ (follicular prominence)[a] **Kidney**: renal cell carcinoma (M)/− **Thymus**: −/epithelial hyperplasia **Uterus, mammary gland**: −/−(atrophy)[a]
Tofacitinib	−/−	NA	**Testis**: Leydig cell tumor/ Leydig cell hyperplasia **Brown adipose tissue**: malignant hibernoma/− **Thymus**: benign thymoma/− **Mesenteric lymph node**: hemangioma/−

−: negative; HPRF: histopathologic risk factor; NA: no data available or not done.

a, Changes other than HPRF that may be factors in tumorigenesis (altered endocrine environment).

b, Female C3H/HeJ MMTV^{+} (high titer to murine mammary tumor virus) mice.

c, Changes other than HPRF that may be factors in tumorigenesis (chronic inflammation and/or tissue damage).

Table 11. Tumor/HPRF of Category (4) rasH2-Tg Mouse-negative Non-genotoxic Rodent-specific Carcinogens

Compound	Tumors/HPRF		
	6M rasH2-Tg mouse study	2Y mouse study	2Y rat study
Abiraterone	**Testis**: −/ Leydig cell hyperplasia (testicular atrophy)[a]	NA	**Testis**: Leydig cell tumor/focal Leydig cell hyperplasia (atrophy of male reproductive organs)[a]
Aliskiren	**Intestine**: −/mucosal hypertrophy and hyperplasia	NA	**Intestine**: colonic adenoma, cecal adenocarcinoma/large intestine mucosal epithelium hyperplasia (erosion, ulceration)[b]
Beclabuvir	−/−	NA	**Liver**: hepatocellular adenoma/basophilic altered foci
Cabozantinib	**Stomach**: −/glandular epithelium hyperplasia **Duodenum**: −/epithelium hyperplasia	NA	**Adrenal**: benign and malignant pheochromocytoma/adrenomedullary hyperplasia
Chlorpromazine	**Liver**: −/hepatocellular hypertrophy	−/NA	**Pancreas**: pancreatic tumor (M)/NA
Filgotinib	**Testis**: −/− (tubular degeneration, atrophy)[a]	NA	**Testis**: Leydig cell tumor/− (testis tubular degeneration, atrophy)[a]
Haloperidol	**Pituitary**: −/hypertrophy **Mammary gland**: −/ development	**Pituitary**: neoplasia/NA **Mammary gland**: neoplasia/NA	−/NA
Indacaterol	**Stomach**: −/mucous neck cell hyperplasia	NA	**Ovary**: equivocal mesovarian leiomyoma/ smooth muscle focal hyperplasia
Maraviroc	−/−	NA	**Thyroid**: follicular cell adenoma/follicular cell hypertrophy and hyperplasia
Metaproterenol	**Liver**: equivocal hepatocellular adenoma/−	**Ovary**: mesovarian leiomyoma/NA **Liver**: hepatocellular adenoma /NA	**Ovary**: equivocal mesovarian leiomyoma/ NA
Phenobarbital	**Liver**: −/hepatocellular hypertrophy	**Liver**: hepatocellular adenoma and carcinoma/NA	**Liver**: benign hepatic neoplasms/NA
Reserpine	**Ovary**: −/increased weight	**Mammary gland**: malignant tumors (F)/−(cystic duct)[a] **Seminal vesicle**: carcinoma/− (chronic inflammation)[b]	**Adrenal**: pheochromocytoma/− **Uterus**:−/− (decreased hyperplasia, cystic, NOS)[a]
Sulfamethoxazole	**Thyroid**: −/follicular cell hypertrophy, hyperplasia	NA	**Thyroid**: nodules and adenomas/NA
Suvorexant	**Liver**: −/hepatocellular hypertrophy	NA	**Liver**: hepatocellular adenoma/ hepatocellular hypertrophy, eosinophilic altered foci **Thyroid**: follicular cell adenoma/follicular cell hypertrophy, focal follicular cell hyperplasia

−: negative; F: female; HPRF: histopathologic risk factor; M: male; NA: no data available or not done; NOS: not otherwise specified.
a, Changes other than HPRF that may be factors in tumorigenesis (altered endocrine environment).
b. Changes other than HPRF that may be factors in tumorigenesis (chronic inflammation and/or tissue damage).

non-genotoxic compounds [Category (2)], five compounds, including bazedoxifene, clofibrate, nilotinib, sunitinib, and vascepa induced ovarian, hepatic, skin, and gastric HPRF at doses close to their tumorigenic doses. Ozanimod and troglitazone resulted in hemangiomas and hemangiosarcomas in rasH2-Tg mouse studies; however, HPRF, which was not associated with these tumors, was observed at 450-fold and 9.9-fold and higher AM, respectively (Tables 2 and 9).

Among 22 rasH2-Tg mouse-negative non-genotoxic rodent carcinogens [Categories (3) and (4)], 14 compounds were associated with HPRF, but did not lead to carcinogenesis. Among these compounds, raloxifene and indacaterol induced HPRF and no tumors at 211-fold AM and 38-fold AM, respectively. Raloxifene was tested in a rasH2-Tg mouse study at a single dose of 211-fold AM and caused ovarian interstitial cell hyperplasia (Table 10); which was presumed to occur at lower doses, as described above. Indacaterol induced HPRF of stomach mucous neck cell hyperplasia, which was unrelated to ovarian tumors occurring in rats (Table 11). Gastroduodenal epithelial hyperplasia induced

Table 12. HPRF of Category (5) rasH2−Tg Mouse−negative Non−carcinogens

Compound	HPRF		
	6M rasH2−Tg mouse study	2Y mouse study	2Y rat study
Asunaprevir	**Liver**: centrilobular hepatocellular hypertrophy	NA	**Liver**: bile duct hyperplasia
Baricitinib	−	NA	−
Bictegravir	−	NA	NA
Bixalomer	−	NA	−
Cholestyramine	−	−	−
Daclatasvir	−	NA	−
Doravirine	−	NA	**Liver**: centrilobular hepatocellular hypertrophy
Etelcalcetide	−	NA	−
Evocalcet	**Stomach**: forestomach squamous cell hyperplasia	NA	**Thyroid**: C-cell hyperplasia **Kidney**: renal urothelial hyperplasia
Glycopyrronium	**Stomach**: forestomach squamous cell hyperplasia	NA	**Olfactory and respiratory tract**: epithelial hyperplasia
Pasireotide	−	NA	−
Rifaximin	−	NA	−
Sulfisoxazole	**Thyroid**: follicular cell hypertrophy and hyperplasia	−	−
Tafamidis	**Liver**: centrilobular hepatocellular hypertrophy	NA	**Liver**: centrilobular hepatocellular hypertrophy, clear cell foci
Telbivudine	−	NA	−[a]
Teneligliptin	**Stomach**: focal forestomach squamous cell hyperplasia **Gall bladder**: focal mucosal hyperplasia **Liver**: diffuse hepatocellular hypertrophy **Urinary bladder**: diffuse transitional cell hyperplasia	NA	**Thymus**: epithelial hyperplasia
Vadadustat	−	NA	−
Velpatasvir	−	NA	−

−: negative; HPRF: histopathologic risk factor; NA: no data available or not done.
a, Pancreatic acinar cell adenoma, pheochromocytoma and mammary fibroadenoma increased at high dose were excluded from statistical analysis, since the high dose clearly exceeded the maximum tolerated dose.

by cabozantinib at 2.5-fold AM was a hyperplasia unrelated to pheochromocytoma occurring in the rat carcinogenicity study (Table 11). Chlorpromazine induced HPRF in the livers of rasH2-Tg mice at 1.0-fold DR or higher, but induced tumors of the pancreas in the 2-year rat study (Table 11). Estrogen (17β-estradiol) induced adrenal subcapsular cell hyperplasia in male rasH2-Tg mice with 9.8-fold DR, which is unrelated to the tumors of the mammary gland, uterus, and pituitary in 2-year studies of mice and rats (Table 10). In addition, HPRF associated with carcinogenesis in rats or mice was observed at doses of 0.3- to 26-fold AM/DR in the thyroid (C cells, follicular cells), testis (Leydig cells), uterus, liver, mammary gland, pituitary gland, ovary, and intestinal tract of rasH2-Tg mice administered the nine compounds (dulaglutide, methapyrilene, abiraterone, aliskiren, haloperidol, phenobarbital, reserpine, sulfamethoxazole, and suvorexant, Tables 10 and 11).

An immunosuppressant (tofacitinib), an antibiotic (ampicillin; induces mononuclear cell leukemia and pheochromocytoma in rats), an antiandrogen (enzalutamide), two CYP inducers (beclabuvir, maraviroc), and a β2-stimulant (metaproterenol; induces mesovarian leiomyoma) (Tables 10 and 11), did not induce HPRF in rasH2-Tg mice when administered at doses of 0.1, 20, 7.3, 1.0, 7.2, 46, and 94-fold AM/DR, respectively. The immunosuppressant filgotinib was associated with testicular seminiferous tubule degeneration at 13-fold AM in rasH2-Tg mice, but not testicular Leydig cell hyperplasia associated with Leydig cell tumors occurring in rats (Table 11).

For the 18 compounds that were negative in both rasH2-Tg mouse and 2-year rat studies, hypertrophy and hyperplasia occurred in the liver, thyroid gland, forestomach, gall bladder, and urinary bladder with 6 compounds in rasH2-Tg mice. Except for one compound (asunaprevir) that caused centrilobular hepatocyte hypertrophy at 350-fold AM, these HPRF occurred at doses of 25-fold AM or less (Table 12).

These results indicate that 1) for the non-genotoxic carcinogens in categories (2), (3), and (4), 21 of 28 (75%) compounds for which HPRF data were available caused the development of HPRF in rasH2-Tg mice. Further, in 14 of those compounds (67%), HPRF was associated with tumorigenesis in the related studies, and occurred at less than 50-

fold AM/DR (26-fold AM/DR or lower); 2) for non-carcinogens of category (5), the frequency of HPRF in rasH2-Tg mice was lower (6/18 compounds, 33%) than that of the non-genotoxic carcinogens of categories (2), (3) and (4); and 3) for non-genotoxic carcinogens and non-carcinogens of categories (2) to (5), 13 of 46 (28%) compounds induced HPRF in rasH2-Tg mice but were not associated with tumorigenesis in the related studies, occurring at less than 50-fold AM (38-fold AM or lower), except for ozanimod (445-fold) and asunaprevir (350-fold).

Carcinogenic doses in 2-year rat or mouse studies (Tables 6, 7)

Twenty-eight of the 35 rodent carcinogens [Categories (1) to (4)] were positive in 2-year rat studies. The carcinogenic doses of the 24 compounds were <25-fold AM/DR. Raloxifene (306-fold AM) and beclabuvir (36-fold AM) were >25-fold at carcinogenic doses in rats; however, drug-induced tumors were found in raloxifene 2-year mouse studies at 0.4 to 21-fold AM. Although beclabuvir has not been subjected to a 2-year mouse study, the tumor observed in rats was a hepatocellular tumor that was not extrapolated to humans[25]. 17β-estradiol and chlorpromazine were not available in the comparative rat studies. In addition, indacaterol and metaproterenol induced an equivocal increase in mesovarian leiomyoma at 14-fold AM and 124-fold DR, respectively.

A 2-year mouse study was conducted for 17 of the 35 tumorigenic compounds. With the exception of ampicillin, clofibrate, chlorpromazine, and metaproterenol, drug-induced tumors were observed at <25-fold AM/DR (9.9-fold or lower) in 13 compounds. The 2-year mouse studies of ampicillin and clofibrate were negative at 2.6- to 5.2-fold DR and 0.4- to 0.9-fold DR, respectively, whereas the 2-year rat studies showed drug-induced tumors at 2.6- to 5.2-fold DR and 1.2- to 1.9-fold DR, respectively. No data on the study doses were available for chlorpromazine. In a 2-year mouse study of metaproterenol, drug-induced and human-irrelevant mesovarian leiomyoma and liver tumors were observed at 31 to 62-fold DR, while the 2-year rat study showed an equivocal increase in mesovarian leiomyoma at 124-fold DR. Among the five compounds (nilotinib, ozanimod, troglitazone, cyclosporin, and haloperidol) that were negative in the rat studies, drug-induced tumors were found in 2-year mouse studies of troglitazone, cyclosporin, and haloperidol at 9.9- to 12-fold AM, 0.1-fold DR, and 0.3- to 1.2-fold DR, respectively. Nilotinib and ozanimod were not tested in the 2-year mouse study and were positive in the rasH2-Tg mouse study.

These results confirm that the carcinogenic risk of 29 compounds, except for chlorpromazine, for which data could not be confirmed; for indacaterol, metaproterenol, and beclabuvir for equivocal increase or increase in human-irrelevant tumors at doses above 25-fold AM; and for nilotinib and ozanimod, for which no 2-year mouse study was conducted, can be identified in 2-year rodent studies at 25-fold AM/DR or less.

In this study, we investigated the relationship between dose levels tested and tumor and HPRF development for 53 compounds that were tested in rasH2-Tg mouse studies. The findings revealed the following:

1) The tumorigenic doses of all 13 compounds that were positive in the rasH2-Tg mouse model were positive at <50-fold AM/DR, and higher than 25-fold AM in three compounds (bazedoxifene, nilotinib, and ozanimod).

2) Although relative tumorigenic sensitivity can vary between rasH2-Tg mouse and 2-year rodent bioassay models, the rasH2-Tg mouse model is not inherently less sensitive than either the 2-year rat or mouse models. Similar sensitivities were most apparent among the six genotoxic carcinogens.

3) The 2-year rat, 2-year mouse, and 6-month rasH2-Tg mouse models can yield a lone positive response to non-genotoxic carcinogens when the other models are negative, which may be due to differences in tolerability, pharmacologic responsiveness, or metabolism.

4) Approximately 75% of the non-genotoxic carcinogens (categories (2) to (4)) that were positive for rasH2-Tg mice or rasH2-Tg mouse-negative rodent carcinogens developed HPRF in rasH2-Tg mice, 67% of which were associated with these tumors at less than 50-fold AM/DR.

5) Approximately 28% of the non-genotoxic carcinogens and non-carcinogens (categories (2) to (5)) developed HPRF that was not associated with tumorigenesis in related studies at less than 50-fold AM/DR, except for two compounds (ozanimod and asunaprevir) that first yielded HPRF at doses exceeding 50-fold (350-fold and higher) AM.

In conclusion, when high dose exposures are tolerated in rasH2-Tg mice, exceeding 25-fold might be of value; however, the overall evidence indicates that there is no benefit of exceeding a 50-fold exposure margin.

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