Safety & Environmental Assurance Centre

METABOLISM CONSIDERATIONS IN NEXT GENERATION RISK ASSESSMENT: CHALLENGES OF FIT-FOR-PURPOSE EXPERIMENTAL DESIGN USING COUMARIN AS A CASE STUDY

Spriggs S, Baltazar MT, Reynolds G, Cubberley R and Hatherell S
Unilever Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedfordshire MK44 1LQ, UK;

Introduction

Next Generation Risk Assessment (NGRA) integrates estimations of internal exposure (e.g. plasma Cmax) with measures of bioactivity detected across a range of in vitro bioassays to inform the safety of a new chemical for a defined consumer use scenario. Within this framework, there is a need to consider metabolism-driven toxicity. Thus, when in silico tools predict the potential formation of reactive metabolites, based on the chemical structure of the parent compound alone, a tiered approach is taken to confirm metabolite formation and define the best model for bioactivity detection. To demonstrate the application of this tiered approach for assessing the safety of parent and metabolites in the context of a cosmetic risk assessment, we used coumarin as a case study chemical present at 0.1% in a hypothetical face cream.

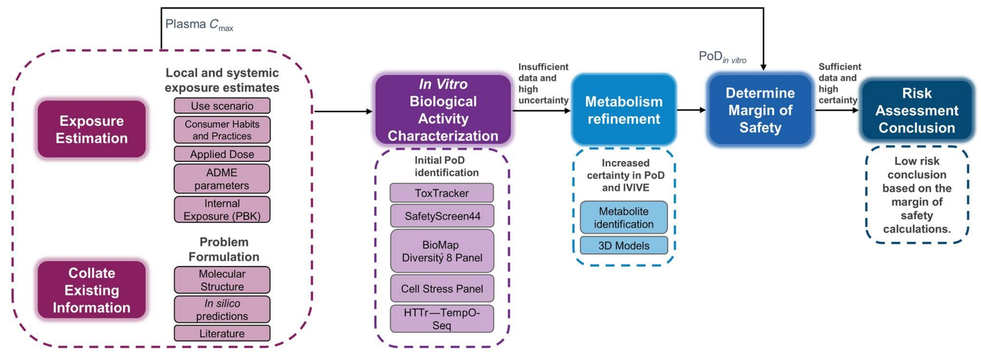

Figure 1. Next-Generation Risk Assessment case study workflow for 0.1% coumarin in consumer products.

Metabolism consideration in Risk assessment and associated in vitro assays

The battery of in vitro biological assays (Toolbox) uses cell lines that do not possess relevant metabolic competency (HepG2, MCF-7, HepaRG 2D).
Here, we present a tiered approach where metabolism considerations can be addressed in a fit-for-purpose manner (i.e. when is there a need to consider metabolite(s) separately from the parent compound for Risk Assessment?)
A mixture of in vitro assays covering parent chemical clearance rate, formation and identification of metabolites and , when relevant, cellular effect of metabolites formed in situ in complex 3D models (HepaRG spheroids, organs on-a-chip…) can be used.

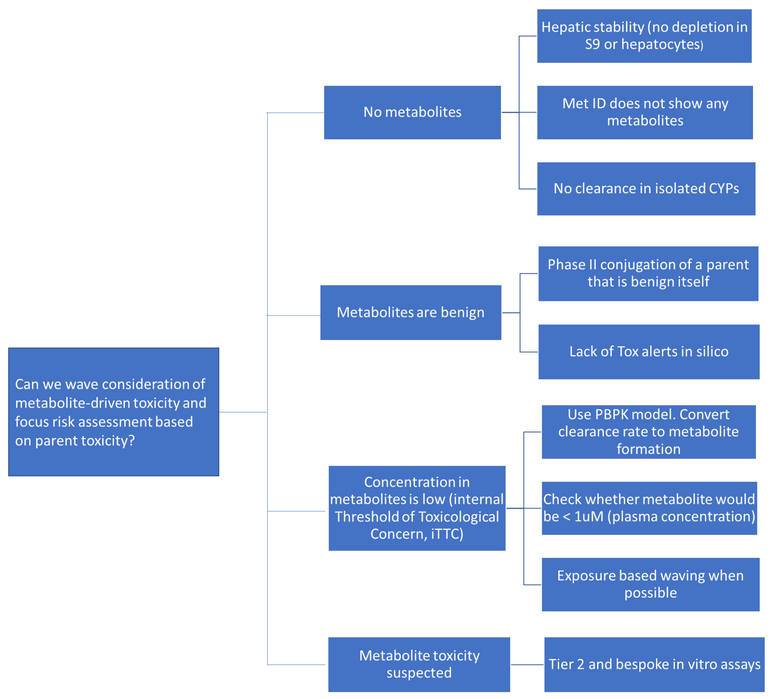

Figure 2. Metabolism considerations in Risk Assessment. Tier 1 assessment: Proposed experimental design to demonstrate that a risk assessment based on parent is appropriate and conservative (under evaluation).

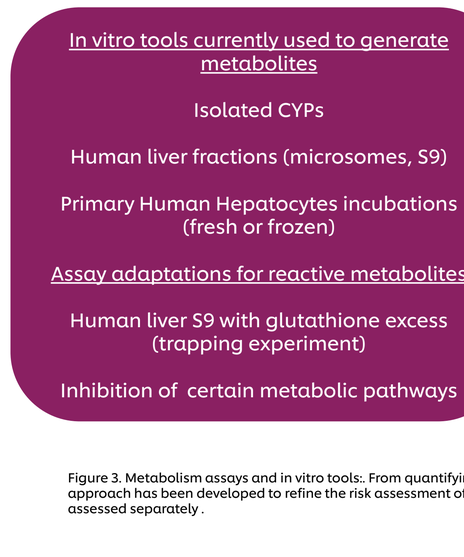

Figure 3. Metabolism assays and in vitro tools:. From quantifying metabolite formation to measuring cellular effect, a fit-for purpose tiered approach has been developed to refine the risk assessment of parent chemical and determine whether the metabolite(s) should be risk assessed separately .

Coumarin case study

In silico predictions

The in silico tools ToxTree, OECD Toolbox, Derek Nexus, Meteor Nexus, TIMES, and molecular initiating events (MIE) ATLAS were run to predict the potential biological activity of coumarin, identify the active groups and predict its metabolic fate.

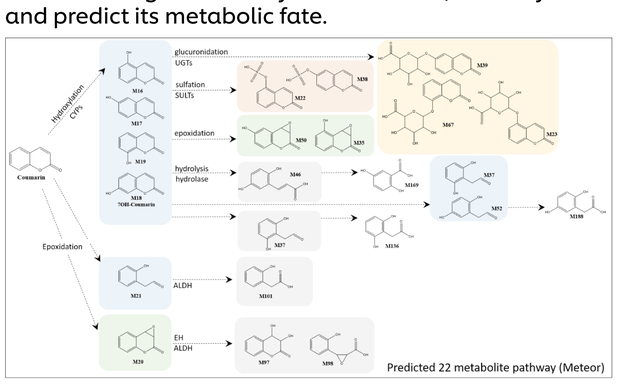

Figure 4. Predicted metabolic pathway by METEOR highlighting possible formation of reactive metabolites (i.e. epoxides).

Coumarin metabolism in vitro

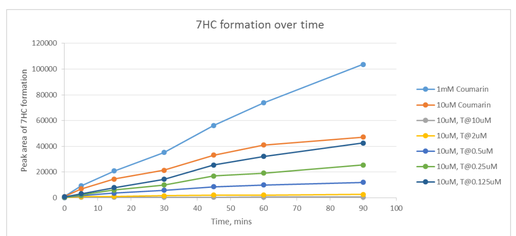

Figure 5. Effect of tranylcypromine on the production of 7-hydroxycoumarin.

Reactive metabolites (coumarin-3,4-epoxide and o-hydroxyphenylacetaldehyde) were only formed if the pathway for metabolism of coumarin to 7-hydroxycoumarin was saturated (1mM Coumarin).

Pooled human cryopreserved primary hepatocytes (50 multidonors, BioIVT, lot no. YQV) in suspension at a cell density of 0.5 million cells per ml, were incubated at 37°C up to 90 min with 10 µM coumarin (final concentration of 0.25% DMSO) without and with tranylcypromine (0.5, 2 µM) to inhibit the CYP2A6-specific reaction. A second experiment was conducted at a higher concentration of coumarin (1 mM) without the inhibitor, to saturate the 7-hydroxycoumarin pathway; all experiments were performed in duplicate. Full scan liquid chromatography-mass spectrometry data were acquired using multienergy time-of-flight acquisition (Waters Xevo G2 Q-ToF in MSE mode).

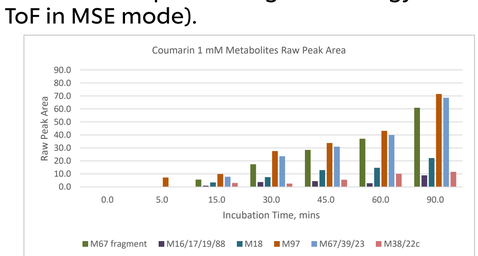

Figure 6. Coumarin 1 mM Metabolite Identification Incubations – positive ion mode. Intensity of the peak expressed as Raw peak areas.

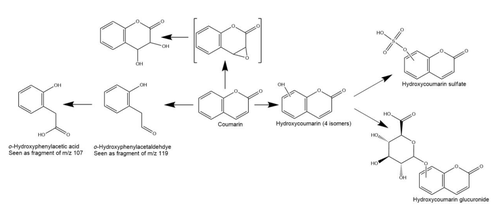

Figure 7. Coumarin's proposed metabolic pathway based on the in vitro experiments.

Skin exposure refinement

There was little evidence of metabolism of coumarin in ex vivo cultured skin (20µL of 500mM Coumarin applied topically, 24h) through minimal formation of 7-hydroxycoumarin (<0.01% of the dose applied and no further 7-hydroxycoumarin glucuronide formation observed.), providing supporting evidence that safety data obtained from parent alone would be sufficient for the risk assessment.

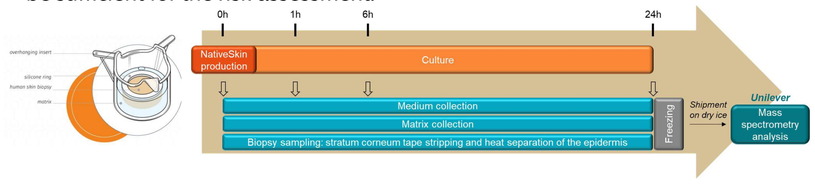

Figure 8. Experimental workflow for coumarin topical exposure on NativeSkin, ex vivo human cultured skin model (Genoskin, France). Mass spectrometry analysis of tape strips extracts, epidermis, dermis, media, gel matrix, cell insert extracts and original dosing solution were carried out on a Waters Xevo TQ-XS LC/MS system with a Waters Acquity UPLC inlet, where quantification against calibration standards commercially available was performed using Multiple Reaction Monitoring m/z 146.97>103.02 (collision energy 16eV) for coumarin, m/z 162.90>106.99 (collision energy of 20eV) for 7-hydroxy coumarin and m/z 339.3>163.0 (collision energy 30eV) for 7-Hydroxy coumarin glucuronide.

Coumarin in other in vitro assays (Cellular stress pathways and transcriptomics)

There was no significant difference in points of departure, obtained from a range of assays (cellular stress pathways and transcriptomics), between 2D HepaRG (24h), 3D HepaRG (7 days) and less metabolically active cell lines such as HepG2 or NHEK, when the dosing regimen was below 1mM.

Cell Model	HepG2	MCF7	HepaRG 2D	HepaRG 3D
Pathway-level tests PoD_T (µM)	(308 pathways)	(0 pathways)	(17 pathways)	(2 pathways)
20 pathways with the lowest *p* value Reactome	70	NA	58*	46*
20 pathways with the lowest BMD Reactome	44	NA	58*	46*
BMD of Reactome pathway with lowest BMD that meets significance threshold criteria	31	NA	38	41
Gene-level tests PoD_T (µM)	(1570 genes)	(47 genes)	(87 genes)	(9 genes)
Mean BMD of 20 genes with largest fold change	6	3	54	55
Mean BMD of genes between 25th and 75th percentile	17	1	59	46*

Highlighted (*) are values where the number of pathways or genes was below the recommended number (ie, 20) for grouping. Abbreviation: NA, not applicable.

Table 1. PoDT Values (mM) for Coumarin Treated Across 4 Cell Models for 24 h Using a Subset of Proposed Approaches for Gene Selection Based on Those Proposed by Farmahin et al. (2017). Highlighted (*) are values where the number of pathways or genes was below the recommended number (ie, 20) for grouping. Abbreviation: NA, not applicable.

Biomarker	Cell Type	Stress Pathway	PoD (µM)	Effect	CDS
ATP (6 h)	HepG2	Cell health	**794** (363–977)	Down	0.98
ATP (24 h)			**617** (282–891)	Down	1
Phospholipidosis (24 h)	HepG2	Cell health	**759** (437–977)	Down	0.93
GSH (24h)	HepG2	Oxidative stress	**851** (301–1000)	Up	0.92
IL-8 (24h)	HepG2	Inflammation	**912** (575–1000)	Down	0.61
OCR (1 h)	NHEK	Mitochondrial toxicity	**62** (2.6–776)	Down	0.6
OCR (6 h)			**468** (214–794)		1
OCR (24 h)			**309** (138–1000)		0.52
Reserve capacity (1 h)	NHEK	Mitochondrial toxicity	**44** (23–96)	Down	1
Reserve capacity (6 h)			**759** (302–1000)		0.9
Reserve capacity (24 h)			**794** (295–1000)		0.55
Caspase 3–7 (72 h)	HepaRG 3D	Cell health	**741** (245–977)	Up	0.95
Cell membrane permeability (168 h)	HepaRG 3D	Cell health	**55** (26–141)	Up	0.99
ATP (72h)	HepaRG 3D	Cell health	**186** (129–288)	Down	1
ATP (168h)			**135** (85–195)	Down	
Phospholipidosis (168h)	HepaRG 3D	Cell health	**776** (234–1000)	Up	0.86
GSH (168 h)	HepaRG 3D	Oxidative stress	**776** (275–1000)	Down	0.92
Mitochondrial mass (72 h)	HepaRG 3D	Mitochondrial toxicity	**871** (234–1000)	Down	0.65
Mitochondrial mass (168 h)			**831** (275–1000)	Down	0.73

Table 2. To increase our confidence in the initial PoDs from the 2D cell models using the cell stress panel, coumarin was tested for longer exposure durations (7 days) in a 3D HepaRG model with potentially higher metabolic capacity and in vivo-like physiology than HepG2 cells. Even though spheroid size was unchanged throughout the duration of the experiment, early signs of cell damage were observed at low concentrations with a dose-dependent increase in cell permeability at 168 h, and ATP decrease at 72 and 168 h.

Conclusions and next steps

The safety decision that 0.1% coumarin in a hypothetical face cream would be safe to use, obtained using new approach methodologies, was comparable to the traditional decision which would have been made using toxicity data from published animal studies.

Future work to evaluate tools for the generation of reactive metabolites in situ, to refine risk assessments of chemicals designed for cosmetic use, include multiple exposure of HepaRG organoids over 7 days (simplified Cell Stress pathways analysis as output).

References:
1. Baltazar MT, Cable S, Carmichael PL, Cubberley R, Cull T, Delagrange M, Dent MP, Hatherell S, Houghton J, Kukic P, Li H, Lee MY, Malcomber S, Middleton AM, Moxon TE, Nathanail AV, Nicol B, Pendlington R, Reynolds G, Reynolds J, White A, Westmoreland C. A Next-Generation Risk Assessment Case Study for Coumarin in Cosmetic Products. Toxicol Sci. 2020 Jul 1;176(1):236-252. doi: 10.1093/toxsci/kfaa048. PMID: 32275751; PMCID: PMC7357171.
2. Reynolds G, Reynolds J, Gilmour N, Cubberley R, Spriggs S, Aptula A, Przybylak K, Windebank S, Maxwell G, Baltazar MT. A hypothetical skin sensitisation next generation risk assessment for coumarin in cosmetic products. Regul Toxicol Pharmacol. 2021 Dec;127:105075. doi: 10.1016/j.yrtph.2021.105075. Epub 2021 Oct 30. PMID: 34728330.

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