How to perform risk assessment of a new cosmetic ingredient using new approach methodologies?

Systemic exposure assessment and SAR- based read across applied to oxidative hair dyes

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6th German Pharm-Tox Summit
Advanced course: Scientific Workshop on Alternative Methods to Animal Computational/in silicoToxicology/QSARs – Implementation and Assessment in the Regulatory Context
1 March 2021

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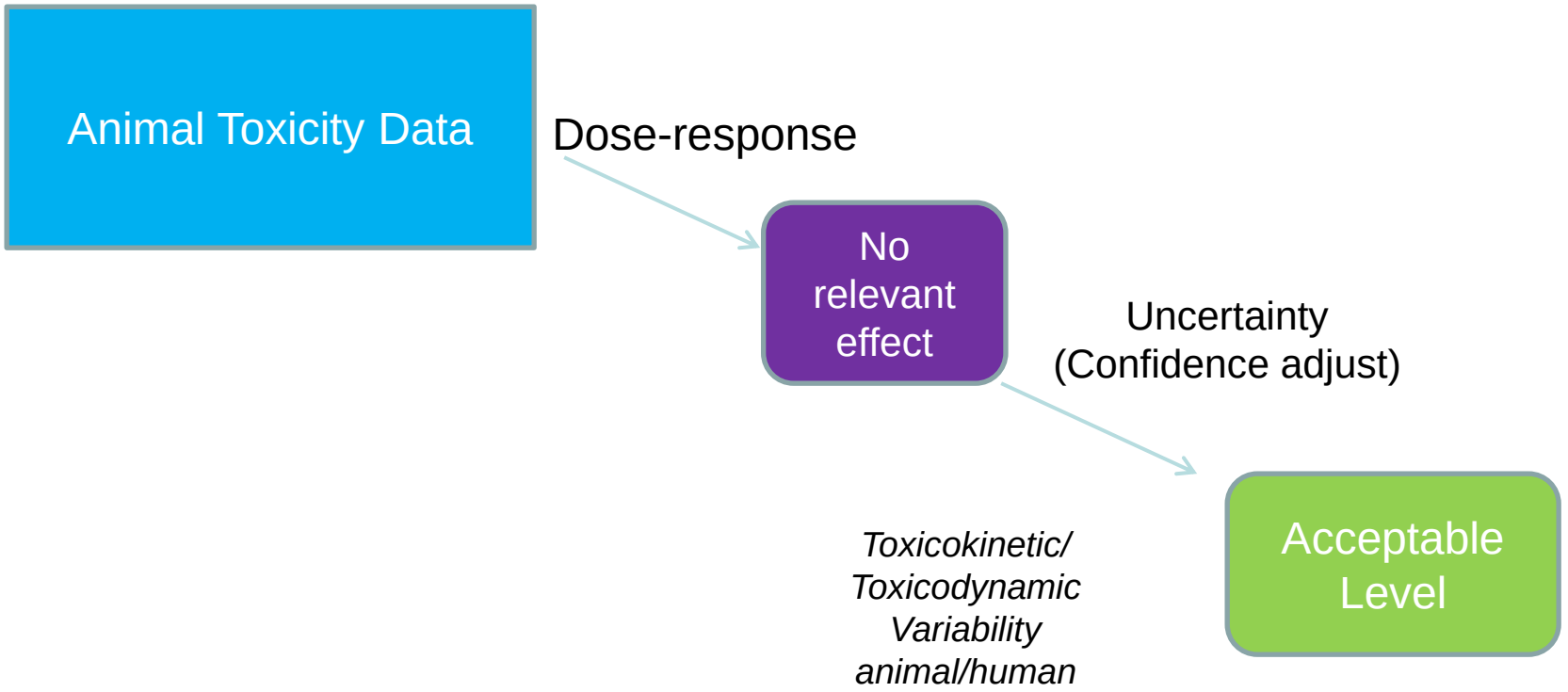
Risk Assessment (traditional)



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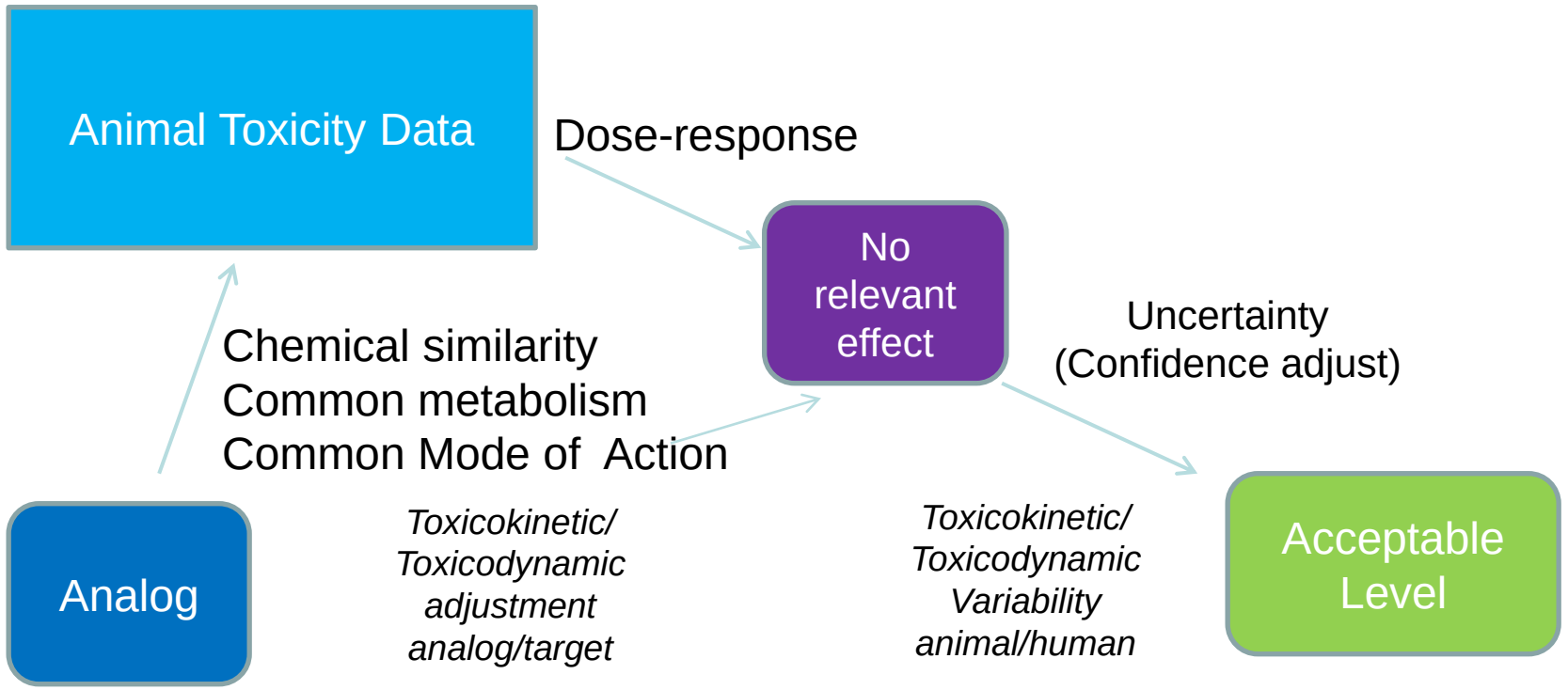
Risk Assessment by Analogy



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Guiding questions for Read Across

- robust group of chemicals which includes the target chemical?
- relevant members to fill a data gap considering endpoint under assessment?
- appropriate toxicology studies of sufficiently high quality for the source chemical(s) to allow a meaningful Read Across?
- uncertainties defined and acceptable in order to use the read across prediction(s) to fill the data gap(s) for a specific regulatory purpose?

References: Schultz and Cronin, 2017; Przybylak et al., 2017

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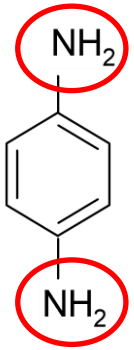
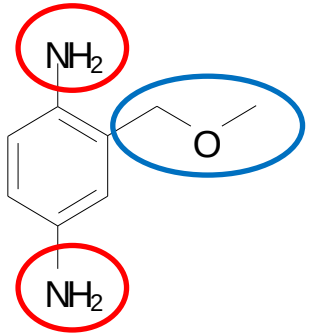
Group of chemicals

Sources

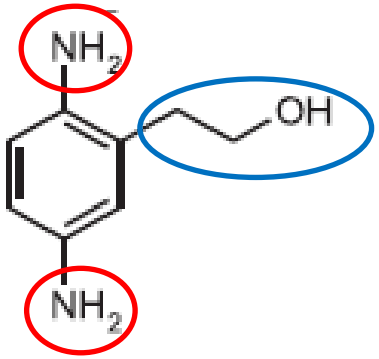
Target



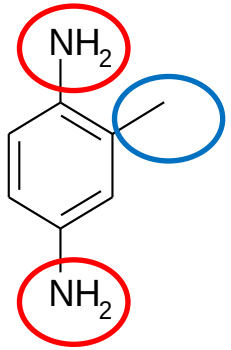
p-phenylenediamine
(**PPD**)



2-methoxymethyl-
p-phenylenediamine
(**ME-PPD**)



hydroxyethyl-
p-phenylenediamine
(**HE-PPD**)



toluene-2,5-diamine
(PTD)

Prediction of oral 90-days repeated dose toxicity NOAEL for target PTD'

- structural similarity < 50 % using ChemIDplus for 1,4-benzenediamine (PPD) alone and combined with 2-methyl-side chain and target structure
- manually filtered for benzenediamine with amino groups in positions 1 and 4, side chains in position 2 for potential biological reactivity
- compounds with relevant repeated dose toxicity data

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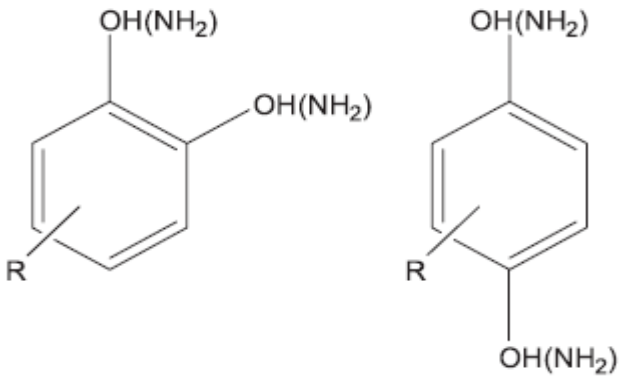
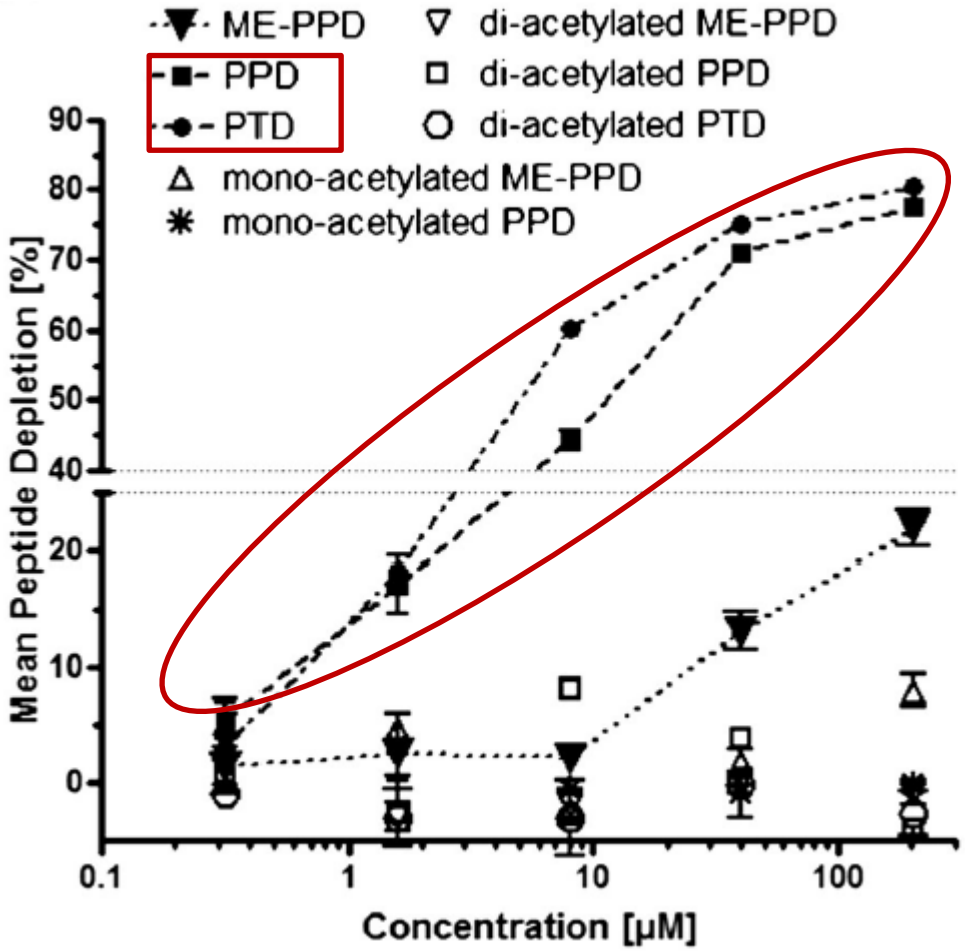
Mechanism of toxicity/reactivity:

Pre-Michael Acceptor (aromatics with ortho- or para- hydroxyl- and/or amino-groups) {9}



Structure-based prediction supported by GSH reactivity data
Reference: Schultz et al., 2009

Cysteine peptide reactivity of ME-PPD, PPD and PTD and their N-acetylated derivatives.



Percent depletion of cysteine peptide monomer, expressed in terms of mean (±SD) of triplicate incubations for 24h.
Reference: Goebel et al., 2014

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Target and source substances: Data compilation I

Predict oral 90-days repeated dose toxicity NOAEL for PTD

Chemical (INCI) (abbreviation)	*p*-phenylenediamine (PPD)	2-methoxymethyl-PPD (ME-PPD)	hydroxyethyl-PPD (HE-PPD) sulfate	toluene-2,5-diamine (PTD)
Reference	SCCS opinion 2012	SCCS opinion 2013	SCCS opinion 2010	SCCS opinion 2012
CAS# free base	106-50-3 624-18-0 (dihydrochlor.)	337906-36-2 337906-37-3	- 93841-25-9 (sulfate)	95-70-5 615-50-9 (sulfate)
MW [g/mol]	Free base 108.14 Dihydrochloride 181.07	Free base 152.20 Sulfate 250.28	Free base 152.20 Sulfate 250.28	Free base 122.17 Sulfate 220.25
Phys-Chem properties **Water solubility [g/l]** **Log Pow:**	 Free base ~10 Free base -0.31	 Free base 284 Free base:-0.65	 Sulfate 51.2 Sulfate 0.07	 Sulfate 5 Sulfate 0.74
OECD guideline	408	408	408	
Dosing [mg/kg bw/day]	0, 2, 4, 8 and 16	0, 10, 30 and 90	0, 25, 35 and 55	
Toxicity	At 16: liver and kidney weight increase, myodegenerative effects in two animals	At 90: marginally increased activity in liver enzymes (AST), increased liver weight/hepatocellular hypertrophy	At 55: increased activity in liver enzymes (AST, ALT)	
NOAEL	8 (free base)	90 (sulfate)	35 (sulfate)	
Oral bioavailability	High (rats)	High (rats)	High (*in vitro AB permeability*)	High (*in vitro AB permeability)**
Cystein reactivity at 200 µM**	High (80% depletion)	Medium (25% depletion)	n.d.	High (80% depletion)

*References (other than SCCS opinions): *Obringer et al., 2015; **Goebel et al., 2014)*

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Target and source substances: Data compilation II: first pass and systemic metabolism

Molecule	Human liver metabolism (hepatocytes)	Human skin metabolism (skin ex vivo/ keratinocytes/HaCaT)	Major Metabolic pathway in rats	Major Metabolic pathway in humans (hair dyeing)
PPD	- Mono/Di-acetylation - No evidence of oxidative metabolism, transformation to N-hydroxylated derivatives	- Mono/Di-acetylation - No evidence of oxidative metabolism, transformation to N-hydroxylated derivatives	Mono/Di-Acetylation following dermal exposure	Mainly Di-Acetylation following topical exposure
HE-PPD	n.d.	Indication for Mono/Di-acetylation	n.d.	n.d.
ME-PPD	- Mono-acetylation - No evidence of potentially biologically reactive oxidized metabolites	- Mono-acetylation, - No evidence of potentially biologically reactive oxidized metabolites, *(Di-acetylation in 1 human skin donor)*	Mono/Di-Acetylation following oral and dermal exposure	n.d.
PTD	- Mono/Di-acetylation - No evidence of potentially biologically reactive oxidized metabolites	- Mono/Di-acetylation - No evidence of potentially biologically reactive oxidized metabolites		
Summary	*No evidence of potentially biologically reactive oxidized metabolites*	*Mono/Di-acetylation, no evidence of potentially biologically reactive oxidized metabolites*	*Mono/Di-Acetylation following oral and dermal exposure*	*High likelihood for N-acetylation*

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References: Skare et al., 2009; SCCS 2012, 2013; Goebel et al 2014; Manwaring et al., 2015,

Example – ***non-animal skin and liver metabolism data for PTD (TDA) and PPD***

Comparison of *in vitro* and *in vivo* metabolism data.

1	2	3				4			5
Compound/ MW [Da]	Exposure/ metabolism pathway	Human keratinocytes (HaCaT)				Human hepatocytes			Human skin *ex vivo*
		In vitro culture[a]				*In vitro* culture[a]			Hair dye use exposure[b]
TDA	*Dose unit:*	*µg/ml*				*µg/ml*			*mg/cm²*
MW: 122.1	*Dose:*	*0.35*	*0.70*	*2.8*	*5.6*	*0.872*	*8.72*	*87.22*	*2.22*
	Pathway(s):								
	Mono–*N*–acetylation [%]	54	60	57	52	35	71	100	21
	Di–*N*–acetylation [%]	46	40	12	5	65	3	x	69
									Human skin model (EpiDerm)[e]
PPD	*Dose unit:*	*µg/ml*				*µg/ml*			*mg/cm²*
MW: 108.1	*Dose:*		*0.59*	*5.91*		*0.77*	*7.7*	*77.0*	*Dose equivalent to 3.5*
	Pathway(s):								
	Mono–*N*–acetylation [%]		11	15		x	40	3	10
	Di–*N*–acetylation [%]		89	11		100	16	x	47

a) in the medium after 24 h incubation; b) metabolite percentages in receptor fluid after 60 min skin exposure collected for 24 h; data taken from e) Hu et al. (2009);

Reference: Manwaring et al., 2015

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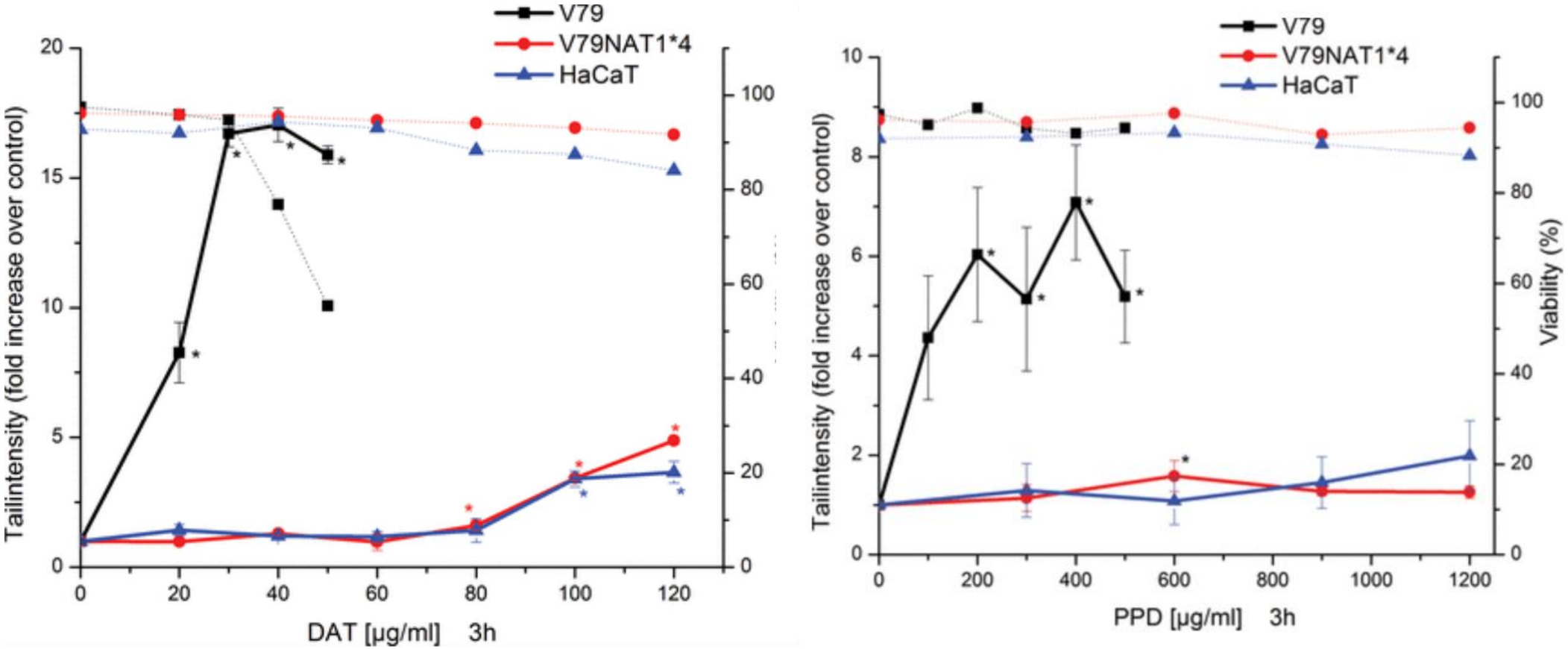
No relevant biological activity when *N*-acetylated:

DNA reactivity of PTD (DAT) and PPD in absence and presence of *N*-acetyltransferase activity in Comet assay



Comet assay with DAT in three different cell lines. Left hand ordinate: Solid lines represent primary DNA damage given as fold increase of % tail intensity over control. Average and standard deviations are shown. Right hand ordinate: dotted lines represent viability, measured via trypan blue dye exclusion, as % of control. Asterisk indicates different from control (P ≤ 0.05). Numerical values of % tail intensity available as supplementary material in the publisher's website.

Mutagenesis, Volume 29, Issue 1, 25 November 2013, Pages 37–48, https://doi.org/10.1093/mutage/get053

No relevant biological activity when *N*-acetylated:

DNA reactivity of PTD (DAT) and PPD in absence and presence of N-acetyltransferase activity

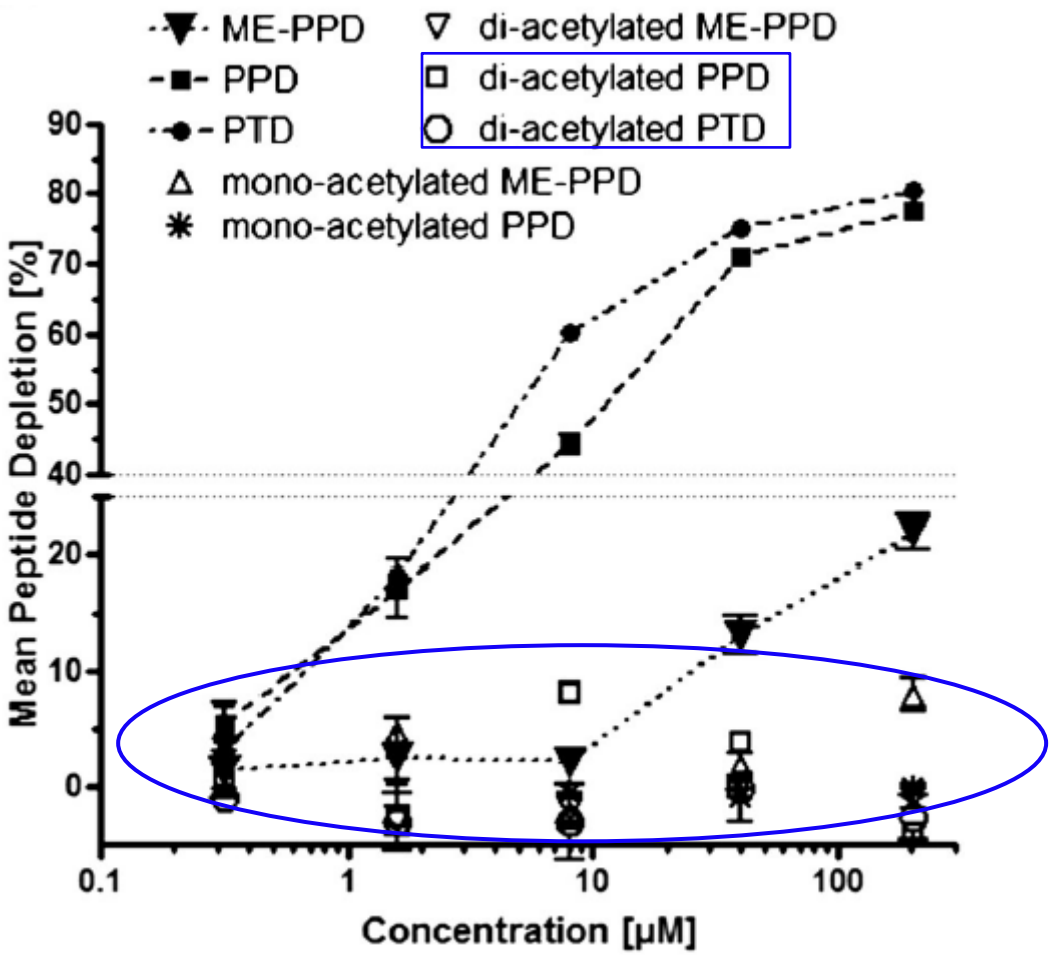
Table II. Overview of genotoxicity test results from our studies and those of Garrigue *et al.* (14)

	Ames	MN/CA	V79 Comet
DAT			
2-Mono-Ac-DAT			
5-Mono-Ac-DAT		NT	
Di-Ac-DAT			
PPD		*	
Mono-Ac-PPD		*	

Test substances are listed in the left column, test results under the respective column heads: green, negative result; red, positive result; NT, not tested.
*Data from Garrigue *et al.* (14).

No relevant biological activity when *N*-acetylated:

No relevant cysteine peptide reactivity of ME-PPD, PPD and PTD, when mono or di-acetylated.



Percent depletion of cysteine peptide monomer, expressed in terms of mean (±SD) of triplicate incubations.

Reference: Goebel et al., 2014

Similarity Assessment/Confidence

Assessment:

- Structure
- Chemical property (e.g. MW)
- Constituents (e.g. amino groups)
- Metabolism (N-acetylation)

- Mechanistic plausibility (Michael addition, toxicological findings)
- Other endpoints (Genetox, Cystein reactivity)
- Toxicokinetic parameters (more details in part 2)

Confidence/WoE:

- high, similar 2 D structures
- high, narrow range
- high, consistent across members

- high, consistent across members in skin and liver: *N-acetylation*
- high, *similar tox symptoms, similar cysteine reactivity,* PTD close to PPD regarding reactivity
- high, consistent results *in vitro*, PTD close to PPD regarding reactivity

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Part II

Reference: Manwaring et al., 2015

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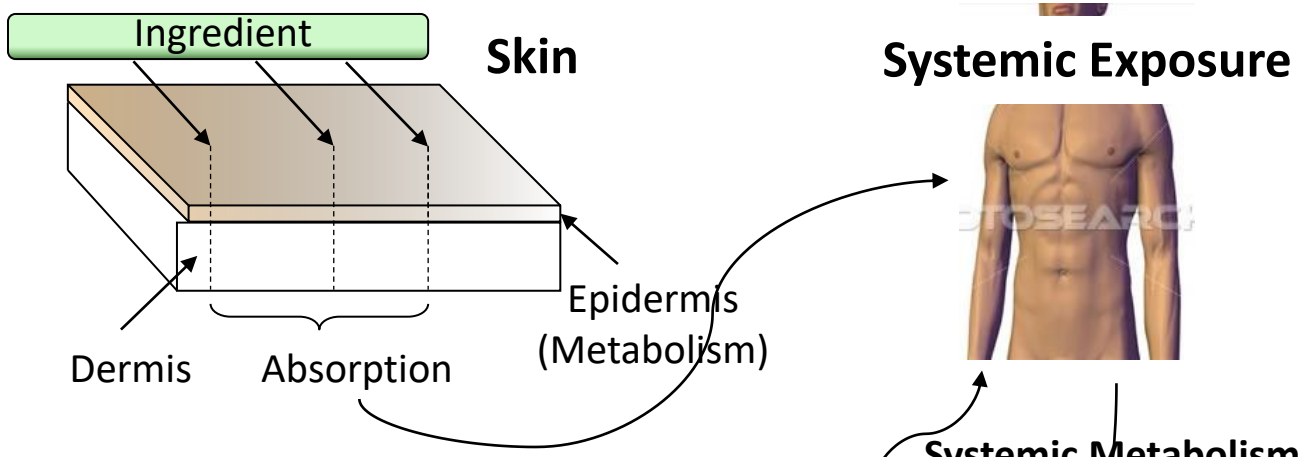
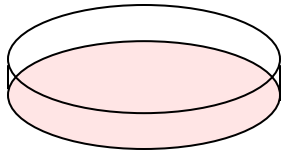
Non-animal approach for predicting Systemic Exposure

Example: hair dye use conditions



Penetration/
Survival through Skin
(skin 1st pass effect)

Methods: *in vitro in vitro metabolism*



keratinocyte cell line
(HaCaT)/
human hepatocytes

0 - 24 h

Detection of metablite(s)/parent by HPLC/MS

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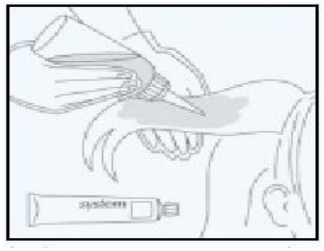
in vitro skin penetration

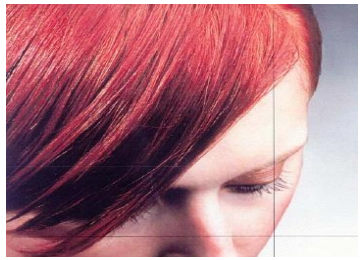
product

+



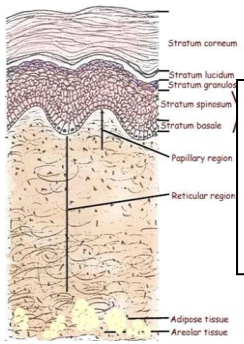
Application

30mins



Rinse/Dry

approx. 30 min



Removal with water/ shampoo

- Surface excess (rinsings)
- Stratum corneum (SC)
- Living skin
- **Receptor fluid (RF)**

Combination of *in vitro* data to predict to skin toxicokinetics

Keratinocytes	Skin penetration
▸ V_{max} – metabolism reaction velocities (converted to 'µg/min/cm²' using MW and cell density of viable epidermis of 6.02 x 10^6 cells/cm²)	▸ K_p (cm/hr) skin penetration constant for hair dye
▸ Thickness of viable epidermis H_{epid} = 4.88 µm	▸ Amt_{abs} (µg/cm²)= amount of dose absorbed through skin to receptor fluid (parent + metabolites)

- $K_{elm} = V_{max}/Amt_{abs}$
- **MRT** (mean residence time) = H_{epid}/K_p in viable epidermis
- *parent hair dye penetrating into blood* = $Amt_{abs} \times e^{(-Kelm \times MRT)}$

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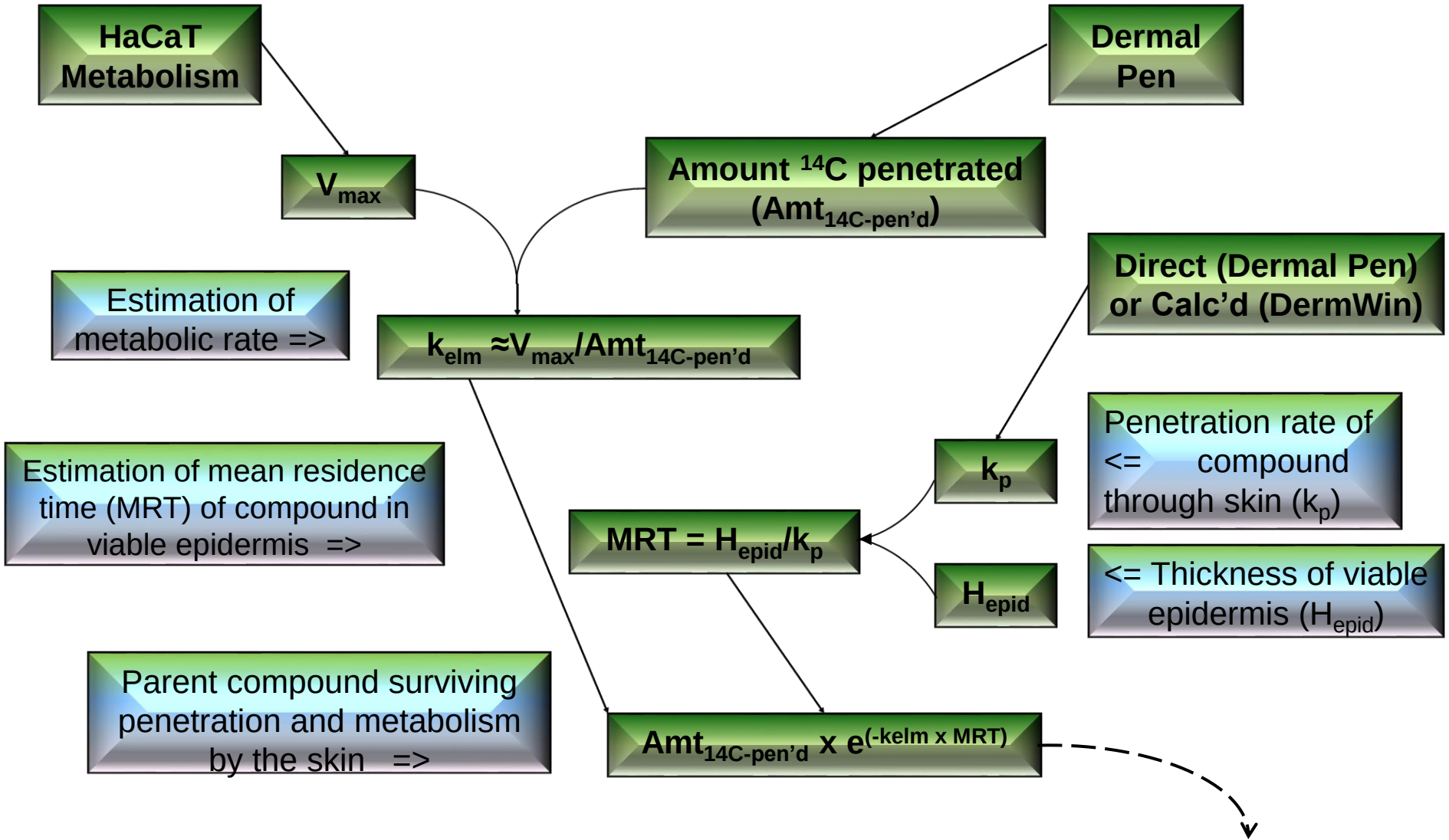
Penetration/Survival Through Skin



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Skin toxicokinetics

Hair dye	Vmax[1] (µg/min /cm²)	Exposure per hair dye event, after rinsing (µg eq/cm²)	Amt[2] absorbed (parent+ metab.) (µg eq /cm²)	k_{elm} V_{max}/ Amt pen'ted (hr^{-1})	Kp_{corr}[3] (cm/hr)	MRT (hr)	Calculated Amt of Parent surviving metabolism[4] (µg/cm²)	Measured Amt of Parent surviving metabolism[5] (µg/cm²)
PTD	0.0152	51.2	15.5	0.048	4.09E-04	11.93	8.72	1.61 (Factor 5 lower than calculated)
PPD	0.0052	36.7	5.5	0.056	2.45E-04	19.94	1.78	1.84

Foot notes:

1) Fitting of data to Michaelis–Menten curve using GraphPad Prism v4.00.
2) Amount of radioactive dose absorbed through skin to receptor fluid (parent + metabolites) from skin penetration and metabolism studies respectively.
3) Corrected value from DermWin v2.01 (EPA and SRC)
4) Rowland M, and Tozer TN, "Clinical Pharmacokinetics: Concepts and Applications", Lea & Febiger, Philadelphia, (1989), p. 66.
5) Accumulative amount of parent in receptor fluid measured after 24 h exposure period to *metbolically active living* human skin *ex vivo*

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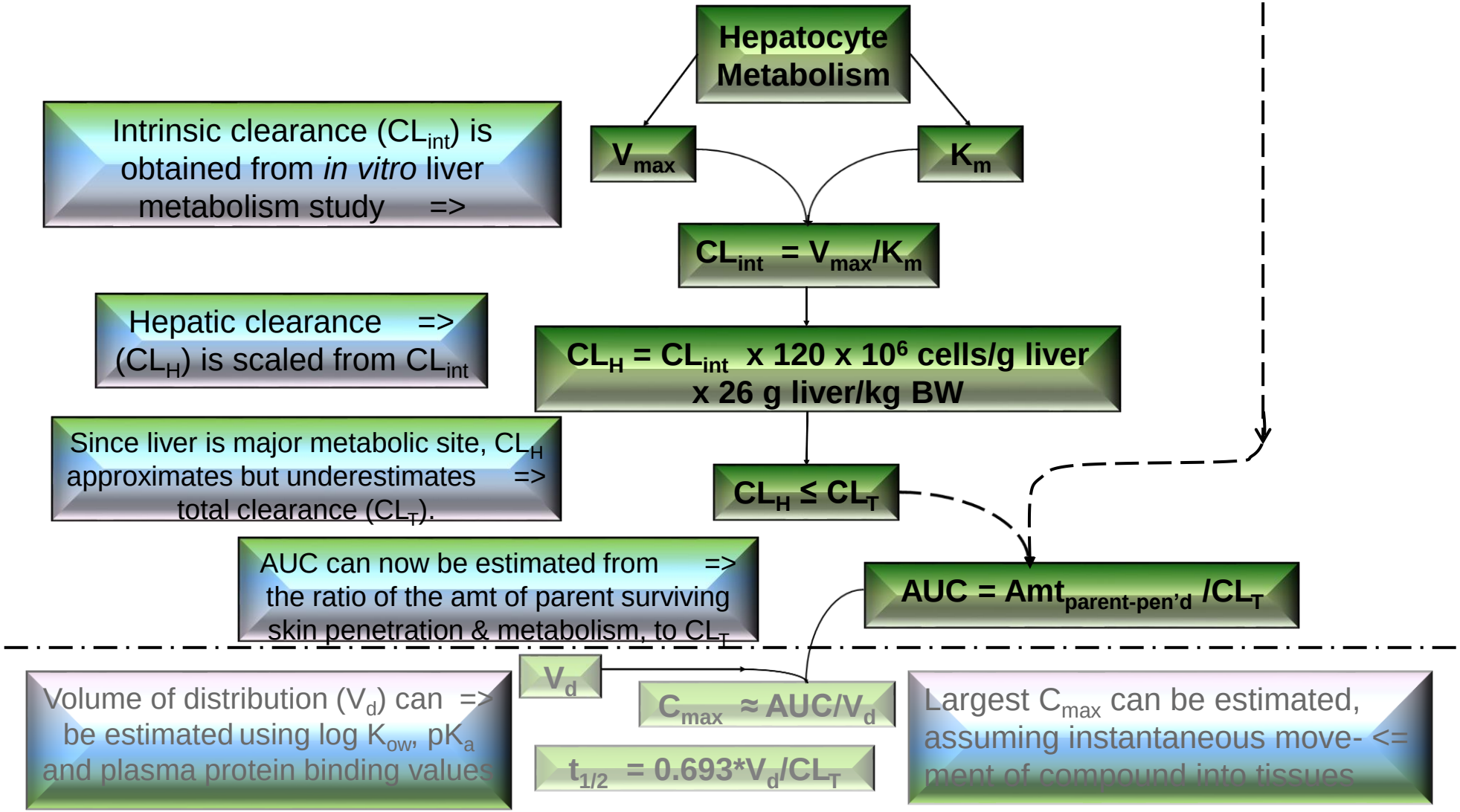
Systemic Exposure



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Liver toxicokinetics

Compound	V_{max} [hepatic] (nmol/min/10^6 cells)	K_m (µM)	Intrinsic clearance V_{max}/K_m (ml/min/10^6 cells)	**Hepatic Clearance** CL_H (ml/min/kg)	Total Amt Parent surviving skin metabolism[a] (µg)	Parent AUC[b] (µg·h/ml)
***N*-acetylation**						
PTD	65.52	60906	1.08×10^{-3}	1.23	5058	0.98
PPD	10.28	21252	4.84×10^{-4}	0.61	1035	0.41

[a]Calculated Amt of Parent surviving skin metabolism multiplied by average scalp application area of 580 cm^2,
[b]calculated for an average weight of 70 Kg

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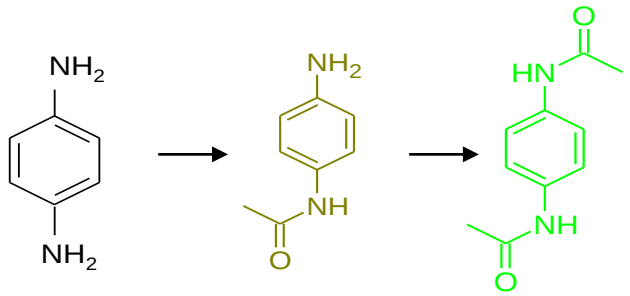
In vitro estimation/in vivo data comparison

PK Parameter	Estimated	Measured *In Vivo**	N-Acetyaltion
PPD AUC	0.41 µg-h/ml (parent)	1.4 µg eq-h/ml (^{14}C) *To be divided by 10 to account for metabolism*	**Parent present as < 10% of ^{14}C due to N-acetylation**
PPD C_{max}	0.027 µg/ml (parent)	0.13 µg eq/ml (^{14}C) *To be divided by 10 to account for metabolism*	
PTD AUC	0.98 µg-h/ml (parent)	1.2 µg eq-h/ml (^{14}C) *Metabolism to be considered*	**Same assumption as above**
PTD C_{max}	0.13 µg/ml (parent)	0.27 µg eq/ml (^{14}C) *Metabolism to be considered*	

*SCCS (Scientific Committee on Consumer Safety), 2012. Opinion on p-phenylenediamine, 26-27 June 2012, Opinion on toluene-2,5-diamine, 26-27 June 2012

Human metabolism data for PPD:



Urinary concentrations of PPD, N-monoacetyl- or N,N'-diacetyl-PPD determined by HPLC/MS/MS in study subjects after hair dyeing with a 1.0% [14C]-PPD-containing oxidative hair dye

SAMPLE	**PPD** **(ng/mL)** **Mean ± S.D.**	**N-monoacetyl-PPD** **(ng/mL)** **Mean ± S.D.**	**N,N'-diacetyl-PPD** (ng/mL) Mean ± S.D.	% of PPD metabolized
Pre-study (blank)	<1.28	<1.0	25.84 ± 9.86[a]	-
0 – 12 hours	6.91 ± 8.86[b]	3.50 ± 2.04[c]	2265 ± 1283	> 99
12 – 24 hours	<1.28	1.19 ± 0.06[d]	578.5 ± 348.8	> 99
24 – 48 hours	<1.28	<1.0	96.81 ± 83.86	> 99

[a]Calculated from 8/16 subjects, [b]Calculated from 9/16 subjects, [c]Calculated from 14 of 16 subjects, [d]Calculated from 2 of 16 subjects

Reference: Nohynek et al., 2015

Conclusion for systemic exposure prediction

For the aromatic amine hair dye molecules assessed, the non-animal approach based on *in vitro* assessment of metabolism and skin absorption

- provided reasonable, conservative estimations of *in vivo* systemic availability (AUC).
- is suitable for systemic exposure estimations
- supports the “read across” of systemic toxicity data

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Read across assessment considerations

- **Chem. properties**: in line for target and group
- **Metabolism**: N-acetylation as major pathway
- **Mode of action**: defined, Michael acceptor reactivity – closer to PPD than to ME-PPD
- **Toxicokinetics**: sufficiently similar for prediction, human data for PPD available
- **Available data**: high quality
- **Confidence**: overall high
- Read across of 90 day repeated dose tox NOAEL from group member (PPD) with similar reactivity to target (PTD) justified by overall Weight of Evidence:
  use 8 mg/kg bw/day

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Studies for evaluation:

- Chem. properties, see SCCS opinions
- All relevant *in vivo* data for group members (detailed study reports)

Detailed study reports for target and group members for comparative similarity assessment:

- Relevant *in silico* predictions on SAR/QSAR
- Reactivity studies, i.e. protein reactivity supporting mode of action
- Metabolism skin/liver studies
- Toxicokinetics assessment based on skin penetration and metabolism data
- Confidence assessment addressing similarity and differences regarding group members/target

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References (without SCCS opinions)

Part I

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Part II

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Data compilation including PTD in vivo repeated dose data

Chemical (INCI) (abbreviation)	*p*-phenylenediamine (PPD)	2-methoxymethyl-PPD (ME-PPD)	hydroxyethyl-PPD (HE-PPD) sulfate	toluene-2,5-diamine (PTD)
Reference	SCCS opinion 2012	SCCS opinion 2013	SCCS opinion 2010	SCCS opinion 2012
CAS# free base	106-50-3 624-18-0 (dihydrochlor.)	337906-36-2 337906-37-3	- 93841-25-9 (sulfate)	95-70-5 615-50-9 (sulfate)
MW [g/mol]	Free base 108.14 Dihydrochloride 181.07	Free base 152.20 Sulfate 250.28	Free base 152.20 Sulfate 250.28	Free base 122.17 Sulfate 220.25
Phys-Chem properties **Water solubility [g/l]** **Log Pow:**	 Free base ~10 Free base -0.31	 Free base 284 Free base:-0.65	 Sulfate 51.2 Sulfate 0.07	 Sulfate 5 Sulfate 0.74
OECD guideline	408	408 (sulfate)	408 (sulfate)	408 (sulfate)
Dosing [mg/kg bw/day]	0, 2, 4, 8 and 16	0, 10, 30 and 90	0, 25, 35 and 55	0, 2.5, 5, 10, and 20
Toxicity	At 16: liver and kidney weight increase, myodegenerative effects in two animals	At 90: marginally increased activity in liver enzymes (AST), increased liver weight/hepatocellular hypertrophy	At 55: increased activity in liver enzymes (AST, ALT)	At 10: Increase in AST levels, yodegenerative effects
NOAEL	8 (free base)	90 (sulfate)	35 (sulfate)	10 (sulfate)
Oral bioavailability	High (rats)	High (rats)	High (*in vitro AB permeability*)	69% (rats) High (*in vitro AB permeability)**
Cystein reactivity at 200 µM**	High (80% depletion)	Medium (25% depletion)	n.d.	High (80% depletion)

*References (other than SCCS opinions): *Obringer et al., 2015; **Goebel et al., 2014)*

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