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Research Article

Assessment of Mechanistic Data for Hexavalent Chromium-Induced Rodent Intestinal Cancer Using the Key Characteristics of Carcinogens

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ABSTRACT

Oral exposure to hexavalent chromium (Cr[VI]) induces intestinal tumors in mice. Mutagenic and nonmutagenic modes of action (MOAs) have been accepted by different regulatory bodies globally, the latter involving cytotoxicity-induced regenerative cell proliferation. However, concerns persist that all possible MOAs have not been fully considered. To address the potential for alternative MOAs, mechanistic data *not* represented in the existing two MOAs were evaluated. Relevant data were identified and organized by key characteristics of carcinogens (KCCs); literature related to epigenetics, immunosuppression, receptor-mediated effects, and immortalization were reviewed to identify potential key events associated with an alternative MOA. Over 200 references were screened for these four KCCs and further prioritized based on relevance to the research objective (ie, *in vivo*, oral exposure, gastrointestinal tissue). Minimal data were available specific to the intestine for these KCCs, and there was no evidence of any underlying mechanisms or key events that are not already represented in the two proposed MOAs. For example, while epigenetic dysregulation of DNA repair genes has been demonstrated, epigenetic effects were not measured in intestinal tissue, and it has been shown that Cr(VI) does not cause DNA damage in intestinal tissue. High-throughput screening data related to the KCCs were also evaluated, with activity generally limited to the two recognized MOAs. Collectively, no plausible alternative MOAs (or key events) were identified in addition to those previously proposed for Cr(VI) small intestine tumors.

Key words: chromium compounds; hexavalent chromium; key characteristics of carcinogens; mode of action; intestinal tumor; data integration; systematic review; threshold-based dose-response; cancer risk assessment; cancer.

Hexavalent chromium (Cr[VI]) has long been recognized as a lung carcinogen via inhalation exposure (Glaser *et al.*, 1986; IARC, 1990; Proctor *et al.*, 2016), and more recently recognized as an oral carcinogen in rodents exposed to very high concentrations in drinking water (NTP, 2008; Stout *et al.*, 2009). In mice, adenomas and carcinomas occurred in the proximal small intestine (SI) beginning at 30 ppm, whereas squamous cell carcinomas of the oral cavity occurred in rats at 180 ppm. Shortly after the release of the 2-year cancer bioassay for sodium dichromate dihydrate (SDD; NTP, 2008), a mutagenic mode of action (MOA) based almost entirely on nontarget tissue and *in vitro* data (McCarroll *et al.*, 2010) was published, and an oral cancer slope factor was published based on the intestinal tumors in male mice (Stern, 2010). The California Office of Environmental Health Hazard Assessment used this slope factor as the basis of setting a public health goal for Cr(VI) of 0.02 ppb. For context, the mean and median and 95th percentile Cr(VI) concentrations in U.S. water supplies are 1 and 3 ppb, respectively (U.S. EPA, 2017).

Based on evidence that the intestinal tumors in mice were preceded by cytotoxicity and regenerative cell proliferation (Bucher, 2007; NTP, 2008), a research effort was undertaken to

provide additional data to better inform whether the MOA for the tumors observed in the 2-year cancer bioassay were more consistent with mutagenic or nonmutagenic mechanisms (Thompson *et al.*, 2011a). This research resulted in numerous publications (Supplementary Table 1; https://cr6study.info/), with much of the data synopsized in two reviews summarizing evidence that unreduced Cr(VI) enters the intestinal lumen and is absorbed into intestinal villous enterocytes, thereby leading to villous enterocyte cytotoxicity, compensatory crypt enterocyte hyperplasia, and eventually tumorigenesis due to a lifetime of increased cell turnover in the intestine (Thompson *et al.*, 2013a, 2017b). These targeted studies have been used by several regulatory agencies to develop threshold-based toxicity criteria for Cr(VI), in many cases resulting in safe water levels ranging from approximately 30–100 ppb (Food Safety Commission of Japan, 2019; Health Canada, 2016; TCEQ, 2016; WHO, 2019). Notably, the current maximum contaminant level is 100 ppb. With typical water levels of 1 ppb in the United States, it is a critical public health question to understand whether the science supports safe levels at 30–100 ppb or cancer risk at $\geq$0.02 ppb.

Although there appears to be growing consensus by regulatory bodies, including the WHO (2019) and Health Canada (2016) that the available science supports the derivation of threshold-based toxicity criteria, some entities remain hesitant to support such an approach. As a recent example, the Secretaries' Science Advisory Board (SSAB) to the North Carolina Department of Environmental Quality recommended that, despite the merits of the threshold-based nonmutagenic MOA, state regulators strongly consider linear low-dose extrapolation in the derivation of toxicity criteria until the U.S. Environmental Protection Agency (U.S. EPA) releases their assessment of Cr(VI) (NCSSAB, 2020). In a public meeting, the same SSAB stated that research focused on demonstrating a cytotoxic MOA and evidence for the lack of genotoxicity in target tissue was perhaps myopic and had not considered other possible MOAs. Similar concerns about hypothesis-driven research have been used as support for the proposed key characteristics of carcinogens (KCCs) approach which, "should introduce objectivity that could reduce reliance on expert opinion, as well as facilitate comparisons across agents…[and]…may afford a broad consideration of the mechanistic evidence rather than focusing narrowly on independent mechanistic hypotheses or pathways in isolation" (Smith *et al.*, 2016), and "avoids a narrow focus on specific pathways and hypotheses and provides for a broad, holistic consideration of the mechanistic evidence" (NASEM, 2017). More directly, the authors of the original KCC publication state that the approach is "in contrast to more narrow, reductionist approaches such as adverse outcome pathway and MOA frameworks that focus on singular events" (Smith *et al.*, 2020). To address these concerns for Cr(VI) specifically and also, more broadly, criticisms of targeted research despite its requirement in risk assessment, we used the KCC approach to identify potential alternative MOAs for Cr(VI)-induced intestinal cancer in mice. Specifically, mechanistic data *not* directly related to the two MOAs already identified (mutagenic [McCarroll, *et al.*, 2010] and cytotoxicity/regenerative cell proliferation [Thompson *et al.*, 2013b, 2017a]) were investigated by leveraging the organizational approach afforded by the KCCs to identify potential alternative MOAs and/or related key events from published mechanistic data for Cr(VI).

The KCC, ten characteristics that represent mechanistic events common across human carcinogens, have been proposed for the identification and organization of mechanistic data in assessments of known or suspected human carcinogens (Smith *et al.*, 2016). The use of the KCC as an organizational strategy for mechanistic data is being readily used by authoritative bodies globally (Guyton *et al.*, 2018b; Iyer *et al.*, 2019), and is specifically being implemented by the U.S. EPA Integrated Risk Information System (IRIS) program for chromium (U.S. EPA, 2019). KCCs *not* directly related to the aforementioned MOAs include: epigenetics, immunosuppression, receptor-mediated effects, and immortalization (discussed further in the Materials and Methods section). Electrophilicity, genotoxicity, DNA damage response, oxidative stress, chronic inflammation, and cell death/cell proliferation were not specifically reviewed in the present assessment due to the extensive existing and ongoing study of such mechanisms in regard to Cr(VI) and the well-characterized involvement of these KCCs in the proposed MOAs. Because use of the KCC has been associated with systematic review methods, the approach applied herein utilized aspects of systematic review where possible. This investigation is informative not only to the evaluation of Cr(VI), but also generally broadly related to the utility and challenges of the organizational concepts of KCC relative to the specificity required in MOA evaluations.

MATERIALS AND METHODS

Selection of key characteristics that could possibly inform alternative MOAs. The focus of the analyses presented herein was on data that could inform alternative MOAs (ie, that not previously evaluated as part of existing MOAs; Figure 1). Much of the mechanistic evidence base for Cr(VI) has been evaluated previously in the context of key events/underlying mechanisms related to two published MOAs for Cr(VI): the mutagenic MOA (McCarroll *et al.*, 2010) or to the proposed/accepted MOA involving cytotoxicity and regenerative hyperplasia (Thompson *et al.*, 2011b). The two KCCs that are directly related to these MOAs, KCC 2 (is genotoxic) and KCC 10 (alters cell proliferation, cell death, or nutrient supply), have been previously comprehensively evaluated and therefore are not a focus of this assessment. Readers are referred elsewhere for the data supporting mutagenic (McCarroll *et al.*, 2010; Zhitkovich, 2011) and nonmutagenic (Bhat, 2020; Thompson *et al.*, 2013b, 2017a) MOAs which address KCCs 2 and 10, respectively.

The potential role of electrophilic intermediates (KCC 1) and oxidative stress (KCC 5) has been evaluated previously: it is well understood that Cr(VI) is nonenzymatically reduced intracellularly to potentially DNA- and protein-reactive Cr(III) (KCC 1), an event that could potentially result in oxidative stress (KCC 5; IARC, 2012), and it was initially hypothesized that oxidative stress may play a key role in the MOA (Thompson *et al.*, 2011a). However, oxidative DNA damage has not been observed in the duodenum following Cr(VI) exposure (De Flora *et al.*, 2008; Thompson *et al.*, 2011b) despite some transcriptomic evidence for this, such as induction of genes involved in glutathione synthesis (Kopec *et al.*, 2012a). Relatedly, KCC 3 (alters DNA repair/genomic stability) was not prioritized due to the direct relationship with DNA damage, and the aforementioned reasons for not reviewing genotoxicity studies. KCC 6 (chronic inflammation) does not appear operable based on the absence of chronic inflammation in the intestinal in tissues from NTP cancer bioassay. As such, only KCCs 4, 7, 8, and 9, epigenetics, immunosuppression, receptor-mediated effects, and immortalization, respectively, were selected for review herein.

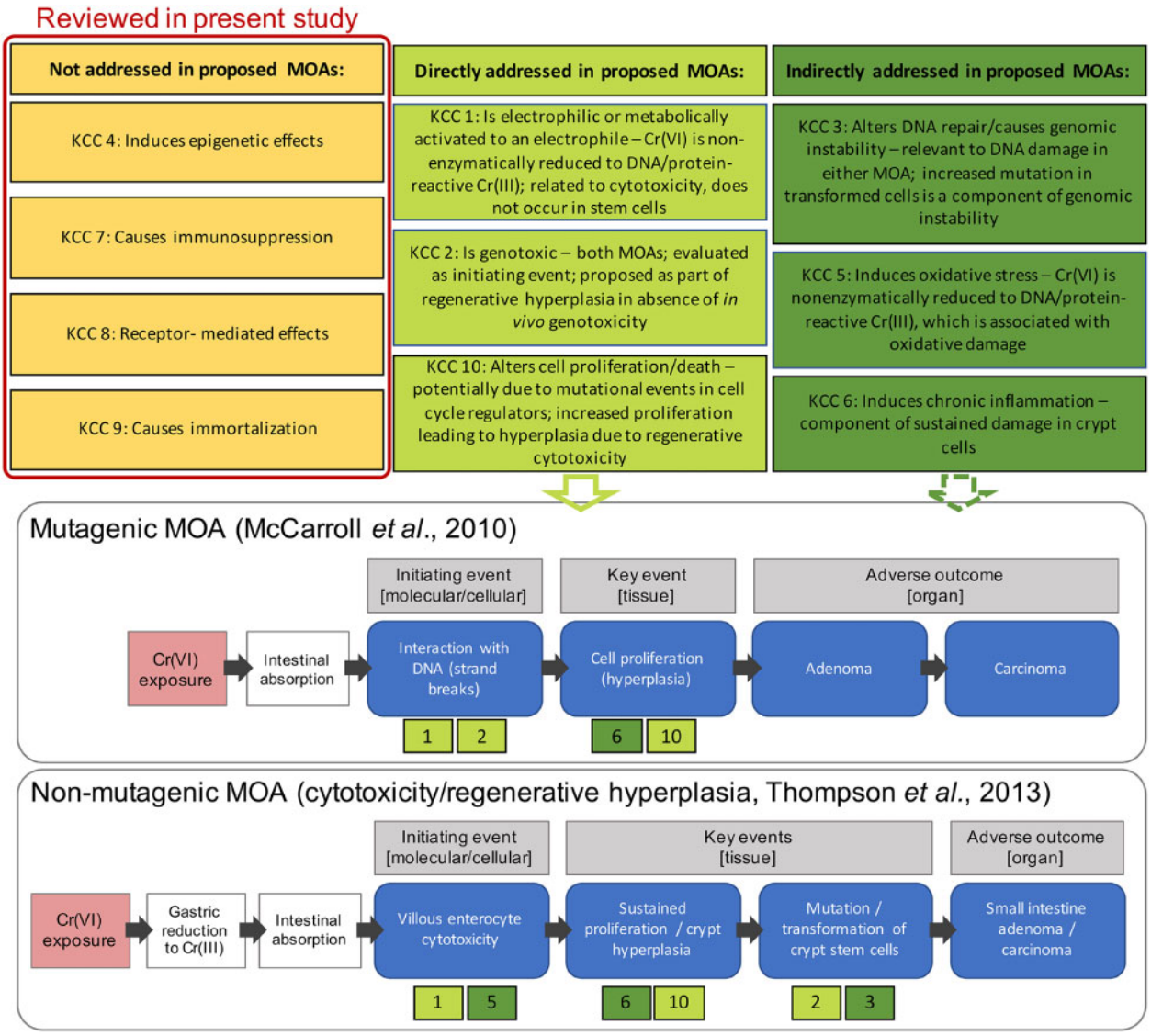

Figure 1. Schematic supporting prioritization of key characteristics of carcinogen (KCC) data reviewed for potential alternative modes of action (MOAs). KCCs were categorized in relation to existing MOAs (not addressed, directly addressed, or indirectly addressed), to determine which characteristics have not been accounted for and thus may provide information to identify alternative MOAs.

Literature identification. Mechanistic data for Cr(VI) are voluminous; in order to conduct a targeted assessment, studies that were systematically identified by the U.S. EPA as relevant the risk assessment and subsequently categorized by KCCs were utilized as the primary evidence base (U.S. EPA, 2019). Articles identified in the U.S. EPA's systematic review of Cr(VI) preliminary assessment materials, as listed and organized in the Health Assessment Workplace Collaborative (HAWC) dashboard (https://hawcprd.epa.gov/assessment/100500006/), were downloaded on October 30, 2019. All articles identified within the "Cr(VI) (mechanistic) (2018)" category that were also marked as "Prioritized for inventory," "Cancer," and assigned to at least one of the ten KCCs were downloaded. The KCC assignment as presented in the HAWC assessment was retained for each reference.

During article review, some relevant references were identified within the references cited in the studies reviewed and/or based on prior knowledge of the Cr(VI) literature base that were not included in the HAWC library. Where relevant, such articles are discussed herein. Some such studies were marked as "Potentially relevant supplemental material" within the U.S. EPA's Health & Environmental Research Online (HERO) database for Cr(VI) (https://hero.epa.gov/hero/index.cfm/project/page/project_id/2233; last accessed July 20, 2020).

Literature screening. All references were imported into the DistillerSR software (Evidence Partners, Ottawa, USA). The KCC categorization assigned within HAWC was applied to all references using the tagging feature in Distiller SR. Studies were prioritized for review based first on the level of directness, or relevancy to the topic of investigation (ie, *in vivo*, oral exposure, and measurements conducted in gastrointestinal tissue), and secondarily by KCC (Figure 2). Many studies include toxicological endpoints related to more than one KCC; as such, screeners extracted data for all toxicological endpoints within the studies that were tagged for the 4 prioritized KCC. For example, because

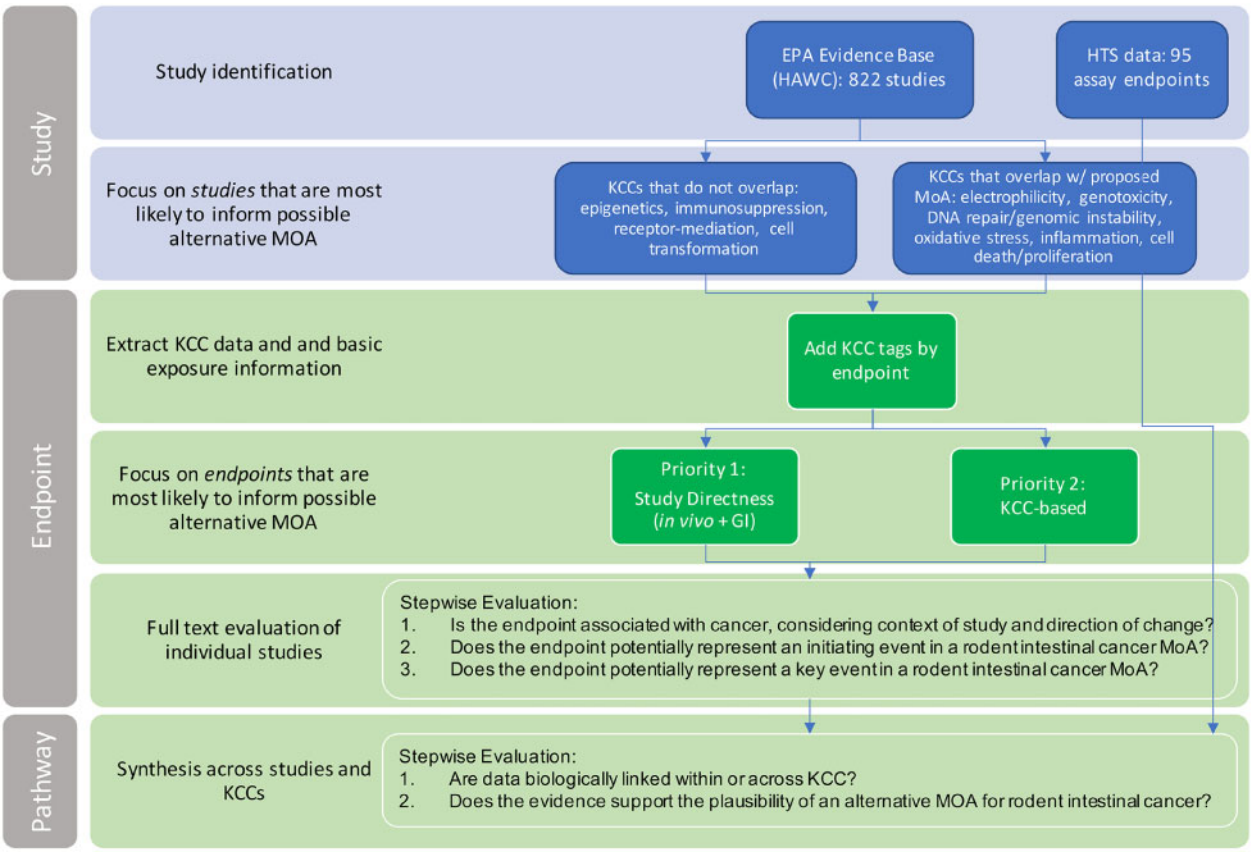

Figure 2. Flowchart of overall approach to the evaluation of potential alternative modes of action via assessment of key characteristics of carcinogen data.

many of the epigenetics studies (KCC 4) reviewed epigenetic regulation of DNA repair genes, and alteration of such genes was tagged in the present assessment as KCC 3 (alters DNA repair/genomic instability), a subset of KCC 3 data were extracted and reviewed herein owing to the connection with epigenetic mechanisms.

Because KCC 8 (modulates receptor-mediated effects) represents a broad range of potential effects due to the number and variety of nuclear receptors, we selected articles with highest relevance by mining titles and abstracts for the words "mouse," "mice," "rat," "intestine," "intestinal," "stomach," and "oral." Further, we searched peer-reviewed literature for other mechanisms or MOAs for intestinal cancer that include receptor-mediated effects as a means to identify potentially relevant receptors to be queried within the Cr(VI) literature.

Evidence mapping. Independent KCC categorizations were assigned at the individual endpoint level and were used for all further data categorization and characterization. A comparison was made to identify discrepant KCC assignment between the assignments in HAWC for the U.S. EPA IRIS assessment versus those assigned in this work. This comparison was made noting that the KCC tag information in HAWC is on the study level, whereas the assignments conducted herein were at the assay endpoint level. "Endpoints" for the purposes of this assessment are defined as the output of a single assay (eg, activation of a nuclear receptor) or measurement (eg, neutrophil level, DNA repair gene expression, cytokine release, etc.). Nonetheless, the complete absence of a KCC tag in one or the other assessments (HAWC versus that presented herein) represents a discrepancy.

Data were tabulated according to KCC assignment (by endpoint), type of study (*in vitro* versus *in vivo*), route of exposure (*in vivo* only; inhalation vs. oral, etc.), and cell type or tissue studied, among other attributes.

High-throughput screening data. High-throughput screening (HTS) data for SDD in KCC-relevant assays within the ToxCast/Tox21 database were also reviewed to further identify and/or confirm signal for KCCs related to potential alternative MOAs (U.S. EPA ToxCast Summary Files database; https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data; invitrodbv_v3.2; released August 2019, accessed October 2019 for this study). The "mapping" of HTS data to KCCs is not yet a consistent practice (Iyer *et al.*, 2019). Herein, we took a comprehensive approach to mapping and evaluated all KCC-relevant HTS assay data for SDD, based on previously published assay mappings (Chiu *et al.*, 2018; Iyer *et al.*, 2019), plus additional assay mapping using detailed assay information and expert judgment (HTS assay-KCC mapping included in Supplementary Table 2). Activity/inactivity in KCC-relevant assays was reviewed according to "hit-calls" provided in the ToxCast summary files, with contextualization of potential cytotoxic interference in activity assignments for these assays, as described further in Supplementary Table 2.

Evidence integration. Using a stepwise approach, data were evaluated in the context of the potential relationship to

Table 1. Characterization of Evidence Base According to the 4 Key Characteristics of Carcinogen (KCCs) Reviewed Herein

Key Characteristic	Number of Articles (as Tagged by U.S. EPA in HAWC)	Number of Articles (as Tagged in This Study)	Number of Endpoints Independently Tagged in This Study	Number of Endpoints Measured in Target Tissue Following Oral Exposure
4. Epigenetics	24	23	55	0
7. Immunosuppression	6	19[a]	57	2[a]
8. Receptor-mediated effects	195	Selectively reviewed[b]	NA	0[b]
9. Immortalization	58	44	104	0

[a]Two additional studies were identified and included in the assessment presented herein, which were marked as "potentially relevant supplementary material" in the EPA IRIS assessment. Thus, these two studies are not included in the 6 tagged in the HAWC assessment.
[b]No articles were identified that were conducted in rodents orally exposed to chromium compounds and measurements taken in intestine, based on targeted keyword searching of titles and abstracts within the HAWC-tagged KCC 8 database, as described in the Materials and Methods section.

carcinogenesis, including specific consideration of the direction of activity, consistency of findings, and concordance with rodent SI tumorigenesis. *In vivo* oral exposure rodent studies were prioritized, with those that measured effects in the target tissue (intestine) representing the studies considered the most relevant to the study objective. *In vitro* studies were also considered for potentially relevant contextual mechanistic information (see Figure 2). Data were synthesized within and across KCCs to determine biological plausibility of an alternative MOA for rodent intestinal cancer. This approach is similar to that described by the U.S. EPA in the protocol for the conduct of a systematic review to support the IRIS evaluation (U.S. EPA, 2019).

RESULTS

Literature Screening

A total of 226 titles and abstracts were reviewed across KCCs 4, 7, 8, and 9. Table 1 shows the breakdown of KCC tagging by the U.S. EPA in HAWC download, as well as the number of *endpoints* tagged for each KCC as independently assigned in this study. From the screened studies, over 200 endpoints were extracted for the 4 KCCs reviewed. Details on all extracted endpoints can be found in Supplementary Table 1.

KCC 4—Induce Epigenetic Alterations

A total of 23 articles were identified as containing information pertinent to epigenetic mechanisms, which included a total of 60 endpoints (ie, various measures of epigenetic marks or alterations). None of the studies of epigenetic mechanisms were conducted in the target tissue (intestine) in the target species (mice and rats). Data were available for surrogate tissues, such as peripheral blood or serum, from *in vivo* studies of exposure to Cr(VI) compounds in rats and humans. Only one study measured epigenetic changes following oral exposure, in which there was no change in promoter hypermethylation of the tumor-suppressor gene p16 in plasma from Sprague Dawley rats exposed to potassium dichromate in the drinking water at concentrations up to 300 mg/L for four weeks, and there was a dose-dependent reduction in global DNA methylation in the plasma (Wang *et al.*, 2016). The human studies in which epigenetic alterations were measured (DNA methylation or histone methylation in blood) were specific to inhalational occupational exposure. Although the involvement of epigenetic mechanisms in Cr(VI)-induced cancer has been reported, particularly lung cancer in humans occupationally exposed via inhalation (Browning *et al.*, 2017; Chen *et al.*, 2019; Rager *et al.*, 2019), the potential relevance or involvement of the same or similar epigenetic alterations in Cr(VI)-induced intestinal cancer is not clear.

The majority of the epigenetics studies involved Cr(VI)-induced posttranslational histone modifications measured in *in vitro* and *in vivo* models (Supplementary Table 1). The only histone mark that was consistently measured (ie, the same mark evaluated across multiple studies) was methylation of histone 3 at lysine 9 (H3K9). Specifically, H3K9 dimethylation (H3K9me2) and trimethylation (H3K9me3) were measured both *in vitro* and *in vivo*, using study models aimed at evaluating effects in the lung (either lung tissue or lung-derived cells; Sun *et al.*, 2009; Wang *et al.*, 2018b; Zhou et al., 2009), and in mouse spermatogonial stem cells (Lv *et al.*, 2018). H3K9 methylation is a hallmark of heterochromatin and represents a mark of transcriptional repression (Black *et al.*, 2012; Zhao and Shilatifard, 2019). Generally, reduced heterochromatin is a hallmark of cancer cells (Dejardin, 2015), while the heterochromatin-maintaining H3K9me2/3 was globally increased in all studies of Cr(VI) in which it was measured (Lv *et al.*, 2018; Sun *et al.*, 2009; Wang *et al.*, 2018b). Histone modifications within the promoter region of specific genes directly influences their expression. An increase in the repressive mark H3K9me2 within the promoter of the mismatch repair gene *MLH1* was associated with its down-regulation *in vitro* (Sun *et al.*, 2009). Overall, there are insufficient data to characterize the ability of Cr(VI) to modify histone marks in the intestine following oral exposure.

MicroRNA expression and gene-specific DNA methylation changes were also reported in the database, but were not measured in the target tissue. These studies primarily reviewed post-transcriptional regulation of genes involved in DNA damage response and repair mechanisms, and would thus be most informative if measured in the target tissue or in a confirmed predictive surrogate tissue. Few reports of alterations to micro RNAs (miRNAs) were identified in the Cr(VI) evidence base. The "oncomir" miR-21 was increased in Cr(VI)-transformed cells, in lung tumors of intranasally exposed mice, and in mouse xenograph tumors following injection of Cr(VI)-transformed cells (Pratheeshkumar *et al.*, 2016, 2017) (The research group that conducted these studies is the subject of an investigation for research misconduct, with many publications retracted [https://www.the-scientist.com/news-opinion/university-of-kentucky-to-fire-professors-for-research-misconduct-66352]). These data

support the possibility that over-expression of miR-21, specifically activation of the miR-21/programmed cell death protein 4 (PDCD4) signaling pathway, may be involved in Cr(VI)-induced cell transformation *in vitro* and in lung tumors. Data are insufficient to determine if changes in miRNA expression occur in the intestine following oral exposure to Cr(VI).

Although mixed reports of Cr(VI)-induced gene-specific DNA methylation were identified, global DNA hypomethylation was more consistently reported. Global DNA hypomethylation induces genomic instability and contributes to the carcinogenic transformation of cells (Kulis and Esteller, 2010). Several studies measured methylation of the tumor-suppressor gene p16, with 2 studies reporting increased methylation in lung tissue from occupationally exposed chromate workers and in human bronchial epithelial cells (Hu *et al.*, 2018; Kondo *et al.*, 2006), and the other 2 reporting a lack of change in the plasma of rats exposed to Cr(VI) via drinking water and in two human cell lines (lung carcinoma and lymphoblastoid; Lou *et al.*, 2013; Wang *et al.*, 2016). The methylation status of other tumor suppressor (TP53) and DNA repair (HOGG1 and RAD51) genes was evaluated. The hypermethylation of the DNA repair genes was increased in a correlative fashion with decreased mRNA expression (Hu *et al.*, 2018). In the Hu *et al.*, study, hypermethylation was measured both *in vitro* in human bronchial epithelial cells (HBE16 cell line), as well as in the blood of occupationally exposed workers. The alterations to the methylation and expression of DNA repair genes were also correlated with DNA damage in the workers. One caveat to these data is that several studies exposing rodents to up to 180 ppm Cr(VI) orally have failed to detect micronuclei at sites of contact or in blood except by intraperitoneal injection (De Flora et al., 2006; Bucher, 2007; O'Brien *et al.*, 2013; Thompson *et al.*, 2015b). As such, it is uncertain how exposure to permissible levels of Cr(VI) would induce blood micronuclei in humans, and the attribution of micronuclei (and perhaps the epigenetic findings) to Cr(VI) by Hu *et al.* is, in our opinion, uncertain. Overall, data are insufficient to determine if changes in DNA methylation, whether global changes or gene-specific, occur in the intestine following oral exposure to Cr(VI).

Because many of the studies of Cr(VI)-induced epigenetic alterations focused on regulation of DNA damage repair machinery and/or genomic stability, many of the epigenetics studies were also tagged herein for containing data relevant to KCC 3 (alters DNA repair/genomic stability). Although KCC 3 was not one of the prioritized KCCs, these data were considered for their relationship to a possible alternative MOA. For example, the Hu *et al.* (2018) study discussed above conducted in blood samples collected from occupationally exposed humans demonstrated down-regulation of expression of DNA repair genes (Hu *et al.*, 2018), linking the epigenetic effects with a response relevant to DNA damage. Other studies of epigenetic regulation of DNA repair genes/mechanisms contained endpoints marked herein as related to KCC 3 (details in Supplementary Table 1). Importantly, in the gastrointestinal tract, low doses of Cr(VI) are reduced in the stomach to inert Cr(III), thereby limiting entry into intestinal enterocytes (Buttner and Beyersmann, 1985; Collins *et al.*, 2010; Zhitkovich, 2005). Higher oral doses of Cr(VI) increase the likelihood of Cr(VI) entering intestinal enterocytes; however, a lack of DNA damage has been demonstrated in the intestinal tissue of rats and mice administered high oral doses of Cr(VI) (Aoki *et al.*, 2019; O'Brien *et al.*, 2013; Thompson *et al.*, 2015c, 2017c). Thus, the relevance of alterations to DNA repair genes in circulating blood following exposure to Cr(VI) via inhalation to the intestine is unclear, considering the lack of direct genotoxicity in the SI in rodents orally exposed to Cr(VI).

Taken together, the available data support Cr(VI)-induced epigenetic alterations that influence the expression of DNA repair genes in blood or lung tissue in humans exposed to Cr(VI) by inhalation, which is in agreement with the conclusions of several reviews of epigenomic effects of Cr(VI) (Chen *et al.*, 2019; Chervona et al., 2012; Rager *et al.*, 2019). The relevance of such epigenetic alterations in regard to mechanisms associated with intestinal tumor response in rodents is not clear; while the ability of Cr(VI) and related chromium compounds to alter the epigenetic state of a cell is evident, the ability of Cr(VI) to enter intestinal cells at concentrations high enough to induce such changes is not as clear. Ultimately, while epigenetics may play a role in the existing proposed MOAs, the evidence does not support identification of key events or pathways that would indicate an alternative MOA involving epigenetics.

KCC 7—Be Immunosuppressive

Eighteen articles containing a total of 58 endpoints (ie, various measures of immune response) were identified as containing information pertinent to immunomodulatory activity (or lack thereof). Many of the endpoints were measured in *in vitro* systems, in many cases related to interleukins or other cytokines that have specific relevance to either inflammatory or immune response. Such measures may be relevant to both inflammatory and immune response, depending on the timepoint and system. In addition to the *in vitro* studies, a recent immunotoxicity study was identified that was conducted in mice and rats orally exposed to Cr(VI) (Shipkowski *et al.*, 2017), which was not included in the HAWC library. Another *in vivo* oral exposure study conducted in rodents (mice) measured transcriptomic alterations in the intestine. We focused on these rodent studies conducted *in vivo* with an exposure route of direct relevance to the research question. A human occupational exposure study was also identified and evaluated; however, the relevance of inhalational exposure and alterations to blood biomarkers to rodent intestinal tumorigenesis is unclear.

The immunotoxicity study was conducted in B6C3F1 mice and Fischer 344/N rats, the same strains used in 2-year cancer bioassays conducted by the NTP, as well as Sprague Dawley rats (Shipkowski *et al.*, 2017). In this study, rats were exposed to concentrations of 0, 5, 20, 60, or 180 Cr(VI), and mice were exposed to concentrations of 0, 5.5, 11, 22, 44, or 88 ppm Cr(VI) in the drinking water for 28 days. Immunotoxicological endpoints measured at the end of the exposure period included T-cell proliferation, formation of IgM antibodies in the blood and spleen, changes in splenic lymphocyte sub-populations, mixed leukocyte response in the spleen, and natural killer cell activity. The study generally evaluated increases in these endpoints, as a means to characterize potential immunotoxicity, whereas decreases in these measures would be indicative of immunosuppression. Nonetheless, no significant and consistent changes in any of the immunological parameters were observed in either species. The authors concluded that SDD administered in the drinking water for 28 days produced minimal toxicological and immunotoxic effects in female F344/N rats, Sprague Dawley rats, and B6C3F1 mice.

Significant alterations in the expression of immune response genes were observed in mouse (B6C3F1) and rat (Fisher 344) SI (duodenum and jejunum) following 7 and 90 days of exposure to 0.3–520 mg/l SDD in the drinking water (Kopec *et al.*, 2012a,b). Specifically, the epithelium was scraped from the duodenal and jejunal sections, and then homogenized (duodenum and jejunum epithelia were homogenized and processed/analyzed separately). Individual genes assigned to the functional category of "immune response" were both up- and down-regulated, with some exhibiting intestinal region- and/or timepoint-specific directionality. For example, the chemokine *Ccl24* is up-regulated in the duodenum and down-regulated in the jejunum at both 7 and 90 days of exposure in mice, and Annexin A2 gene (*Anxa2*) is down-regulated in both the duodenum and jejunum at day 7, and up-regulated in both tissues at day 90 (Kopec *et al.*, 2012a). However, the changes in the genes classified by the authors as related to "immune response" were concluded by the authors to be related to inflammatory response that may be generally related to the effects on redox status and histopathology. Thus, these transcriptomic data likely represent a secondary effect related to key events in the proposed MOA for Cr(VI)-induced SI cancer (Thompson *et al.*, 2011b).

In a human observational study, leather tanning workers and chromeplaters who were regularly exposed to chromium (Katiyar et al., 2008). The levels of Th1/Th2 cytokines interleukin (IL)-2, IL-4, tumor necrosis factor-alpha (TNF-α), IL-10, and IL-6 were evaluated in the sera and in phytohaemagglutinin and lipopolysaccharide (PHA/LPS)-stimulated culture supernatants of peripheral blood mononuclear cells. IL-6 levels were significantly lower in PHA/LPS-stimulated culture supernatants from chromium exposed groups as compared with unexposed healthy volunteers. IL-4 and IL-10 could not be detected, and the decrease in IL-2 was not statistically significant. There was no difference in TNF-α levels in sera samples of chromium exposed individuals as compared with controls. The relationship between the apparent suppressive action of inhaled Cr(VI) on immune-related cytokines in the blood and intestinal effects is not clear.

Overall, the mechanistic evidence do not indicate a consistent immunosuppressive response across species for Cr(VI). Although there is evidence that overt immunotoxicity does not occur following oral exposure to Cr(VI) in mice and rats for 28 days, it is possible that more subtle (ie, mRNA changes) alterations that result in decreased antigen presenting capability of cells could potentially contribute to the ability of transformed cells to survive and proliferate.

KCC 8—Modulates Receptor-Mediated Effects

Based on the keyword mining of titles and abstracts for the references tagged as KCC 8 as described in the Materials and Methods, none of the 196 articles tagged as KCC 8 in the HAWC assessment contained information relevant to an *in vivo* rodent exposure. Thus, none of the studies of receptor-mediated effects appear to have been conducted in rodents orally exposed to a chromium compound, limiting the relevance of these studies to the understanding of MOA for SI tumorigenesis. The Wnt/β-catenin signaling pathway, which involves signaling through cell surface receptors, plays an important role in many intestinal and colorectal cancers (Rizk and Barker, 2012; Takahashi-Yanaga and Sasaguri, 2007) and was thus considered as a potential mechanism related to KCC 8 in the present assessment. There were no data specific to Wnt/β-catenin signaling in the articles tagged as KCC 8 in the HAWC data. Further, aberrant proliferative foci, which are the consequence of increased levels of β-catenin, were not observed in the SIs of rodents exposed to Cr(VI) for up to 90 days (Thompson *et al.*, 2013b, 2015a). Overall, there is no evidence of a nuclear receptor binding initiating event for Cr(VI)-induced intestine tumorigenesis, nor is there evidence that any other receptor-mediated effects represent a key event in an alternative MOA.

KCC 9—Causes Immortalization

In this study, 59 articles were tagged for KCC 9 in the U.S. EPA IRIS HAWC assessment. All of these articles were conducted in *in vitro* models, with the exception of one *in vivo* assay conducted in rats exposed to chromium-containing materials by intrabronchial implantation (Levy and Venitt, 1986). In the *in vivo* study, rats exposed to Cr(VI) materials, but not Cr(III) materials, developed squamous metaplasia in the lung. Although the authors of this study considered squamous metaplasia to be a transformed state from which squamous carcinoma may arise, squamous metaplasia can be an adaptive change that occurs in response to chronic irritation or injury, and is typically not preneoplastic (Cesta *et al.*, 2015). Many studies reviewed the potential of chronic exposure to low levels of Cr(VI) compounds to result in cellular transformation *in vitro*, with varying results across cell model type, concentration, and chromium compound (see Supplementary Table 1). The relevance of this deliberate *in vitro* transformation to *in vivo* exposure and effects in intestinal cells, specifically regarding immortalization, is unclear. Overall, the specific endpoints that are relevant to this KCC aside from oncogenic viral immortalization are not clear. No evidence of immortalization was available for intestinal tissues in orally exposed rodents.

HTS Data

SDD was tested in a total of 235 HTS assays as of October 2019 (invitrodb_v3.2), 95 of which represent primary read-outs (in contrast to background or contextual measures, such as cell viability) and are mapped to one or more of the KCCs. Among those 95 assay endpoints that were mapped to KCCs, SDD was active at subcytotoxic concentrations in 18 assays. Overall, the activity profile for KCC-relevant HTS data for SDD corroborated previous data in regard to the proposed MOAs for rodent SI tumors and/or previous data collected for Cr(VI) compounds in *in vitro* systems: genotoxicity, DNA damage repair, oxidative stress, and cell proliferation/cell death (Supplementary Tables 2 and 3; Figure 3), which are related to the two previously proposed MOAs and do not indicate an alternate MOA. The exception is KCC 8: Receptor-mediated effects, for which activity was reported in seven assays. The effect of SDD was exclusively antagonism, across seven different receptors (androgen receptor, chimeric antigen receptor, estrogen receptor alpha, estrogen receptor beta, progesterone receptor, retinoic acid receptor (RAR)-related orphan receptor gamma, and the thyroid receptor). This broad antagonist activity, coupled with a lack of published involvement of these receptors in SI cancer, suggest a nonspecific signal for SDD for the HTS assay type.

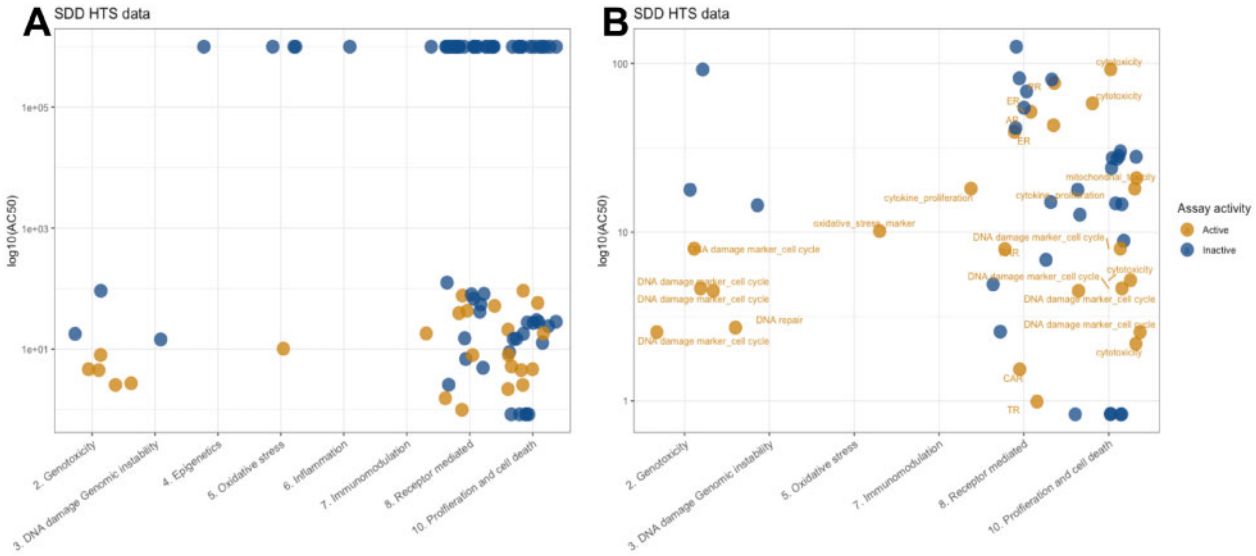

Figure 3. High-throughput screening assay coverage and activity across key characteristics of carcinogens (KCCs). Activity is depicted by colors according to cell viability criteria described in the Materials and Methods section. A, All assays, with assay endpoints with a "hit-call" = 0 according to ToxCast summary data shown at the top at the AC50 value of 1000 μM, designated for all assay endpoints with a hit-call = 0. B, All assays with a "hit-call" = 1 according to ToxCast summary data. Those assays deemed active according to the cell viability criteria described in the Materials and Methods section are labeled with their respective KCC subcategory. KCC that are not displayed on the x-axis had no assay endpoints with a hitcall of 1.

Evidence Integration

Among the KCCs prioritized as potentially representing events in an alternative MOA, KCC 7 (is immunosuppressive) had the greatest amount of data measured in the target tissue in *in vivo* oral exposure rodent studies (Table 1). These studies demonstrated a lack of immunotoxicity for Cr(VI) in the intestine, and there was no indication of immunosuppression as evidenced by transcriptomic analyses in intestinal tissues as well as hematological measures.

Epigenetic dysregulation of DNA repair genes was reported. These data demonstrate a direct link between KCCs 4 and 3 (epigenetic alterations and DNA repair mechanisms/genomic instability), and are inherently linked to KCC 2 (is genotoxic). Chemically induced dysregulation of DNA repair machinery is relevant to tumorigenesis in a scenario with excess DNA damage. Although there is evidence for such Cr(VI)-induced genotoxicity *in vitro* and in humans and via inhalational exposure, data indicate that Cr(VI) does not cause DNA damage in intestinal tissue of rodents orally administered Cr(VI) at both low and high doses (Aoki *et al.*, 2019; O'Brien *et al.*, 2013; Thompson *et al.*, 2015a,b, 2017c).

When the data are considered collectively, the activity observed within individual KCCs was either associated with events in the previously established MOAs, or was not linked to a biological pathway (ie, linked to other KCCs) that would suggest an alternative MOA for rodent SI tumors associated with Cr(VI) exposure (Figure 4).

DISCUSSION

The proposed cytotoxicity/regenerative hyperplasia MOA for Cr(VI)-induced rodent SI cancer has been extensively studied (Thompson, *et al.*, 2011b, 2017a), in addition to a previously proposed mutagenic MOA (McCarroll, *et al.*, 2010). However, it has been argued that evidence collected to support the proposed MOAs may omit other relevant key events that could support an entirely different MOA. The results of the assessment presented herein demonstrate that available data relevant to proposed KCCs do not support an alternative MOA. This is important because the U.S. EPA cancer guidance (2005) recommends linear low-dose extrapolation when the MOA for a chemical-induced tumor is either mutagenic or unknown (or perhaps uncertain), any uncertainty in the MOA might serve as justification to employ default risk assessment approaches (U.S. EPA, 2005). Further, any KCC-related key events that were investigated for biological plausibility for involvement in Cr(VI)-induced rodent intestine tumors (eg, epigenetic alterations, immunosuppression, receptor mediation, or cellular immortalization) would be mediated through threshold-based responses. Nonetheless, the results of the assessment of existing literature demonstrated that there is no evidence that such key events occur in rodent intestine tissue following oral exposure to Cr(VI).

The approach presented herein is similar to that described by the U.S. EPA in the protocol for the conduct of a systematic review to support the IRIS evaluation. Specifically, the protocol describes the use of KCC to inventory data and subsequently to "help identify key events that will be evaluated using the MOA analysis framework described in EPA's cancer guidelines" (U.S. EPA, 2019). Although guidelines or best practices do not exist for the integration of KCC-relevant mechanistic data into MOA analysis, including anchoring to an adverse outcome (Becker *et al.*, 2017; U.S. EPA, 2019; Wikoff *et al.*, 2019), the U.S. EPA describes in the protocol for Cr(VI) that rigorous mechanistic analyses will involve interpretation in the context of pathways anchored to specific health effects (eg, SI tumors in mice)—a key aspect of the evaluation herein. Although MOA research has historically been hypothesis driven, initiatives to more holistically assess carcinogenicity based on chemical-induced effects and/or characteristics with potential relevance to the outcome are becoming more common (NASEM, 2017; Smith *et al.*, 2016). However, MOA analysis is nonetheless an integral component of chemical risk assessment. Here we have

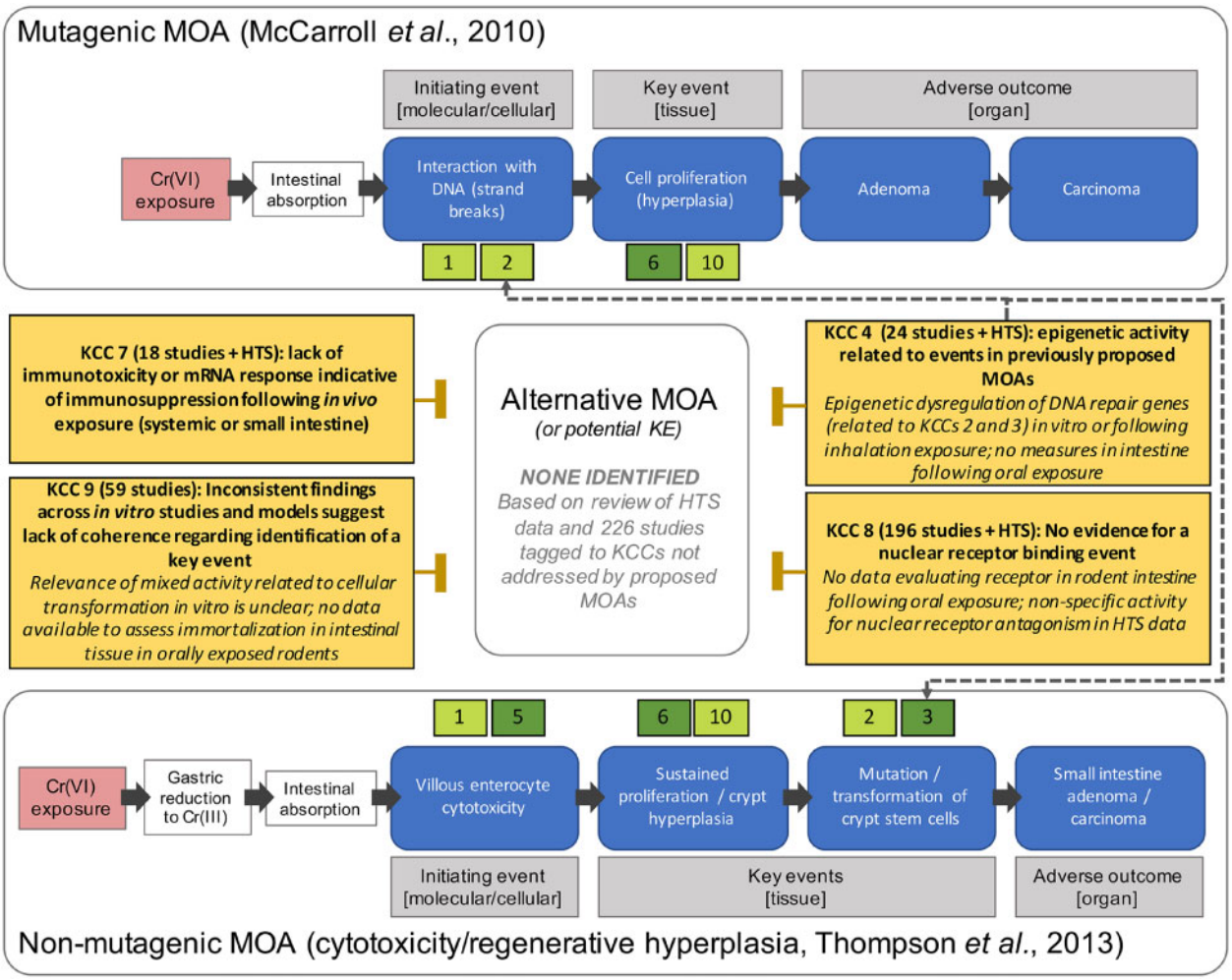

Figure 4. The potential relevance (or lack thereof) of the data for the 4 key characteristics of carcinogen assessed herein with the 2 proposed modes of action (MOAs) for hexavalent chromium-induced small intestine tumors, or any evidence of an alternative MOA or key events, is depicted.

demonstrated both the utility in the KCC approach for organizing mechanistic data, while also demonstrating the necessary further analysis and integration of such data to facilitate risk-based conclusions related to MOA.

As stated by the authors of the proposed KCC, individual KCC do not alone impart carcinogenic potential, but rather act in concert and in many cases with temporal (ie, sequential in nature and/or with essentiality) or even spatial (ie, cell- or tissue-specificity) relationships. This is particularly relevant for epigenetic alterations, most of which are reversible and for which temporality of such changes representing an important consideration. In the present assessment, epigenetic regulation (KCC 4) of DNA repair genes (KCC 3) was reported *in vitro* and in inhalational human exposure to Cr(VI). This regulatory effect is directly relevant to DNA repair mechanisms, which are, by nature, an essential influential component in biological systems in scenarios of exogenous DNA damage (KCC 2). Thus, these 3 KCC are directly relevant to each other and to an outcome related to genotoxicity. For example, loss of expression of important tumor suppressor or DNA damage repair genes due to aberrant DNA methylation within those genes (or within promoters or enhancers of those genes) can result in excess DNA damage without sufficient repair. In the absence of genotoxicity in a specific tissue or at a specific dose level, as is the case in rodent intestine following oral exposure to Cr(VI), and/or without metabolic transformation to a reactive intermediate (KCC 1), the relevance of alterations to epigenetic regulation of repair mechanisms in experimental systems to intestine tumorigenicity is questionable. Using the holistic method to evaluate all KCC may lead to hypothesis generation; for example, the known epigenetic effects of Cr(VI) could represent an underlying mechanism of rodent SI tumorigenesis, as is the case for Cr(VI)-induced lung cancer (Rager *et al.*, 2019). Nonetheless, the investigation of the available data did not result in strong evidence that such is the case. However, such changes occur in sequence or tandem with other important alterations to cells to ultimately result in cancer. As such, it is difficult to delineate which molecular event is key, or if both are key events, or if only a combination of the events results in the carcinogenic process.

Many studies within the Cr(VI) literature that report mechanistic findings are human inhalational epidemiology studies with a focus on lung cancer, with molecular endpoints frequently evaluated in surrogate tissues such as blood and urine to measure biomarkers of exposure or effect, or in lung tissue from Cr(VI)-exposed biopsied patients. Although molecular measures from surrogate tissue represent a potentially valuable source of data, the relationship between molecular changes in surrogate tissues and adverse outcome development in target

tissues (ie, key events or tumors in the SI) remains unclear. This is a topic of ongoing research, including within the field of epigenetics (Lin *et al.*, 2020; Wang *et al.*, 2018a). Relatedly, *in vitro* research of Cr(VI)-induced molecular effects often utilizes human bronchial cells or cell lines. The relevance of data from studies in the lung following inhalational exposure to SI rodent tumors is not clear, and such studies were deprioritized relative to oral exposure studies in the evaluation of KCC activity. Further, it is acknowledged that the doses at which Cr(VI)-induced rodent intestinal cancers were observed in mice (≥30 ppm) are much higher than human exposure levels. The present assessment seeks to further elucidate potential MOAs that could inform the extrapolation method applied (ie, threshold based) in human health risk assessment. Should any alternative MOAs be identified, key events in the pathway could be further explored for relevance in humans.

Assays to measure the KCC as they relate to cancer outcomes are not yet validated, with a lack of consensus on appropriate or predictive measures of KCCs (Smith *et al.*, 2020). For example, KCC 9 (immortalization), which appears to be specific to viral immortalization according to the description of KCC 9 in the original and subsequent publications (Guyton *et al.*, 2018a; Smith *et al.*, 2016), does not have assays validated to indicate transformation/immortalization. Because many assays are conducted in transformed cell lines utilized for the evaluation of other mechanisms associated with exposure and/or carcinogenic process, or low-dose, multi-passage, long-term exposure of the compound of interest (in this case Cr(VI)) is used specifically to transform cells, the applicability of such assays or models are questionable insofar as indicating if a compound indeed immortalizes cells in a way that contributes to the clonal expansion of a transformed cell. The evaluation of activity for this characteristic is complicated by the common utilization of transformed cell lines in evaluation of other mechanisms associated with exposure, and/or low-dose chronic exposure of cell cultures with the intent of oncogenic transformation. In such systems, it is challenging to understand the plausibility of immortalization effects *in vivo*. For example, a recent database of KCC data considered a single study of neoplastic transformation that is included in the IARC monograph for Cr(VI) to represent activity for Cr(VI) for KCC 9 (Al-Zoughool *et al.*, 2019). However, neoplastic transformation is better defined as the cancer outcome itself, as opposed to a mechanism of the tumorigenic process. Notably, in several reviews of mechanistic data for compounds that have been classified as human carcinogens, no compounds have been reported to have strong evidence related to KCC 9 (Guyton *et al.*, 2018a,b; Smith *et al.*, 2016), while such evidence is clear for oncogenic viruses (Birkett *et al.*, 2019; Smith *et al.*, 2016).

This assessment highlights the need for continued efforts to more uniformly identify and select evidence related to key characteristics. Differences in the identification of evidence are commonly due to the approaches for searching—databases queried, search syntax, inclusion/exclusion criteria, etc.—elements that are not yet routinely developed or reported by users of the KCC (eg, IARC). For example, discrepancies in studies that would be included or excluded for the research objective stated herein were identified; specifically, some studies that are relevant to one or more KCCs were not included in the U.S. EPA IRIS HAWC assessment library: only one of 2 transcriptomics studies conducted by Kopec *et al.* (2012a,b) that contain data relevant to KCCs 5, 7, and 10 (oxidative stress, immunosuppression, and cell proliferation/death, respectively), And the study on potential immunotoxicity of Cr(VI) detailed above (Shipkowski *et al.*, 2017) was also not included in the HAWC assessment. These studies were included in the analysis presented herein, but was marked as "Potentially Relevant Supplementary Material" in the HERO database for the EPA IRIS assessment.

In summary, a wealth of mechanistic data exists in the peer-reviewed literature that are relevant to one or more of the KCC for Cr(VI). This review of data for select KCCs that have not been directly related to Cr(VI)-induced SI cancer in rodents was conducted as a means to investigate the possibility that Cr(VI) operates through an alternative mechanism to what has been previously proposed. The results of the review demonstrate that there is not strong evidence support MOAs other than those that have already been proposed and utilized by regulatory bodies globally.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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