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Utilizing Omics Data for Chemical Grouping

Mark R. Viant,[a,b,*] Rosemary E. Barnett,[a] Bruno Campos,[c] John K. Colbourne,[a,b] Marianne Barnard,[b] Adam D. Biales,[d] Mark T. D. Cronin,[e] Kellie A. Fay,[f] Kara Koehrn,[f] Helen F. McGarry,[g] Magdalini Sachana,[h] and Geoff Hodges[c]

[a]Michabo Health Science, Coventry, United Kingdom
[b]School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom
[c]Safety and Environmental Assurance Centre, Unilever, Sharnbrook, United Kingdom
[d]Office of Research and Development, Center for Computational Toxicology and Exposure, US Environmental Protection Agency, Cincinnati, Ohio
[e]School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, United Kingdom
[f]Office of Pollution Prevention and Toxics, US Environmental Protection Agency, Washington, DC
[g]Chemicals Regulation Division, Health & Safety Executive, Redgrave Court, Bootle, United Kingdom
[h]Environment Health and Safety Division, Organisation for Economic Co-operation and Development, Paris, France

INTRODUCTION

Historically, regulatory decisions on the safety of chemicals to both humans and the environment have relied primarily on the availability of in vivo toxicity data to inform hazard and ultimately risk assessment. However, increasing recognition of the benefits of more mechanistically based scientific understanding, together with changing ethical and societal concerns, are driving the development of new approach methodologies (NAMs) that can support robust safety decision-making without animal testing. Grouping and read-across (G/RAx) is one of the most commonly used alternative approaches to animal testing in chemical risk assessment for filling data gaps with existing in vivo toxicity data (European Chemicals Agency [ECHA], n.d.; Organisation for Economic Co-operation and Development [OECD], 2017a). As such, it exemplifies the efficient use of existing data and in some cases new nonanimal data. For example, under REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals regulation) Annex XI, information from one or more analogous (or "source") chemicals can be used to predict missing endpoint data for one or more "target" chemicals (European Commission, 2006). With approximately 100,000 chemicals listed on the European inventory (ECHA, 2023) and approximately 85,000 chemicals listed in the US Environmental Protection Agency's (USEPA's) Toxic Substances Control Act (TSCA) inventory (2024a), the use of G/RAx (described as chemical "categories" under the TSCA; USEPA, 2010) is becoming an increasingly viewed option for addressing regulatory requirements for filling data gaps in chemical safety dossiers for human health and environmental endpoints. Furthermore, grouping of chemicals can facilitate other hazard-assessment practices, for example, the harmonized classification of multiple substances within a group in accordance with the classification, labeling, and packaging regulation (Swedish Chemicals Agency, 2020).

There are numerous approaches for defining groups of chemicals, most often based on chemical similarity (Patlewicz et al., 2018). Notable examples in a regulatory context include the approach documented in the ECHA Read-Across Assessment Framework (RAAF; ECHA, 2017), supporting REACH, and within the TSCA (USEPA, 2010). These existing schemes are traditionally and primarily based on firstly grouping "source" and "target" chemicals into categories based on structural and other physicochemical parameters and, secondly, reading across existing toxicity data (i.e., an apical endpoint) from one or more "source" chemical(s) to predict the toxicity of one or more "target" chemical(s). However, most grouping dossiers still fail to incorporate and utilize absorption, distribution, metabolism, and excretion (ADME)/toxicokinetic and toxicodynamic similarities, with the strong reliance on structure-based similarity often leading to a rejection of the proposed read-across arguments, potentially resulting in regulatory noncompliance. For example, solely relying on structural similarity as the justification for a read-across introduces the potential to misevaluate the hazard of the target because structural similarity does not strongly infer equivalent levels of toxicity. This has prompted new efforts, such as the National Institute of Environmental Health Sciences workshop on clustering and classification (2022), to increase the confidence and consistency of chemical grouping by integrating

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* Address correspondence to mark@michabo.co.uk
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molecular responses, and ideally a mechanistic understanding, into this process (Escher et al., 2019; Pestana et al., 2021).

While NAMs span a wide range of approaches from in vitro testing and novel bioanalytical assays to in silico methods, in our study we focus on the application of omics technologies to generate molecular data that can be used to quantitatively determine group membership, thereby offering a solution to a significant limitation of conventional structure-based G/RAx approaches. This approach to forming chemical groups involves quantitatively comparing "profiles" of biological response data, derived from omics technologies such as transcriptomics (measuring gene expression) or metabolomics (measuring downstream metabolic biochemistry), and in concept is not unlike the widely used approaches for comparing structural fingerprints such as Tanimoto similarity (Sperber et al., 2019). Furthermore, with metabolomics possessing the capability to measure substance metabolism, there exists the potential to utilize both ADME/toxicokinetic and toxicodynamic similarities to build reliable groups from this data type. However, progress incorporating omics data into G/RAx has been hampered by a range of factors, including siloing of new scientific developments from regulatory science, to more specific issues such as a lack of standardized assays, reporting templates, and well-constructed case studies.

The overall aim of our study is to demonstrate how grouping using omics data (i.e., biologically based, not physicochemically based) can be relevant and important for chemical risk assessment, with a potentially immediate impact through increasing confidence in the grouping and read-across hypothesis. The four key objectives are as follows:

1. To introduce G/RAx to nonexperts, its importance, its terminology, the legislation through which it operates, and, most importantly, its current limitations.
2. To introduce omics technologies to regulatory scientists, as applied in the context of G/RAx, explaining the value of applying these molecular assays to quantitatively group chemicals.
3. To introduce the reader to some grouping case studies that use omics data, thereby increasing awareness of how these approaches have been used to group chemicals.
4. To describe some challenges to advancing the incorporation of omics data into chemical grouping and identify next steps toward accelerating this implementation.

To achieve our goal of introducing chemical grouping based on omics data across a range of contexts of use and regulatory jurisdictions, this article is necessarily generalized in places.

Conventional use of grouping to enable read-across predictions

Read-across is routinely used to predict an apical endpoint of a chemical by interpolating or extrapolating the toxicity data of analogous chemicals that are similar in some manner, for example, chemical structure, shared metabolism, and/or mode of action (MoA; which defines a functional cellular change), thereby avoiding further testing. A schematic summarizing the concepts of conventional G/RAx is shown in Figure 1, and relevant terminology is introduced in Textbox 1. It is based on an assumption that a physicochemical, (eco)toxicological, or environmental fate property of a "target" compound can be inferred from test data

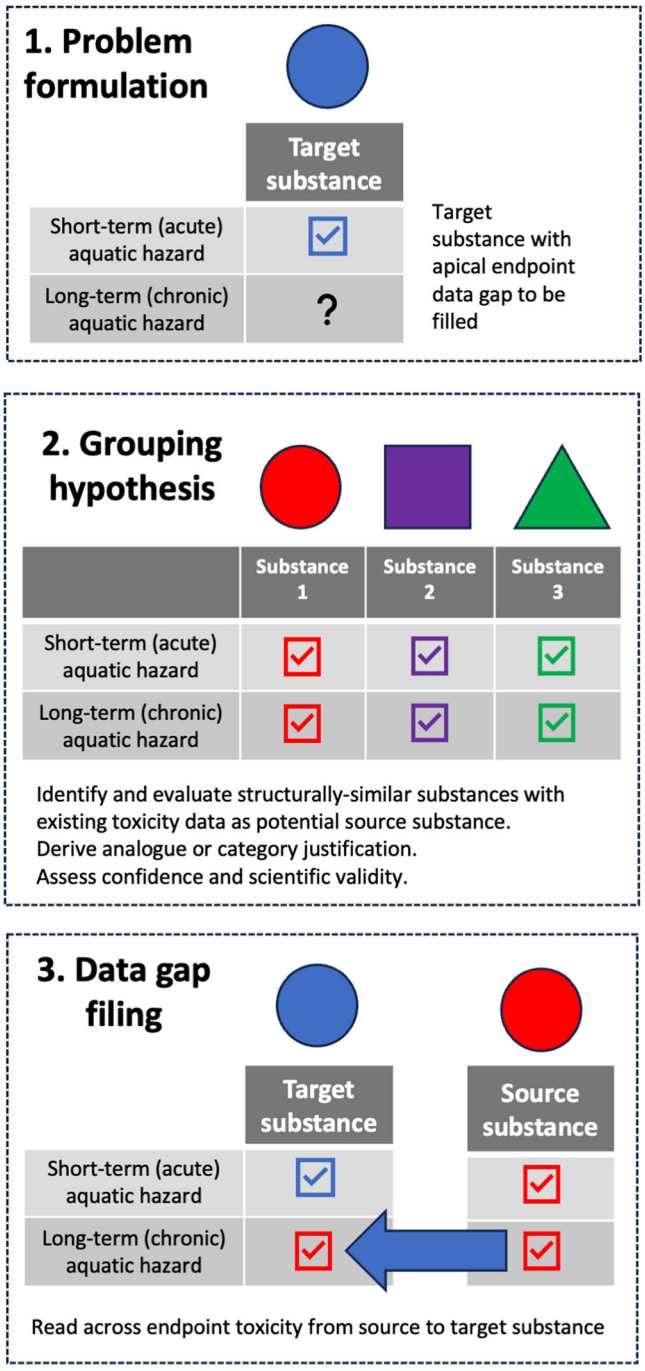

FIGURE 1: Schematic summarizing the concepts of a conventional grouping/read-across alternative method for chemical risk assessment. The approach includes the problem formulation, a grouping hypothesis (indicating which source and target chemicals form a group) with supporting information to justify this hypothesis, and a rationale for the read-across that explains why the (eco)toxicological endpoint can be predicted based on this chemical grouping.

for the same property of similar "source" compounds (OECD, 2017a). There are two distinct approaches to read-across: an *analogue approach* describes read-across from a single or very small number of source chemicals to a target chemical, whereas a *category approach* is used when data from a larger group of source chemicals are read-across to the target(s). Read-across predictions are endpoint-specific; for example, in quantitative read-across a known value(s) of a single endpoint for a source chemical(s) is used to infer a quantitative value of the same endpoint for the target chemical.

TEXTBOX 1

Terminology used in our study to describe grouping and read-across, as an alternative method for chemical risk assessment, for molecular scientists.

- *Grouping*—process of forming groups (or categories) of chemicals that have similar (or follow a regular pattern of) physicochemical, (eco)toxicological, and/or toxicokinetic properties.
- *Read-across*—alternative method for obtaining toxicity data (i.e., endpoint information) for one chemical—the *target*—by using data from the same endpoint from another chemical(s)—the *source* chemical(s), also referred to as an *analogue*—where the source and target chemicals lie within the same group.
- *Endpoint*—definition depends on the context of use. In REACH information requirements, endpoints are described either as a toxicological property (e.g., skin irritation, long-term toxicity to aquatic organisms) or as a type of study (e.g., carcinogenicity study, *Daphnia* chronic assay).
- *Grouping hypothesis*—description of the proposed membership of one (or more) chemical groups, based on the similarities of structural (or other physicochemical), (eco)toxicological, and/or toxicokinetic properties.
- *Category justification*—reasoning and associated evidence to verify the scientific validity of the grouping hypothesis for three or more chemicals. For the specific case of a single source and single target substance, this reasoning would be termed an *analogue justification*.
- *Bridging studies*—comparable studies on the source and target chemicals that allow a direct side-by-side comparison of the chemicals for a particular toxicological property (OECD, 2017a).

Regulatory landscape for chemical grouping

Existing global regulations endorse the use of grouping as the basis for reading across existing toxicity data to fill data gaps for industrial chemicals. For example, in the United States, the TSCA requires consideration of chemical grouping, stating in Section 4(h) that as part of reducing and replacing vertebrate animal testing of chemicals it encourages "the grouping of 2 or more chemical substances into scientifically appropriate categories in cases in which testing of a chemical substance would provide scientifically valid and useful information on the chemical substances in the category" (USEPA, 2018). Under the TSCA, the USEPA already routinely uses chemical grouping techniques such as the classification applied in the Ecological Structure–Activity Relationship (ECOSAR) software, new chemical categories, and analogue identification to fill data gaps in the assessment of new chemical submissions. The USEPA also considers analogues for data gap filling of existing chemicals and in special cases, such as per- and polyfluoroalkyl substances, has developed rules to group chemicals to direct national testing strategies. Associated with these practices, the USEPA is drafting guidance for how to select and use analogue data in ecotoxicology in place of animal data. In parallel, it is noteworthy that TSCA Section 4(h) states that information from "high throughput screening methods and the prediction models of those methods" should be considered "prior to making a request or adopting a requirement for testing using vertebrate animals," providing a legal route toward utilizing molecular (including omics) data for chemical grouping.

Complementing these regulations, extensive international guidance also exists for describing how to assess the hazards of related chemicals as a group, rather than as individual chemicals. Foremost is guidance published by the Chemical Safety Programme of the OECD, which assists member countries in their efforts to protect human health and the environment from hazardous chemicals using the best available science. The OECD is also committed to promoting alternatives to animal testing when suitable methods for evaluating chemical safety can be demonstrated. Of particular note to our study is the OECD Guidance on Grouping of Chemicals (Series on Testing & Assessment No. 194; 2017a), which focuses on considering the hazards of chemicals as a group or category. First published in 2007, it was updated in 2014 to include sections on analogue and category approaches, quantitative and qualitative read-across, justifying read-across, and using "bioprofiling" results (that include molecular data) for grouping chemicals. A new edition of this guidance document is now being prepared, including more extensive guidance on the use of omics data for chemical grouping. A further example of guidance related to chemical grouping, in this case specifically grouping and read-across, is the RAAF published by the ECHA (2017). This document provides both a framework and guidance for describing how G/RAx should be used and presented in a registration dossier in the context of meeting the REACH information requirements. It facilitates the consistent evaluation of the elements within a read-across case, including the grouping hypothesis and category (or analogue) justification.

Case studies represent a further important contributor to defining future regulatory landscapes by helping to establish common and best practices for the use of novel methods for assessing chemicals, including as groups. For example, case studies form the Accelerating the Pace of Chemical Risk Assessment initiative, which is an international government-to-government activity whose aim is to promote collaboration and

dialogue on the scientific and regulatory needs for the application and acceptance of NAMs in regulatory decision making (https://apcra.net/). These include a case study applying multi-omics to chemical grouping (Gruszczynska et al., 2024). The OECD Integrated Approaches for Testing and Assessment (IATA) case studies project aims to provide a forum to exchange information and grow confidence in the application of NAMs to assess chemical hazards in specific regulatory contexts (OECD, n.d.). A total of 34 IATA case studies have been reviewed, discussed, and published on the IATA OECD website (https://www.oecd.org/chemicalsafety/risk-assessment/iata/). Of these, 20 case studies have included aspects related to grouping and read-across, of which two have used transcriptomics data (OECD, 2017b; OECD, 2020).

Structured reporting templates are critical tools for ensuring that standardized information is consistently described, facilitating both the evaluation of chemical toxicity and ecotoxicity data by regulators and data sharing. They also allow end users to assess if the approach may be suitable for other geographical regions, chemical sectors, or regulatory contexts and most importantly allow regulators to become familiar with data derived from NAMs. Reporting formats for documenting conventional chemical grouping and read-across—including the context of use, target chemical(s)/category definition, endpoint, grouping hypothesis, and justification for filling data gaps—are available (see, e.g., Chapter 7 in OECD, 2017a). An OECD project has developed the Omics Reporting Framework (OORF) for describing the acquisition, processing, and analysis of omics data for a range of applications (OECD, 2023). The OORF is currently being extended to include a reporting template for the specific application of molecular (omics) data to chemical grouping.

Current limitations of chemical grouping: Insufficient evidence for robust category formation

The OECD states that "the most compelling evidence in support of a read-across hypothesis is information on a common mode of action of the substances and a mechanistic rationale for their common biological behaviour" (OECD, 2017a). Conventional G/RAx approaches are based on the hypothesis that structurally similar chemicals elicit similar biological responses. Yet there are many groups of chemicals for which this assumed relationship between structure and function does not hold. For example, thalidomide exists as two enantiomers that produce distinct biological responses: one is a sedative, and the other causes fetal malformations. Such "activity cliffs"—where structurally similar compounds have different MoA and/or potencies—demonstrate that structural similarity does not always lead to similar biological responses. By contrast, fentanyl and morphine are structurally dissimilar compounds, yet both share the same MoA, thus producing similar effects. The fact that structure does not always reliably predict function was well articulated by Wallqvist et al. (2006), who reported that "the connection between structure and biological response is not symmetric, with biological response better at predicting chemical structure than vice versa. Structurally and functionally similar compounds can have distinguishable biological responses reflecting different mechanisms of action." Furthermore, such differences in the toxicodynamic properties (i.e., MoA) of analogous compounds may also manifest as different acute and/or chronic toxicity outcomes.

This limitation is acknowledged in ECHA's RAAF, which states "structural similarity alone is not sufficient to justify the possibility to predict properties of the target substance by read-across." It is therefore no surprise that Schultz et al. (2019) identified one of the main sources of uncertainty in read-across as the *category justification* (i.e., justifying group membership). If the source chemical(s) is not sufficiently similar to the target chemical(s) in terms of structure, to support similarity in their MoA, read-across will not be justifiable. For this reason, uncertainty surrounding the category justification must be addressed because it weakens confidence in the grouping, discussed further below.

Under REACH, if read-across within a registration dossier does not provide sufficient evidence for a robust grouping of source(s) and target(s) chemicals, the dossier could be deemed noncompliant. Specifically, the ECHA has reported that G/RAx studies can be rejected in the absence of supporting data to substantiate the grouping hypothesis, for example, lack of knowledge of MoA and/or bridging studies between the source and target chemicals (ECHA, 2020). A second cause of registration dossiers that incorporate G/RAx being rejected is when the rationale for the read-across is missing or weak; for example, no explanation is provided linking structural similarity with the predicted toxicological endpoint. Another common problem when forming chemical groups based on ECOSAR and other quantitative structure–activity relationship ([Q]SAR) predictions is when many chemicals fall into multiple groups or into none at all. This is a particular problem where there are no or too few measured data to enable any benchmarking of the predicted values. There is clearly a need to improve our confidence in the formation of chemical groups or categories such as those efforts undertaken by the Board of Scientific Counselors in updating the TSCA (USEPA, 2022) and to quantify that level of confidence to facilitate the use of grouping in various risk-assessment contexts.

Omics data to increase confidence in the grouping of chemicals

The use of biological effects data as a basis for providing evidence to support group (or category) formation—via calculating the similarity of responses to chemical exposure—is widely viewed as a feasible solution for reducing uncertainty in grouping. However, we must ensure that the biological data will provide sufficient confidence for this application. It is important to emphasize that the generation of meaningful biological data requires a consideration of both the biological test system and the applied molecular assay.

Several options are available for measuring molecular responses to exposure. For the generalized case of a data-poor

target (i.e., limited experimental toxicity data), a biological effects comparison of source and target chemicals should examine a broad range of possible MoAs or mechanisms of action (MechoAs) to ensure that any significant toxicological effects are covered. In contrast, if the question is whether a target chemical behaves similarly to a group of chemicals with a relatively well-defined MoA or MechoA (which defines a specific target or pathway), a greater weighting could be placed on assessing the similarity of biological effects specific to that MechoA/MoA. Molecular measurements from omics technologies can be used to help address both of these situations, as illustrated in Figure 2, with introductory terminology presented in Textbox 2. For the former case, "untargeted" omics technologies measure a broad range of molecular responses (thus informing a broad range of MechoAs/MoAs); for the latter, a defined panel of "key event" molecular biomarkers could be measured that, for example, were derived from an adverse outcome pathway (AOP) describing the MechoA/MoA (https://aopwiki.org/). This first step in the application of omics technologies to chemical grouping is depicted in Figure 3 (right side).

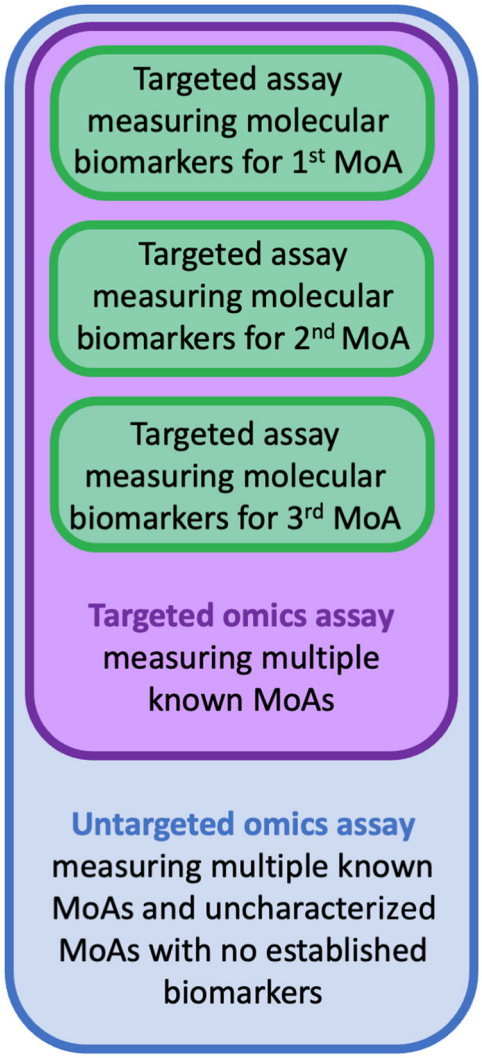

FIGURE 2: Interrelationships between the different measurement strategies (illustrated in green—targeted assays each measuring biomarker[s] linked to a single mode of action [MoA]; purple—a targeted omics assay measuring biomarkers linked to multiple MoAs; and blue—an untargeted omics assay measuring biomarkers linked to multiple MoAs and wider effects) that can generate molecular data for chemical grouping. Terminology is further explained in Textbox 2.

TEXTBOX 2

Omics terminology used in our study, for regulatory scientists.

- *Ome*—a broad collection of biomolecules, for example, genome (all genetic material), transcriptome (all gene transcripts), proteome (all proteins), and metabolome (all small-molecule metabolites).
- *Omics*—technologies that can be used to measure a broad range of molecular responses in the genome, transcriptome, proteome, or metabolome of a biological test system following chemical exposure.
- *Transcriptomics*—systematic study of expression of many genes in a cell, tissue, or organism, providing information on the molecular responses following chemical exposure.
- *Metabolomics*—systematic study of levels of many small-molecule metabolites and the biochemical processes that they are involved in, within a cell, tissue, or organism, providing information on downstream functional molecular responses to exposure.
- *Targeted assay*—measurement method that predefines the analytes, for example, to measure a specific MechoA/MoA (if few analytes are targeted) or multiple MechoAs/MoAs (if many analytes are targeted). Measurements using omics technologies can be targeted (see Table 1).
- *Untargeted assay*—measurement method that does not predefine the analytes. This approach attempts to measure the broadest range of molecular responses and hence gain insights into multiple MechoAs/MoAs simultaneously. Measurements using omics technologies can be untargeted (see Table 1).
- *Key event molecular biomarker*—a measurable marker that serves as an indicator of a specific MechoA/MoA, for example, derived from an AOP.
- *Molecular effects data*—a type of NAM data derived from either targeted or untargeted molecular assays that is used to calculate the bioactivity similarity of two or more chemicals and which can provide insights into MechoA(s)/MoA(s).
- *Bioactivity similarity*—a quantitative measure of similarity of two or more chemicals that is derived by comparing the molecular effects data from omics assays.

It is important to highlight that measuring and using molecular data in regulatory toxicology are not new. Of the three measurement strategies for grouping, described in Table 1, the

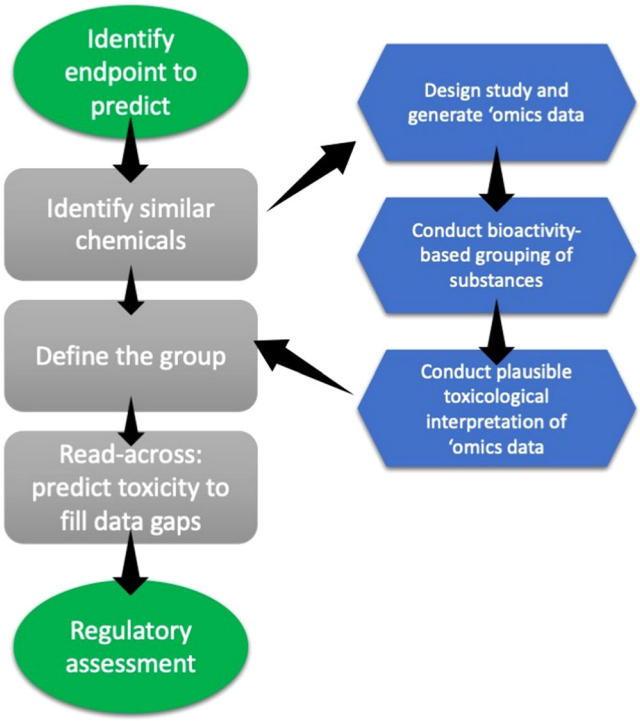

FIGURE 3: Schematic showing an example of how omics-derived molecular data (blue shapes) can be integrated into conventional grouping and read-across, thereby contributing to the category (or analogue) justification via demonstrating bioactivity similarity between the source and target substances.

first—targeted molecular measurements—is already part of internationally accepted OECD test guidelines. The second strategy multiplexes (or parallelizes) many targeted molecular measurements, and the third approach additionally introduces untargeted measurements. Examples are provided in Table 1 to support the reader's understanding of these measurement strategies for a generalized MechoA/MoA assessment.

Having generated either targeted or untargeted omics data in a relevant biological test system, the next step is to calculate the bioactivity similarity of the molecular responses to the source and target chemicals (see definitions in Textbox 2), in what can be referred to as a bridging study (Textbox 1). This typically involves applying multivariate statistical analyses and can enable assigning a quantitative level of confidence to the grouping hypothesis (Figure 3, right side). The third step is to interpret the molecular data in an attempt to identify the MechoA/MoA/AOP to build evidence for the grouping hypothesis; that is, if the omics data identify shared MechoA/MoA/AOP(s) that are perturbed as a result of exposure to a series of chemicals, then this provides a mechanistic justification to group those chemicals (Figure 3, right side). Minimally, evidence of shared molecular effects (without a clear understanding of mechanism) can be used to provide some justification for the chemical category. Here, resources that associate molecular changes with pathway perturbations, MechoA(s)/MoA(s)/AOP(s), and/or hazard are important parts of the toolkit. Many open-access and commercial resources exist, including the AOP Wiki, the Comparative Toxicogenomics Database, the S1500+ gene panel, the MTox700+ metabolite panel, BASF's MetaMapTox, and Qiagen's Ingenuity Pathway Analysis.

By using omics data to substantiate a grouping hypothesis, the conventional G/RAx workflow (e.g., OECD, 2017a) need only be slightly altered to include this additional supporting evidence (Figure 3, left side). The initial steps—identifying the target compound and selecting source chemicals based on structural and physicochemical similarity—can remain largely the same. This satisfies current requirements for read-across to

TABLE 1: Measurement strategies for deriving molecular data for chemical grouping

Measurement strategy	Purpose	Limitations	Examples
Targeted assay measuring a single (or few) KE molecular biomarker	Indicator for a specific MechoA/MoA/AOP	Only provides information on one biomarker, hence multiple assays in parallel are required to investigate the MechoA(s)/MoA(s)/AOP(s) of a chemical	Measurement of triiodothyronine, thyroxine, and thyroid-stimulating hormone in OECD TG408 as a predictor of thyroid toxicity (2018) Measurement of ornithine and cystine (devTOX quickPredict™ assay) to support the prediction of developmental toxicity (https://stemina.com/products-and-services/devtox-quickpredict/)
Targeted ("multiplexed") omics assay measuring many (typically hundreds) molecular biomarkers	Indicator for many MechoAs/MoAs/AOPs simultaneously	Currently insufficient knowledge of robust molecular biomarkers for all MechoAs/MoAs/AOPs	US National Toxicology Program S1500+ gene panel (US Department of Health and Human Services, 2023) Michabo Health Science/ECHA MTox700+ metabolite panel (https://michabo.co.uk/resources/mtox)
Untargeted omics assay measuring many (typically thousands) genes, proteins, or metabolites	Indicator for many MechoAs/MoAs/AOPs simultaneously, also can detect uncharacterized toxicities	More complex type of molecular data, more expertise currently required for data interpretation	High-throughput transcriptomics (USEPA, 2024b) High-throughput mass spectrometry metabolomics (Viant et al., 2019)

KE = key event; MechoA = mechanism of action; MoA = mode of action; AOP = adverse outcome pathway; OECD TG = Organisation for Economic Co-operation and Development Test Guideline; ECHA = European Chemicals Agency.

be based on structural similarity between source and target chemicals. The next step would typically be to refine the categories using mechanistic (MechoA/MoA/AOP) and/or endpoint-specific profilers (e.g., using the OECD [Q]SAR Toolbox). It is at this stage that the omics data can be introduced; that is, in addition to using these profilers, chemical categories are also refined based on bioactivity similarity using omics-derived molecular data. Source chemicals that are shown through a bridging study to *not* be biologically similar to the target can be excluded from the group. This approach should provide more confidence that the source and target chemicals share a common MechoA/MoA/AOP and thus elicit similar toxicological endpoints. Once the final list of source chemicals has been produced, the available toxicity data can be read across to fill the data gap for the target chemical. In effect, this is a weight-of-evidence approach that utilizes structural and bioactivity similarity to derive the chemical categories. In the future, if a structure-based chemical group is inadequate, it may be useful to create an *alternative* grouping hypothesis based solely on omics-derived molecular data. Such a change to regulatory practice would first require greater confidence in bioactivity profile–based grouping using omics data than exists today and would be best facilitated by the availability of a database of omics responses to enable comparison to data generated for the target substance.

Benefits of incorporating omics data into grouping

Several bioactivity profile–based grouping (and read-across) studies using omics data have been published, demonstrating the interest in evaluating this approach for supporting effective decision-making. We selected five studies and mapped them against a series of questions to highlight what each study sought to achieve, what approaches were used including whether any guidelines were followed, and to what extent uncertainties were considered to enable the reporting of the confidence of the grouping (Supporting Information, Table S1). Although such a mapping exercise is helpful for highlighting emerging "acceptable practice," in our study we focus on examining the benefits (this section) and challenges (next section) of incorporating omics data into chemical grouping (summarized in Textbox 3).

The key benefit is that grouping using omics data does offer a solution to the well-recognized problem that grouping hypotheses typically do not include biological effects data; that is, they lack sufficient mechanistic underpinning. Furthermore, by measuring a broad swathe of biological response space (i.e., multiple MechoAs/MoAs/AOPs) simultaneously, and without bias, the untargeted omics measurements enable a data-driven assessment of not only which chemicals group together but also what molecular perturbations are driving that grouping. This knowledge could provide insights into hazards and, consequently, could aid the selection of further targeted assays.

Further benefits of bioactivity profile–based grouping using omics data arise from the inherent capabilities of the technologies and computational approaches being applied. For example, metabolomics analyses can be conducted on biofluids, thereby enabling repeated measurements of some biological test systems, which could be valuable in cases where extensive metabolism or bioactivation occurs. Furthermore, the data analysis workflows are able to provide *quantitative* measures of similarity between two (or more) omics profiles,

TEXTBOX 3

Summary of top five benefits and challenges of incorporating omics data into chemical grouping.

Benefits of using omics data for grouping

- To provide targeted measurements of a broad range of MechoAs/MoAs simultaneously and an untargeted assessment of uncharacterized MechoAs/MoAs (dependent on the biological test system).
- To enable grouping by quantitatively comparing the similarities and differences of omics bioactivity profiles across chemicals, using statistically derived probability estimates, as a complement to structural similarity.
- In addition, the potential to provide mechanistic insights alongside statistically derived bioactivity-based grouping, together contributing to the category (or analogue) justification to support read-across to fill data gaps.
- Can be applied in high-throughput in vitro studies to screen and group the effects of multiple substances, enabling the triggering of higher-tier testing of a few sentinel substances.
- Can be used to group substances even when their structures are ill-defined, for example, UVCBs and polymers.

Challenges of using omics data for grouping

- A complex type of molecular data that requires specialist expertise for data analysis and interpretation.
- Currently, level(s) of bioactivity similarity has not been determined to assess "how similar is similar enough" to place two or more substances into the same group, nor have bioactivity thresholds been determined to delineate a molecular effect from no effect.
- It can be difficult to interpret the molecular changes and associate them to a MechoA/MoA.
- Currently, there has been only a limited assessment of the reliability of bioactivity profile–based grouping using omics data across laboratories, although there is ongoing work to address this challenge.
- There is currently no agreed "best practice" for grouping using omics data, although progress is underway at the OECD.

and confidence in the grouping can be based on statistical probabilities, facilitating robust decision-making (Gruszczynska et al., 2024). Although we are proposing that bioactivity profile–based grouping be considered as part of the weight of evidence toward a grouping hypothesis based on structural similarity, this assumes that structures can be defined for the test substances. However, there are many substances, for example, those of unknown or variable composition, complex reaction products and biological materials (UVCBs), that are poorly characterized in terms of component chemicals and their proportions, for which structure-based grouping is difficult, if not impossible. Applying an untargeted omics assay to an appropriate biological test system and calculating the bioactivity similarity from the biological response profiles can in principle still be used to group these challenging substances.

Finally, while this introductory study has not explicitly defined that grouping based on the responses of one biological test species is typically used to read-across endpoint data in the same species, the ability of omics technologies to reveal insights into the MechoA(s)/MoA(s) of a group of chemicals opens new possibilities for cross-species extrapolation, particularly within ecotoxicity testing. This involves duplicating the grouping hypothesis derived from an omics study in one test species to another species, based on evidence that the molecular pathways associated with the MechoA/MoA that underpins the category formation are conserved (Colbourne et al., 2022). A benefit of such an approach would be the avoidance of further testing (relevant to human and environmental assessments). Conversely, the approach could be used to identify where there are significant differences between species. Realistically, while evidence of homology of key toxicological mechanisms between some species is already well established and may be demonstrable in some cases, to fully exploit this knowledge more broadly for the purpose of read-across would require further evidence and case studies to provide sufficient robustness for regulatory acceptance.

Challenges and next steps toward accelerating the use of bioactivity profile–based grouping using omics data in regulatory toxicology

For a screening approach, the challenge is to select appropriate test systems and a panel of molecular assays to ensure that a broad biological response space can be *perturbed* and subsequently *measured, respectively. Either an inappropriate test system or molecular assay may cause a G/RAx study to fail*. Arguably one of the greatest current challenges for an in vitro G/RAx study is determining how many, and of what cell types, a panel of in vitro test systems should comprise. For G/RAx, the test system should be capable of exhibiting changes at the molecular level that are either directly associated with the endpoint to be read across or associated with a MechoA/MoA that is known to manifest in that endpoint. There also remains the legitimate concern, which goes beyond the scope of our study, that a single cell type may not respond in the same manner as the affected cell type within an organism.

The challenge for the omics assay is to ensure that it does actually measure a very wide range of molecular perturbations, that is, that it delivers on its greatest strength: full transcriptome RNA sequencing can largely achieve this, although both targeted transcriptomics (using reduced gene sets) and mass spectrometry metabolomics assays cannot observe all genes and metabolites. There are several reasons for metabolomics assays not being able to measure all polar metabolites and lipids, in particular the wide concentration range of these small molecules (exceeding the dynamic range of the mass spectrometer) and the high diversity of their physicochemical properties (limiting their ionization in a mass spectrometer). This must be considered during the design of a G/RAx study, to ensure that the assay(s) can measure molecular changes that are either associated with the endpoint being read across or associated with a MechoA/MoA that is known to manifest in that endpoint.

A further experimental design challenge is the exposure duration, from acute to chronic; but again, this is more about whether the biological response has had time to be *perturbed* in the test system rather than a particular challenge for omics measurements (USEPA, 2023). The selection of appropriate exposure concentrations should also be considered carefully to ensure that sufficient information can be extracted from the study to provide evidence of a shared MechoA/MoA. This will require testing at multiple concentrations, although there is no guidance yet on how many concentrations. The relatively high cost of omics assays may limit the number of treatment groups that can be measured. One option is therefore to utilize dose range-finding to carefully select a minimal number of concentrations for the omics measurements, for example, three, which is the minimum required in several OECD test guidelines.

Ensuring the relevance and reliability (including laboratory reproducibility) of omics data is key to its regulatory acceptance. Regulators and the wider scientific community need to be confident in omics data in the same way as when presented with conventional toxicity test data measured according to recognized test guidelines. Confirming high *reliability* of bioactivity profile–based grouping is the easier of these challenges. Recently, the Cefic Long-Range Research Initiative MATCHING international metabolomics ring trial confirmed the high reproducibility of metabolomics data when applied to chemical grouping, demonstrating in a blinded investigation that all of the five laboratories that passed data quality standards could correctly and consistently group eight test substances into three groups (Viant et al., 2024). What is particularly striking about this ring trial is that the consistent chemical grouping was achieved without prescribing strict standard operating procedures to each laboratory; instead, they applied their own mass spectrometry metabolomics methods for data acquisition, processing, and statistical analysis. An important conclusion from that investigation is the need to more clearly define international quality assurance and quality control practices in metabolomics when applied in a regulatory context. An equivalent international study could be considered for grouping using transcriptomics data.

Demonstrating the *relevance* of omics measurements in the specific context of G/RAx is not as great a challenge as for the case of predicting apical endpoints from molecular data. For G/RAx, omics technologies are employed in bridging studies to allow a direct side-by-side comparison of the multidimensional responses to source and target chemicals, to define similarities or probabilities of risk to reach a certain apical endpoint. This does not require the molecular data to directly predict the endpoint, though a "plausible toxicological interpretation" describing how the molecular changes may be associated with an underlying MechoA/MoA is desirable and would strengthen a grouping justification (OECD, 2017a). It is not unreasonable to expect that at least some of an omics response is interpretable given the current knowledge of molecular biomarkers, including in existing OECD test guidelines, such as markers of endocrine disruption. Furthermore, the continuously developing AOP Wiki (to name one such knowledgebase) has the capability to associate "key event" molecular biomarkers (measured within the omics profile) with specific adverse outcomes, which could also be used to interpret some of the omics responses and thereby add confidence to the analogue or category justification.

Another consideration of using omics technologies is that multiple methods exist to generate, process, and statistically analyze these data types, depending on their regulatory application; hence, it is not feasible to define a single method to be validated. In the specific context of G/RAx, the recent MATCHING international ring trial has demonstrated that we do not require a highly prescribed method. Instead, the community could focus attention on defining quality assurance and quality control practices as well as criteria for validating the use of targeted and untargeted omics data. Well-established criteria for validating single molecular biomarkers, such as by the US Food and Drug Administration and the European Medicines Agency, provide a basis for defining criteria for more complex assays, including the development of fit-for-purpose tiered validation criteria dependent on the regulatory use case (Sarmad et al., 2023). In the short term, deriving "acceptable practices" from recognized use cases (preferably codeveloped by regulators, industry, and researchers) and working toward "best practices" for data quality standards, interpretation, and integration with existing regulatory frameworks should remain a key goal. In addition to deriving acceptable or best practices for conducting a study, guidelines to describe how to report a bioactivity profile–based grouping study that uses omics data are also highly desirable. Considerable progress has been made recently, as described above, with ongoing efforts focused specifically on developing OECD guidance on how to report a bioactivity profile–based grouping study (as part of updating OECD Series on Testing & Assessment No. 194; 2017a).

The final set of challenges are more technically oriented, relating to the complexity of interpreting the results from bioactivity profile–based grouping studies. For example, what bioactivity threshold(s) should be used for concluding that the magnitude of an omics response to chemical exposure is large enough for reliable grouping? And what level (s) of bioactivity similarity should be used for concluding that omics responses to multiple chemicals are similar enough to justify placing them in a single group? Can the lack of an omics response be interpreted as a reliable indication of "no effect" (though this is heavily dependent on the biological test system)? These challenges can be addressed through multistakeholder case studies. For example, researchers are well positioned to provide statistical confidence for decision-making because this is ingrained in their daily practices, but academic scientists do not set regulatory limits. Hence, for bioactivity profile–based grouping using omics data and NAMs more generally, close collaboration between developers, users, and scientists in regulatory agencies is essential. Furthermore, two-way training is also urgently required not only to educate risk assessors and managers in NAMs approaches but also to educate NAM experts in the chemical safety problem space so that solutions are codesigned. Herein lies a significant practical challenge, that the day jobs of NAM experts and regulators have traditionally been siloed from each other. However, the value of participating in collaborative case studies, though not perhaps yet sufficiently recognized, is starting to increase, with examples such as the initiative of the Interagency Coordinating Committee on the Validation of Alternative Methods helping to guide NAMs in chemical safety and medical products (2018).

CONCLUSIONS

The importance of chemical G/RAx as an alternative test method for the hazard assessment of chemicals has been introduced, including general concepts, terminology, and the legislation through which this approach can operate. In addition, omics technologies and terminology have been introduced to a wide audience, allowing bioactivity profile–based grouping to be described in several steps: first, designing the study, including the choice of biological test system and omics assays; second, generating the omics data; third, calculating the bioactivity similarity between chemicals via statistical analysis of the omics data and contributing these results toward justifying a grouping hypothesis; and fourth, attempting to provide a plausible toxicological interpretation of the omics data, toward building stronger evidence for the analogue or category justification along with other data sources including chemical structure. An optional additional step is to duplicate the grouping hypothesis derived from an omics study in one test species to (an)other species, based on compelling evidence that the molecular pathways underpinning the MechoA/MoA defining the category are conserved across the species being considered. We have then described several benefits of applying omics to grouping, primarily by providing a solution to the well-recognized problem that a chemical structure–based grouping hypothesis is insufficiently robust, that is, by providing rigor through introducing shared molecular effects and, potentially, a mechanistic underpinning. However, several challenges remain, including the need to ensure the relevance and reliability of omics data for chemical

grouping, including to define fit-for-purpose tiered validation criteria. While some challenges associated with interpreting bioactivity profile–based grouping results remain, other barriers that were identified previously are actively being addressed through several current activities, including updating the OECD's principal guidance on chemical grouping (OECD Series on Testing & Assessment No. 194; 2017a), an active OECD project to define how to report omics data in a G/RAx regulatory study, extension of the MATCHING project to more thoroughly investigate how a "plausible toxicological interpretation" can be derived from metabolomics grouping data, and projects within the EU Partnership for the Assessment of Risks from Chemicals initiative, to name a few. In conclusion, the outlook for the future of bioactivity profile–based grouping using omics data is highly encouraging, with a need for continuing case studies to build confidence in this approach.

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