Regulatory Toxicology and Pharmacology 81 (2016) 242–249

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Prediction of carcinogenic potential of chemicals using repeated-dose (13-week) toxicity data

Ruud A. Woutersen [a, b, *], Ans E.M.F. Soffers [b], E. Dinant Kroese [a], Cyrille A.M. Krul [a, e], Jan Willem van der Laan [d], Jan van Benthem [c], Mirjam Luijten [c]

[a] TNO Innovation for Life, P.O. Box 360, 3700 AJ, Zeist, The Netherlands
[b] Division of Toxicology, Wageningen University and Research Centre, P.O. Box 8000, 6700 EA, Wageningen, The Netherlands
[c] National Institute for Public Health and the Environment (RIVM), Centre for Health Protection, P.O. Box 1, 3720 BA, Bilthoven, The Netherlands
[d] Medicines Evaluation Board, Section on Pharmacological, Toxicological and Biotechnological Assessment, P.O. Box 8275, 3503 RG, Utrecht, The Netherlands
[e] University of Applied Sciences Utrecht, Institute for Life Sciences & Chemistry, P.O. Box 12011, 3501 AA, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form
30 August 2016
Accepted 5 September 2016
Available online 8 September 2016

Keywords:
Rat
Sub-chronic toxicity
Carcinogenicity
Non-genotoxic carcinogens
Preneoplastic lesions
Tumours
Predictivity
Risk assessment

ABSTRACT

Sub-chronic toxicity studies of 163 non-genotoxic chemicals were evaluated in order to predict the tumour outcome of 24-month rat carcinogenicity studies obtained from the EFSA and ToxRef databases. Hundred eleven of the 148 chemicals that did not induce putative preneoplastic lesions in the sub-chronic study also did not induce tumours in the carcinogenicity study (True Negatives). Cellular hypertrophy appeared to be an unreliable predictor of carcinogenicity. The negative predictivity, the measure of the compounds evaluated that did not show any putative preneoplastic lesion in de sub-chronic studies and were negative in the carcinogenicity studies, was 75%, whereas the sensitivity, a measure of the sub-chronic study to predict a positive carcinogenicity outcome was only 5%. The specificity, the accuracy of the sub-chronic study to correctly identify non-carcinogens was 90%. When the chemicals which induced tumours generally considered not relevant for humans (33 out of 37 False Negatives) are classified as True Negatives, the negative predictivity amounts to 97%. Overall, the results of this retrospective study support the concept that chemicals showing no histopathological risk factors for neoplasia in a sub-chronic study in rats may be considered non-carcinogenic and do not require further testing in a carcinogenicity study.

1. Introduction

Cancer is one of the leading causes of death in industrialized countries. In those countries the cancer rate has risen from 1 in 10 in 1930 to 1 in 3 today. According to the American Cancer Society, almost 45% of men and 38% of women will be diagnosed with cancer at some point in their lives (American Cancer Society, 2014). Cancer is responsible for 7.6 million deaths worldwide per year, with 3 million new cancer cases per year in Europe alone (WHO, 2011).

Whilst there is no single cause of cancer, evidence is emerging that exposures to chemical substances in our everyday life may be contributing to the increasing cancer burden. Past occupational exposure to known or probable carcinogens is estimated to account for 5.3% (8023) of cancer deaths and 4% of cancer registration occurring each year in Great Britain (HSE, 2014).

Industry currently uses thousands of substances that have not yet been tested for their effect on human health. The EU REACH (Registration, Evaluation, Authorization and restriction of CHemicals) (EC, 2007) aims to evaluate any substance produced or imported in significant quantities unless sufficient safety information already exists. When applying the traditional toxicity tests this will cost more than 200 years to be completed. In the worst-case scenario, 2.9 million animals would be needed for testing all these chemicals (Van der Jagt et al., 2004). This is ethically and economically not defensible. Therefore, alternative methods for toxicity testing are warranted.

Much of what we know about chemicals and cancer comes from studies with animals, long-term follow-up of workers exposed to chemicals at their workplace, and epidemiological studies in

* Corresponding author. TNO Innovation for Life, Utrechtseweg 48, 3704 HE, Zeist, The Netherlands.
E-mail addresses: ruud.woutersen@tno.nl, ruud.woutersen@iCloud.com (R.A. Woutersen).

http://dx.doi.org/10.1016/j.yrtph.2016.09.003

communities where residents are exposed to hazardous agents. Although a number of factors have been related to the induction of cancer, it is not possible to predict with complete certainty from animal studies alone which chemicals under which exposure circumstances will be carcinogenic in humans. The current 2-year rodent carcinogenicity study (OECD, 2009) has been the regulatory standard in safety assessments of environmental chemicals, and is presumed to forecast potential long-term human cancer risks of exposure to industrial chemicals. Under REACH, relevant factors that may trigger a two-year carcinogenicity bioassay in this respect include: i) genotoxicity (germ cell mutagen category 2); ii) evidence of treatment-related hyperplasia and/or preneoplastic lesions in the sub-chronic study; iii) previous demonstration of carcinogenic potential in the chemical class or product that is considered relevant to humans; iv) widespread use or evidence of frequent long-term human exposure.

A positive test result in an *in vivo* genotoxicity test is generally considered indicative of the carcinogenic potential of a chemical. For pharmaceuticals, agrochemicals and most consumer products, a positive result in an assay for DNA reactivity will usually preclude further development (ICH, 1995; Snyder and Green, 2001). Under REACH (EC, 2007), the requirement to perform a carcinogenicity study is conditional, since the default presumption is that if a substance is classified as germ cell mutagen category 1A or 1B, a genotoxic mechanism for carcinogenicity is likely.

There is, however, considerable scientific doubt about the reliability of the carcinogenicity bioassay, since too many false positive outcomes have been observed (Van Oosterhout et al., 1997; Cohen, 2004; Boobis et al., 2009) concerns raised about the predictive value of *in vivo* studies in general (Jacobson-Kram et al., 2004; Anisimov et al., 2005; Billington et al., 2010; Osimitz et al., 2013) and the push for refinement, reduction, and replacement of animal studies, resulted in the recommendation to re-evaluate the 2-year rodent bioassay as the best approach to predict human disease (Cohen, 2004; Ward, 2007; Boobis et al., 2009; Friedrich and Olejniczak, 2011; Sistare et al., 2011; Benigni, 2012; Doktorova et al., 2012; Gori, 2013).

Reddy et al. (2010) and Sistare et al. (2011) concluded that pharmaceuticals that showed absence of histopathological risk factors, such as hyperplasia, hypertrophy, foci of cellular alteration, and cell proliferation, for neoplastic lesions in any tissue in rats in the 6- or 12-month study may be considered non-carcinogens and do not require testing in a two-year carcinogenicity study, provided that these pharmaceuticals lack genotoxic potential and fail to induce hormonal perturbations.

The sub-chronic (13-week) rat study (OECD, 1998), which includes endpoints as clinical chemistry and histopathology of a wide range of organs and tissues, is currently required by regulatory bodies worldwide as the first tier in safety testing of foods and chemicals. In the present paper, we further investigated the hypothesis of Reddy et al. (2010) and Sistare et al. (2011) that chemicals showing no histopathological risk factors for carcinogenicity in an extended sub-chronic toxicity study may be considered non-carcinogenic (the so called “whole animal negative predictivity hypothesis”), using 202 chemicals for which adequate data from sub-chronic (13-week) and carcinogenicity (24-month) studies were present in the ToxRef EPA- and EFSA database.

2. Materials and methods

2.1. Data sources, and data inclusion and exclusion criteria

The rat sub-chronic toxicity and chronic carcinogenicity data used for this evaluation came from two different sources: the European Food Safety Agency (EFSA) database (www.efsa.europa.eu/publications) and the ToxRef EPA database (www.epagov/pesticides/science/comptox-glossary.html#toxrefdb). The data used are all publicly available and have been derived from studies performed according to OECD Test Guidelines.

This evaluation focused on sub-chronic (13-week) toxicity and carcinogenicity (24-month) studies conducted in rats. The criteria applied for inclusion of the studies were similar to those reported previously by Reddy et al. (2010) and Sistare et al. (2011). These are based on the study outcomes (putative preneoplastic lesions in sub-chronic studies, and tumours in carcinogenicity studies, resp.) and the dose levels used. Apart from a few exceptions, the short- en long-term studies were performed with the same strain of rats.

In short, studies were included in the evaluation when:

- the dose levels in the sub-chronic study showed any overlap with those of the carcinogenicity study;
- the sub-chronic study was negative (did not demonstrate putative preneoplastic lesions) at doses higher than those used in the carcinogenicity study;
- the sub-chronic study was positive (demonstrated putative preneoplastic histopathological changes) even if the doses were less than 75% of the doses used in the carcinogenicity study; or
- the sub-chronic study was negative and the top dose was less than 75% of the top-dose used in the carcinogenicity study, but lower doses in the carcinogenicity study matched those in de sub-chronic study and positive tumour findings occurred.

Substances were excluded on the basis of two criteria:

- the sub-chronic study was positive (demonstrated putative preneoplastic lesions) only at a dose that was over 25% higher than the highest dose used in the carcinogenicity study, and
- the highest dose in the sub-chronic study was negative (demonstrated no putative preneoplastic lesions), but this dose was less than 25% of the lowest dose in the carcinogenicity study.

2.2. Toxicological evaluation of substances

All substances present in the database were evaluated for the following parameters: body weight, organ weights, presence of putative preneoplastic histopathological changes in the sub-chronic study, treatment-related increased tumour incidences in the 24-month carcinogenicity studies, and genotoxicity. We have not included the mode or mechanism of action (e.g. hormonal perturbation) of the chemicals evaluated.

2.2.1. Histopathological changes

Substances were evaluated for the following histopathological changes in sub-chronic (13-week) toxicity studies:

- cellular hyperplasia,
- presence of altered hyperplastic foci of cellular alteration (atypical) cell foci (basophilic; acidophilic foci), and
- cellular proliferation.
- cellular hypertrophy.

In case of induction of cellular hypertrophy, this was recorded but not included as a putative preneoplastic lesion since an International ESTP Expert Workshop recently concluded that cellular hypertrophy without histopathological or clinical alterations are generally considered as an adaptive and non-adverse reaction and not as a step toward carcinogenicity (Hall et al., 2012).

Substances were scored negative when any of the

aforementioned histopathological changes was not mentioned in the database. Substances were scored positive when incidences were higher than in controls, and treatment-related. With no access to the original study reports, no attempts were made to re-evaluate the data. When the histopathological changes were incorporated in the database, they were considered to be related to treatment and significantly increased.

2.2.2. Organ and body weights

Increased organ weights, expressed as an organ-to-body weight ratio, were collected from 13-week repeated-dose toxicity studies and considered statistically significantly increased when mentioned as such in the database. Statistical analyses were not performed as individual data were not available.

2.2.3. Carcinogenicity

Substances were considered positive for carcinogenicity when a treatment-related increase in benign and/or malignant tumour incidence in the 2-year bioassay was reported in any of the databases used. Substances were considered negative for carcinogenicity when no increase in tumour incidence in the two-year bioassay was reported.

2.2.4. Genotoxicity

Genotoxicity data available through TOXNET (http://toxnet.nlm.nih.gov) were evaluated to assess the genotoxic potential of substances examined in this study. Data from the following *in vitro* genotoxicity assays were taken into consideration: the Ames test (OECD, 1997a) and/or mammalian cell gene mutation tests (OECD, 1997b) for mutagenicity; the *in vitro* micronucleus test (OECD, 2014a) and/or the chromosome aberration test (OECD, 2014b) for clastogenicity. For evidence of *in vivo* genotoxicity, data from the following assays were collected: the chromosome aberration test (OECD, 2014c) and the micronucleus test (OECD, 2014d) in rodents for chromosomal alterations, the *in vivo* transgenic rodent gene mutation assay (OECD, 2013) for mutagenicity, and the *in vivo* alkaline comet assay (OECD, 2014e) as indicator for DNA damage.

Substances with a negative *in vitro* test result for mutagenicity and clastogenicity were considered to have no genotoxic potential. Substances with a positive test result in any of the aforementioned *in vitro* genotoxicity assays were only scored positive for genotoxicity if confirmed in an adequate *in vivo* genotoxicity test. According to the OECD Test Guidelines, positive results seen only at concentrations associated with high levels of cytotoxicity would not be considered positive. Substances with a positive outcome in any of the *in vivo* genotoxicity assays mentioned above that was not otherwise explained as an irrelevant finding were considered genotoxic. Substances with conflicting data were scored as "inconclusive".

3. Results

In total, 233 substances were evaluated, of which 202 met the criteria described in the Materials and Methods section for defining valid pairs of rat sub-chronic and two-year carcinogenicity studies based on matching dose levels. Thirteen compounds are marketed nutrients and the rest are marketed chemicals, mainly pesticides.

Based on the results of the 13-week study, the substances were classified as follows:

- Substances that induced neither putative preneoplastic histopathological changes in the 13-week study, nor a treatment-related increase in benign or malignant tumours in the carcinogenicity study were classified as **True Negative (TN).**
- Substances that induced both putative preneoplastic histopathological lesions in the 13-week study as well as a treatment-related increase in benign and/or malignant tumours in the carcinogenicity study were classified as **True Positive (TP).**
- Substances that induced putative preneoplastic histopathological changes in the 13-week study, but no treatment-related increase in benign and/or malignant tumours in the carcinogenicity study were classified as **False Positive (FP).**
- Substances that induced no putative preneoplastic lesions in the 13-week study but a treatment-related increase in benign and/or malignant tumours in the carcinogenicity study were classified as **False Negative (FN).**

Overall, 163 of the 202 (81%) substances were classified negative for genotoxicity, whereas 39/202 (19%) scored either positive (10%) or inconclusive (9%) in the genotoxicity assays (Table 1).

For the present evaluation, we concentrated on non-genotoxic compounds and excluded substances that were genotoxic or inconclusive with regard to genotoxicity, since the general presumption is that genotoxic compounds are carcinogenic and are presently tested only very occasionally for carcinogenicity in a 24-month study in rats.

Hundred eleven of the 148 (75%) non-genotoxic substances that did not induce putative preneoplastic lesions in the 13-week study, also did not cause treatment-related tumours in the carcinogenicity study (***True Negative compounds***), whereas 37 (23%) non-genotoxic substances that were negative in the 13-week study showed treatment-related tumours in the carcinogenicity study (***False Negative compounds***). Of the 15 non-genotoxic substances that induced putative preneoplastic (hyperplastic) histopathological lesions in the sub-chronic study, thirteen substances (87%) failed to induce treatment-related tumours in the carcinogenicity study (***False Positive compounds***), whereas 2 non-genotoxic substances (13%) also caused tumours in the carcinogenicity study (***True Positive compounds).***

3.1. True Negative (TN) non-genotoxic compounds (Table 2; Annexes A and B)

Of the 111 TN compounds, thirty-one (28%) did neither exhibit any effect on organ weight nor induced cellular hypertrophy in any organ (Annex A).

Eighty of the 111 TN compounds (72%) demonstrated an increase in weight of one or more organs (Annex B; Table 2) such as liver (n = 69; 86%); kidneys (n = 38; 48%); brain (n = 16; 20%); spleen (n = 16; 20%); testes (n = 10; 12%); adrenals (n = 8; 8%); thyroid (n = 4; 5%); pituitary (n = 2; 2%) or lungs (n = 1; 1%), either or not in combination with cellular hypertrophy (n = 50; 55%). Cellular hypertrophy was mainly observed in the liver (n = 48) and/or the thyroid (n = 5).

Table 1
Chemicals evaluated.

Chemicals			Genotoxicity		
Category	N	%	Negative (%)	Positive	Inconclusive
True Negative (TN)	134	66	111 (82)	5	18
True Positive (TP)	8	4	2 (25)	5	1
False Positive (FP)	15	8	13 (87)	2	0
False Negative (FN)	45	22	37 (82)	8	0
Total	202	100	163 (81)	20	19

True Negative: sub-chronic study: negative; carcinogenicity study: negative.
True Positive: sub-chronic study: positive; carcinogenicity study: positive.
False Positive: sub-chronic study: positive; carcinogenicity study: negative.
False Negative: sub-chronic study: negative; carcinogenicity study: positive.

Table 2
True negative compounds: summary of organ weight increase and cellular hypertrophy observed in the sub-chronic studies.

Organ	Organ wt increased; no hypertrophy	Hypertrophy; no increase in organ wt	Organ wt increased and hypertrophy
Liver	27	6	42
Kidneys	38	0	0
Brain	16	0	0
Spleen	15	0	0
Testes	10	0	0
Adrenals	8	1	1
Thyroid	5	4	1
Pituitary	2	1	0

3.1.1. Liver

Forty-eight of the 111 TN compounds (43%) induced hepatocellular hypertrophy, in most cases (n = 42) in combination with an increase in liver weight and occasionally in combination with hypertrophy of the follicular cells of the thyroid, the cells of the zona glomerulosa of the adrenals or pituitary cells. One compound (polixetonium chloride) demonstrated epithelial hypertrophy and inflammation in the brain.

Six compounds (7%) demonstrated hepatocellular hypertrophy without an increase in liver weight.

3.1.2. Thyroid

Six compounds induced an increase in thyroid weight, which in one case was accompanied by hypertrophy of the follicular epithelial cells. Four compounds induced follicular cell hypertrophy in the thyroid without an increase in thyroid weight.

3.2. True Positive (TP) non-genotoxic compounds (Annex C)

Two (Quintozene and Vinclozolin) non-genotoxic substances were classified as True Positive (TP). These substances induced putative preneoplastic lesions in the thyroid (follicular cell hyperplasia) and testes (Leydig cell hyperplasia), respectively, accompanied by an increase in weight of the liver (Quintozene) or liver and adrenals (Vinclozin) in the sub-chronic study. In the carcinogenicity studies, Quintozene and Vinclozolin caused follicular cell carcinomas and Leydig cell carcinomas, respectively.

Six compounds that were positive or inconclusive for genotoxicity induced preneoplastic lesions in the 13-week study in the same organ (stomach; urinary bladder and thyroid) where the tumours developed in the 24-month study.

3.3. False Positive (FP) non-genotoxic compounds (Table 3; Annex D)

Thirteen non-genotoxic compounds caused putative preneoplastic (hyperplastic) histopathological changes in the sub-chronic study, whereas no treatment-related tumours were observed in the carcinogenicity study.

For the non-genotoxic compounds classified as FP, the site of histopathological evidence (cellular hyperplasia) of risk for rat neoplasia was: the urinary tract (7), the gastrointestinal tract (4) and the thyroid (3). Five compounds caused a statistically significant increase in liver weight that was not accompanied by hepatocellular hypertrophy or hyperplasia. One compound induced hyperplasia of the esophageal epithelium and the transitional epithelium of the urinary bladder. In four cases the hyperplasia of the transitional or tubular epithelium of the urinary bladder or the kidneys, respectively, was accompanied by an increase in weight of the kidneys.

3.4. False Negative (FN) compounds (Table 4; Annex E)

Thirty-seven non-genotoxic substances were classified as False Negative (FN) because they did not exhibit putative preneoplastic lesions in the 13-week study, but caused treatment-related benign and/or malignant tumours in the carcinogenicity study.

Eight of these 37 FN compounds induced tumours in the carcinogenicity study without inducing any relevant effect (hypertrophy or organ weight increase) in the sub-chronic study. Four of these 8 compounds induced benign, 2 compounds malignant and 2 compounds benign and malignant tumours in the carcinogenicity study.

Nineteen other compounds induced benign tumours only, whereas 3 other substances induced malignant tumours only. Fifteen compounds induced both benign and malignant tumours. Malignant tumours occurred most frequently in the liver, thyroid, pituitary, breast and occasionally in uterus, and incidentally in nose, Zymbal glands and adrenals.

Four substances (Propyzamide; Tralkoxydim; Imazalil; Chlorpyrifos) induced benign tumours in multiple organs; one substance (C.I. Acid Red) induced malignant tumours in multiple organs and five compounds (Prosulfuron; Thiacloprid Tebufenpyrad; Imazethapyr; Ametryn) induced benign and malignant tumours in multiple organs/tissues.

3.4.1. Benign tumours

The most frequently observed benign tumours were follicular cell adenomas of the thyroid; hepatocellular adenomas; Leydig cell adenomas and adenomas of the pituitary gland. An increased incidence of tubular adenomas of the kidneys, or adenomas of the prostate were isolated findings, each induced by different compounds.

3.4.2. Malignant tumours

Three compounds induced only malignant tumours: i) Spirodiclofen: adenocarcinomas in the uterus; and Cyfluthrin and Tribenuron-methyl: both adenocarcinomas in the mammary gland.

3.4.3. Benign and malignant tumours

Fifteen compounds caused both benign and malignant tumours in the same (9) or in different organs (6).

3.4.4. Liver

Twenty-eight of the 37 FN compounds showed an increase in liver weight, either (11) or not (17) accompanied by hepatocellular hypertrophy in the sub-chronic study. One substance (Tepraloxydim) exhibited hepatocellular hypertrophy only. Six of the 11 compounds, which induced both an increase in weight of the liver and hepatocellular hypertrophy, induced hepatocellular adenomas (Perfluoro-octanesulfonic acid; Dimetheneamide; Tibufenpyrad; Lactofen; Imazalil; 1,3 Dichlorpropene) or hepatocellular adenomas and carcinomas (Diclofop-methyl; Diethylhexylphthalate; C.I. Acid Red 114) in the carcinogenicity study.

Seven compounds induced hepatocellular hypertrophy, all (except Tepraloxydim) accompanied by an increase in liver weight in the sub-chronic study but no development of liver tumours in the carcinogenicity study. Remarkably, all these substances caused tumours in endocrine organs. Thiazopyr and clofentezine caused follicular cell adenomas and adenocarcinomas in the thyroid. Thiacloprid induced thyroid adenomas and uterus adenocarcinomas and propylzamide caused thyroid adenomas and Leydig cell adenomas of the testes. Clodinafop induced prostate adenomas and tepraloxydin caused benign and malignant phaeochromocytomas in the medulla of the adrenals.

Table 3
False Positive compounds: summary of the observations in the sub-chronic studies.

Organ	Organ wt increased; no hyperplasia	Hyperplasia; no increase in organ wt	Increase in organ wt and hyperplasia
Oesophagus/forestomach	0	4	0
Urinary bladder	0	4	0
Thyroid	0	1	2
Kidneys	2	1	2
Liver	5	0	0
Adrenals	1	0	0

3.4.5. Thyroid

Five compounds caused an increase in thyroid weight and follicular cell hypertrophy in the sub-chronic study, which was in 3 cases accompanied by benign and in 2 cases by benign and malignant thyroid tumours in the carcinogenicity study. Overall, thirteen compounds induced follicular cell adenomas (8) or follicular cell adenomas and adenocarcinomas (5) in the thyroid.

3.4.6. Kidneys

Eighteen compounds caused an increase in kidney weight in the sub-chronic study but no kidney tumours in the carcinogenicity study. Only one compound (Imazethapyr) caused an increase in kidney weight in the sub-chronic study accompanied by an increased incidence of tubular cell adenomas in the kidneys in the carcinogenicity study.

3.4.7. Adrenals

Five compounds showed an increase in weight of the adrenals without an increase in the incidence of adrenal phaeochromocytomas in the carcinogenicity study, whereas one compound (Tepraloxydim) induced an increase in incidence of benign and malignant phaeochromocytomas in the carcinogenicity study without an effect on the adrenals in the sub-chronic study.

3.4.8. Testes

One compound (Propyzamide) induced an increase in weight of the testes in the sub-chronic study which was accompanied by an increase in incidence of Leydig cell adenomas in the carcinogenicity study. Four compounds induced Leydig cell adenomas in the carcinogenicity study without an effect on weight of the testes in the sub-chronic study, whereas 2 compounds showed an increase in testes weight in the sub-chronic study without the development of neoplasms in the testes in the carcinogenicity study.

3.4.9. Pituitary

Six compounds induced pituitary adenomas or pituitary adenomas and adenocarcinomas in the carcinogenicity study, without an effect on weight or histopathology of the pituitary in the sub-chronic study.

4. Discussion and conclusions

The present retrospective study using rat data from sub-chronic (13-week) toxicity and carcinogenicity studies obtained from the EFSA and the ToxRef database was performed to support the hypothesis as put forward by Reddy et al. (2010) and Sistare et al. (2011), that the absence of any putative preneoplastic lesion in a sub-chronic (3-month) study may lead to the conclusion that the test compound investigated is negative for carcinogenicity in rats and no further long-term study is needed. They based their so called “whole animal negative predictivity hypothesis” on an evaluation of pharmaceuticals. We focused on industrial chemicals. Overall, the regulatory frameworks for industrial chemicals differ in their information requirements for cancer risk assessment in comparison to pharmaceuticals. The main differences relevant for the present study are: the duration of the rat sub-chronic toxicity study (3 months instead of 6 months), the lack of information on hormonal perturbation and limited information on the mode of action. Since we aimed to test the “whole animal negative predictivity hypothesis” as potential approach for regulatory purposes, we used in the present study only data on genotoxicity and data from 3-months toxicity studies. Hundred sixty-three of the 202 chemicals evaluated in the present study were considered negative for genotoxicity, whereas 39 compounds were considered positive or inconclusive for genotoxicity. Only the non-genotoxic compounds were included in the present evaluation, since the general presumption is that *in vivo* genotoxic compounds are carcinogenic and are presently only incidentally tested in a carcinogenicity study in rats.

4.1. True Negative (TN) compounds

A large number of TN compounds induced an increase in (relative) liver weight. An increase in liver weight may result from a wide variety of causes such as hyperplasia (of any of the resident

Table 4
False Negative compounds: summary of the observations in the sub-chronic and carcinogenicity studies.

	Sub-chronic study			Carcinogenicity study: Tumours	
Organ	Organ wt increased; no hypertrophy	Hypertrophy; no increase in organ wt	Increased organ wt and hypertrophy	Benign	Benign and malignant
Liver	17	1	11	8	4
Kidneys	17	0	0	2	0
Adrenals	4	0	2	0	1
Thyroid	1	3	1	8	5
Spleen	1	0	0	0	0
Brain	7	0	0	0	0
Pituitary	0	0	0	3	3
Testes	3	0	0	4	0
Zymbal gland	0	0	0	0	1
Preputial gland	0	0	0	1	0
Breast	0	0	0	0	4
Nose	0	0	0	0	1
Uterus	0	0	0	1	2
Prostate	0	0	0	1	0

cell types), hypertrophy, inflammation, fibrosis, abnormal storage of metabolism or cleavage products, neoplasia and congestion (Carthew et al., 1996; Greaves, 2007). Typically, these changes do not occur in isolation, so in the absence of overt adverse changes such as inflammation, necrosis or degeneration, it is important to recognize that an increase in liver weight may be induced by hypertrophy, hyperplasia or a combination of the two (Maronpot et al., 2010). A xenobiotic that induces an increase in liver weight of 150% in a sub-chronic study might be considered adverse in the context of dose setting for longer term studies but would not be considered adverse in the context of safety evaluation (Carmichael et al., 1997; Hall et al., 2012). Remarkably, hepatocellular hyperplasia did not occur in the ToxRef or EFSA database for any of the compounds evaluated in the present survey.

In a survey of 139 chemicals used in the agrochemical industry, Carmichael et al. (1997) demonstrated that a relative increase in liver weight of $\geq$150% of control values was correlated with the induction of liver tumours in mice. In a similar review of rat studies, a less statistically significant relationship between liver weight and hepato-carcinogenesis was noted, whereby liver weight increase alone correctly predicted eight of eleven liver carcinogens (but false predicted twenty-six chemicals as positives) and failed to predict three true positives (Carmichael et al., 1997).

Our findings demonstrate that treatment-related changes in organ weights, observed in a sub-chronic study with rats, are most likely non-specific and therefore should not be considered as a risk factor for neoplasia.

The term hypertrophy can have various connotations including an increase in weight of the organ, an increase in the average size of the cells and even enzyme induction (functionally hypertrophy). Allen et al. (2004) evaluated the results for 111 chemicals tested by the National Toxicology Programme. If they applied hepatocellular necrosis, hepatocellular hypertrophy, hepatocellular cytomegaly and increased liver weight as predictors for carcinogenicity, increased liver weight appeared to be the most sensitive parameter. However, chemicals that produced liver tumours frequently induced multiple morphological changes. They concluded that the best single predictor of liver cancer in mice was hepatocellular hypertrophy. They found no false negatives, but numerous false positives in their evaluation.

In the present study, eight TN compounds showed an increase in hepatocellular hypertrophy without an effect on liver weight in the sub-chronic study. In none of the cases, this was associated with the development of hepatocellular adenomas and/or carcinomas in the carcinogenicity study. Fifty-four compounds induced an increase in weight and hepatocellular hypertrophy in the sub-chronic study, which in only five cases was associated with hepatocellular adenomas and/or carcinomas in the carcinogenicity study. These findings support our starting point to classify hyperthrophy as an adaptive rather than an adverse putative preneoplastic lesion. If we had assessed hepatocellular hypertrophy as an indicator for the development of hepatocellular tumours, 55 compounds would have been overpredicted as potential liver carcinogens (False Positive substances). This confirms that liver hypertrophy observed in a sub-chronic (13-week) study is an unreliable predictor of carcinogenicity. This is in agreement with the conclusion from the 3th International ESTP Expert Workshop (Hall et al., 2012) that hepatomegaly as a consequence of hepatocellular hypertrophy without histologic or clinical pathological alterations indicative of liver toxicity is an adaptive and non-adverse reaction.

The negative predictivity, the measure of the compounds evaluated that did not show any putative preneoplastic lesion in de sub-chronic studies and were negative in the carcinogenicity studies, was 75%, whereas the sensitivity, a measure of the sub-chronic study to predict positive carcinogenicity outcome was only 5% (Table 5). In contrast, the specificity, the accuracy of the sub-chronic study to correctly identify non-carcinogens was 90%. Based on the absence of putative preneoplastic lesions in the sub-chronic study, 91% of the 2-year carcinogenicity studies could have been eliminated at the risk of 37 (23%) FN.

4.2. True Positive (TP) compounds

The positive predictivity (13%) is the percentage of compounds that showed putative preneoplastic changes in the sub-chronic study and caused treatment-related tumours in the 24-month carcinogenicity study.

Two non-genotoxic chemicals were classified as TP. They developed putative preneoplastic lesions in thyroid and testes in the sub-chronic study and malignant tumours (follicular cell carcinomas and Leydig cell carcinomas, respectively) in the carcinogenicity study. The number of TP compounds observed in the present study is too low to support the conclusion of Reddy et al. (2010) that the whole animal approach assumes that preneoplastic changes at any organ will be indicative for an increase in tumour incidence in that organ or in any organ at a distant site.

On the contrary, the observation that several of the False Positive compounds (Table 5) demonstrated hyperplastic histopathological changes in the sub-chronic study without development of treatment-related tumours at any site in the carcinogenicity is more in agreement with the observation of Jacobs (2005) who concluded, on the basis of an evaluation of 60 pharmaceuticals, that various short-term indicators for carcinogenicity, such as hyperplasia, do not always result in tumours in that tissue, although such a putative preneoplastic histopathological lesions is generally considered a sign of potential concern for carcinogenicity.

4.3. False Positive (FP) compounds

Thirteen chemicals induced putative preneoplastic (hyperplastic) histopathological changes in the epithelium of the oesophagus/forestomach, the kidneys/urinary bladder or the thyroid, either or not accompanied by an increase in weight of that organ, whereas no tumours occurred in the carcinogenicity study, neither in the same organ nor in an organ at distant site.

The equivocal findings observed in the present paper with TP and FP compounds support the conclusion of Reddy et al. (2010) that more research is needed in order to achieve understanding of the biological links between putative preneplastic lesions

Table 5
Predictivity of the sub-chronic toxicity study for the carcinogenicity of non-genotoxic chemicals.[a]

		Carcinogenicity	
		Positive	Negative
Sub-chronic toxicity	Positive	2	13
	Negative	37	111
	%		
Elimination of studies	91	=[(37 + 111)/(37 + 111+2 + 13)] × 100	
False negatives	25	=[37/(37 + 111)] × 100	
Negative predictivity[b]	75	=[111/(111 + 37)] × 100	
Positive predictivity[c]	13	=[2/(2 + 13)] × 100	
Sensitivity[d]	5	=[2/(2 + 37] × 100	
Specificity[e]	90	=[111/(111 + 13)] × 100	

[a] The subchronic (3-month) study results were used to categorize a compound as True Positive (TP); False Positive (FP); False Negative (FN) and True Negative (TN).
[b] Ability to predict non-carcinogens: [TN/(TN + FN)] × 100.
[c] Ability to predict rat carcinogens: [TP/(TP + FP)] × 100.
[d] Ability to detect rat carcinogens: [TP/(TP + FN)] × 100.
[e] Ability to detect non-carcinogens: [TN/(TN + FP)] × 100.

observed in a sub-chronic study and tumours developing at distant organ sites in the carcinogenicity study.

4.4. False Negative (FN) compounds

Since it is generally accepted that the intention of screening assays should be conservative, it is most important that the number of FNs with respect to human carcinogens should be as low as possible. In the present study, 37 compounds were classified as FN because they did not show putative preneoplastic lesions in the 13-week study, but caused treatment-related tumours in the carcinogenicity study. These compounds are of concern with regard to the acceptability of the negative predictivity of the whole animal approach stating that the absence of evidence of putative preneoplastic lesions in all tissues in the 13-week study may serve as a strong negative predictor of tumour outcome in the carcinogenicity study.

Thirty-three of the 37 FN substances induced benign tumours or benign and malignant tumours which are considered not relevant for the human situation (Williams et al., 2014): phaeochromocytomas of the adrenal medulla (Greim et al., 2009); forestomach tumours (Kroes and Wester, 1986); hepatocellular tumours induced by peroxisome proliferators (IARC, 1995; Williams and Perone, 1996; Klaunig et al., 2003); fibroadenomas of the mammary gland (Russo et al., 1990); pituitary tumours (adenohypophysis tumours) (Gold et al., 2001); Leydig cell (interstitial cell) tumours of the testes (Cook et al., 1999; Prentice and Meikle, 1995); thyroid tumours (Alison et al., 1994; Rice et al., 1999); urinary bladder tumours (Cohen, 1998; Cohen and Ellwein, 1991; Cohen et al., 2004; Rice et al., 1999) and uterus tumours (endometrial stromal polyps) (Davis, 2012).

When the chemicals that gave rise to tumours generally considered not relevant for human risk assessment (n = 33), were moved from the FN to the TN category (leading to figure 37 in Table 5 should be replaced by 4; and 111 by 144), the negative predictivity of the sub-chronic study for the absence of carcinogenicity (ability to predict non-carcinogens) amounts to 97% and the specificity (the ability to detect non-carcinogens) to 92%. This indicates that over 90% of the 24-month carcinogenicity studies could have been avoided when the negative predictivity of the whole animal approach (Reddy et al., 2010; Sistare et al., 2011) would have been applied, which reduced a remarkable number of animals.

The sensitivity (and positive predictivity) of the sub-chronic (13-week) toxicity studies for predicting tumour outcome based on the putative preneoplastic lesions observed in the sub-chronic study was poor, which is in agreement with others who showed that this approach is not useful (Jacobs, 2005; Reddy et al., 2010; Sistare et al., 2011).

Our results demonstrate that sub-chronic (13-week) studies can appropriately classify a non-genotoxic chemical into three categories:

i) highly unlikely to be tumourigenic when no histopathological risk factor for neoplastic lesions is observed in the sub-chronic study in any tissue;
ii) likely tumourigenic in rats, but the putative preneoplastic lesions observed in the sub-chronic study may give rise to a type of tumour in the carcinogenicity study that is not relevant for humans; therefore, a carcinogenicity study has no additional value;
iii) tumourigenicity in humans is uncertain but the putative preneoplastic lesions observed in the sub-chronic study point to the added value of a carcinogenicity study.

Overall, the results of the present retrospective study support the negative predictivity of the whole animal approach as proposed by Reddy et al. (2010) and Sistare et al. (2011): chemicals showing no histopathological risk factors for neoplastic lesions in any tissue in rats in sub-chronic (13-week) toxicity studies may most likely be considered non-carcinogens and do not require further testing in a carcinogenicity study.

This is in agreement with the approach followed by the European Chemical Agency in Helsinki (EC, 2007). Under REACH, a carcinogenicity bioassay is rarely requested. Relevant factors that may trigger a two-year carcinogenicity bioassay in this respect include: i) genotoxicity (germ cell mutagen category 2); ii) evidence of treatment-related hyperplasia and/or preneoplastic lesions in the sub-chronic study; iii) previous demonstration of carcinogenic potential in the chemical class or product that is considered relevant to humans; iv) widespread use or evidence of frequent long-term human exposure.

For risk assessment of plant protection products, however, data from carcinogenicity studies are required (EC, 2013). Modification of these requirements with the 'whole animal negative approach' would save large numbers of animals (up to 400 or 500 per study), time and costs without compromising human safety.

Acknowledgements

The present study was part of the SLiM project ("a smart way from innovations to humans") sponsored by the Dutch Government, Dept Economical Affairs, The Utrecht Province and the Utrecht City Administration (PID101063).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2016.09.003.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2016.09.003.

References

Alison, R.H., Capen, C.C., Prentice, D.E., 1994. Neoplastic lesions of questionable significance to humans. Toxicol. Pathol. 22, 179–186.
Allen, D.G., Pearse, G., Haseman, J.K., Maronpot, R.R., 2004. Prediction of rodent carcinogenesis: an evaluation of prechronic liver lesions as forecasters of liver tumors in NTP carcinogenicity studies. Toxicol. Pathol. 32, 393–401.
American Cancer Society, 2014. Lifetime Risk of Developing or Dying from Cancer. http://www.cancer.org/cancer/cancerbasics/lifetime-probability-of-developing-or-dying-from-cancer.
Anisimov, V.N., Ukraintseva, S.V., Yahin, A.I., 2005. Cancer in rodents: does it tell us about cancer in humans? Nat. Rev. Cancer 5, 807–819.
Benigni, R., 2012. Alternatives to the carcinogenicity bioassay for toxicity prediction: are we there yet? Exp. Opin. Drug Metab. Toxicol. 8, 407–417.
Boobis, A.R., Cohen, S.M., Doerrer, N.G., Galloway, S.M., Haley, P.J., Hard, G.C., Hess, F.G., Macdonald, J.S., Thibault, S., Wolf, D.C., Wright, J., 2009. A data-based assessment of alternative strategies for identification of potential human cancer hazards. Toxicol. Pathol. 37, 714–732.
Billington, R., Lewis, R.W., Mehta, J.M., Dewhurst, I., 2010. The mouse carcinogenicity study is no longer a scientifically justifiable core data requirement for the safety assessment of pesticides. Crit. Rev. Toxicol. 40, 35–49.
Carmichael, N., Enzmann, H., Pate, I., Waechter, F., 1997. The significance of mouse liver tumor formation for carcinogenic risk assessment: results and conclusions from a survey of ten years of testing by the agrochemical industry. Environ. Health Perspect. 105, 1196–1203.
Carthew, P., Nolan, B.M., Edwards, R.E., Smith, L.L., 1996. The role of cell death and cell proliferation in the promotion of rat liver tumours by tamoxifen. Cancer Lett. 10, 163–169.
Cohen, S.M., Ellwein, L.B., 1991. Genetic errors, cell proliferation and carcinogenesis. Cancer Res. 51, 6493–6505.
Cohen, S.M., 1998. Urinary bladder carcinogenesis. Toxicol. Pathol. 26, 121–127.
Cohen, S.M., 2004. Human carcinogenic risk evaluation; an alternative approach to the two-year rodent bioassay. Toxicol. Sci. 80, 225–229.

Cohen, S.M., Klaunig, J., Meek, M.E., Hill, R.N., Pastoor, T., Lehman-McKeeman, L., Bucher, J., Longfellow, D.G., Seed, J., Dellarco, V., Fenner-Crisp, P., Patton, D., 2004. Evaluating the human relevance of chemically induced animal tumors. Toxicol. Sci. 78, 181–186.

Cook, J.C., Klinefelter, G.R., Hardisty, J.F., Sharpe, R.M., Foster, P.M., 1999. Rodent Leydig cell tumorigenesis: a review of the physiology, pathology, mechanisms and relevance to humans. Crit. Rev. Toxicol. 29, 169–261.

Davis, 2012. Endometrial stromal polyps in rodents: biology, etiology and relevance to disease in women. Toxicol. Pathol. 40, 419–424.

Doktorova, T.Y., Pauwels, M., Vinken, M., Vanhaseke, T., Rogiers, V., 2012. Opportunities for an alternative integrating testing strategy for carcinogen hazard assessment? Crit. Rev. Toxicol. 42, 91–106.

EC, 2007. Corrigendum to regulation (EC) No 1907/2006 of the European parliament and of the councel of 18 december 2006 concerning the registration, evaluation, authorization and restriction of chemicals (REACH), establishing a European chemical agency, amending directive 1999/45/EC and repealing council regulation (EEC) No 793/93 and commission regulation (EC) No 1488/94 as well as council directive 76/769/EEC and commission directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Off. J. Eur. Union L136, 3–280.

EC, 2013. Commission Regulation (EU) 248/2013 of 1 March 2013 Setting Out the Data Requirements for Plant Protection Products in Accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council Concerning the Placing of Plant Protection Products on the Market. Available from: http://data.europa.eu/eli/reg/2013/284/oj.

Friedrich, A., Olejniczak, K., 2011. Evaluation of carcinogenicity studies of medical products for human use authorized via the European centralized procedure (1995-2009). Regul. Toxicol. Pharmacol. 60, 225–248.

Gold, L.S., Manley, N.B., Slone, T.H., Ward, J.M., 2001. Compendium of chemical carcinogens by target organ: results of chronic bioassays in rats, mice, hamsters, dogs and monkeys. Toxicol. Pathol. 29, 639–652.

Gori, G.B., 2013. Regulatory forum opinion piece: long-term animal bioassays: is the end near? Toxicol. Pathol. 41, 805–807.

Greaves, P., 2007. Liver and pancreas. In: Histopathology of Preclinical Toxicity Studies, third ed. Elsevier, London, UK, pp. 457–504.

Greim, H., Hartwig, A., Reuter, U., Richter-Reichhelm, H.B., Thielmann, H.W., 2009. Chemically induced pheochromocytomas in rats: mechanisms and relevance for human risk assessment. Crit. Rev. Toxicol. 39, 695–718.

Hall, A.P., Elcombe, C.R., Foster, J.R., Harada, T., Kaufmann, W., Knippel, A., Kuttler, K., Malarkey, D.E., Maronpot, R.R., Nishikawa, A., Nolte, T., Schulte, A., Strauss, V., York, M.J., 2012. Liver hypertrophy: a review of adaptive (adverse and non-adverse) changes – conclusions from the 3rd International ESTP Expert Workshop. Toxicol. Pathol. 40, 971–994.

HSE, 2014. Occupational Cancer in Great Britain. Available from: http://www.hse.gov.uk/statistics/causdis/cancer/cancer.pdf.

IARC, 1995. Peroxisome Proliferation and its Role in Carcinogenesis: Views and Expert Opinion of an IARC Working Group, IARC Tech. Rep. No. 24. International Agency for research on Cancer, Lyon, France.

ICH, 1995. S1A. Need for Carcinogenicity Studies of Pharmaceuticals. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/safety/S1A/Step4/S1a.

Jacobs, A., 2005. Prediction of 2-year carcinogenicity study results for pharmaceutical products: how are we doing? Toxicol. Sci. 88, 18–23.

Jacobson-Kram, D., Sistare, F.D., Jacobs, A.C., 2004. Use of transgenic mice in carcinogenicity hazard assessment. Toxicol. Pathol. 32 (1), 49–52.

Klaunig, J.E., Babich, M.A., Baetcke, K.P., Cook, J.C., Corton, J.C., David, R., DeLuca, J.G., Lai, D.Y., McKee, R.H., Roberts, R.A., Fenner-Crisp, P.A., 2003. PPAR-alpha agonist-induced rodent tumors: modes of action and human relevance. Crit. Rev. Toxicol. 33, 655–780.

Kroes, R., Wester, P.W., 1986. Forestomach carcinogens: possible mechanisms of action. Food Chem. Toxicol. 24, 1083–1089.

Maronpot, R.R., Yoshizawa, K., Nyska, A., Harada, T., Flake, G., Mueller, G., Singh, B., Ward, J.M., 2010. Hepatic enzyme induction: Histopathology. Toxicol. Pathol. 38, 776–795.

OECD, 1997a. Test No. 471: Bacterial Reverse Mutation Test. OECD Publishing.

OECD, 1997b. Test No. 476: in Vitro Mammalian Cell Gene Mutation Test. OECD Publishing.

OECD, 1998. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents. OECD Publishing.

OECD, 2009. Test No. 451: Carcinogenicity Studies. OECD Publishing.

OECD, 2013. Test No. 488: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays. OECD Publishing.

OECD, 2014a. Test No. 487. In: Vitro Mammalian Cell Micronucleus Test. OECD Publishing.

OECD, 2014b. Test No. 473. In: Vitro Mammalian Chromosomal Aberration Test. OECD Publishing.

OECD, 2014c. Test No. 474: Mammalian Erythrocyte Micronucleus Test. OECD Publishing.

OECD, 2014d. Test No. 475: Mammalian Bone Marrow Chromosomal Aberration Test. OECD Publishing.

OECD, 2014e. Test No. 489: in Vivo Mammalian Alkaline Comet Assay. OECD Publishing.

Osimitz, T.G., Droege, W., Boobis, A.R., Lake, B.G., 2013. Evaluation of the utility of the lifetime mouse bioassay in the identification of cancer hazards for humans. Food Chem. Toxicol. 60, 550–562.

Prentice, D.E., Meikle, A.W., 1995. A review of drug-induced Leydig cell hyperplasia and neoplasia in the rat ans come comparisons with ma. Hum. Exper. Toxicol. 14, 562–572.

Reddy, M.V., Sistare, F.D., Christensen, J.S., DeLuca, J.G., Wollenberg, G.K., DeGeorge, J.J., 2010. An evaluation of chronic six- and twelve-month rat toxicology studies as predictors of two-year tumor outcome. Vet. Pathol. 47, 614–629.

Rice, J.M., Baan, R.A., Bletter, M., Genevois-Charneau, C., Grosse, Y., McGregor, D.B., Partensky, C., Wilbourn, J.D., 1999. Rodent tumors of the urinary bladder, renal cortex, and thyroid gland in IARC monographs evaluation of carcinogenic risk to humans. Toxicol. Sci. 49, 166–171.

Russo, J., Russo, I.H., Rogers, A.E., van Zwieten, M.J., Gusterson, B., 1990. Tumors of the mammary gland. IARC Publ. No. 99. In: Turusov, V.S., Mohr, U. (Eds.), Pathology of Tumors in Laboratory Animals. Vol. I. Tumors of the Rat. International Agency for Research on Cancer, Lyon, France, pp. 47–78.

Sistare, F.D., Morton, D., Alden, C., Christensen, J., Keller, D., Jonghe, S., de, Storer, R.D., Reddy, M.V., Kraynak, A., Trela, B., Bienvenue, J.-G., Bjurnström, S., Bosmans, V., Brewster, D., Colman, K., Dominck, M., Evans, J., Hailey, J.R., Kinter, L., Liu, L., Mahrt, C., Marien, D., Myer, J., Perry, R., Potenta, D., Roth, A., Sherratt, P., Singer, T., Slim, R., Soper, K., Fransson-Steen, R., Stolttz, J., Turner, O., Turnquist, S., Heerden, M. van, Woikcke, J., DeGeorge, J.J., 2011. An Analysis of pharmaceutical experience with decades of rat carcinogenicity testing: support for proposal to modify current regulatory guidelines. Toxicol. Pathol. 39, 716–744.

Snyder, R.D., Green, J.W., 2001. A review of the genotoxicity of marketed pharmaceuticals. Mutat. Res. 488, 151–169.

Van der Jagt, K., Munnn, S., Törslóv, de Bruijn, J., 2004. Alternative Approaches Can Reduce the Use of Test Animals under REACH, Addendum to the Report "Assessment of Additional Testing Needs under REACH. Effects of (Q)SARs, Risked Based Testing and Voluntary Industry Initiatives". JRC Report EUR 21405 EN. European Commission. Joint Research Centre, Ispra, Italy, p. 25. http://ecb.jrc.it.

Van Oosterhout, J.P.J., Van der Laan, J.W., De Waal, E.J., Olejniczak, K., Hilgenfeld, M., Schmidt, V., Bass, R., 1997. The utility of two rodent species in carcinogenic risk assessment of pharmaceuticals in Europe. Regul. Toxicol. Pharmacol. 25, 6–17.

Ward, J.M., 2007. The two-year rodent carcinogenesis bioassay-will it survive? J. Toxicol. Pathol. 20, 13–19.

WHO, 2011. Environmental and Occupational Cancers. Fact sheet No 350. http://www.who.int/mediacentre/factsheets/fs350/en/.

Williams, G.M., Perone, C., 1996. Mechanism-based risk assessment of peroxisome proliferating rodent hepatocarcinogens. In: Reddy, J.K., Suga, T., Mannaerts, G.P., Lazarow, P.B., Subramani, S. (Eds.), Peroxisomes: Biology and Role in Toxicology and Disease, 804. The New York Academy of Sciences, New York, pp. 554–572.

Williams, G.M., Iatropoulos, M.J., Enzmann, H.G., Deschl, U.F., 2014. Carcinogenicity of chemicals: assessment and human extrapolation. In: Hayes, A.W., Kruger, C.L. (Eds.), Hayes' Principles and Methods in Toxicology, sixth ed. CRC Press, pp. 1251–1304.