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A comprehensive weight of evidence assessment of published acetaminophen genotoxicity data: Implications for its carcinogenic hazard potential

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ABSTRACT

In 2019, the California Office of Environmental Health Hazard Assessment initiated a review of the carcinogenic hazard potential of acetaminophen, including an assessment of its genotoxicity. The objective of this analysis was to inform this review process with a weight-of-evidence assessment of more than 65 acetaminophen genetic toxicology studies that are of widely varying quality and conformance to accepted standards and relevance to humans. In these studies, acetaminophen showed no evidence of induction of point or gene mutations in bacterial and mammalian cell systems or in *in vivo* studies. In reliable, well-controlled test systems, clastogenic effects were only observed in unstable, p53-deficient cell systems or at toxic and/or excessively high concentrations that adversely affect cellular processes (e.g., mitochondrial respiration) and cause cytotoxicity. Across the studies, there was no clear evidence that acetaminophen causes DNA damage in the absence of toxicity. In well-controlled clinical studies, there was no meaningful evidence of chromosomal damage. Based on this weight-of-evidence assessment, acetaminophen overwhelmingly produces negative results (i.e., is not a genotoxic hazard) in reliable, robust high-weight studies. Its mode of action produces cytotoxic effects before it can induce the stable, genetic damage that would be indicative of a genotoxic or carcinogenic hazard.

1. Introduction

The genotoxicity data related to acetaminophen (4-hydroxyacetanilide, or N-acetyl-*p*-aminophenol, CAS No. 103-90-2; molecular weight 151.163 g/mol; structure shown in Fig. 1) has been extensively reviewed and analyzed by a number of independent institutions, including IARC (1990a, 1999) and NTP (1993). The potential genotoxicity of acetaminophen became a topic of discussion in the 1990s, following several publications on the potential of a metabolite, N-acetyl-p-benzoquinone imine (NAPQI) to bind to DNA, and the results of various *in vitro* and *in vivo* genotoxicity studies. These data were reviewed in detail by a panel of European regulatory genetic toxicology experts Bergman et al., 1996, who concluded that acetaminophen:

1. Did not induce gene mutations either in bacteria or in mammalian cells *in vitro* and
2. Did induce chromosomal damage *in vitro* in mammalian cells at high concentrations, and similar effects could occur *in vivo* at high dosages.

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Abbreviations

CA	chromosomal aberrations
Hprt	hypoxanthine-guanine phosphoribosyl transferase
i.p.	intraperitoneal
IQ	2-amino-3-methylimidazo[4,5-f]quinoline
MN	micronucleus or micronuclei
MoA	mode of action
NAPQI	N-acetyl-p-benzoquinone imine
NQO	4-nitroquinoline-N-oxide
ROS	reactive oxygen species
SCE	sister chromatid exchanges
6 TG	6-thioguanine
Tk	thymidine kinase
UDS	unscheduled DNA synthesis
WoE	weight of evidence

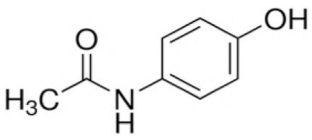

Fig. 1. Structure of acetaminophen.

Chromosome damaging effects have only been observed at high concentrations that were cytotoxic to the test system. Since the detailed review of Bergman et al., 1996 several papers related to the potential genotoxicity of acetaminophen have been published. Since the focus of the Bergman paper was the relevance of genotoxicity at therapeutic doses, the assessment presented here builds on the review of Bergman et al., 1996 and considers how the data previously reviewed, or any new data, impact the genotoxic hazard assessment for acetaminophen at therapeutic as well as doses above the maximum labelled dose of 4 g/day. In terms of whether the genotoxicity data are indicative of a genotoxic or cancer hazard, the specific focus herein is placed on:

1. Relevance of genotoxic endpoints towards assessing potential cancer hazard
2. Conditions of genotoxic effects and whether the type of damage is stable or persistent
3. Likely modes of genotoxic action and relevance towards the carcinogenic process, and
4. Strength of the weight of evidence (WoE) for genotoxic potential indicative of a carcinogenic hazard

In 2019, the California Office of Environmental Health Hazard Assessment (OEHHA) initiated a review process on the carcinogenic hazard potential of acetaminophen. OEHHA developed a hazard identification document that summarizes the genetic toxicology studies, but does not provide a weight of evidence assessment. To provide input to this review process, a broader scientific and weight of evidence evaluation of the carcinogenicity hazard potential of acetaminophen that included assessments of animal carcinogenicity (Murray et al., 2020), mechanisms of toxicity (Jaeschke, 1990), and Quantitative Systems Toxicology modeling (Eichenbaum et al., 2020) was conducted. The objective of this analysis was to provide a comprehensive weight of evidence assessment of the genotoxic potential of acetaminophen.

2. Methods

2.1. Data sources

The published literature was searched for studies that evaluated the genotoxicity of acetaminophen in different test systems for various endpoints through November 2019. The PubMed database was queried for potentially relevant articles using combinations of the following keywords: acetaminophen, 4-hydroxyacetanilide, N-acetyl-*p*-aminophenol, mutagenicity, genotoxicity, DNA damage, DNA repair, chromosome aberrations, micronucleus, gene mutation, Ames, or comet assay. This search identified 549 articles. Articles that were in foreign languages, conference abstracts, and non-peer reviewed reports with limited methodology were excluded. Studies reviewed in regulatory documents such as (IARC, 1990b, IARC, 1999) and (OEHHA, 2019) were also compared against those from the database search, and additional pertinent articles were retrieved and evaluated. Foreign language articles and industrial reports unavailable for review were included if they were reviewed and included in a regulatory document; the source of information is noted in the description of the study. This then left a total of 69 articles that were reviewed in detail to investigate the genotoxicity of acetaminophen (Table 2 to Table 10).

2.2. Weight of evidence (WoE) approach

2.2.1. General principles

The published literature assessing the genotoxicity of acetaminophen includes data from 69 studies using different test systems and investigating different endpoints. Such an extensive, diverse set of studies with varying degrees of relevance to humans, quality and conformance to accepted standards requires a robust WoE approach rather than a systematic review.

The test systems used and endpoints studied in genetic toxicology tests provide information with varying levels of relevance of responses. For example, mutation endpoints are considered more important in determining potential risk than endpoints that are reversible (i.e., DNA breakage) or not associated with a known adverse effect (i.e., sister chromatid exchange). As recommended by Brusick et al. (2016) and Eastmond (2017), in a WoE approach, studies are evaluated based on quality, reproducibility and consistency, significance of the genetic alteration, phylogenetic relevance to humans, type of test system (*in vivo* vs. *in vitro*, cell type, p53 status etc.), and relevance of the route of administration for *in vivo* studies. This is not a quantitative "points--based" approach but (as discussed in detail in Brusick et al., 2016) relies on assessing the contribution the endpoint, test system and methodology make towards the overall evidence.

Genetic effects identified *in vivo* are generally considered more important than responses from *in vitro* tests, particularly *in vitro* tests employing p53-deficient cell lines that are susceptible to misleading positive results, or from non-mammalian systems (other than the Ames test) for which no recommended testing guidelines are available. As stated in the recent OECD Genetic Toxicology Guidance Document "*assays conducted in mammalian cells are preferred because they are considered more relevant*" (OECD, 2015). Therefore, responses in non-mammalian test systems such as mussels, insects, plants, yeasts and acellular systems should not be considered as being as relevant (i.e., not be given the same weight) as results from mammalian systems and the Ames test.

Data from indicator tests such as DNA strand breaks, or from endpoints such as sister chromatid exchanges (SCE), where the biological relevance of the effects is not understood and OECD guidelines have been deleted, should contribute negligible or very low weight. As stated in the OECD Genetic Toxicology Guidance document (2015) "*[i]ndicator tests detect primary DNA damage (i.e., the first in the chain of events leading to a permanent change), but not the consequences of this genetic damage. They are called indicator tests because the measured endpoint does not*

always lead to mutation, a change that can be passed on to subsequent generations", and "*[w]hen evaluating potential genotoxicants, more weight should be given to the measurement of permanent DNA changes than to DNA damage events that are reversible*". Most regulatory bodies therefore rely on a set of core endpoints that are known or suspected to be associated with neoplastic initiation in somatic cells or alteration of the genetic information in germ cells (EFSA, 2011; ICH, 2011; Kirkland et al., 2011).

2.2.2. Evidence weighting

The approach we have applied to evidence weighting uses an empirical framework in which the weights assigned to individual assays are based on the strength of evidence assigned to an endpoint, i.e., the extent to which validation studies support the endpoint's involvement in carcinogen prediction as well as its relevance to mechanisms involved with initiation of malignancy (ICH, 2011). Human and *in vivo* mammalian systems were assigned the highest test system weight, with a lower degree of weighting applied to *in vitro* mammalian cell systems and *in vivo* non-mammalian systems, and the lowest weight to *in vitro* non-mammalian systems (with the exception of the well validated bacterial reverse mutation "Ames" tests using mammalian metabolic activation) (Brusick et al., 2016). Other considerations that were used include response reproducibility or GLP compliance. GLP compliance indicates a high degree of, and standard for, detailed documentation of experimental conditions and data. Based on the above, the endpoints given the greatest weight include chromosomal aberration (CA) or micronucleus (MN) formation *in vivo* and gene mutation *in vitro* in bacteria (Ames) or *in vivo*. The published studies on the genotoxicity of acetaminophen have therefore been considered in terms of their weighted contribution to an overall indication of genotoxic hazard employing the method for weighting genetic toxicology test methods as described above and in Brusick et al. (2016) (Table 1). In summary a test's weighted contribution to a classification of hazard potential was determined based on information regarding its relevance and reliability, predictivity, the endpoint's reversibility, susceptibility to false responses, and its mechanistic role in initiation of malignancy.

2.2.3. Impact of cytotoxicity, concentrations tested and cell type

Bergman et al. (1996) concluded that acetaminophen induced chromosomal damage (clastogenicity) *in vitro* in mammalian cells at high cytotoxic concentrations, and that similar effects could occur *in vivo* at high toxic doses. As will be discussed later, more recent publications confirm this conclusion. However, to understand the relevance of such genotoxic activity it is important to understand the relationship between clastogenicity and cytotoxicity, and the importance of the different cell types in which clastogenicity has been observed.

Table 1
Description of weighted contribution categories (adapted from Brusick et al., 2016).

Weight Descriptor	Definition
Negligible weight	The endpoint is not linked to any adverse effect relevant to genetic or carcinogenic hazard/risk (e.g., SCE).
Low weight	The end point is indicative of primary DNA damage, not directly linked to mechanisms of tumorigenicity (e.g., DNA breakage or computer-based SAR results), or the endpoints are evaluated in non-mammalian test systems (other than the Ames test).
Moderate weight	The endpoint may be: (a) only potentially relevant to tumor initiation, (b) subject to secondary effects (cytotoxicity), (c) subject to threshold dependent mechanisms of induction (aneugens) or (d) the test system exhibits a high rate of false responses with respect to carcinogenicity predictivity (e.g., mammalian cell *in vitro* clastogenicity and gene mutation tests, particularly in p53-deficient cells).
High weight	The endpoint is one that has been demonstrated to play a critical role in the process of tunorigenicity (e.g., gene mutation in bacteria [Ames test] or *in vivo*, chromosome aberrations, or micronuclei *in vivo*).

Heddle et al. (1981), concluded that the types of "*aberrations that involve a rearrangement of gene order rather than a direct loss of a gene are not cell lethal events and, hence, are not contributors to cellular toxicity. In contrast, those aberrations that lead directly to the loss of a section of genetic information are usually cell lethal events and do contribute directly to cellular toxicity*". Thus, chromosome breakage (clastogenicity) and cell death are inextricably linked. It is now accepted that positive chromosomal aberration or micronucleus results at high levels of cytotoxicity could be a misleading indicator of the genotoxic potential of a test substance (Kirkland, 1992). This is prominently discussed in ICH S2(R1) (ICH, 2011):

- As cytotoxicity increases, mechanisms other than direct DNA damage by a compound or its metabolites can lead to 'positive' results that are related to cytotoxicity and not genotoxicity.
- In cytogenetic assays *in vitro*, even weak clastogens that are known to be carcinogens are positive without exceeding a 50% reduction in cell proliferation. On the other hand, compounds that are not DNA damaging, mutagenic or carcinogenic may induce chromosome breakage but only at toxic concentrations.

Hence, OECD guidelines now recommend use of cell proliferation measurements to determine cytotoxicity, careful control of the extent of cytotoxicity in genotoxicity tests, and urge caution in interpreting positive results only observed at levels of cytotoxicity close to or above the recommended maximum.

Further, ICH recommends a maximum concentration of 1 mM or 0.5 mg/mL to be tested in mammalian cell assays, when not limited by solubility (ICH, 2011). This maximum concentration recommended by ICH for human pharmaceuticals is lower than recommended for other chemicals in OECD guidelines (10 mM or 2 mg/mL). The higher concentration limits recommended for testing non-pharmaceuticals are based mainly on the fact that data from other toxicological and carcinogenicity studies are often not available, assessing systemic exposures in humans is difficult, and there are no therapeutic benefits. According to ICH S2(R1), the rationale for this lower limit was "*[t]here is a very low likelihood of compounds of concern (DNA damaging carcinogens) that are not detected in Ames test or in vivo genotoxicity assay, but are detectable in an in vitro mammalian assay only above 1 mM*" and that the "*limit of 1 mM maintain[ed] the element of hazard identification, being higher than clinical exposures to known pharmaceuticals, including those that concentrate in tissues, and [was] also higher than the levels generally achievable in preclinical studies in vivo*" (ICH, 2011). Since acetaminophen is clearly negative in the Ames test (Section 3.1.1) and is a human medicine that provides a therapeutic benefit, we believe it is appropriate to consider the concentrations of acetaminophen tested in mammalian cells when assessing the relevance of the results to predict human hazard.

It is well established that induction of chromosome breaks will lead to cell death. However, chromosome breaks can rejoin leading to stable rearrangements that may be inherited by daughter cells after division. These types of rearrangements are associated with a mutagenic or carcinogenic hazard. Such rearrangements would need to be induced at low levels of cytotoxicity, such that affected cells would survive. Stable chromosome rearrangements are not usually scored in chromosomal aberration tests, because it requires specialized banding techniques, but the induction of unstable rearrangements (complete and incomplete inter- and intra-chromatid exchanges) are an indication of the potential to induce stable rearrangements.

It is also now known that p53-deficient rodent cells are more likely to produce "misleading" positive results (i.e., with substances that are not genotoxic or carcinogenic *in vivo*), particularly for clastogenicity, than p53-competent human cells (Fowler et al., 2012). It is therefore not uncommon to find positive clastogenicity results in p53-deficient Chinese hamster cell lines (CHO, CHL, V79) with substances that are negative in p53-competent human lymphocytes or human TK6 cells, or to find positive results at lower concentrations in Chinese hamster cells

than in human cells. Thus, more weight should be given to results in p53-competent human cells than in p53-deficient hamster cells. Taking these 2 aspects together, if only chromatid and chromosome breaks (but no unstable rearrangements) are induced by a test substance, and only under cytotoxic conditions, the cells will not survive and therefore this type of genotoxic damage does not indicate a clear genotoxic or carcinogenic hazard.

2.2.4. Route of exposure

According to ICH and OECD guidelines, the route of administration should be the anticipated route of human or clinical exposure (ICH, 2011; OECD, 2016a, 2016b, 2016c). In the case of acetaminophen, the expected clinical route is oral or intravenous administration. While intraperitoneal (i.p.) injection has been used to deliver large bolus doses of acetaminophen in numerous rodent studies (Giri et al., 1992; Hazleton Microtest, 1992; Hongslo et al., 1994; King et al., 1979; Markovic et al., 2013; Oshida et al., 2008; Severin and Beleuta, 1995; Sicardi et al., 1991), according to OECD 474, 475, and 489 Guidelines, i.p. is generally not recommended for testing since it is not a typical relevant route of human exposure (OECD, 2016a; 2016b; 2016c). For example, OECD 489 Guideline for In Vivo Mammalian Alkaline Comet Assay stated that *"[i]ntraperitoneal injection is generally not recommended since it is not a typical relevant route of human exposure, and should only be used with specific justification (*e.g. *some positive control substances, for investigative purposes, or for some drugs that are administered by the intraperitoneal route)*" (OECD, 2016a). One major challenge with correlating results from i.p. exposure compared to oral or intravenous routes is the potential differences due to pharmacokinetic considerations and associated toxicity. For example, i.p. administration of a micronized suspension of acetaminophen in rats was shown to cause hepatic and renal toxicity at doses that were non-toxic when administered orally, likely due to a higher dose and rate of acetaminophen delivery to the liver (i.e., first pass metabolism) when administered via i.p. (Colin et al., 1986). Although this rat study would suggest that i.p. administration is less relevant for human exposure, rats are considered a poor model for the human pathophysiology (McGill et al., 2012). On the other hand, the kinetics of bioactivation in mice, the most relevant species for human toxicity, does not seem to differ between the routes of administration when given acetaminophen dissolved in saline (McGill et al., 2016). Therefore, careful consideration is given to the rodent species used for the *in vivo* studies; in general, results from *in vivo* studies using oral or intravenous administration are considered more relevant.

3. Results - WoE assessment of the studies

The genetic toxicology studies that have been reviewed are summarized in Table 2 - Table 10, along with a corresponding WoE assessment of the results. The weight (High, Moderate, Low, Negligible etc.) is based on the criteria given in Table 1. The WoE assessments given in Tables 2–10 are based not only on the weight applied to the study type and endpoint but also on factors such as response reproducibility or GLP compliance, the endpoint's reversibility, and the susceptibility of the test system to false or misleading positive responses. For example, a positive result at high levels of cytotoxicity in p53-deficient Chinese hamster cells would be given a lower WoE assessment than a positive result at moderate levels of cytotoxicity in p53-competent human cells. Similarly, a positive result only observed at concentrations exceeding the current limit of 10 mM would be given a lower WoE assessment than a positive result at lower "likely not mutagenic concentrations", or the biological relevance of the result is questionable, according to our WoE assessment. The factors driving such different conclusions are described as "Considerations" in Tables 2–10.

3.1. Mutagenicity studies

Acetaminophen has been tested for mutagenicity under *in vitro* conditions utilizing both bacterial and mammalian cell systems (Tables 2 and 3, respectively) as well as under *in vivo* conditions (Table 4). Many of the *in vitro* studies were reviewed by Bergman et al. (1996) and will not be discussed in detail here.

3.1.1. In vitro studies

Although most of the bacterial mutagenicity studies (Table 2) did not include all strains currently recommended in OECD Guideline 471 (OECD, 1997), Burke et al., 1994 and Dybing et al. (1984) obtained negative results in TA102 and a more recent study by (Martinez et al., 2000) obtained negative results in *E. coli* WP2*uvrA*. Therefore, the required panel of tester strains have been used across different publications and acetaminophen clearly does not have the potential to induce point mutations in bacteria.

Only one *in vitro* mammalian cell gene mutation study reviewed by Bergman et al., 1996 reported a positive response. (Clements, 1992) reported induction of *tk* mutations in mouse lymphoma cells in the absence of metabolic activation. However, a biologically relevant response was observed only at 13.2 mM, which far exceeds the current recommended limit of 1 mM (ICH, 2011) and would be considered not biologically relevant by today's standards. Moreover, there was no sizing of mutant colonies, and since acetaminophen does induce chromosomal damage at concentrations also exceeding 1 mM (Section 3.2), it is highly likely the mutant colonies were due to chromosome damage and not true gene mutations.

In a study by Shimane (1985), V79 cells were treated with acetaminophen at 100, 200 and 400 μg/mL for 24 h, or 50, 100 and 200 μg/mL for 48 h in the absence of metabolic activation. Solvent control treatments were only included for the 24-h treatments. After an appropriate expression time, cultures were assessed for mutations to 6-thioguanine (6 TG) and ouabain resistance. At 200 μg/mL, cytotoxicity (reduction in colony forming ability) was around 25% for the 24-h treatment and around 40% for the 48-h treatment, but at 400 μg/mL cytotoxicity was >50% for both treatment times. 6 TG mutant frequencies increased at 200 (>2-fold) and 400 μg/mL (>4-fold) following 24-h treatment, but there was no statistical analysis, and no historical control data. Mutant frequencies were low and may have been within the historical control range. Moreover, both of these concentrations exceed the current upper limit for testing (1 mM) according to ICH recommendations (ICH, 2011). 6 TG mutant frequencies appeared also to increase at all 3 concentrations following 48-h treatment, but since there was no solvent control for the 48-h treatments it is not possible to assess their biological relevance. Ouabain-resistant mutant frequencies increased at 100 and 400 μg/mL, but not at 200 μg/mL following 24-h treatment, so there was no dose-response. It should be noted that V79 cells are p53-deficient, and highly susceptible to misleading positive results (Fowler et al., 2012), and as such these results would be considered only of moderate weight and of questionable relevance (Section 2.2.2). Moreover, these results are in conflict with other studies where *Hprt* and ouabain mutations were not induced (Patierno et al., 1989; Sasaki et al., 1983; Sawada 1985). Thus, there is no clear evidence that acetaminophen induces gene mutations in other mammalian cell studies.

Table 2
Overview of relevant non-mammalian *in vitro* mutagenicity studies.

Study	Strain	Concentrations Tested	Response	WoE Assessment	Weight[a]
Burke et al. (1994)	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	2,500 μg/plate	Negative	Not mutagenic	High
Bartsch et al. (1980)	*S. typhimurium* TA100	408 μg/plate	Negative	Not mutagenic	High
Camus et al. (1982)	*S. typhimurium* TA98, TA100	Not reported	Negative	Not mutagenic	High
Dybing et al. (1984)	*S. typhimurium* TA98, TA100, TA102	151–3,023 μg/mL	Negative	Not mutagenic	High
Jasiewicz and Richardson (1987)	*S. typhimurium* TA97, TA98, TA100, TA1535, TA1537, TA1538	100-50,000 μg/plate	Negative	Not mutagenic	High
Haworth et al. (1983)	*S. typhimurium* TA98, TA100, TA1535, TA1537	100-10,000 μg/plate	Negative	Not mutagenic	High
Imamura et al. (1983)	*S. typhimurium* TA100	250-5,000 μg/plate	Negative	Not mutagenic	High
King et al. (1979)	*S. typhimurium* (5 unspecified strains); *E. coli* K12/343/113	3,628 or 4,535 μg/plate	Negative	Not mutagenic	High
Kuboyama and Fujii (1992)	*S. typhimurium* TA98, TA100	100 μg/plate	Negative	Not mutagenic	High
Nohmi et al. (1985)	*S. typhimurium* TA100	Not Reported	Negative	Not mutagenic	High
Oldham et al. (1986)	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	5-5,000 μg/plate	Negative	Not mutagenic	High
NTP (1993)	*S. typhimurium* TA98, TA100, TA1535, TA1537	100-10,000 μg/plate	Negative	Not mutagenic	High
Wirth et al. (1980)	*S. typhimurium* TA98, TA100	25-1,000 μg/plate	Negative	Not mutagenic	High
Martinez et al. (2000)	*E. coli* ICI88/*E. coli* WP2uvrA, *E. coli* IC203	1,500 μg/plate	Negative	Not mutagenic	High

[a] Weight attributed to each study as discussed in the Methods.

Table 3
Overview of *in vitro* mutagenicity studies in mammalian cells.

Study	Cell Type/ Concentration/ Duration	Response	Considerations	WoE Assessment	Weight[a]
Mammalian cell gene mutation (Tk locus, 6 TG, HPRT)					
Clements (1992)	Mouse/lymphoma cells/3.3–33 mM/Not Reported	Positive; Increases in mutations at TK locus for doses ranging from 3.3 to 33 mM; 3-fold spontaneous level observed at 13.2 mM	• Report was unavailable for review; details included were from Bergman et al. (1996). • Positive in the range 3.3–33 mM, exceeding the ICH recommended upper limit, and therefore likely to cause physiological disruption and stress-related damage. • No conclusions could be drawn on the type of damage that acetaminophen caused since the size of the mutant colonies was reportedly not analyzed (Bergman et al., 1996). It is possible that small increases in mutation frequencies at high concentrations in this assay can be attributed to chromosomal damage rather than point mutations (Bergman et al., 1996).	Likely not mutagenic; Responses only at high concentrations call into question the biological relevance of the result	Moderate
Shimane (1985)	Chinese hamster lung (V79)/0.66–2.6 mM/ 24 h	Weakly positive 6 TG mutant frequencies increased at 100 or 400 μg/mL, depending on exposure period	• Mutant frequencies were low and may have been within the historical control range. • There was no dose-response with 48 h exposure. • Moreover, V79 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g. primary human) cells.	Likely not mutagenic; Use of p53-deficient cells calls into question the biological relevance of the result	Low-Moderate
Mammalian cell gene mutation (Ouabain resistance)					
Patierno et al. (1989)	Mouse Fibroblast (C3H/ 10T1/2 clone 8)/3.3-13.2 mM/24 h)	Negative	• None	Not mutagenic	Moderate
Sasaki et al. (1986; 1983)	Chinese Hamster Ovary (CHO–K1)/up to 3.3 mM/2 h	Negative	• None	Not mutagenic	Moderate
Shimane (1985)	Chinese hamster lung (V79)/0.66–2.6 mM/ 24 h	Weakly positive Ouabain resistant mutant frequencies increased at 100 or 200 μg/mL, depending on exposure period	• Mutant frequencies were low and may have been within the historical control range. • There was no dose-response with 24 h exposure. • Moreover, V79 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g. primary human) cells.	Likely not mutagenic; Use of p53-deficient cells calls into question the biological relevance of the result	Low-Moderate

[a] Weight attributed to each study as discussed in the Methods.

3.1.2. *In vivo studies*

No *in vivo* gene mutation studies were available at the time of the Bergman et al. (1996) review, but 4 studies (summarized in Table 4) have been published since then:

- Kanki et al. (2005) tested acetaminophen (10,000 ppm in diet for 13 weeks, equivalent to 525 mg/kg/day or 140 mg/rat/day) for induction of gene mutations (6 TG resistance) in female transgenic *gpt* delta rats. The treatment resulted in a statistically significant increase in liver/bodyweight ratio, but there was no increase in GST-P positive liver cell foci, and no increase in *gpt* mutant frequency, even though other substances tested at the same time (IQ and *N*-nitrosopyrrolidine) were positive for both markers.
- These negative results were confirmed by Matsushita et al. (2013) in male *gpt* delta rats fed acetaminophen at 6,000 ppm in diet for 4 weeks, where no increase in *gpt* mutant frequency was found. The mutation spectra in acetaminophen-treated rats were also similar to

Table 4
Overview of mammalian *in vivo* mutagenicity studies.

Study	Animal Model	Dose/Route of Exposure/Duration	Response	Considerations	WoE Assessment	Weight[a]
Transgenic mutations						
Matsushita et al. (2013)	Rat (F344/NSlc *gpt* delta transgenic); liver	6000 ppm/diet/4 weeks	Negative	None	Not mutagenic	High
Kanki et al. (2005)	Rat (Sprague-Dawley *gpt* delta transgenic); liver	525 mg/kg/day/diet/13 weeks	Negative	None	Not mutagenic	High
Pig-a/Pig-ret						
Suzuki et al. (2016)	Rat (Sprague-Dawley); red blood cells and reticulocytes	500–2000 mg/kg/p.o./single administration	Negative	None	Not mutagenic 1, 2 or 4 weeks after dosing	High
van der Leede et al. (2020)	Rat (Sprague-Dawley); total red blood cells and reticulocytes	250–1000 mg/kg/day/p.o./3, 15 or 29 days	Negative	None	Not mutagenic	High

[a] Weight attributed to each study as discussed in the Methods.

those in controls. Acetaminophen inhibited the formation of GST-P positive liver cell foci, as was also shown by Ito et al. (1988).

- Suzuki et al. (2016) showed that single oral doses of acetaminophen at 500, 1,000 or 2,000 mg/kg did not induce *Pig-a* mutations in either erythrocytes or reticulocytes of rats, sampled 1, 2 or 4 weeks after dosing. By contrast, the positive control chemical, *N*-nitroso-*N*-ethylurea, induced a clear time-related response.
- In a more recent study in rats (van der Leede et al., 2020), acetaminophen at oral doses up to 2,000 mg/kg/day for 3 or 29 days, and up to 1,000 mg/kg/day for 15 days, including a 1 month recovery phase, did not induce biologically relevant increases in *Pig-a* mutations in reticulocytes or erythrocytes, but only slight to minimal hepatotoxicity was seen particularly after extended dosing or recovery.

Thus, acetaminophen does not induce gene mutations *in vivo* using transgenic or *Pig-a* endpoints; all studies were negative, even after extended dosing (13 weeks in Kanki et al., 2005).

3.2. Clastogenicity studies

3.2.1. In vitro studies

The *in vitro* studies investigating induction of micronuclei (MN), chromosomal aberrations (CA), and sister chromatid exchanges (SCE) are summarized in Table 5. The majority of studies in Table 5 will not be reviewed here because they were covered/reviewed by Bergman et al. (1996). While the MN and CA studies are given moderate weight (according to Brusick et al., 2016) the SCE studies are considered to contribute negligible weight. As stated in the Overview of the set of OECD Genetic Toxicology Test Guidelines and updates performed in 2014–2015 OECD (2015) "*TG 479 (in vitro sister chromatid exchange test for mammalian cells) was deleted because of a lack of understanding of the mechanism(s) of action of the effect detected by the test.*" This has also been discussed above (Section 2.2.2). For the WoE assessment, results in p53-competent human cells, at concentrations below the 1 mM top concentration recommended by ICH (2011) or below 50% cytotoxicity when determined by effects on cell proliferation, were considered most relevant. As discussed above, positive responses in p53-deficient cells (even if below 1 mM), at concentrations exceeding 1 mM or exceeding 50% cytotoxicity were considered of questionable relevance.

Two studies published since the Bergman et al. (1996) review are considered to be worthy of particular comment:

- Matsushima et al. (1999) showed that acetaminophen induced MN in Chinese hamster lung (CHL) cells after extended (24- and 48-h) treatments in the absence of metabolic activation, but significant effects were seen at lower concentrations than in other studies (from about 20 μg/mL (0.13 mM) and above). However, there was no concurrent measure of cytotoxicity reported. The induction of MN at low concentrations in CHL cells is consistent with induction of CA in the same cells reported by Ishidate et al. (1988), with induction of CA in V79 cells in the absence and presence of metabolic activation, at concentrations ranging from 25 to 200 μg/mL (Shimane, 1985), and induction of CA in CHO–K1 cells at similar low concentrations (Sasaki 1986; Sasaki et al., 1983). However, all of these studies used p53-deficient Chinese hamster cell lines, which are known to be more sensitive to cytotoxic and genotoxic chemicals than p53-competent human cells (Fowler et al., 2012), particularly in the absence of detoxification processes.
- Ibrulj et al. (2007) confirmed the ability of acetaminophen to induce CA in cultured human lymphocytes, exposed continuously for 72 h, but at a higher concentration than required in Chinese hamster cell lines (200 μg/mL = 1.3 mM), whereas negative results were obtained at 50 and 100 μg/mL. However, no MN were induced at any of the concentrations tested. Although cytotoxicity would be expected at concentrations >1 mM, the effect on nuclear division index was small (in the region of 20% at 200 μg/mL). The CA results are similar to those reported by Hongslo et al. (1991) in human lymphocytes exposed to acetaminophen for the last 24 h of a 72-h incubation. It is important to note that almost all induced aberrations in both studies were chromatid breaks which, as discussed earlier, are associated with cell lethality.

Therefore, as can be seen from Table 5 and as discussed above, in the most relevant MN and CA studies in p53-competent cells, no clastogenic effects were induced at acceptable concentrations (<1 mM, 151.16 μg/ml in the case of acetaminophen) or levels of cytotoxicity (<50%). Thus, clastogenic effects are not induced unless higher concentrations are employed that affect cellular processes and induce cytotoxicity. These higher concentrations are not expected to lead to viable cells containing stable genetic damage indicative of a clear genotoxic hazard in humans.

3.2.2. In vivo studies

Many *in vivo* chromosomal damage (MN, CA and SCE) studies were reviewed by Bergman et al. (1996), and together with more recent studies are summarized in Table 6. As discussed in Section 3.2, the biological relevance of induced SCE is not understood, and therefore the one *in vivo* SCE study is considered to contribute negligible weight to the overall assessment.

Certain *in vivo* MN and CA studies are worthy of detailed comment. Bergman et al. (1996) described 2 previously unpublished MN studies in rats (by Marshall and Thomas, summarized in Table 6) where slightly increased MN frequencies were seen, but only at oral doses of acetaminophen (3 × 900 mg/kg at 4 h intervals, or 3 × 500 mg/kg at 4 h intervals, or 1 × 1500 mg/kg) causing marked liver and bone marrow toxicity.

Markovic et al. (2013) administered acetaminophen intraperitoneally (i.p.) at 60 mg/kg to pregnant BALB/c mice consecutively on days 12 and 14 of pregnancy. The dose is equivalent to a normal 50 kg human

Table 5
Overview of *in vitro* clastogenicity and SCE studies in mammalian cells.

Study	Cell Type/Concentration/ Duration	Response	Considerations	WoE Assessment	Weight[a]
Micronucleus (MN) test					
Ibrulj et al. (2007)	Human Lymphocyte/0.33–1.3 mM/72 h	Negative	• MN frequencies in acetaminophen-treated cultures (3.5, 5.5, 5.75 per 1000 binuclear cells) were similar to control (5 per 1000 binuclear cells), and there were no significant differences.	Not genotoxic	Moderate
Simkó et al. (1998)	Human Amniotic Fluid (AFC)/ 0.3–2.5 mM/24, 48, 72 h	Positive; Increases in MN frequency after exposure to ≥1 mM	• MN frequencies in controls were high, and background data for AFC cells not available so it is not clear whether this was normal. • Slides were not coded so potential bias cannot be excluded. • Time and concentration dependence not consistent for cytotoxicity. • Results are questionable - inconsistent results reported for MN formation across plots for similar concentrations.	Multiple factors call into question the biological relevance of the result	Moderate
Müller-Tegethoff et al. (1995)	Primary Rat Hepatocyte/ 0.01–1.0 mM/48 h	Negative	• None	Not genotoxic	Moderate
Dunn et al. (1987)	Rat Kidney Fibroblast (NRK-49F)/5–20 mM/1 h	Positive Increase in MN frequency after exposure to 10 and 20 mM	• Positive, but only at very high concentrations (10 & 20 mM), exceeding ICH and OECD recommended limits, likely to cause physiological disruption and stress-related damage. Such results should be considered irrelevant and discounted.	Responses at excessive concentrations indicate result is not biologically relevant	Moderate
Matsushima et al. (1999); Matsuoka et al. (1993)	Chinese Hamster Lung (CHL/ IU)/0.099–0.79 mM/24, 48, 72 h	Positive Increase in MN frequency after exposure to ≥0.0993 mM	• CHL cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells. • Cytotoxicity not assessed.	Multiple factors call into question the biological relevance of the result	Low-Moderate
Chromosomal aberrations (scored as %)					
Watanabe (1982)	Human Lymphocytes/1–4 mM/ 72 h	Positive Increases in CA at concentrations ≥ 200 μg/mL (1.32 mM) after 72-h treatment	• Weakly positive at 200 μg/mL (1.32 mM), positive at 400 and 600 μg/mL (2.65 and 3.97 mM) after 72-h treatment. All concentrations giving positive response exceed the ICH recommended 1 mM limit, and all induced >50% mitotic inhibition, so aberrations could be due to severe cytotoxicity. • Gaps were included, and this is not normal convention. • Unclear whether slides were coded, so potential scorer bias cannot be excluded.	Use of high, cytotoxic concentrations indicate result is not biologically relevant	Moderate
NTP (1993)	Chinese Hamster Ovary (CHO)/ 8.3–33 mM/2, 18 h	Positive Increase in CA after 20-h treatment -S9 for concentrations ≥8.32 mM	• Only tested above 1 mM, exceeding ICH recommended limit, likely to cause physiological disruption and stress-related damage. Such results should be considered irrelevant and discounted. • No concurrent measure of cytotoxicity. • CHO cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Responses at excessive concentrations and use of p53-deficient cells indicates result is not biologically relevant	Low-Moderate
Shimane (1985)	Chinese Hamster Lung (V79)/ 0.17–1.32 mM//48 h	Positive Increases in CA for cells exposed to concentrations ranging from 25 to 200 μg/mL in the absence and presence of metabolic activation	• No concurrent measure of cytotoxicity, but probably <50% in this range, based on other data in the paper. • Unclear if slides were coded. • V79 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Multiple factors call into question the biological relevance of the result	Low-Moderate
Müller et al. (1991)	Chinese Hamster Lung (V79)/ 0.1–10 mM/2–24 h	Positive Increases in CA, particularly after 6 and 12-h continuous treatments in the absence of metabolism, or when co-cultured with hepatocytes; mainly positive at concentrations >1 mM	• Positive at concentrations >1 mM, exceeding ICH recommended limit, likely to cause physiological disruption and stress-related damage. Such results should be considered irrelevant and discounted. • No concurrent measure of cytotoxicity. • V79 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Responses at high concentrations and use of p53-deficient cells indicates result is not biologically relevant	Low-Moderate
Matsuoka et al. (1993)	Chinese Hamster Lung (CHL)/ 0.083–0.66 mM/24, 48 h	Positive Increase in CA after exposure to ≥0.33 mM	• CHL cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells. • Cytotoxicity not assessed.	Multiple factors call into question the	Low-Moderate

(*continued on next page*)

Table 5 (*continued*)

Study	Cell Type/Concentration/ Duration	Response	Considerations	WoE Assessment	Weight[a]
			• Unclear if slides were coded.	biological relevance of the result	
Chromosomal aberrations (scored as #)					
Ibrulj et al. (2007)	Human Lymphocyte/0.33–1.3 mM/72 h	Positive Increases in CA at 1.3 mM	• Positive only at 1.3 mM, exceeding ICH recommended limit. Such results should be considered irrelevant and discounted.	Response at a high concentration indicates result is not biologically relevant	Moderate
Hongslo et al. (1991)	Human Lymphocyte/0.75–3.0 mM/24 h	Positive including gaps Increases in CA at concentrations ≥ 0.75 mM, if gaps are excluded	• If gaps are excluded, weakly positive at 0.75 mM, and positive at 1.5 and 3 mM. However, abnormal chromosome morphology at 3 mM. • Slides were coded, but no concurrent measure of cytotoxicity, so chromosome breaks could be associated with toxic effects.	Multiple factors call into question the biological relevance of the result	Moderate
Hongslo et al. (1990)	Mouse Mammary (TA3H)/ 1.0–10 mM/2 h	Weakly positive Increases in CA at concentrations ≥1 mM	• Positive, but only at concentrations >1 mM, inducing >50% reduction in cell growth, both of which exceed the ICH recommended limits. Such results are therefore irrelevant and should be discounted. • There were high frequencies of acentric fragments even in control cells. • There are no background data on these mouse mammary tumor cells, and their p53 status and genomic stability are unknown.	Responses at high, cytotoxic concentrations, and high frequency of acentric fragments, indicates result is not biologically relevant	Moderate
Sasaki et al. (1980)	Chinese Hamster (Don-6)/ 0.5–1.0 mM/26–30 h	Positive Increases in CA at concentrations 75 and 151 μg/mL (0.5 and 1 mM) after 26–30 h continuous treatment	• No concurrent measure of cytotoxicity. • No background data on chromosome damage in Don-6 cells, which are probably also p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Multiple factors call into question the biological relevance of the results	Low-Moderate
Sasaki et al. (1986; 1983)	Chinese Hamster (CHO–K1)/ 0.066–0.66 mM/2, 6, 24 h	Positive Increases in CA at concentrations 70 and 100 μg/mL (0.63 and 0.662 mM, respectively) with 24 h treatment (excluding gaps)	• Not stated that slides were coded, so potential scorer bias cannot be excluded. • CHO–K1 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Multiple factors call into question the biological relevance of the results	Low-Moderate
Ishidate et al. (1978; 1983)	Chinese Hamster Lung Fibroblast (CHL-IU)/0.1–0.4 mM/24 h	Positive Increases in CA at 60 μg/mL (0.4 mM) after 48-h treatment in the absence of S9	• No concurrent measure of cytotoxicity. • Not clear whether slides were coded. • CHL cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Multiple factors call into question the biological relevance of the results	Low-Moderate
Sister chromatid exchange (SCE)					
Hongslo et al. (1991)	Human lymphocyte/1.0–10 mM/2 h	Positive Increases in SCE at concentrations ≥1 mM	• Positive, but only at concentrations ≥1 mM. • No concurrent measure of cytotoxicity. SCE results contribute negligible weight to genotoxic hazard assessment.	Relevance of SCE endpoint is not understood, and in conjunction with other factors, result is not biologically relevant	Negligible
Wilmer et al. (1981)	Human Fibroblast/0.1–10 mM/ 2 h	Negative	• Negative at concentrations from 0.1 to 10 mM	Not genotoxic	Negligible
Hongslo et al. (1990)	Mouse (TA3H)/1.0–10 mM/2 h	Positive Increases in SCE at concentrations ≥1 mM	• Positive, but only at concentrations ≥1 mM, inducing >50% reduction in cell growth, both of which exceed the ICH recommended limits. Such results are therefore irrelevant and should be discounted. • There are no background data on these mouse mammary tumor cells, and their p53 status and genomic stability are unknown.	Relevance of SCE endpoint is not understood, and in conjunction with other factors, result is not biologically relevant	Negligible
Holme et al. (1988)	Chinese Hamster Lung (V79)/ 1.0–10 mM/2 h		• Positive, but only at concentrations ≥1 mM.	Relevance of SCE endpoint is not	Negligible

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Table 5 (*continued*)

Study	Cell Type/Concentration/ Duration	Response	Considerations	WoE Assessment	Weight[a]
		Positive Increases in SCE at concentrations ≥1 mM	• V79 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	understood, responses at high concentrations, and use of p53-deficient cells indicate result is not biologically relevant	
Hongslo et al. (1988)	Chinese Hamster Lung (V79)/ 1.0–10 mM/2 h	Positive Increases in SCE at concentrations ≥3 mM	• Positive, but only at concentrations >1 mM. • V79 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Relevance of SCE endpoint is not understood, responses at high concentrations, and use of p53-deficient cells indicate result is not biologically relevant	Negligible
Hongslo et al. (1989)	Chinese Hamster Lung (V79)/ 3.0 mM/2 h	Positive Increases in SCE at concentration of 3 mM	• Positive, but only at concentrations >1 mM. • V79 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Relevance of SCE endpoint is not understood, responses at high concentrations, and use of p53-deficient cells indicate result is not biologically relevant	Negligible
Shimane (1985)	Chinese Hamster Lung (V79)/ 0.08–0.66 mM/24 h	Positive Increases in SCE at 50 and 100 μg/mL (0.33 and 0.66 mM, respectively) in the absence & presence of metabolic activation	• No concurrent measure of cytotoxicity, but probably <50% in this range, based on other data in the paper. • Unclear whether slides were coded. • V79 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Relevance of SCE endpoint is not understood, and in conjunction with multiple other factors, result is not biologically relevant	Negligible
NTP (1993)	Chinese Hamster Ovary/ 0.033–1.3 mM/1.5–2, 26 h	Positive/weakly positive Increase in SCE at concentrations ≥0.992 mM and 33.1 mM in the absence and presence of metabolic activation, respectively	• No concurrent measure of cytotoxicity. • CHO cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Relevance of SCE endpoint is not understood, and in conjunction with other factors, result is not biologically relevant	Negligible
Sasaki (1986)	Chinese Hamster Ovary (CHO–K1)/0.066–1.3 mM/27 h	Positive Increases in SCE at concentrations ≥0.662 mM	• No concurrent measure of cytotoxicity. • CHO cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Relevance of SCE endpoint is not understood), and in conjunction with other factors, result is not biologically relevant	Negligible

[a] Weight attributed to each study as discussed in the Methods.

Table 6
Overview of *in vivo* clastogenicity, aneugenicity and SCE studies.

Study	Animal Model	Dose/Route of Exposure/Duration	Response	Considerations	WoE Assessment	Weight[a]
Micronucleus (MN) test						
Marshall (1993)	Rat	900 mg/kg/p.o./3 doses at 4-h intervals	Positive; Weak (mainly 2-3-fold but up to 7-fold) increase in MN frequency after 24 and 40 h oral dosing of 3 doses of 900 mg/kg	• Report not available. Results taken from Bergman et al. (1996). • Results confounded by clear bone marrow toxicity due to very high dose.	Bone marrow toxicity calls into question the biological relevance of the weak result	High
Thomas (1995)	Rat	25–500 mg/kg/p.o./3 doses at 4-h intervals	Positive; Increase in MN frequency after 22 h of oral dosing of 3 doses of 500 mg/kg in male and female rats or 1,500 mg/kg in male rats	• Report not available. Results taken from Bergman et al. (1996). • The percentage of erythroblasts decreased dramatically at 500 mg/kg and above in non-purified preparations. • Increase in centrilobular necrosis in the liver and increased GOT, GPT, LDH, and creatinine plasma levels for males dosed with $\geq 3 \times 175$ mg/kg and for females dosed with 3×500 mg/kg.	Liver toxicity calls into question the biological relevance of the weak result	High
King et al. (1979) (oral)	Mouse	453 mg/kg/p.o./2 doses at 24-h intervals	Negative	• None	Not genotoxic	High
King et al. (1979) (i.p.)	Mouse	453 mg/kg/i.p. 2 doses at 24-h intervals	Negative	• None, but i.p. route not as relevant as oral or intravenous routes.	Not genotoxic	High
van der Leede et al. (2020)	Rat; reticulocytes	250–1,000 mg/kg/p.o./3, 28 days	Positive; Increases in MN frequency only after 1 month of dosing of 500 and 1,000 mg/kg/day	• Statistically significant increases in MN in reticulocytes after 1 month of dosing at 500 and 1,000 mg/kg/day were attributed to rebound erythropoiesis in response to marked hematotoxicity (severe bone marrow toxicity was seen 4 days after the start of dosing), and therefore the increased MN were concluded to be due to a non-genotoxic mode of action.	Not genotoxic; Severe bone marrow toxicity and rebound erythropoiesis indicate non-genotoxic mode of action	High
Sicardi et al. (1991)	Mouse	5–200 mg/kg/i.p./single administration	Positive; Weak positive (2.4-fold increase) in mice dosed with 100 or 150 mg/kg; no response at 200 mg/kg	• i.p. route not as relevant as oral or intravenous routes. • No dose-response. • No cytotoxicity data reported. • Results questionable.	Multiple factors call into question the biological relevance of this result	High
Markovic et al. (2013)	Mouse (dam)	60 mg/kg/i.p./3 doses at 24-h intervals on gestation day 12–14	Negative; 3.25-fold increase in MN frequency in pregnant BALB/c mice 48 h after i.p. dosing of 60 mg/kg (not statistically significant)	• i.p. route not as relevant as oral or intravenous routes. • Weak positive (3.25-fold) in pregnant BALB/c mice 48 h after i.p. dosing at 60 mg/kg on days 12 and 14 of pregnancy; however, the increase was not statistically significant. • Slides were not coded so potential scorer bias cannot be excluded.	Not genotoxic	High
Markovic et al. (2013)	Mouse (newborn)	60 mg/kg (dams)/i.p. (dams)/ Dams dosed at 3 doses at 24-h intervals on gestation day 12–14; newborns were not directly dosed	Positive; 2.28-fold increase in MN frequency in the blood of the pups (statistically significant, $p < 0.05$)	• Treatment of pregnant mice as above, but i.p. route not as relevant as oral or intravenous routes. • Weak positive (2.28-fold) in the blood of the pups that was statistically significant ($p < 0.0.5$), but associated with evidence of oxidative stress and hepatotoxicity. • Slides were not coded so potential scorer bias cannot be excluded.	Multiple factors call into question the biological relevance of this result	High
Chromosomal aberrations (scored as %)						
Reddy and Subramanyam (1985)	Mouse (Swiss albino)	25–100 mg/kg/p.o./single administration and 3 administrations at 24-h intervals	Negative/Positive; No increases in structural aberrations; Increased univalents and polyploidy in testes	• Increased univalents and polyploidy in testes; however, the biological relevance of univalents and polyploidy is unclear and can occur spontaneously.	Not clastogenic Multiple factors call into question the biological relevance of the univalents and polyploidy result	High
Laxminarayana et al. (1980)	Mouse (strain C3H/ He)	25–100 mg/kg/p.o./single administration and 3 administrations at 24-h intervals	Positive; Increased univalents and polyploidy in testes	• Increased univalents in testes; however, the biological relevance of univalents and polyploidy is unclear and can occur spontaneously.	Not clastogenic Multiple factors call into question the biological	High

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Table 6 (*continued*)

Study	Animal Model	Dose/Route of Exposure/Duration	Response	Considerations	WoE Assessment	Weight[a]
					relevance of the univalents result	
Giri et al. (1992)	Mouse	50–400 mg/kg/i.p./single administration	Positive; 2- to 4-fold increases in CA in mice dosed with 200 or 400 mg/kg, accompanied by dose-response	• i.p. route not as relevant as oral or intravenous routes. • Slides were coded and gaps were excluded. • Mitotic index showed no bone marrow toxicity. • Based on literature, these doses would be expected to be near or above hepatotoxicity Nayak et al. (2011).	Multiple factors call into question the biological relevance of this result	High
Chromosomal aberrations (scored as #)						
Reddy (1984)	Mouse	25–100 mg/kg/p.o./single administration and 3 administrations at 24-h intervals	Weakly positive; Very small increases in breaks (max. 3/205 cells) (if gaps and polyploid cells excluded)	• Same doses and dosing schedule as in Laxminarayana et al. (1980) (same laboratory), but bone marrow sampled at 24, 48 and 72 h. • Results highly questionable - if gaps and polyploid cells excluded (which is recommended practice), very small increases in breaks (max. 3/205 cells), but vehicle controls had 0 or only 1 break at all sampling times, which is unusually low. Increases from 0 or 1 in controls to 2 or 3 breaks in treated groups would not be considered biologically relevant. • No measure of cytotoxicity.	Multiple factors call into question the biological relevance of this result	High
Severin et al. (1995)	Mouse	800 mg/kg/p.o./3 doses at 4-h intervals	Positive; Clear increases in breaks after dosing with 3 × 800 mg/kg, particularly at 24 h after dosing	• No measure of cytotoxicity. • Slides not coded, so potential scorer bias cannot be excluded. • Hepatotoxicity was likely induced based on previous literature Uchida et al. (2017).	Multiple factors call into question the biological relevance of this result	High
Severin et al. (1995)	Mouse	100–400 mg/kg/i.p./single administration	Positive; Increases in breaks at doses of 200 or 400 mg/kg that decreased by 72 h	• i.p. route not as relevant as oral or intravenous routes. • Increases in breaks at all doses were 2–9 fold below the positive control and decreased by 72 h. • No measure of cytotoxicity. • Slides not coded, so potential scorer bias cannot be excluded. • Based on other studies, 250–300 mg/kg caused severe hepatotoxicity in mice and therefore, the small changes in CA could be due to toxic response Nayak et al. (2011).	Multiple factors call into question the biological relevance of this result	High
Aneuploidy						
Tsuruzaki et al. (1982)	Rat (embryos)	500–1,000 mg/kg/p.o./Dams dosed 2 weeks prior to mating to 11.5 days after mating,	Negative/Positive; No increases in structural aberrations. Increases in aneuploidy were reported for female rats dosed with 500 or 1000 mg/kg.	• Rat fetuses showed no increase in structural aberrations. • Results questionable since it is unclear how the slides were prepared (Japanese paper) and, it is not known whether the chromosome loss/gain was due to hypotonic treatment or a true effect on the spindle. • No measure of cytotoxicity. • Not known if slides were coded, so potential scorer bias cannot be excluded.	Not clastogenic. Multiple factors call into question the biological relevance of the aneuploidy result	High
Sister Chromatid Exchange (SCE)						
Giri et al. (1992)	Mouse	50–400 mg/kg/i.p./single administration	Positive; Increases in SCE for concentrations ≥50 mg/kg	• The i.p. route is not as relevant as oral or intravenous routes. • The SCE assay was deleted as a test guideline due to a poor understanding of the mechanisms of action that can be detected by the test and the biological relevance of SCE is not understood. SCE results therefore contribute negligible weight to genotoxic hazard assessment. Based on other studies the top dose at least would be expected to be hepatotoxic Nayak et al. (2011).	Multiple factors call into question the biological relevance of this result	Negligible

[a] Weight attributed to each study as discussed in the Methods.

Table 7
Overview of human clastogenicity, aneugenicity and SCE studies.

Study	Dose	Response	Considerations	WoE Assessment	Weight[a]
Micronucleus test					
Kocisová and Sram (1990)	3 × 1 g over 8 h	Negative	• The frequencies of MN in lymphocytes at all sampling times were similar to the pre-dose frequency, and not significantly different.	Not genotoxic	High
Šrám et al. (1990); Topinka et al. (1989)	3 × 1 g over 8 h	Positive; Statistically significant increase	• 2 studies, probably in the same group of volunteers, one with ascorbic acid, the other without. • In both cases MN in buccal cells increased transiently at 72 h but not at earlier or later sampling times. The MN frequencies were low, and, based on other publications, probably within the normal range.	Questionable biological relevance; responses likely within normal range.	High
Chromosomal aberrations					
Hantson et al. (1996)	3 × 1 g over 8 h	Negative	• Negative, even after suicidal doses.	Not genotoxic	High
Hongslo et al. (1991)	3 × 1 g over 8 h	Positive; Increase in gaps and chromatid breaks	• Increase in chromatid breaks in lymphocytes to 2.16% was significant but probably within normal range, and not considered biologically relevant.	Questionable biological relevance; chromatid break frequency likely within normal range.	High
Kocisová et al. (1988)	3 × 1 g over 8 h	Positive; Increase in chromatid breaks at certain sampling times.	• 2 studies in the same group of volunteers, one with ascorbic acid, the other without. • In both cases CA (only chromatid breaks) in lymphocytes increased transiently but at different times, and were normal either before and after, or after the increase. • Some individuals showed an increase in CA whereas others did not or showed a decrease (Bergman et al. (1996); Kirkland et al. (1992)). • Individuals who had shown a comparatively large increase in chromatid break frequency in the first study showed a small increase or even a decrease in the second study, and vice versa. It is therefore highly likely the increases in CA were due to chance Kirkland et al. (1992).	Multiple factors call into question the biological relevance of this result	High
Kirkland et al. (1992)	3 × 1 g over 8 h	Negative	• Double blind and placebo-controlled study.	Not genotoxic	High
Sister Chromatid Exchange (SCE)					
Hongslo et al. (1991)	3 × 1 g over 8 h	Positive	• Small increases in SCE (<2-fold) not considered biologically relevant. • The biological relevance of SCE is not understood, and the OECD guideline has been deleted. SCE results therefore contribute negligible weight to genotoxic hazard assessment.	Relevance of SCE endpoint not understood; small increases in SCE (<2-fold) not biologically relevant	Negligible

[a] Weight attributed to each study as discussed in the Methods.

taking 3 g of acetaminophen in a day. Blood samples were taken from the dams on day 12 of pregnancy and 48 h after drug administration for *in vivo* MN assays. In each litter, blood samples from 6 animals were analyzed for MN. Anti-oxidant activity (glutathione peroxidase in blood) and an indicator of lipid peroxidation (malondialdehyde in liver) were also measured in the pups. For each of the MN assays, 1000 acridine orange-stained reticulocytes per animal were assessed. This is a much smaller population of cells than is currently recommended in OECD guidelines. Importantly, it is not stated that the slides were "blinded" before scoring, and therefore scorer bias cannot be excluded. MN frequencies in vehicle control animals were normal (0.86/1000 reticulocytes) and were significantly increased by the positive control chemical (cyclophosphamide). MN frequencies in the dams treated with acetaminophen were increased slightly (3.25-fold) above vehicle control frequencies at 48 h after dosing but were not statistically significantly different. On the other hand, MN frequencies in the blood of the pups showed a smaller increase (2.28-fold) above vehicle controls, but this was statistically significant ($p < 0.05$). Glutathione peroxidase activity in the hemolysate of the new-born pups, and malondialdehyde levels in the livers of the pups, were significantly lower than in vehicle control pups. The authors speculate that the reduction of glutathione peroxidase reflected systemic oxidative stress. They state that this reduction is known to occur with acetaminophen treatment, while the reduction of malondialdehyde in the liver can be interpreted as an unspecific reaction to drug treatment that might have cytotoxic, and in particular hepatotoxic, effects associated with oxidative stress and lipid peroxidation. Given that mice are more sensitive than rats to the hepatotoxic effects of acetaminophen (Davis et al., 1974), and the increases in MN frequency in the dams were higher than in pups, yet were not statistically significant, and that the slides were not reported as having been "blinded" before scoring, these results should be viewed with caution. The results are consistent with the variable *in vivo* MN results in mice summarized in Bergman et al. (1996).

In a more recent study van der Leede et al. (2020) rats were given acetaminophen at oral doses up to 2,000 mg/kg/day for 3 and 29 days and 1,000 mg/kg for 15 days, including a 1 month recovery phase following the 29 day treatment. Statistically significant increases in MN in reticulocytes after 1 month of dosing at 500 and 1,000 mg/kg/day were attributed to rebound erythropoiesis in response to marked hematotoxicity (severe bone marrow toxicity was seen 4 days after the start of dosing), and therefore the increased MN were concluded to be due to a non-genotoxic mode of action.

Thus, acetaminophen was found to induce chromosomal damage *in vivo*, but many of the positive findings are questionable due to factors such as inappropriate route of administration, impact of toxicity and slides not "blinded" before scoring. In animal species more resistant to the hepatotoxic effects of acetaminophen, chromosomal damage only occurs at extreme and/or toxic exposures.

3.2.3. Human studies

The various human studies in which CA, MN and SCE have been measured are summarized in Table 7. As discussed in Section 3.2, the biological relevance of induced SCE is not understood, and therefore the one human SCE study is considered to contribute negligible weight to the overall assessment.

Bergman et al. (1996) previously reviewed all except one of these studies (Hantson et al., 1996, was published after the review), but since human data are considered to have high weight (Section 2.2.2), they are worthy of some detailed comment. Kocisová et al. (1988) reported on 2 studies. In the first study, acetaminophen was administered (3 × 1 g during 8 h) to 11 volunteers (3 males/8 females), and a small but statistically significant ($p < 0.05$) increase (from 1.68% pre-dose to 2.77% at 24 h after the first dose) in the proportion of lymphocytes with CA (excluding gaps) was observed. However, CA frequencies were not significantly different from pre-dose levels at later sampling times (72 or 168 h), and had returned to below pre-dose levels by 168 h. Thus, the increase in the proportion of cells with CA was transient, which is unusual since in other longitudinal studies CA levels tend to remain increased for periods of weeks or months (Kucerova et al., 1980; Schmid et al., 1985). The transient nature of the response could indicate that the damage was lethal, and that the damaged/dead cells had disappeared by the later sampling times. In the same publication a second study with the same volunteers was performed 1 week later with the same dosing schedule, except that each dose of acetaminophen was given together with 1 g of the anti-oxidant, ascorbic acid. A small but statistically significant ($p < 0.05$) increase (from 1.09% pre-dose to 2.22% 72 h after the first dose) in the proportion of cells with CA was reported. CA levels were not significantly different from pre-dose at 24 or 168 h, so again the increase in the proportion of cells with CA was transient. It is unclear whether the co-administration of ascorbic acid delayed the appearance of CA, or whether the time difference was due to chance. It should be noted that in both studies the increased CA levels were due entirely to chromatid breaks; there were no increases in chromosome breaks or exchanges. The results of the individual volunteers in these 2 studies, not included in the Kocisová et al. (1988) paper, was provided to Kirkland et al. for their 1992 paper. Since the same 11 volunteers were treated in each study it was possible to compare the CA responses in each individual. In the first (acetaminophen only) study 7 individuals showed an *increase* in CA frequency, and 4 showed *no increase or a decrease* in CA frequency. However, in the second (acetaminophen plus ascorbic acid) study 6 individuals showed an *increase* in CA frequency whereas 5 showed *no increase or a decrease* in CA frequency. Importantly, those individuals that showed increased CA levels with acetaminophen alone were not the same as those showing increased CA levels with acetaminophen plus ascorbic acid. Also, those individuals who had shown a comparatively large increase in chromatid break frequency in the first study showed a small increase or even a decrease in the second study, and vice versa. It was clear that no specific sub-group of the volunteers showed a consistent (increased or decreased) response, and that the variations in CA frequency in these 2 studies were due to chance and not related to treatment with acetaminophen.

Hongslo et al. (1991) administered acetaminophen (3 × 1 g during 8 h) to 9 volunteers and reported a small (from 2.38% pre-dose to 5.03% 24 h after the first dose) but insignificant ($p < 0.1$) increase in the proportion of cells with CA, including gaps. When gaps were excluded (as is normal practice), the increase was much smaller from 2.16% to 3.43% (this was not analyzed for statistical significance). Excluding gaps, the increase was primarily due to a 6-fold increase (significant, $p < 0.005$) in chromatid breaks (i.e. similar to the observations of Kocisová et al. (1988), although no blood samples were taken at later sampling times). As in the Kocisová et al. (1988) study, not all volunteers showed an increase in the levels of aberrant cells, excluding gaps (7/9 volunteers showed an increase but 2/9 showed a decrease), but in the absence of data from the same individuals at a later sampling time, we do not know whether these increases/decreases were persistent.

The study by Kirkland et al. (1992) was considered by Bergman et al. (1996) to be the most carefully controlled of these human studies since it was, unlike the other studies, a double-blind study (i.e. acetaminophen, 3 × 1 g during 8 h, and placebo groups) in 24 volunteers (12 males/12 females). The study therefore not only compared pre- and post-dose samples from each individual, but also compared acetaminophen-treated with placebo-treated groups. Blood samples for the determination of CA frequencies in peripheral lymphocytes were taken 24 h prior to dosing and at 24 h, 3 days and 7 days after administration of the first dose. Although a larger number of cells than in the 2 other studies was analyzed, no significant increases in % cells with CA (excluding gaps) were found either (a) when CA levels in the acetaminophen-treated individuals (men or/and women) were compared post-dose with pre-dose, or (b) when treated groups at any sampling time were compared with the placebo groups. There was no evidence that any individual responded to acetaminophen or that a group response was masked by non-responders. The study also included

determinations of plasma concentrations of acetaminophen; C_{max} after the third dose was 0.08 mM in men and 0.11 mM in women.

The findings of Kirkland et al. (1992) were confirmed in a study by Hantson et al. (1996), that was published after the Bergman et al. (1996) review. This showed that in volunteers who had been administered a single oral dose of 3 g acetaminophen, patients who had received 2 g of acetaminophen by intravenous infusion every 6 h for at least 7 days, and in self-poisoned patients who, for suicidal reasons, had ingested more than 15 g acetaminophen, there were no increases in the frequency of structural CA in the circulating lymphocytes. The lack of increased CA in suicide patients suggests that even toxic doses will not always lead to chromosome breakage.

Bergman et al. (1996) noted that a genetic polymorphism with respect to glutathione transferase has been described for Caucasians, a minor proportion of which lack glutathione transferase genes (13.31% for *GSTT1* null genotypes but 42–60% for *GSTM1* null genotypes in Europeans, (Sharma and Mehta, 2014) and this may render them more susceptible to genotoxic compounds. However, as discussed above, the individual data on the volunteers of the Kocisová et al. (1988) studies do not indicate that individual differences affected the increased chromosomal damage that they observed. In the large, double-blind, carefully controlled study of Kirkland et al. (1992), individuals possibly at higher risk were also probably included (based on the frequencies of null genotypes mentioned above), yet this study found no indications of a clastogenic effect at maximum therapeutic dosage.

Two publications described measurement of MN in humans given 1 g of acetaminophen orally 3 times during an 8-h period. Due to lack of methodological detail in these papers Bergman et al. (1996) were unable to reach any firm conclusions. These 2 studies are discussed briefly. Topinka et al. (1989) administered acetaminophen (3 × 1 g during 8 h) to 11 volunteers (3 males, 8 females). Another group (or maybe the same group of volunteers, since the design is identical that of Kocisová et al. (1988), and the average age was the same) were co-administered acetaminophen and ascorbic acid. Buccal cells were sampled at 0 (presumably equivalent to pre-dose), 24, 72 and 168 h after the first of the 3 doses. Slides were made, stained with light green, coded (for blinded scoring) and 2,000 cells/sample scored for presence of MN. No data on the individual volunteers was presented. A statistically significant 2-fold increase in the group mean frequency of micronucleated buccal cells was seen at 72 h but not at 24 or 168 h. A slightly smaller, but still statistically significant increase, was seen at 72 h in the acetaminophen + ascorbic acid group, but again there were no increases at 24 or 168 h. Since no historical data are given, it is unclear whether the raised MN frequencies (0.38% in each case) were within normal control ranges. However, the authors note that "*the statistically significant increase of micronuclei is low in comparison with other groups presented by* Stich et al. (1983)". The pre-dose MN frequencies in this study were 0.19 and 0.23%. However, a survey of multiple publications by Holland et al. (2008) revealed baseline MN frequencies ranging from 0.05 to 1.15%. Thus, a frequency of 0.38% would be well within the observed normal range and may simply represent "background noise". In the same study the authors observed decreased UDS at all sampling times, but notably at 168 h, and so the MN may result from inhibition of ribonucleotide reductase, as discussed by Bergman et al. (1996). However, it is therefore curious that increased MN frequencies were only seen at 72 h and not also at 168 h. The biological relevance of these results is therefore highly questionable.

Kocisová and Sram (1990) used the same treatment and sampling regimens as described by Topinka et al. (1989) and Kocisová et al. (1988) but with 12 volunteers (3 males/9 females). Blood samples were taken at 0 (presumably equivalent to pre-dose), 24, 72 and 168 h after the first of the 3 doses. Lymphocytes were stimulated to divide by phytohemagglutinin, and Cytochalasin B was added to the cultures 44 h later. Cultures were harvested at 72 h, cells were gently swollen, fixed and stained with Giemsa. It is not stated whether slides were blinded before scoring, but 1,000 binucleate cells/sample were scored for MN. The frequencies of MN at all sampling times were similar to the pre-dose frequency, and not significantly different, whereas the MN frequency in an elderly group of volunteers (included as a "positive control" group since MN frequencies increase with age) was significantly different. Thus, under the same conditions as this research group found increased CA in blood lymphocytes Kocisová et al. (1988) and reported increased MN in buccal cells (although, as discussed above, the biological relevance is debatable; Topinka et al., 1989), there were no increases in MN frequency in blood lymphocytes. Thus, in well conducted human studies, negative results for CA and MN in lymphocytes were observed at therapeutic doses and also in overdose situations (in self-poisoned persons). Increased MN in buccal cells of humans at therapeutic doses are not considered biologically relevant.

3.3. DNA damage studies

DNA damage and repair studies are considered "indicator tests" by OECD since they do not measure stable genetic damage OECD (2015). DNA damage may be reversible or may be lethal and not lead to mutations. As discussed in the methods section, these types of studies conducted *in vitro* contribute only low weight to the assessment of genotoxic potential, but *in vivo* studies could be considered to contribute moderate weight. The various DNA damage and repair studies with acetaminophen are summarized in Table 8, Table 9, and Table 10 for *in vitro*, *in vivo* and human studies, respectively.

3.3.1. In vitro studies

Many of the *in vitro* DNA damage studies were reviewed by Bergman et al. (1996), and, as can be seen from Table 8, were either negative, or were positive at high concentrations (>1 mM), or associated with high cytotoxicity, or used p53-deficient cells, or used non-guideline methods such as alkaline elution. Since there is no recommended guidance for such studies, and no historical control data to define an acceptable study, the results are of questionable biological relevance. Two more recent studies (Bandi et al., 2014; Jetten et al., 2014) used the comet assay, but again positive responses were only found at concentrations exceeding the ICH limit of 1 mM (ICH, 2011), and are therefore of questionable biological relevance.

As can also be seen in Table 8, all except one of the *in vitro* UDS studies used scintillation counting to measure radioactivity in DNA. This method was acceptable under OECD Guideline 482 (OECD, 1986), but only if S-phase synthesis is blocked (to avoid artifacts), and in some publications it is not clear whether this was done. The autoradiographic method is preferred by many since it is easy to identify and exclude cells in S-phase, but this was only used by Sasaki (1986). The OECD guideline for the *in vitro* UDS test (TG 482) was deleted since it is rarely used in various legislative jurisdictions. The results from these studies were very inconsistent with as many authors reporting negative results or decreased UDS as reported weak or positive responses. In any case, these studies are of low weight and considered of questionable biological relevance.

The repair of single-strand DNA breaks induced by UV or NQO was studied in several publications by the same research group, and acetaminophen was shown to inhibit the induced DNA damage (Brunborg et al., 1995; Hongslo et al., 1993). However, impairment of nucleotide excision repair occurs at cytotoxic concentrations and exhibits a threshold, but cytotoxicity was not measured in these studies. Such inhibition of repair is likely to be associated with depletion of nucleotide pools (as discussed by Kunz 1982, 1988, 1994), and it is interesting that addition of deoxyribonucleosides reversed the inhibitory effect of acetaminophen.

3.3.2. In vivo studies

Many of the *in vivo* DNA damage studies, summarized in Table 9, were performed using the i.p. route which is not considered as relevant as oral or intravenous routes, or clinically relevant. Some studies were

Table 8
Overview of *in vitro* DNA damage/repair studies in mammalian cells.

Study	Cell Type/Concentration/ Duration	Response	Considerations	WoE Assessment	Weight[a]
DNA damage					
Andersson et al. (1982); Nordenskjold and Moldeus (1983)	Cultured skin fibroblasts/0.05 mM/Not Reported	Negative	• Negative; DNA strand breaks are an "indicator test" and contribute only low weight to hazard assessment.	Not genotoxic	Low
Jetten et al. (2014)	Human liver tissue/2.5–10 mM/24 h	Positive; Increase in DNA damage at concentrations ≥ 2.5 mM	• The concentrations of acetaminophen used to treat the liver slices ranged from 2.5 to 10 mM, and therefore exceeded recommended upper limits for mammalian cell tests. Such results are irrelevant and should be discounted. • Benchmark dose analysis suggested some donors showed increased comets, but these were only seen at high cytotoxicity (>50%). DNA strand breaks are an "indicator test" and contribute only low weight to hazard assessment.	Use of excessive and cytotoxic concentrations indicates result is not biologically relevant	Low
Bandi et al. (2014)	Human peripheral blood mononuclear cells/10 mM/ 72 h	Positive; Increase in comets and γH2AX at concentration 10 mM	• Positive for comets and γH2AX, but data only given for 10 mM, which exceeds the recommended upper limit for mammalian cell tests. • Viability was reduced to approximately 50% at this concentration. Such results are irrelevant and should be discounted. DNA strand breaks are an "indicator test" and contribute only low weight to hazard assessment.	Use of excessive concentrations indicates result is not biologically relevant	Low
Dybing et al. (1984)	Reuber rat hepatoma/10 mM/1 h	Negative	• Used alkaline elution method for which there is no OECD guideline, so no recommendations for what constitutes an adequate test or how to interpret the results. • 10 mM acetaminophen did not induce strand breaks. • The p53 status of Reuber hepatoma cells is unknown. DNA strand breaks are an "indicator test" and contribute only low weight to hazard assessment.	Not genotoxic	Low
Sasaki (1986)	Hamster ovary (CHO–K1)/ 3.3–33.1 mM/0.5, 1, 2 h	Weakly positive; Increase in DNA damage at concentration 5,000 μg/mL (33.1 mM)	• Used alkaline elution method for which there is no OECD guideline, so no recommendations for what constitutes an adequate test or how to interpret the results. • Weak positive response at 5,000 ug/ml to 33.1 mM, exceeding the recommended upper limit for mammalian cell tests. Such results are irrelevant and should be discounted. • No details are given, so the extent of cytotoxicity at these high concentrations is not clear. • CHO cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells. • DNA strand breaks are an "indicator test" and contribute only low weight to hazard assessment.	Multiple factors call into question the biological relevance of this result	Low
Hongslo et al. (1988)	Chinese Hamster Lung (V79)/ 1.0–10 mM/2 h	Weakly positive; Increase in DNA damage for 3 and 10 mM concentrations	• Used alkaline elution method for which there is no OECD guideline, so no recommendations for what constitutes an adequate test or how to interpret the results. • Weak positive response at 3 and 10 mM, exceeding the recommended upper limit for mammalian cell tests. Such results are irrelevant and should be discounted. • Cytotoxicity as measured by colony forming ability was only slight. • V79 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g. primary human) cells. DNA strand breaks are an "indicator test" and contribute only low weight to hazard assessment.	Multiple factors call into question the biological relevance of this result	Low
Unscheduled DNA synthesis (UDS)					
Binkova et al. (1990)	Peripheral blood lymphocytes/0.05–10 mM/ 0.5 h	Weakly positive; Slight increase in UDS over a wide concentration range	• UDS was measured by scintillation counting, which is only acceptable if S-phase synthesis is blocked, and it is not clear if this was done. • Statistically significant increase in UDS over a wide concentration range, but small increases probably not biologically relevant. • UDS is an "indicator test", the *in vitro* test guideline has been deleted, and this endpoint is considered low weight.	Various factors call into question the biological relevance of this result	Low
Dybing et al. (1984)	Mouse hepatocytes/0.1–10 mM/18–19 h	Positive; UDS was induced at concentrations ≥ 5 mM	• UDS was measured by scintillation counting, which can be susceptible to artifacts. • UDS was induced at 5 mM and above, which exceeds the recommended upper limit for mammalian cell tests. Such results are irrelevant and should be discounted. • UDS is an "indicator test", the *in vitro* guideline has been deleted, and this endpoint is considered low weight.	Responses at high concentrations indicate result is not biologically relevant	Low
Holme and Søderlund (1986)	Mouse hepatocytes/0.1–10 mM/18–19 h	Positive; UDS was induced at concentrations ≥ 5 mM	• UDS was measured by scintillation counting, which can be susceptible to artifacts. • UDS was induced at 5 mM and above, which exceeds the recommended upper limit for mammalian cell tests. Such results are irrelevant and should be discounted. • Also, cytotoxicity was around 50% or higher at these concentrations.	Various factors call into question the biological relevance of this result	Low

(*continued on next page*)

Table 8 (*continued*)

Study	Cell Type/Concentration/ Duration	Response	Considerations	WoE Assessment	Weight[a]
			• UDS is an "indicator test", the *in vitro* guideline has been deleted, and this endpoint is considered low weight.		
Holme and Søderlund (1986)	Rat hepatocytes/0.5–10 mM/ 18–19 h	Weakly positive; UDS was induced at concentrations ≥ 2.5 mM	• UDS was measured by scintillation counting, which can be susceptible to artifacts. • UDS was slightly increased at 2.5 mM and above, which exceeds the recommended upper limit for mammalian cell tests. Such results are irrelevant and should be discounted. • UDS is an "indicator test", the *in vitro* guideline has been deleted, and this endpoint is considered low weight.	Various factors call into question the biological relevance of this result	Low
Milam and Byard (1985)	Rat hepatocytes/3.0–7.0 mM/24 h	Negative	• UDS was measured by scintillation counting, which can be susceptible to artifacts. No induction of UDS at 3 and 7 mM. • UDS is an "indicator test", the *in vitro* guideline has been deleted, and this endpoint is considered low weight.	Not genotoxic	Low
Sasaki (1986)	Rat hepatocytes/0.83–3.3 mM/Not Reported	Negative; Decrease in repair at concentrations ≥ 1.66 mM	• Significant decrease in nuclear granules likely due to toxicity. UDS is an "indicator test", the *in vitro* guideline has been deleted, and considered low weight.	Multiple factors call into question relevance and use of study for hazard assessment	Low
Holme and Søderlund (1986)	Hamster hepatocytes/0.1–10 mM/18–19 h	Negative; Decrease in repair	• UDS was measured by scintillation counting, which can be susceptible to artifacts. • The decrease in UDS is probably due to cytotoxicity. UDS is an "indicator test", the *in vitro* guideline has been deleted, and this endpoint is considered low weight.	Not genotoxic	Low
Holme and Søderlund (1986)	Guinea pig hepatocytes/ 0.1–10 mM/18–19 h	Negative; Decrease in repair	• UDS was measured by scintillation counting, which can be susceptible to artifacts. • The decrease in UDS is probably due to cytotoxicity. UDS is an "indicator test", the *in vitro* guideline has been deleted, and this endpoint is considered low weight.	Not genotoxic	Low
Hongslo et al. (1988)	Chinese Hamster Lung (V79)/0.25–10 mM/2 h	Negative; Decrease in repair	• UDS was measured by scintillation counting, which is only acceptable if S-phase synthesis is blocked, and it is not clear if this was done. • The decrease in UDS is probably due to toxicity associated with inhibition of replicative DNA synthesis. UDS is an "indicator test", the *in vitro* guideline has been deleted, and this endpoint is considered low weight.	Not genotoxic	Low
Impairment of nucleotide excision repair					
Hongslo et al. (1993)	UV-pretreated mononuclear blood cells, T-lymphocytes, B-lymphocytes & monocytes/ 0.1–0.3 mM/0.75, 2.5 h	Positive; Inhibited repair of UV-induced DNA strand breaks at concentration of 0.1 mM (mononuclear blood cells) and 0.3 mM (all cells tested)	• No OECD guideline for this technique, so no recommendations for what constitutes an adequate test or how to interpret the results. Inhibitory effects reversed by addition of deoxyribonucleosides. • Impairment of nucleotide excision repair occurs at cytotoxic concentrations and exhibits a threshold; however, cytotoxicity not measured.	Various factors call into question the biological relevance of these results	Low
Brunborg et al. (1995)	UV, X-ray, NQO, or MMS-pretreated mononuclear blood cells/0.3 mM/0.5–2.5 h	Positive/Negative; Inhibited repair of UV- and NQO-induced DNA strand breaks at concentration of 0.3 mM; no effect on MMS- or X-ray-induced DNA strand breaks	• No OECD guideline for this technique, so no recommendations for what constitutes an adequate test or how to interpret the results. Inhibitory effects reversed by addition of deoxyribonucleosides. • Impairment of nucleotide excision repair which occurs at cytotoxic concentrations and exhibits a threshold; however, cytotoxicity not measured.	Various factors call into question the biological relevance of these results	Low
Brunborg et al. (1995)	UV-pretreated HL-60 cells or rat hepatocytes/0.3–1 mM/ 0.75–2.5 h	Positive; Inhibited repair of UV-induced DNA strand breaks at concentrations 0.3 mM (HL-60) and 1 mM (rat hepatocytes)	• No OECD guideline for this technique, so no recommendations for what constitutes an adequate test or how to interpret the results. • Impairment of nucleotide excision repair which occurs at cytotoxic concentrations and exhibits a threshold; however, cytotoxicity not measured.	Various factors call into question the biological relevance of these results	Low
Brunborg et al. (1995)	UV-pretreated fibroblast cells or rat testicular cells/1 mM/ 0.75–2.5 h	Negative	• No OECD guideline for this technique, so no recommendations for what constitutes an adequate test or how to interpret the results. • Impairment of nucleotide excision repair which occurs at cytotoxic concentrations and exhibits a threshold; however, cytotoxicity not measured.	Not genotoxic	Low
Brunborg et al. (1995)	NQO-treated rat testicular cells/1 mM/0.75–2.5 h	Positive; Inhibited repair of NQO-	• No OECD guideline for this technique, so no recommendations for what constitutes an adequate test or how to interpret the results.	Various factors call into question the biological relevance of these results	Low

(*continued on next page*)

Table 8 (*continued*)

Study	Cell Type/Concentration/Duration	Response	Considerations	WoE Assessment	Weight[a]
		induced DNA strand breaks at concentration of 1 mM	• Impairment of nucleotide excision repair which occurs at cytotoxic concentrations and exhibits a threshold; however, cytotoxicity not measured.		
Hongslo et al. (1988)	UV-pretreated Chinese Hamster lung (V79)/1–10 mM/1 h	Positive; Inhibited repair of UV-induced DNA strand breaks at concentrations 3 and 10 mM	• Repair inhibited at 3 and 10 mM, which did not significantly reduce colony formation. However, these concentrations exceed the recommended upper limit, and such results are irrelevant and should be discounted. • No OECD guideline for this technique, so no recommendations for what constitutes an adequate test or how to interpret the results. • V79 cells are p53-deficient and susceptible to misleading positive responses not found in p53-competent, genomically stable (e.g., primary human) cells.	Use of excessive concentrations and p53-deficient cells indicate results are not biologically relevant	Low
Impairment of DNA repair					
Wan et al. (2004)	Rat/C6 glioma cells/2.5–5.0 mM/24, 48 h	Positive; Inhibition of 8-oxodG incision at concentration 5 mM	• Inhibition of repair of oxidative damage by OGG1 at 5 mM, associated with reactive oxygen species (ROS) production and GSH depletion. This concentration exceeds the recommended upper limit for testing in mammalian cells. Such results are irrelevant and should be discounted.	Use of excessive concentrations indicates results are not biologically relevant	Low
Oxidation of DNA (8-oxodG)					
Wan et al. (2004)	Rat/C6 glioma cells/2.5–5.0 mM/24, 48 h	Positive; Formation of 8-oxodG at concentrations ≥2.5 mM	• Induction of 8-oxodG at 2.5 mM and above, associated with ROS production and GSH depletion. These concentrations exceed the upper limit for testing in mammalian cells. Such results are irrelevant and should be discounted.	Use of excessive concentrations indicates results are not biologically relevant	Low

[a] Weight attributed to each study as discussed in the Methods.

negative, but a few reported weak or positive results. However, these were only under conditions where hepatotoxicity was or would have been induced. Many of these studies were reviewed by Bergman et al. (1996), but 2 more recent studies are worthy of more detailed comment:

- Oshida et al. (2008) investigated the induction of DNA strand breaks (comets) in the livers, kidneys and bone marrow of mice given a single intraperitoneal dose of acetaminophen. No DNA damage was induced in kidneys or bone marrow, and comets were only induced in liver at the highest dose (300 mg/kg) where hepatotoxicity was also observed.
- In the recent study of van der Leede et al. (2020) small increases in liver comets were seen in 2 out of 6 male rats dosed orally at 1000 mg/kg/day for 1 month, but single cell and focal necrosis were observed in the liver of these rats, so it is highly likely that these histopathological findings influenced the DNA damage response. Therefore, the small increases in DNA damage levels were not considered biologically relevant.

3.3.3. Human study

Only 1 human study investigated effects of acetaminophen on DNA repair (Table 10). A decrease in UDS was observed, which would indicate a toxic rather than genotoxic effect.

The weight of evidence demonstrates that acetaminophen does not cause DNA damage in reliable, well-controlled test systems using a physiologically relevant route of administration in the absence of cytotoxicity or hepatotoxicity. Such results are consistent with other endpoints (e.g., chromosomal damage) showing that genotoxicity resulting from acetaminophen exposure only occurs at high, toxic doses which are not likely to result in viable cells containing stable genetic damage that would be indicative of a clear genotoxic hazard.

3.4. Cell transformation studies

Although not considered genotoxicity assays, cell transformation assays can provide supporting information, and can be considered as indicative of carcinogenic potential, so are included here. Patierno et al. (1989) studied *in vitro* cell transformation of C3H/10T½ clone 8 mouse embryo fibroblast (10T½) cells exposed to acetaminophen. These cells are considered to be similar to BALB/3T3 and Swiss/3T3 cells, as they are stable in culture and highly sensitive to post-confluence inhibition of cell division (Reznikoff et al., 1973). C3H/10T½ cells, together with other immortalized aneuploid mouse cells, represent one of the 2 major types of systems used for *in vitro* cell transformation assays, the other type being primary diploid cells, such as Syrian Hamster Embryo cells (Creton et al., 2011).

In this study, Patierno et al. (1989) treated 10T½ cells with acetaminophen at concentrations ranging from 0.5 to 2.0 mg/mL (3.3–13 mM) for either 24 h without S-9 or 3 h with Aroclor 1254-induced hamster liver S-9. In the absence of S-9 acetaminophen induced a small, but dose-dependent increase in the number of type II morphologically transformed foci. A greater number of type II transformed foci were induced by acetaminophen in the presence of S-9. Similar cell transformation results were observed with the carcinogen phenacetin (of which acetaminophen is a major metabolite). Several metabolites of acetaminophen (and phenacetin) were also tested in C3H/10T½ cells (NAPQI, PAP, p-benzoquinone), and each was found to be inactive in the cell transformation assay. Patierno et al. (1989) characterized the type II foci induced by acetaminophen and phenacetin as atypical (weak) non-neoplastic morphologically transformed cells that *"did not exhibit any other classical parameters of neoplastic transformation, such as increased saturation density or anchorage independence"*. Patierno et al. (1989) indicated that the *"results suggest that metabolic intermediates of high concentrations of phenacetin and acetaminophen induce a low frequency of nonneoplastic morphological transformation of 10T½ mouse embryo cells"*. Therefore, the results by Patierno et al. (1989) suggest that

acetaminophen does not cause neoplastic transformation in this *in vitro* assay.

4. Discussion

4.1. Weight of evidence assessment

The Weight of Evidence assessment of the available genotoxicity and supporting cell transformation studies presented herein demonstrates that there is no clear evidence that acetaminophen induces gene mutations in bacteria or cultured cells *in vitro*, or in rodents *in vivo*. It can induce genotoxic effects (chromosomal and DNA damage) in cultured cells and animals, but in genomically stable p53-competent cells and in animal species more resistant to the hepatotoxic effects of acetaminophen, this only occurs at extreme and/or toxic exposures. Thus, the clastogenic effects of acetaminophen in relevant systems only occur at cytotoxic exposures, such that the cells containing these CA will not be able to survive to produce stable or persistent genetic damage that could pre-dispose to genetic disease or cancer.

It is interesting to compare the pattern of genotoxicity results observed for acetaminophen with the pattern of results that would be expected for a typical genotoxic carcinogen. This comparison is summarized in Table 11 below, where it can be seen quite clearly that acetaminophen does not present a profile that is typical of a clear genotoxic carcinogenic hazard.

4.2. Mechanistic considerations

There is increasing evidence that many substances producing genotoxic responses, particularly *in vitro* in tests detecting chromosomal or DNA damage, exhibit thresholds that are tied to cytotoxicity. Several publications have described modes of action and circumstances that would define such a threshold-mediated genotoxic response (Muller and Kasper, 2000; Scott et al., 1991; Thybaud et al., 2007). Some examples of indirect mechanisms that might not be relevant for humans or might exhibit a threshold are given in the ICH genotoxicity guidance, ICH S2 (R1) (ICH, 2011) and include:

- Inhibition of DNA synthesis
- Reactive oxygen species produced only at high concentrations
- Aneuploidy due to spindle poisons
- Indirect induction of DNA damage secondary to damage to non-DNA targets (e.g. at high levels of cytotoxicity or at high concentrations)
- Substances metabolically detoxified or rapidly excreted *in vivo*.

Bergman et al. (1996) proposed 3 possible mechanisms (discussed below) to explain acetaminophen-induced genotoxicity that is observed in certain model test systems, and stated that all would involve dose thresholds. New insights have emerged on the pathways for acetaminophen toxicity (Ramachandran and Jaeschke, 2018), which suggest that there are alternative or additional pathways that help to explain the data and these are discussed in the context of the 3 mechanisms proposed by Bergman et al. (1996).

4.2.1. Inhibition of ribonucleotide reductase

Ribonucleotide reductase is an enzyme that catalyses the reduction of ribonucleotides to deoxyribonucleotides, and so provides the cell with a balanced supply of the 4 deoxyribonucleotides required for DNA synthesis. Hongslo et al. (1990) hypothesized that acetaminophen inhibits ribonucleotide reductase *in vitro* by destroying a tyrosyl free radical localised on one of the subunits of the enzyme. One proposed way for this to occur is through acetaminophen transformation to a free radical (e.g. a radical phenoxy species), which may be formed non-enzymatically or catalysed by peroxidases (Fischer et al., 1986; Hongslo et al., 1990; Richard et al., 1991).

As discussed in Section 3.3, and summarized in Table 8 through Table 10, studies have reported that acetaminophen can inhibit replicative DNA synthesis, and there are reports of both increased and decreased reparative DNA synthesis. It has been proposed that decreased reparative DNA synthesis occurs through inhibition of ribonucleotide reductase, since this inhibition leads to decreases in, or depletion of, the cellular deoxyribonucleotide pool. It has been well documented that disruption of the deoxyribonucleotide pool leads to aberrant DNA replication or repair which in turn leads to a multitude of genotoxic effects including mutation, recombination, DNA strand breakage, CA, MN and SCE (Kunz, 1982, 1988; Kunz et al., 1994).

While this mechanism is plausible, the following factors suggest that there may be alternative reasons for these effects and that there is insufficient evidence to support that these effects are due to inhibition of ribonucleotide reductase. Also, it is suggested that these effects do not have any relevance to the carcinogenicity hazard potential of acetaminophen based on the following:

- There are no studies showing direct binding of acetaminophen to ribonucleotide reductase (Hinson et al., 2004).
- No data was identified demonstrating that acetaminophen inhibits ribonucleotide reductase or disrupts the ribonucleotide pool *in vivo*.
- Studies claiming that there is inhibition of ribonucleotide reductase have been conducted in *in vitro* model test systems that have highly questionable relevance to humans or animals (e.g., mouse mammary immortalized tumor cell line with mutations introduced (Hongslo et al., 1990); in multiple *in vitro studies* the conditions tested are implausible in humans (i.e., high concentrations for 48 h in a static system).
- The reduced thymidine uptake is transient, reversing *in vivo* within 2–4 h (Hongslo et al., 1994; Lister and McLean, 1997).
- There is no evidence that the effects are sustained with multiple dosing at therapeutic or non-toxic supratherapeutic doses and lead to sustained DNA effects at non-toxic concentrations.
- If there is *in vitro* inhibition of ribonucleotide reductase, it occurs at lower concentrations than are required for the induction of genotoxicity, indicating that a threshold for genotoxicity exists. The downstream disruption of nucleotide pools would likely lead to genotoxicity without any interaction between the chemical itself and DNA. Therefore, concentrations of chemicals below the no-effect concentration (NOEC) for nucleotide pool disruption would not be genotoxic (there would be a threshold, as discussed in Thybaud et al., 2007).

There are other potential mechanisms, besides direct inhibition of ribonucleotide reductase, that could cause the effects seen in these *in vitro* model systems by Hongslo et al. (1990). One potential alternative mechanism for the effects observed on DNA repair could be acetaminophen induced mitochondrial permeability transition *in vitro* that occurs in 2 phases (glutathione depletion/covalent binding followed by mitochondrial dysfunction). Mitochondrial dysfunction can drive toxicity, inhibit ribonucleotide reductase function in the cytosol (Desler et al, 2007, 2010) and lead to the *in vitro* effects on DNA that were observed.

When viewed in the context of the negative carcinogenicity studies Murray et al. (2020) and other genetic toxicology studies, the data support that if there is inhibition of ribonucleotide reductase, which may not be the case, the inhibition does not represent a genotoxic or carcinogenic hazard to humans.

4.2.1.1. Increase in cytosolic and intranuclear Ca^{2+} levels. Acetaminophen metabolism forms a reactive metabolite, which binds to cellular proteins. One of the proteins adducted is Ca^{2+} ATPase (Tsokos-Kuhn et al., 1988), which leads to increase in cytosolic Ca^{2+} levels. Elevated cytosolic Ca^{2+} concentrations can contribute to mitochondrial dysfunction and ATP depletion (Jaeschke, 1990), and after

Table 9
Overview of *in vivo* DNA damage studies.

Study	Animal Model	Dose/Route of Exposure/Duration	Response	Considerations	WoE Assessment	Weight[a]
Comet assay						
Oshida et al. (2008)	Mouse (liver)	12–300 mg/kg/i.p./ single administration	Positive; Increase in comets at sampling times of 4 and 24 h after administration of the highest dose of 300 mg/kg	• i.p. route is not as relevant as oral or intravenous routes. • Plasma AST and ALT levels indicated hepatotoxicity. Therefore, the DNA damage may have been secondary to tissue toxicity.	Response at high, hepatotoxic dose calls into question the biological relevance of this result	Moderate
Oshida et al. (2008)	Mouse (kidney)	12–300 mg/kg/i.p./ single administration	Negative	• None, but i.p. route not as relevant as oral or intravenous routes.	Not genotoxic	Moderate
Oshida et al. (2008)	Mouse (bone marrow)	12–300 mg/kg/i.p./ single administration	Negative	• None, but i.p. route not as relevant as oral or intravenous routes.	Not genotoxic	Moderate
van der Leede et al. (2020)	Rat (peripheral blood and liver)	250–1,000 mg/kg/p.o./29 days	Weak positive in liver; Small increase in liver comets were seen in 2 out of 6 male rats dosed at 1,000 mg/kg/day for 1 month	• Single cell and focal necrosis were observed in the liver of these rats, so it is highly likely that these histopathological changes influenced the DNA damage response.	Response at high, hepatotoxic dose calls into question the biological relevance of this result	Moderate
DNA damage by other methods						
Hongslo et al. (1994)	Rat (kidney)	600 mg/kg/i.p/single administration	Negative	• None, but i.p. route is not as relevant as oral or intravenous routes.	Not genotoxic	Moderate
Hongslo et al. (1994)	Rat (liver)	600 mg/kg/i.p./single administration	Negative	• None, but i.p. route is not as relevant as oral or intravenous routes.	Not genotoxic	Moderate
Hongslo et al. (1994)	Mouse (kidney)	600 mg/kg/i.p/single administration	Negative	• None, but i.p. route is not as relevant as oral or intravenous routes.	Not genotoxic	Moderate
Hongslo et al. (1994)	Mouse (liver)	300–600 mg/kg/i.p./ single administration	Positive; Clear increase in DNA strand breaks after 600 mg/kg dose, but not at 300 mg/kg.	• i.p. route is not as relevant as oral or intravenous routes. • Used alkaline elution method for which there is no OECD guideline, so no recommendations for what constitutes an adequate test or how to interpret the results. • Based on other studies the 600 mg/kg dose would be hepatotoxic, so DNA damage likely secondary to tissue toxicity.	Various factors call into question the biological relevance of this result	Moderate
Oxidation of DNA						
Wang et al. (2015)	Mouse (serum)	400 mg/kg/p.o./single administration	Positive; Slight increase in 8-OHdG levels (50%)	• Mice given single dose of 400 mg/kg - presumably oral, but not clear. • AST and ALT levels increased markedly and GSH decreased, indicating hepatotoxicity.	Response at hepatotoxic dose calls into question the biological relevance of this result	Moderate
Impairment of nucleotide excision repair						
Hongslo et al. (1994)	NQO-treated Rat (liver, kidney, spleen)	300 mg/kg/i.p./single administration	Positive; Inhibited repair of NQO-induced DNA strand breaks in all 3 tissues	• i.p. route is not as relevant as oral or intravenous routes. • Impairment of nucleotide excision repair which occurs at cytotoxic concentrations and exhibits a threshold response. • Based on other studies, a single i.p. dose of 300 mg/kg would be likely to be hepatotoxic.	Multiple factors call into question the biological relevance of this result	Moderate
Hongslo et al. (1994)	NQO-treated Mouse (liver, kidney, spleen)	300 mg/kg/i.p./single administration	Positive; Inhibited repair of NQO-induced DNA strand breaks in all 3 tissues	• i.p. route is not as relevant as oral or intravenous routes. • Impairment of nucleotide excision repair which occurs at cytotoxic concentrations and exhibits a threshold response. • Based on other studies a single i.p. dose of 300 mg/kg would be likely to be hepatotoxic.	Multiple factors call into question the biological relevance of this result	Moderate

[a] Weight attributed to each study as discussed in the Methods.

Table 10
Overview of human DNA damage studies.

Study	Dose	Response	Considerations	WoE Assessment	Weight[a]
Unscheduled DNA Synthesis (UDS)					
Topinka et al. (1989)	3 × 1 g over 8 h	Decrease in repair	• UDS in lymphocytes was measured by scintillation counting, which can be susceptible to artifacts. • Decrease in UDS is probably due to toxicity.	Not genotoxic	Low

[a] Weight attributed to each study as discussed in the Methods.

Table 11
Comparison of test response profiles from acetaminophen to the profile characteristics of confirmed genotoxic carcinogens (adapted from Brusick et al. (2016); based on Bolt et al. (2004) and Petkov et al. (2015)).

Characteristic	Carcinogens with a proven genotoxic mode of action	Acetaminophen
Profile of Test Responses in Genetic Assays	Positive effects across multiple key predictive endpoints (i.e. high weight studies such as gene mutation in bacteria or *in vivo*, chromosomal aberrations or micronuclei *in vivo)*.	No valid evidence for gene mutation in bacteria, mammalian cells or *in vivo*; no convincing evidence of chromosomal aberrations in humans; chromosomal damage in rodents only at hepatotoxic doses.
Structure Activity Relationships	Positive for structural alerts associated with genetic activity.	Expected to result in a structural alert for clastogenicity based on the hydroxyanaline moiety. (Blagg, 2003)
DNA binding	Agent or breakdown product are typically electrophilic and exhibit direct DNA binding.	No unequivocal evidence that metabolically activated acetaminophen or NAPQI form DNA adducts in cells *in vitro* at concentrations that do not also cause cytotoxicity; no reliable evidence of DNA adduct formation in animals or humans *in vivo* at any dose level.
Consistency	Positive test results are highly reproducible both *in vitro* and *in vivo*.	Conflicting and/or non-reproducible responses in the same test or test category both *in vitro* and *in vivo*.
Response Kinetics	Responses are dose dependent over a wide range of exposure levels.	Any positive responses in robust, reliable test systems are generally non-linear, exhibiting a threshold.
Susceptibility to Confounding Factors (e.g. Cytotoxicity)	Responses are typically found at non-toxic exposure levels.	Positive responses in robust, reliable test systems typically associated with evidence of overt toxicity.

accumulation in the nucleus (Ray et al., 1990) can promote endonuclease activation and DNA fragmentation (Collins and Rivas, 1993; McConkey and Orrenius, 1994). This is supported by data showing that Ca^{2+} antagonists such as chlorpromazine, verapamil and diltiazem protect against acetaminophen-induced hepatotoxicity (Ray et al., 1993; Satorres et al., 1995; Shen et al., 1991, 1992). Increased Ca^{2+} levels have also been shown to lead to mitochondrial dysfunction, increased levels of reaction oxygen species, and cytotoxicity via necrosis (Miao et al., 2019; Orrenius et al., 2010, 2015). Bergman et al. (1996) argued that since any genotoxic effects are most likely a consequence of cytotoxic events, affected cells would not survive.

Thus, since high concentrations/doses of acetaminophen induce a marked increase in intranuclear Ca^{2+}, resulting in endonuclease activation and DNA fragmentation, and such increased Ca^{2+} levels are associated with high cytotoxicity, any resultant genotoxicity will exhibit a threshold.

4.2.2. DNA damage caused by NAPQI after glutathione depletion

The major detoxification pathways for acetaminophen are the formation of sulfate and glucuronide conjugates with the parent compound. However, acetaminophen can be converted to a reactive electrophile and oxidising agent, N-acetyl-*p*-benzoquinone imine or NAPQI (Dahlin et al., 1984; Guengerich and Liebler, 1985) by liver microsomal CYP2E1, 1A2, and 3A4 (Raucy et al., 1989; Thummel et al., 1993). NAPQI (structure shown in Fig. 2) is normally detoxified by conjugation with glutathione (see Fig. 3). At high doses of acetaminophen, the sulfation and glucuronidation pathways are saturated, and glutathione is depleted (Athersuch et al., 2018 for review), resulting in NAPQI-mediated cytotoxicity.

As an electrophile, NAPQI could theoretically have the potential to bind to nucleophilic sites in DNA. Rogers et al. (1997) showed that there is ^{3}H-label bound to the liver and kidney DNA of male mice given ^{3}H-labelled acetaminophen at intraperitoneal doses of 10 mg/kg and higher. However, it is not known where the label was located on acetaminophen, and the ^{3}H label can be easily lost through exchange processes, can enter the nucleotide pool and thereby incorporated into DNA. The authors only measured radioactivity in the DNA and assumed this reflected binding of acetaminophen *to* DNA, but the radioactivity could have resulted from "metabolic incorporation". No adducts were identified or characterised, and the presence of radioactivity in DNA does not prove that adducts have been formed (Phillips et al., 2000). As commented by Bergman et al. (1996), *"[d]efinite proof that the covalent binding of radioactivity from ^{3}H-labelled paracetamol to DNA represents the formation of true DNA adducts would require chemical structural analysis"*. There was also no clear induction of adducts in liver DNA using the ^{32}P-postlabeling technique. In addition, comparison of the relative binding of the tritiated label to the DNA, chromatin and nucleus, demonstrate that almost all of the label was on the chromatin and nucleus and not on the DNA, which would suggest that the label is binding to histones and protamines rather than the DNA itself. These results therefore do not provide sufficient evidence that DNA adducts are formed at any dose level *in vivo*. Rogers et al. (1997) also showed that ^{14}C-labelled NAPQI, and also acetaminophen in the presence (but not in the absence) of rat liver microsomes, bound to calf thymus DNA. However, this was only evident at low pH, whereas there was no significant binding at physiological pH. Dybing et al. (1984) also found ^{3}H-labelled acetaminophen bound to mouse liver DNA *in vitro* in the presence of mouse liver microsomes. It also bound to liver DNA of phenobarbital pre-treated mice given an intraperitoneal dose of 500 mg/kg ^{3}H-labelled acetaminophen. Again, the authors only measured radioactivity in DNA, and no adducts were identified or characterised.

NAPQI is considered to be a "soft" electrophile, binding

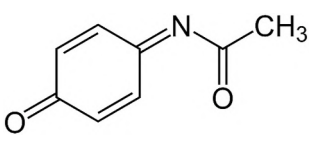

Fig. 2. Structure of NAPQI.

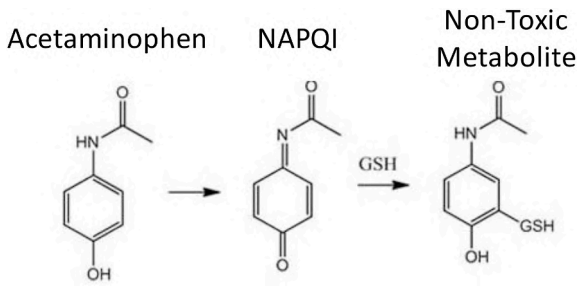

Fig. 3. Metabolic pathway for NAPQI formation and detoxification by conjugation with glutathione.

preferentially to soft nucleophilic sites in proteins (-SH groups) rather than to the harder nucleophilic groups in nucleic acids (Guengerich and Liebler, 1985). This is confirmed by the covalent binding studies of Dybing et al. (1984) and Hongslo et al. (1993), where metabolically-activated acetaminophen and NAPQI bound much more extensively (40–80x greater) to proteins than to DNA *in vitro* and *in vivo*. Also, in the studies of Rogers et al. (1997), binding of NAPQI to calf thymus DNA was reduced to background levels in the presence of 1 mM cysteine. More recently, Klopčič et al. (2015) showed that NAPQI reacts much faster with glutathione than it does with DNA, confirming the preferential binding to thiol groups. In fact, Potter et al. (1974) showed that covalent binding of acetaminophen to hepatic protein in i.p.-dosed hamsters was only evident when glutathione became depleted, indicating that binding to DNA would only occur with extensive glutathione depletion. When Hasegawa et al. (1988) used the ^{32}P-postlabelling technique, no DNA adducts were observed in male F344 rats given acetaminophen at various concentrations (0. 1–1.5%) in the diet for one week, or given a single slightly hepatotoxic intragastric dose of 1 g/kg 6 h prior to death. However, at least 6 adducts were seen when NAPQI was incubated with isolated calf thymus DNA. Also, in cultures of human granulocytes and HL-60 leukaemia cells, ^{14}C-labelled acetaminophen bound covalently to both DNA and RNA if the cells were stimulated by phorbol myristate acetate to undergo 'respiratory burst', but no adducts were identified or characterised (Corbett et al., 1992).

Typically, binding of a substance to DNA is necessary but not sufficient to result in mutations or tumours. The formation of DNA adducts by aflatoxin B1 is linear, whereas the formation of GGT-positive pre-neoplastic foci was non-linear and therefore exhibited a threshold (Root et al., 1997). Also, the induction of biological effects such as DNA strand breaks, mutations, pre-neoplastic lesions and tumours has been elegantly shown to be non-linear and to occur at higher doses than the induction of DNA adducts for heterocyclic amines, nitrosamines (Fukushima, 2010) and 2-acetylaminofluorene (Williams et al., 2004). Thus, it is predicted that higher doses of NAPQI would be required to induce biological changes such as genotoxicity or tumours than are required to produce DNA adducts. Furthermore, although metabolically-activated acetaminophen and NAPQI bind to DNA in cultured cells, and to naked DNA, where normal detoxification processes will be less efficient (or non-existent) than in whole animals, and no competing substrates are present, covalent binding to DNA *in vivo* has been observed only after the administration of hepatotoxic doses to mice.

Thus, the ability of NAPQI or metabolically-activated acetaminophen to associate with DNA has been shown in cultured cells *in vitro* and in isolated DNA, where detoxification by glutathione conjugation would be limited or absent. Moreover, some of the methods are of questionable reliability (e.g. use of ^{3}H label), and no adducts have been identified or characterised. Any of the cases where there may be been an association of NAPQI with nuclear DNA *in vivo* on (based on measurement of radioactivity without structural confirmation) were only observed at hepatotoxic doses, and would therefore exhibit a threshold.

4.2.3. Mitochondrial dysfunction and oxidative stress

When glutathione conjugation of NAPQI is depleted, it reacts with cellular macromolecules (primarily to soft nucleophilic sites in proteins -SH groups), and initiates cell death due to mitochondrial damage, increased oxidative/nitrosative stress and DNA fragmentation. The associated molecular signaling mechanisms of the cell death and transcriptomics that accompany this pathway have been reviewed (Chang et al., 2004; Ramachandran and Jaeschke, 2018, 2019; Stamper, 2015). Significant evidence has been generated over the past 15–20 years in animals and humans demonstrating that the dominant mechanism for DNA effects in the cell following high exposures to acetaminophen is through inhibition of mitochondrial respiration leading to mitochondrial dysfunction (McGill et al., 2013). Mitochondrial dysfunction and mitochondrial-dependent DNA fragmentation occur when cellular glutathione is depleted, and mitochondrial protein adducts are formed. The resulting NAPQI-associated protein adducts can be detected in the cytosol and in mitochondria, and cause mitochondrial dysfunction by increasing the generation of superoxide and peroxynitrite (Ramachandran and Jaeschke, 2018). The mitochondrial oxidative/nitrosative stress leads to the mitochondrial permeability transition pore opening with matrix swelling and release of intermembrane endonucleases, which translocate to the nucleus and cause DNA fragmentation as the cell dies (McGill et al., 2013). These processes ultimately result in acetaminophen-metabolite mediated cytotoxicity resulting in organ dysfunction. Thus, nuclear DNA fragmentation is completely dependent on mitochondrial dysfunction and represents the point of no-return for cell death. Under conditions where significant DNA fragmentation occurs, the cell has passed the point of no-return to necrosis, which makes it impossible that such a cell survives and initiates carcinogenesis. Therefore, any genotoxic effects observed as a result of this process would also exhibit a threshold. This pathway is described in more detail in a companion manuscript in this issue (Jaeschke et al., 2020).

Thus, from all of the available mechanistic data there is no evidence that acetaminophen acts via a non-threshold mechanism and nuclear DNA effects are only evident under conditions of cellular toxicity.

5. Conclusion

We have carefully applied an accepted, empirical, weight of evidence approach to evaluate the results of 69 studies investigating the genotoxic potential of acetaminophen. Data from humans and *in vivo* mammalian systems were given the highest weight, and the strength, relevance and reliability of the evidence, including information regarding the reversibility of an endpoint and its susceptibility to false or misleading positive responses, quality and control of the study, and impact of confounding factors were also taken into account. Through this comprehensive weight of evidence assessment acetaminophen consistently produces negative results in reliable, robust high weight studies. While some genotoxic effects (clastogenicity) are seen in moderate weight studies, in relevant, robust test systems, these are only seen at unacceptably high concentrations or under cytotoxic conditions that are associated with cell lethality. As discussed in detail above, the likely mechanisms leading to DNA and/or chromosomal damage are invariably associated with cell-lethal processes, and cells experiencing such damage will not survive to initiate carcinogenesis. The failure to induce gene mutations is consistent with the genotoxic damage being associated with lethality. Therefore, from all of the available data, it is extremely unlikely that acetaminophen induces the stable, genetic damage that would be indicative of a clear genotoxic carcinogenic hazard in humans.

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Declaration of competing interest

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