Building scientific confidence in metabolic similarity in read-across through the use of *in vitro*, *in silico* and analytical data

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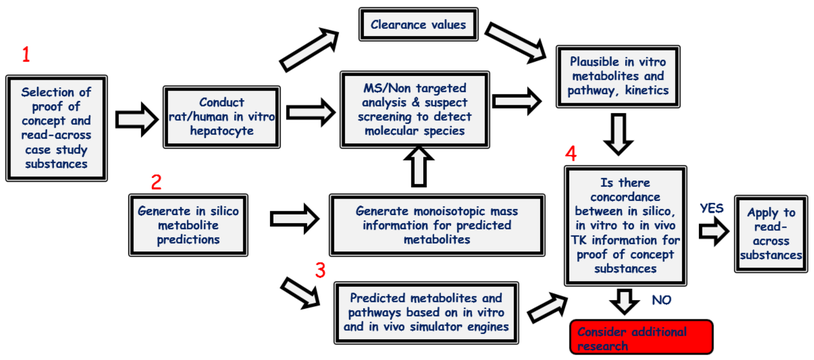
Abstract

The underlying principle of read-across is that the biological activity of a chemical is inherent in its molecular structure. Analogues are typically identified by structural similarity then evaluated on the basis of their bioavailability, reactivity and metabolic similarity. While structural similarity is the major tenet in read-across, a critical consideration is whether structural differences impact biological activity. This source of uncertainty can potentially be addressed with toxicokinetic (TK) information. Here we report progress on a case study to investigate the feasibility of using *in vitro* high-throughput metabolism experiments in concert with *in silico* metabolism predictions to substantiate biological similarity to enable quantitative read-across. Parent chemicals were incubated with a suspension of primary rat hepatocytes. Possible metabolites were predicted *in silico* using expert systems (Meteor Nexus and the simulators within TIMES). Suspect screening analysis was performed using liquid chromatography mass spectroscopy (LC/MS) and the *in silico* predicted metabolites. Four target chemicals representing different read-across scenarios were identified: 2 proof-of-concept examples and 2 test cases. Candidate source analogues were identified based on structural similarity and information availability for the two proof-of-concept chemicals (similar metabolism (methyl eugenol & estragole) and different metabolism (2-nitrotoluene & 4-nitrotoluene), whereas for the two test cases, the experimental and *in silico* results will be integrated to substantiate the validity of the source analogues to inform selection of the most appropriate analogue. To date, *in silico* metabolism predictions have been generated for the proof-of-concept chemicals and compared to the *in vivo* metabolic profiles reported in the literature to assess their agreement. The metabolism predictions from the different tools complemented each other in capturing the primary pathways, and in particular the pathway that has been associated with the genotoxicity (nitrotoluenes) but no tool correctly captured all the metabolites observed.

Study Workflow



In vivo toxicokinetics information if available is often relied upon to substantiate biological similarity for the purposes of justifying a read-across
This information is usually extracted from the literature
In this pilot study we sought to investigate the feasibility and utility of using *in vitro* toxicokinetics data to substantiate biological similarity.

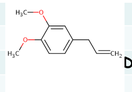
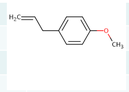
Step 1 – Target/Source analogue identification

Chemicals	Category
Methyl eugenol (CASRN 93-15-2), Estragole (CASRN 140-67-0)	Proof-of-concept: Known similar metabolism; methyl eugenol showed metabolic clearance in the Wetmore *et al.*, (2015) studies
2-nitrotoluene (CASRN 88-72-2), 4-nitrotoluene (CASRN 99-99-0)	Proof-of-concept: Known different metabolism; 4-nitrotoluene showed metabolic clearance in Wetmore *et al.*, (2015) studies
Target: 4-Methyl-2-Pentanol (CASRN 108-11-2) Analogues: 4-methyl-2-pentanone (CASRN 108-10-1), 2-propanol (CASRN 67-63-0), 2-propanone (CASRN 67-64-1)	Application to Read-across: Metabolism considerations form the basis for analogue identification and selection of most appropriate surrogate chemical
Target: 3,5-Dinitroaniline (CASRN 618-87-1) Analogues: 2-Nitroaniline (CASRN 88-74-4), 3-Nitroaniline (CASRN 99-09-2), 4-Nitroaniline (CASRN 100-01-6)	Application to Read-across: no information on target compound; exploring the utility of metabolism in informing analogue selection

Step 2: Generate *in silico* metabolite predictions

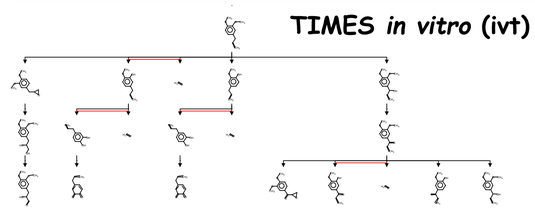
***In silico* metabolism tools**
A selection of available commercial and freely available software tools were used to simulate plausible metabolites. Results are illustrated for one proof-of-concept pair only.
TIMES – TIssue MEtabolism Simulator – Commercial tool from Laboratory Mathematical Chemistry (LMC), Bourgas, Bulgaria. Metabolism and autoxidation simulators can be used in vacuo though they have been "trained" to reproduce the metabolic maps and their associated metabolites for genotoxicity and skin sensitization endpoints. Provides a qualitative estimate of quantities of metabolites and their relative probability of transformation.
OECD Toolbox – Freely available, Contains the same simulators as in TIMES but only provides a list of metabolites.
Derek Nexus – Commercial, LHASA Ltd. Default option is site of metabolism scoring with mass variance

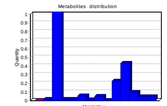
Step 3: Predicted *in silico* metabolism pathways

		Methyl eugenol		Estragole
Role		Target		Source
CAS		[93-15-2]		[140-67-0]
DTXSID		DTXSID5025607		DTXSID0020575
Average Mass		178.23		148.21
LogKow (predicted)		2.61		3.02
Boiling pt (predicted)		252.16 deg C		219.74 deg C
Melting pt (predicted)		30.59 deg C		-7.93 deg C
Reactivity		Potential Michael acceptor		Potential Michael acceptor

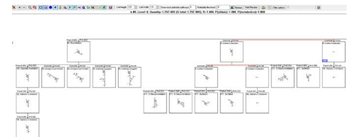
Step 3: Predicted *in silico* metabolism pathways

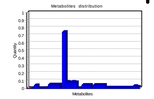
TIMES *in vitro* (ivt) map for methyl eugenol



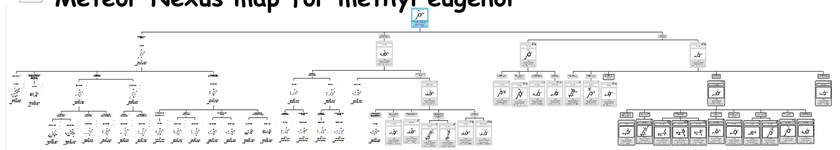


TIMES *in vivo* (iv) map for methyl eugenol

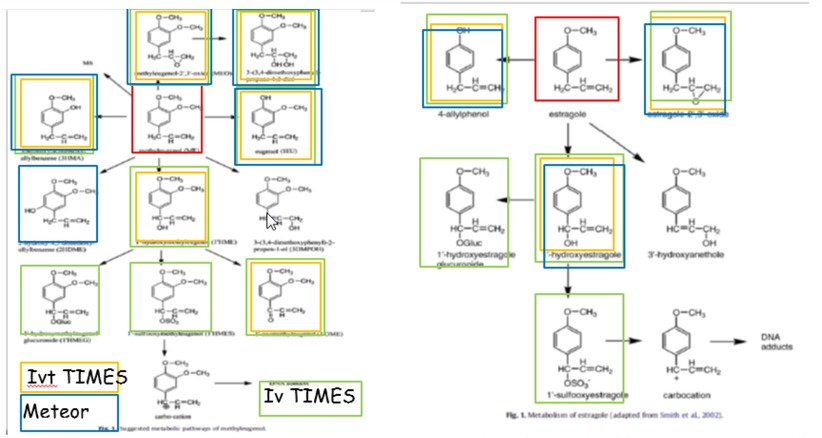




Meteor Nexus map for methyl eugenol



Step 4: Concordance between *in silico* and *in vivo* data



The OECD toolbox metabolites presented a subset of those identified by TIMES (results not shown). *In vitro* TIMES predictions presented an incomplete picture of the metabolism pathway of target and analogue substances. *In vivo* TIMES metabolism simulator was able to replicate the majority of the experimental *in vivo* metabolites. Similar results were found for the nitrotoluenes proof-of-concept pair.

Preliminary findings and Next steps

For target 3-Nitroaniline, the first Meteor predicted metabolite, 3-nitroacetanilide was detected in greater abundance than the parent chemical itself.
Compare concordance with *in vitro* experimental data to evaluate whether a combination of *in silico* & *in vitro* data best represents the *in vivo* metabolism profile of a given target/analogue.