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Methods to evaluate *in vitro* models of skin metabolism

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Agenda

- Physiology of the skin
- What is metabolism?
- Why does skin metabolism matter?
- Simple *in vitro* tools to assess skin metabolism
- Metabolic competence of 3D skin models
- Summary

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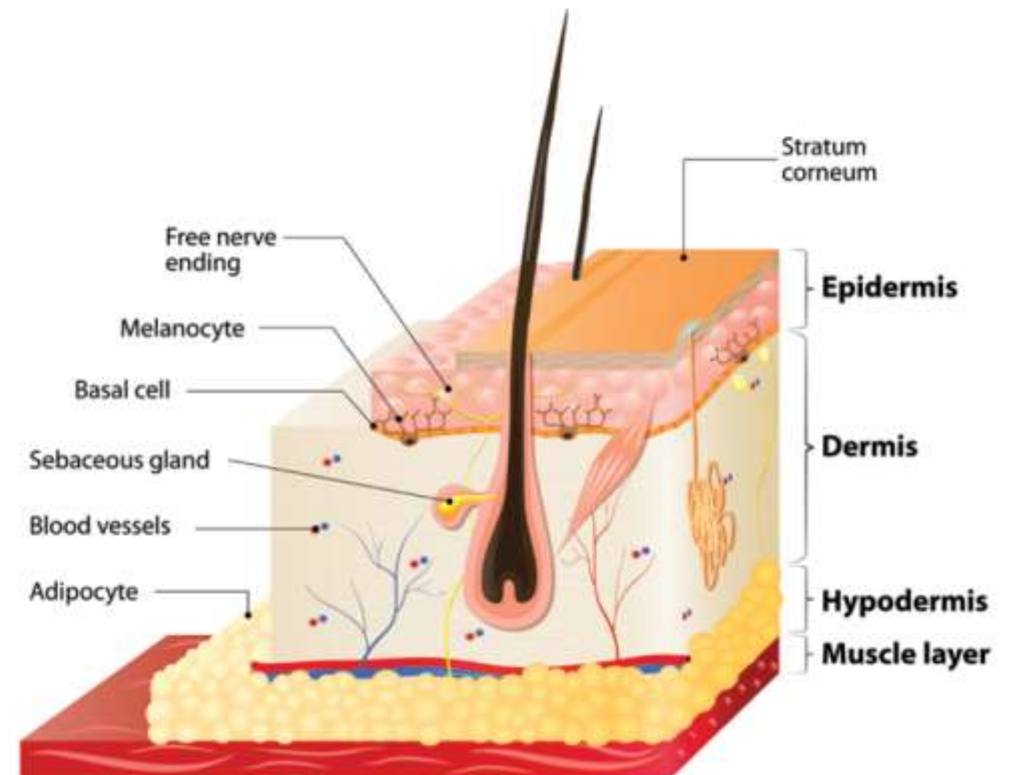
Physiology of the skin

- Largest organ in human body
- Three main functions:
  - Protection:
    - Sentinel role in protecting body from bacterial, fungal and viral pathogens
  - Regulation:
    - Critical in thermoregulation
  - Sensation:
    - Tactile sensitivity to immediate surroundings
- Made up of three main sublayers:
  - Epidermis
  - Dermis
  - Hypodermis



Svensson et al., 2009. DMD 37 (2), 247-253

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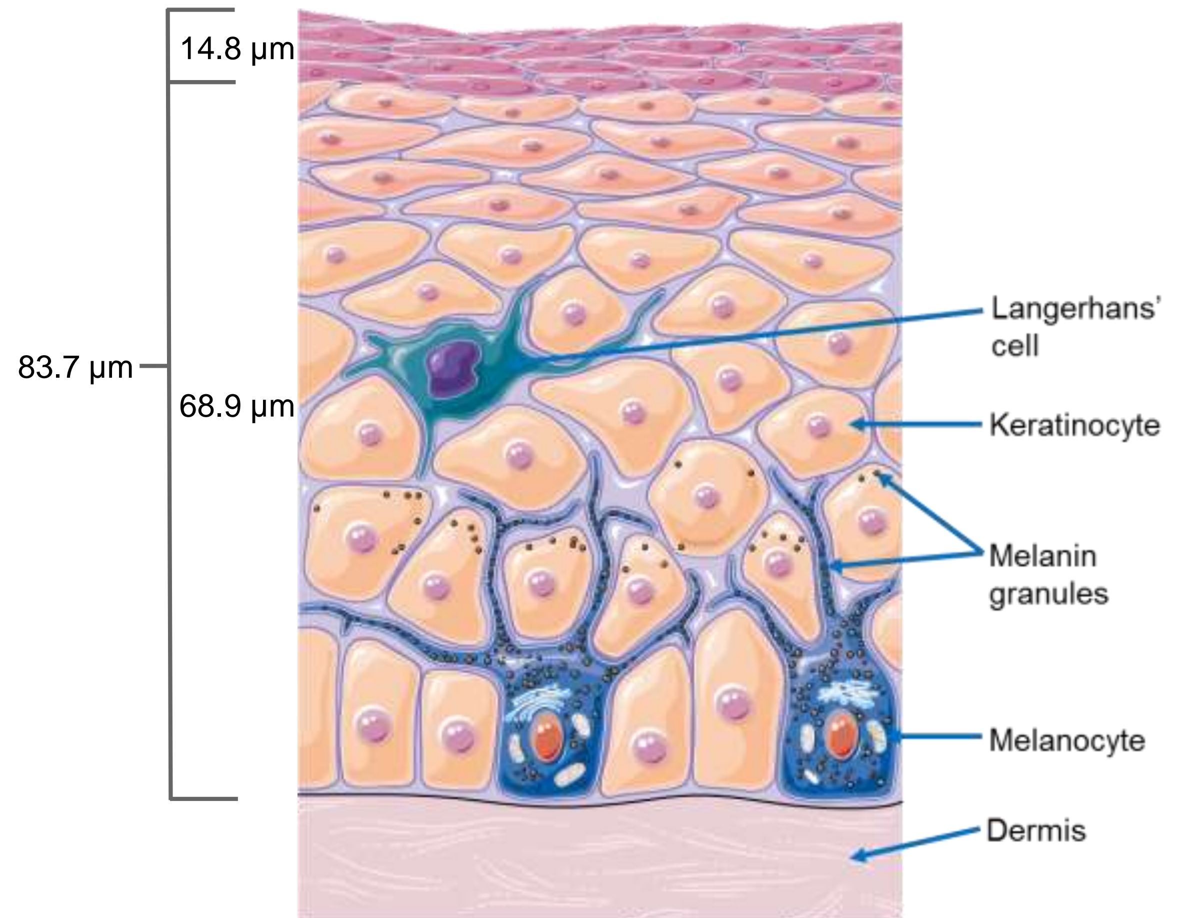
Physiology of the skin

Epidermis

Stratum corneum:
- Non-viable layer
- "Brick and mortar" structure

Viable epidermis:

- **Keratinocytes**
  - Make up 90-95% of cells in this layer of skin
  - Undergo programmed differentiation → stratification of cell phenotype
- **Langerhans cells**
  - Key role in skin immune response, acting as antigen-presenting cells
- **Melanocytes**
  - Melanin producing cells
    - Skin pigmentation
    - Protection from UV light
- **Merkel cells**
  - Mechanoreceptors
    - Density of these cells is highest in regions of skin that provide greatest tactile response e.g. fingertips



Svensson et al., 2009. DMD 37 (2), 247-253; Dumont et al., 2015. App In Vitro Tox 1(2), 147-164; Haake et al., 2000. The Biology of the Skin, 19-45; Servier Medical Art by Servier

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Physiology of the skin

Dermis, hypodermis and skin appendages

- **Dermis**
  - 300-4000 µm thick
  - Extensive vascular network, lymphatics, nervous system
  - Tensile strength that provides mechanical resistance
  - Primarily comprised of fibroblasts – secrete extracellular matrix that creates dense fibril network
  - Also contains mononuclear phagocytic system (dendrocytes, mast cells, macrophages)

- **Hypodermis**
  - Up to several millimetres thick
  - Also houses vascular network, lymphatics and nervous system
  - Composed primarily of adipocytes, also possesses apocrine and eccrine sweat glands
  - Subcutaneous fat tissue
  - Insulation, cushioning, energy supply, connects underlying structure

- **Skin appendages**
  - Set in dermis
  - Mainly hair follicles and sweat glands

Svensson et al., 2009. DMD 37 (2), 247-253; Dumont et al., 2015. App In Vitro Tox 1(2), 147-164

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What is metabolism?

- Biotranformation process for endogenous compounds and xenobiotics
- Increase excretion by increasing hydrophilicity
- Phase I:
  - Introduction of functional group
  - Oxidation, reduction, hydrolysis
  - Active metabolites
    - Increased efficacy/undesirable off-target effects
    - Pro-drugs
  - Reactive metabolites
    - Irreversible binding to macromolecules → toxicity
- Phase II:
  - Conjugation of functional group and endogenous substrate
  - Glucuronidation, sulphation, acetylation, methylation
  - Inactive metabolites → detoxification mechanism

Phase I	Phase II
Cytochrome P450 (CYP)	Uridine 5'-diphospho-glucuronyl transferase (UGT)
Flavin-containing mono-oxygenase (FMO)	Glutathione S-transferase (GST)
Monoamine oxidase (MAO)	N-acetyltransferase (NAT)
Alcohol/aldehyde dehydrogenase (ADH/ALDH)	Sulphotransferase (SULT)
Aldehyde oxidase (AO)	
Esterase (e.g. hCE)	
Amidase	
Epoxide hydrolase	
Reductase	

- Enzyme expression/function affected by:
  - Inhibition/induction (environment, drugs, diet)
  - Genetic polymorphisms
  - Age
  - Gender
  - Disease state

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Skin enzymology

Enzyme	Evidence of mRNA expression or enzyme activity (native skin)
Cytochrome P450 (CYP)	CYP1, 2B6, 3A mRNA CYP3A activity CYP1, CYP2B6 activity not detected
Flavin-containing mono-oxygenase (FMO)	FMO3, FMO5 mRNA only
Alcohol/aldehyde dehydrogenase (ADH/ALDH)	ALDH2, ADH1B*, ALD7A1 mRNA only
Epoxide hydrolase	EH1* mRNA only
Cyclooxygenase (COX)	COX-2 mRNA + activity
Glutathione S-transferase (GST)	GST Pi, omega – mRNA + activity GST alpha, theta – mRNA only
N-acetyltransferase (NAT)	NAT10 – activity only NAT1 –mRNA only
Sulphotransferase (SULT)	SULT2B1 – mRNA only
Uridine 5'-diphospho-glucuronyl transferase (UGT)	UGTs 1A mRNA + activity UGT2B mRNA not detected

Based on Hewitt et al., 2013
* Dermis only

See also: Oesch et al., 2014, Manevski et al., 2015

Hewitt et al., 2013. Toxicol Sci 133(2), 209-217; Oesch et al., 2014. Arch Toxicol 88 (12), 2135-2190; Manevski et al., 2015. DMD 43(1), 126-139

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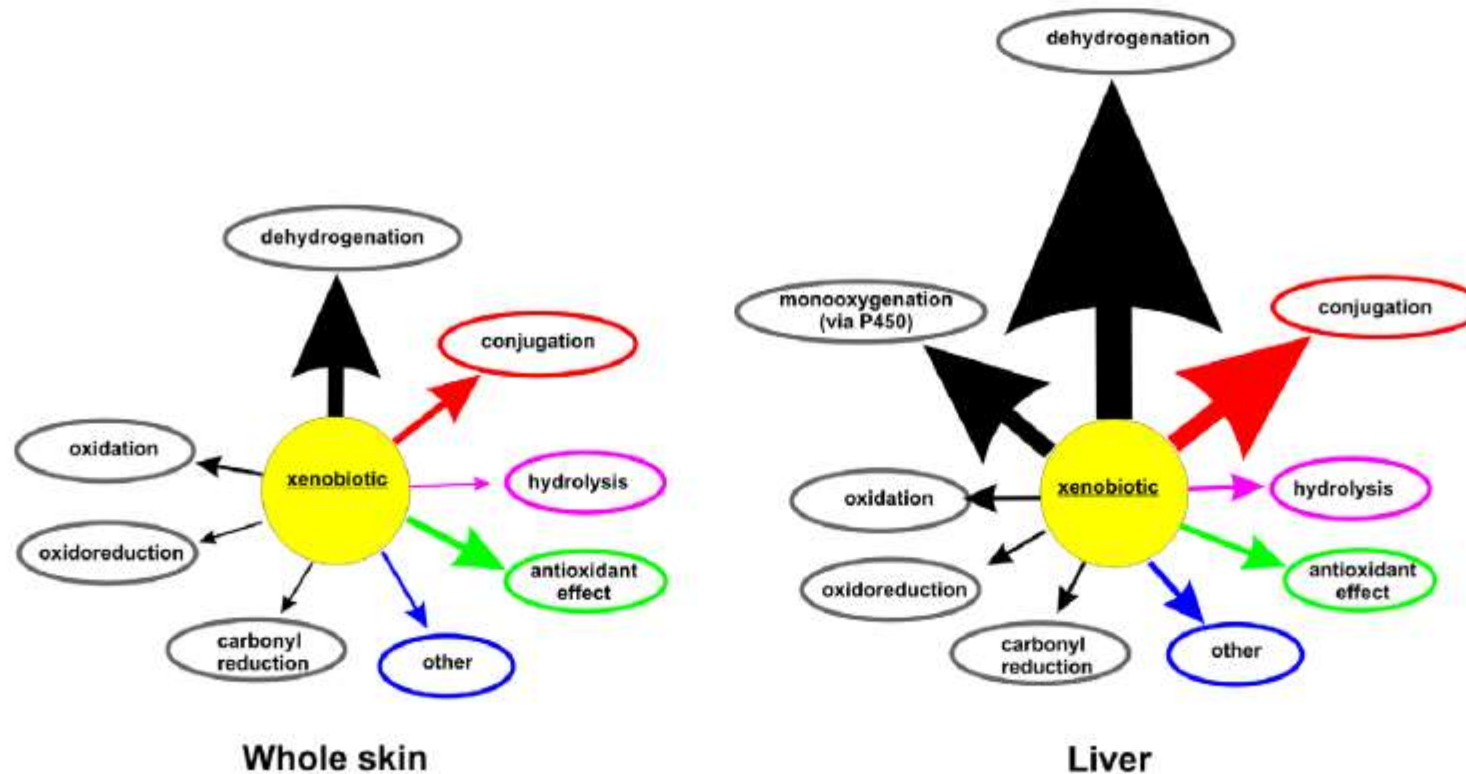
Skin enzymology



- Potential routes of xenobiotic metabolism in skin and liver
- Size of each arrow is proportional to the number of XMEs detected that may catalyse each bioconversion indicated
- Reproduced from van Eijl et al., 2012

Van Eijl et al., 2012. PLoS One 7(7), e41721

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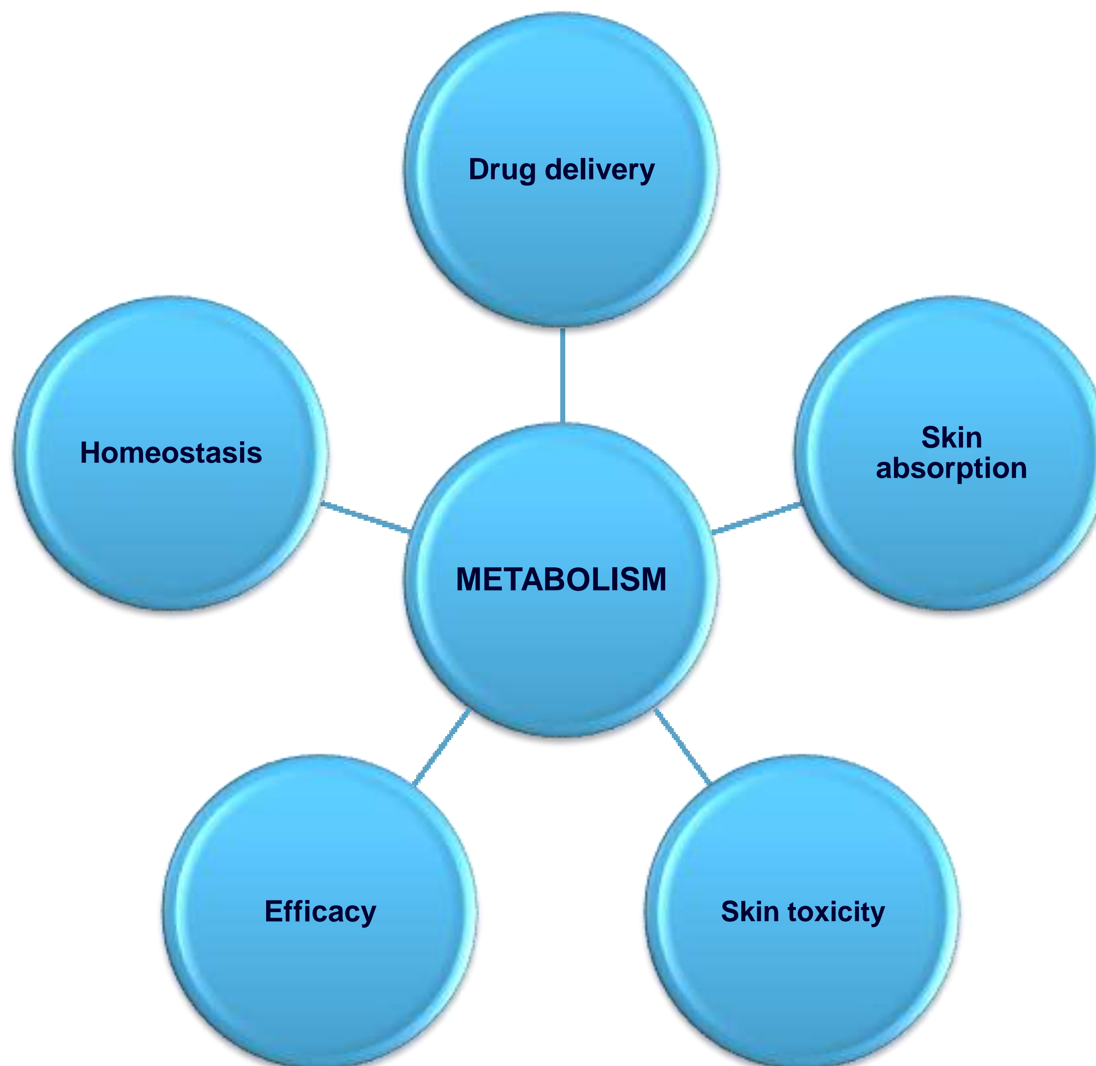
Why does skin metabolism matter?



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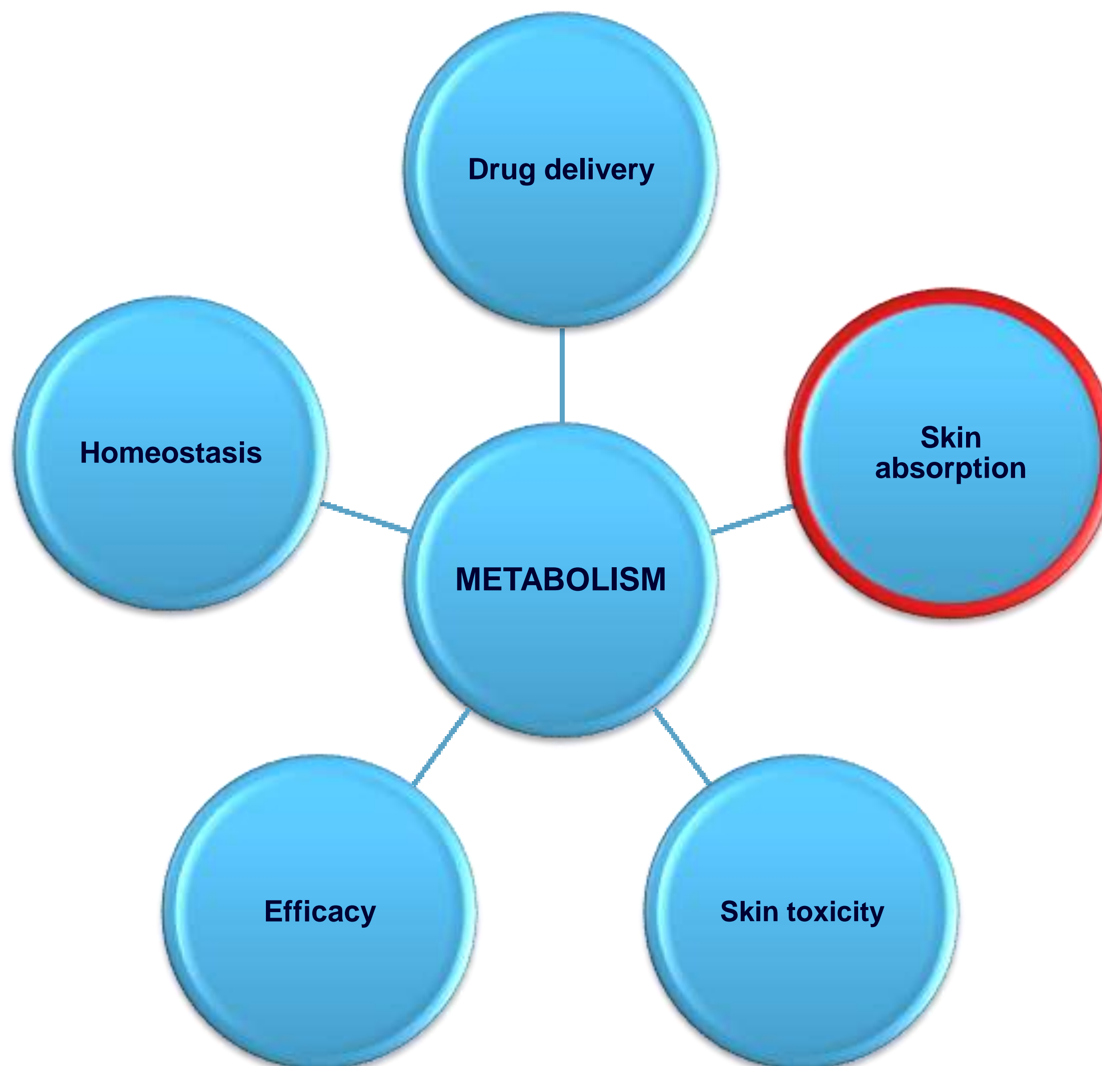
Why does skin metabolism matter?



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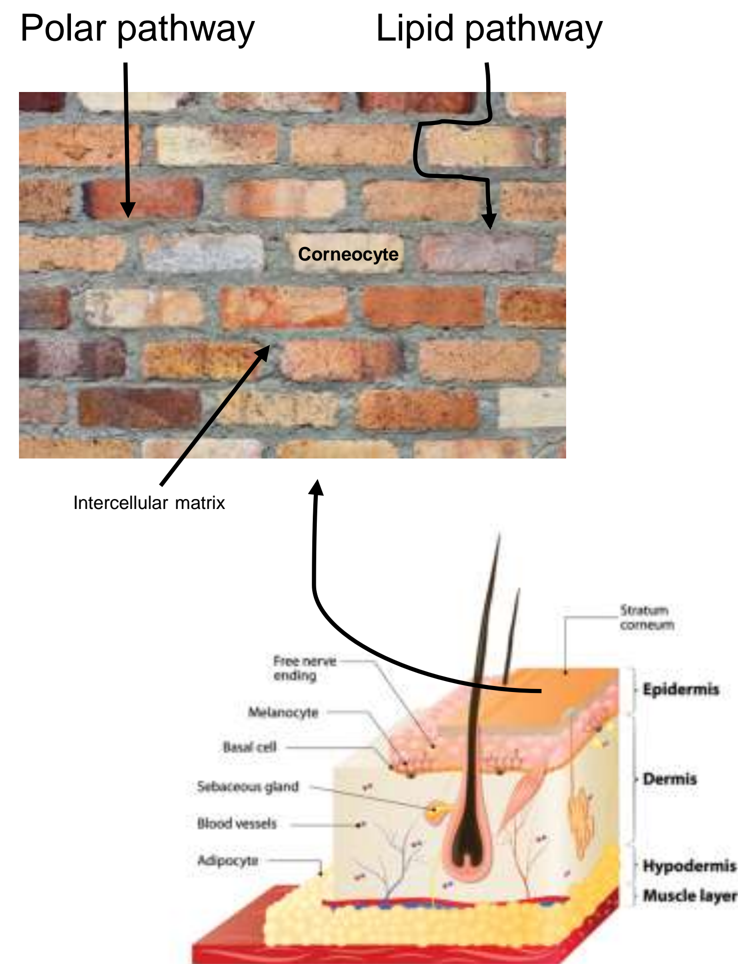
Role of metabolism in skin absorption

- Dermal absorption occurs predominantly by passive diffusion – influenced by size and lipophilicity of xenobiotic

Epidermis:

- Stratum corneum is a lipophilic barrier. Many highly hydrophilic xenobiotics will not pass this first barrier
- Skin penetration occurs in 3 main ways:
  - **“Polar pathway”** – through corneocytes by partitioning into and out of the cell membrane.
    Hydrophilic xenobiotics pass mainly via this transcellular route
  - **“Lipid pathway”** – transfer around corneocytes in lipid-rich extracellular region.
    Lipophilic xenobiotics pass mainly via this intracellular route
  - **Transappendageal (shunt) pathway** – through the sweat glands, sebaceous glands and hair follicles



Svensson et al., 2009. DMD 37 (2), 247-253; Dumont et al., 2015. App In Vitro Tox 1(2), 147-164

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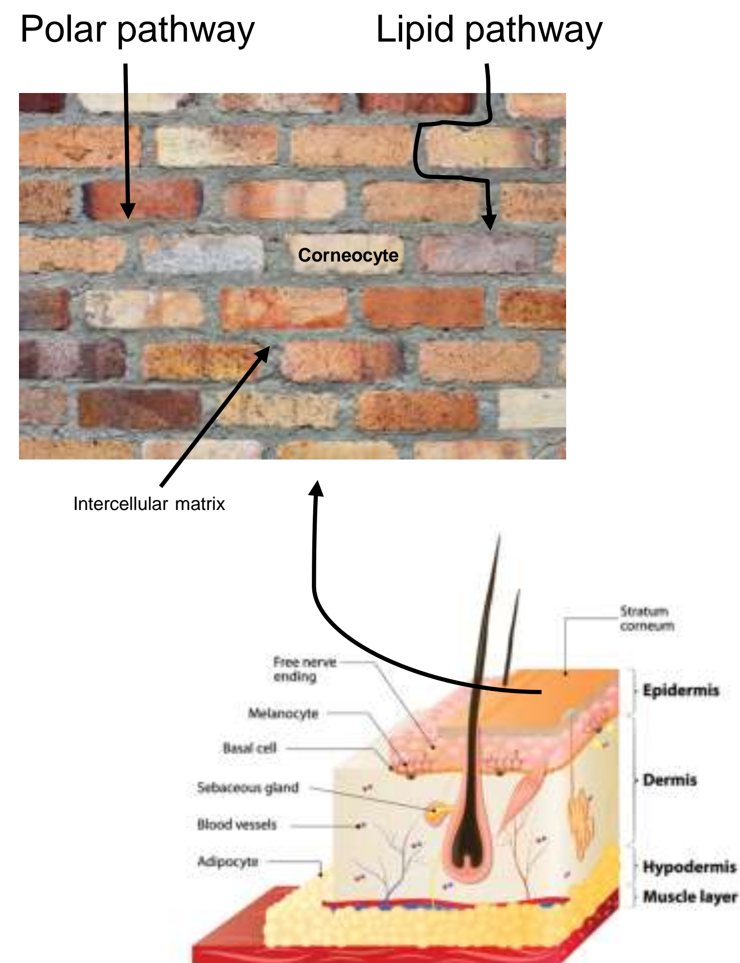
Role of metabolism in skin absorption

Dermis:

- Mainly aqueous environment – high percentage of water means a more effective barrier against lipophilic chemicals
- Contributes significantly to transport and distribution. Presence of blood vessels and the lymphatic and nervous systems means may reach systemic circulation



Substance may remain in the skin and eventually released over time – impacts on bioavailability

Stratum corneum, viable epidermis, dermis and hair follicles all capable of “reservoir effect”

Dependent on desquamation for emptying of reservoir

Svensson et al., 2009. DMD 37 (2), 247-253; Dumont et al., 2015. App In Vitro Tox 1(2), 147-164

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Role of metabolism in skin absorption

- Metabolism may impact on rate of absorption by changing physicochemical properties
- Dermal absorption of lipophilic xenobiotics may increase if metabolised to more polar, water-soluble structures
- Biotransformation may reduce systemic availability of parent or increase availability of metabolite
- Metabolism is key component in determining local and systemic concentrations of both parent and metabolites

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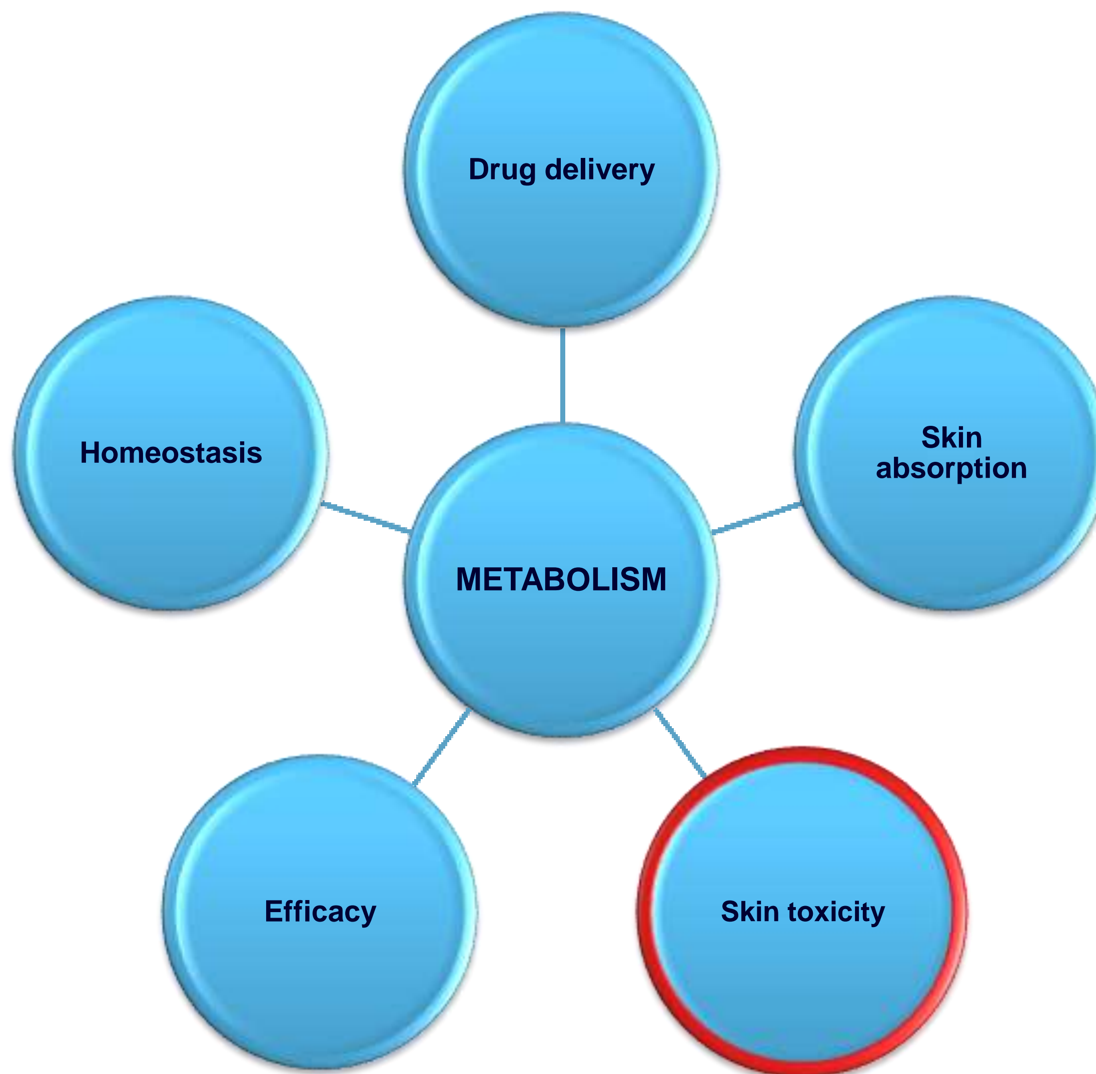
Why does skin metabolism matter?



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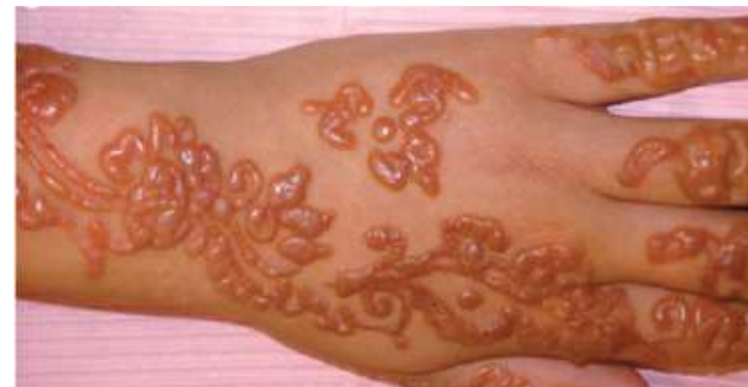
Role of metabolism in skin toxicity

Skin sensitisation

- ‘A substance that will induce an allergic response following skin contact’
- Allergic contact dermatitis (ACD) is most common manifestation of immunotoxicity in humans
- Adverse skin condition developed on repeated contact to chemical allergens
- Approximately 20% of adults in the general population are allergic to one or more skin sensitiser
- Skin sensitisation is the toxicological endpoint associated with chemicals capable of causing ACD
- ACD is an adaptive immune response and symptoms also known as allergic reactions are driven by specific T cells



- Symptoms include:
  - Rash/skin lesions/blisters/redness of the skin
  - Oozing/draining/crusting of the skin
  - Itchiness, inflammation, desquamation, localised swelling

Evans and Fleming, 2008. N Engl J Med 7, 627; Global harmonized system of classification and labelling of chemicals (GHS), 2013. Fifth edition; Frosch et al., 2006. Contact dermatitis. 4th Edition; Nielsen et al., 2002. Br J Dermatol 147, 487-492

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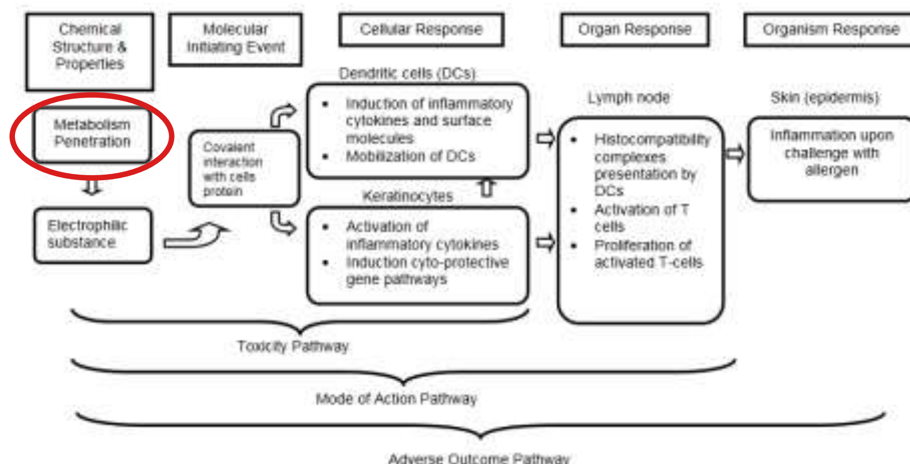
Role of metabolism in skin toxicity

Skin sensitisation

- Chemical sensitisers that can cause ACD are known as haptens, pre-haptens or pro-haptens

Haptens	Pre-haptens	Pro-haptens
Require no transformation to become electrophilic	Undergo auto-activation outside of skin → Limonene, PPD	Undergo bioactivation within the skin → Eugenol

- May react with nucleophilic amino acid residues in proteins within the skin. Resulting conjugates are fully immunogenic
- 20% of known skin allergens would not react with proteins without previous metabolic activation (bioactivation)



Adverse Outcome Pathway for the manifestation of skin sensitisation

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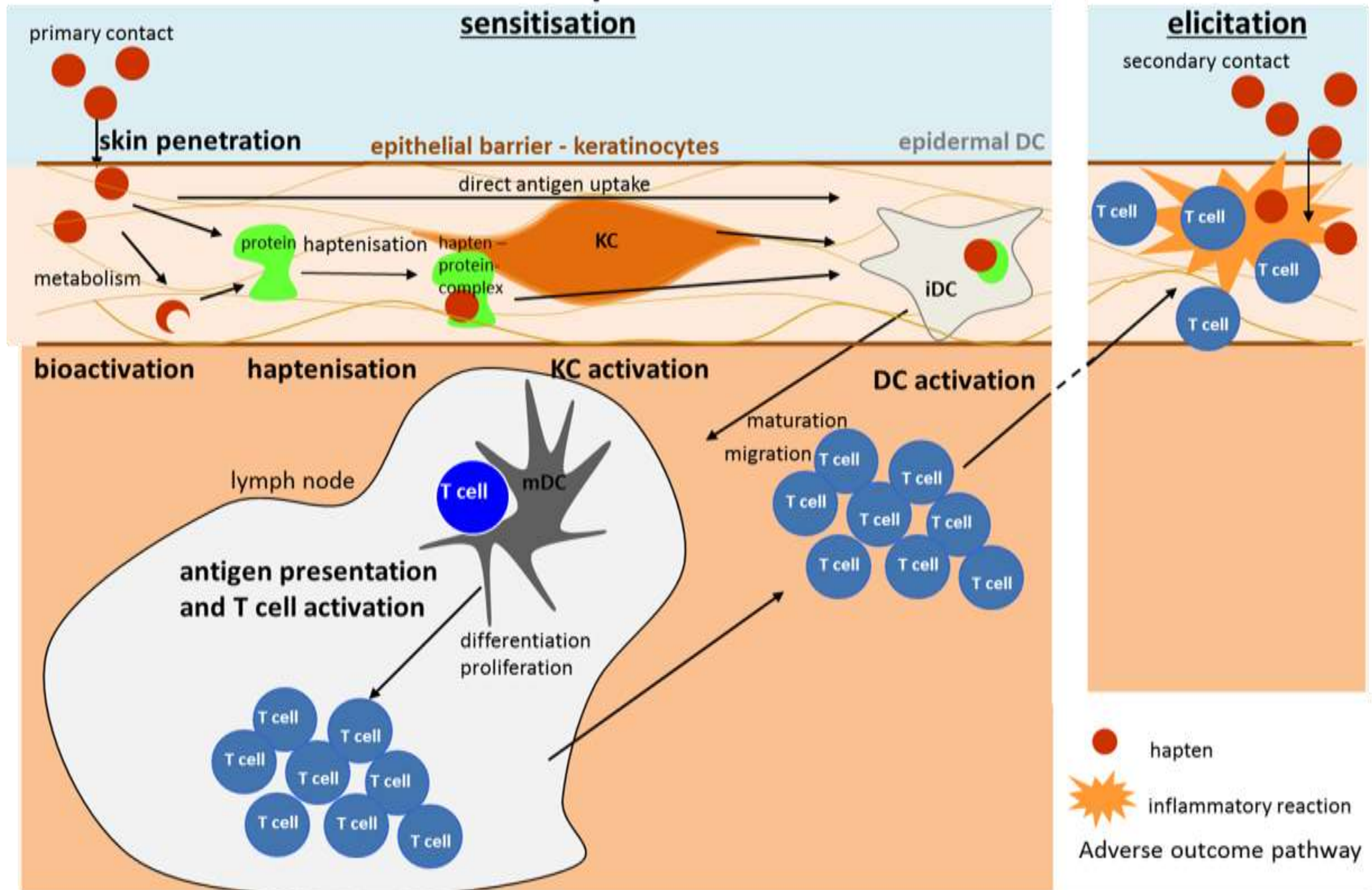
Role of metabolism in skin toxicity

Cellular process of ACD



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Role of metabolism in skin toxicity

Potential for cutaneous bioactivation to contribute to skin toxicity

Additionally, generation of genotoxins, ROS → carcinogenesis

Enzymatic activity will influence predictivity of skin irritation when irritant is metabolically produced/detoxified

Role of skin in detoxification

- Detoxification of potentially toxic metabolites → defence against potentially harmful chemicals
- Enhanced absorption may reduce toxicity

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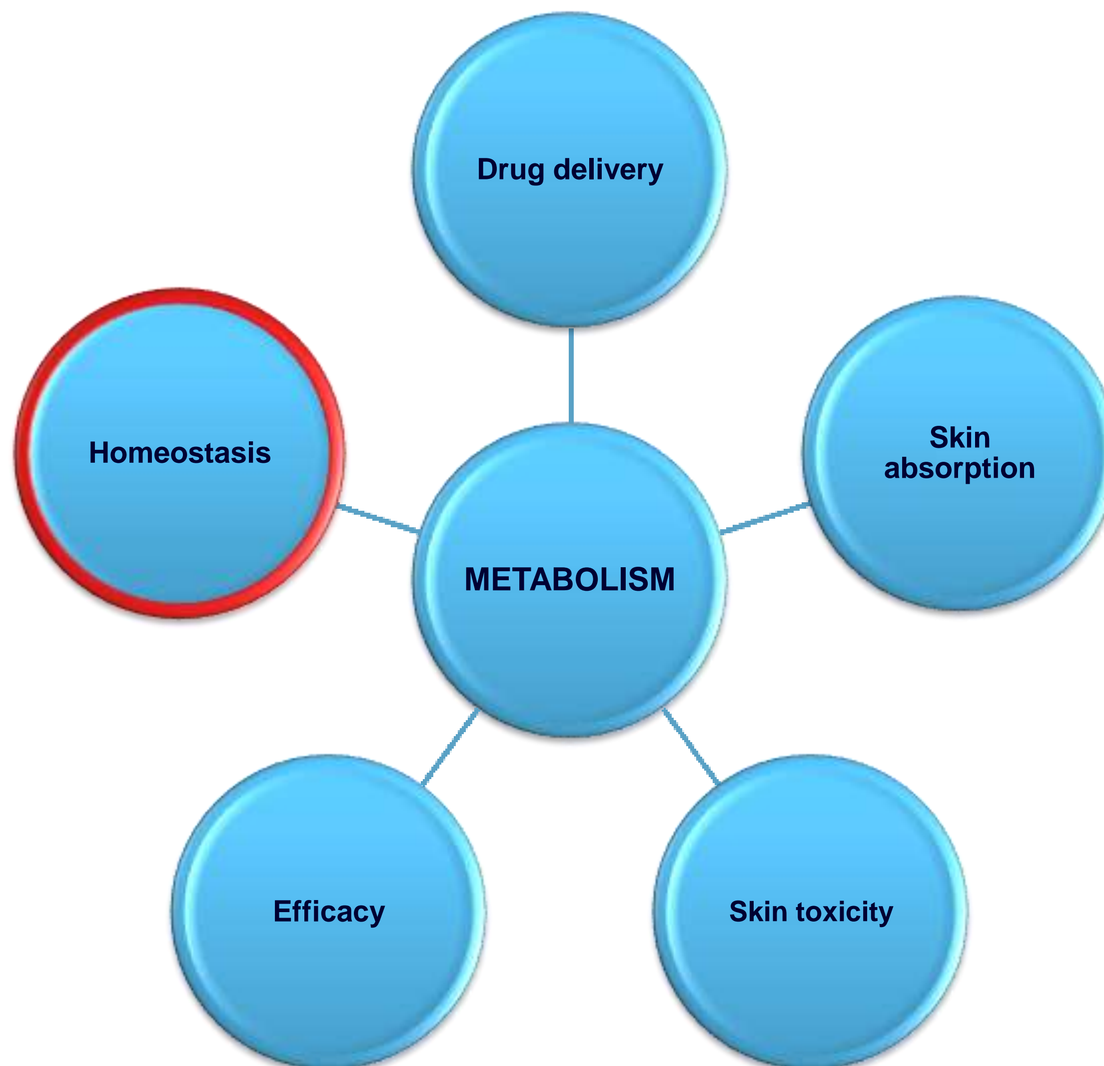
Why does skin metabolism matter?



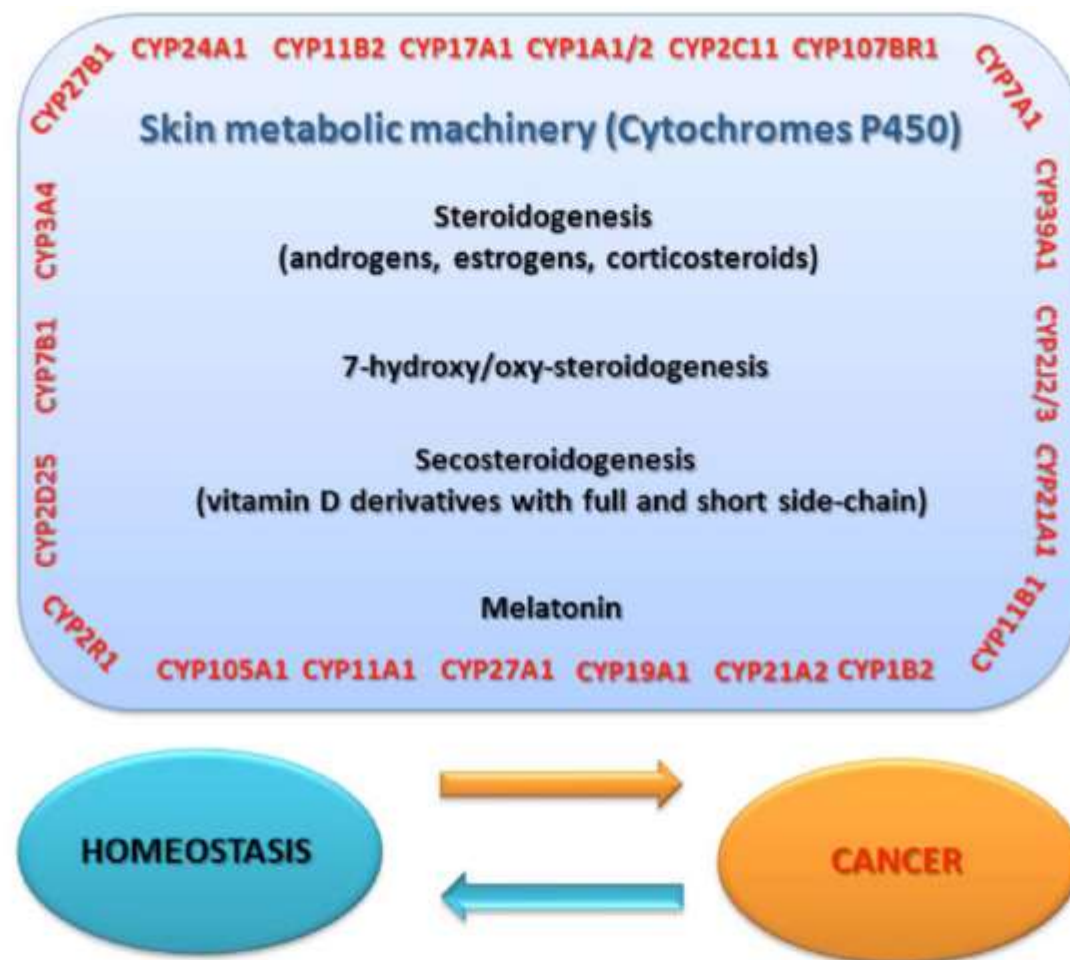
Skin metabolism and cancer

- Skin is important barrier to environment
- Exposed, acutely and chronically, to variety of factors including ultraviolet radiation (UVR), topically applied drugs and cosmetics, environmental pollutants
- In addition to xenobiotic metabolism, CYP enzymes also involved in key metabolic pathways regulating the activities of sex hormones, corticosteroids, secosteroids and melatonin

Loss of proper CYP function by genetic and epigenetic factors

Destabilisation of internal homeostasis

Cancer progression



Slominski et al., 2014. Anticancer Agents Med Chem 14(1), 77-96

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In vitro tools to assess skin metabolism

Aims

- Evaluate simple *in vitro* systems to assess skin metabolism
  - Can this provide a quick assessment of bioactivation potential or metabolic lability?
  - Differences between hepatic and cutaneous metabolism
- Initial focus on characterising metabolic capability utilising archetypal enzyme-specific substrates
- Comparison of skin S9 and HaCaT cells with liver S9
  - Skin S9
    - Supernatant fraction obtained from skin homogenate by centrifuging at 9000g. Contains cytosol and microsomes
    - Pool of 3 donors
  - HaCaT cells
    - Immortalised human keratinocyte cell line
    - Commonly used tool in dermal toxicology

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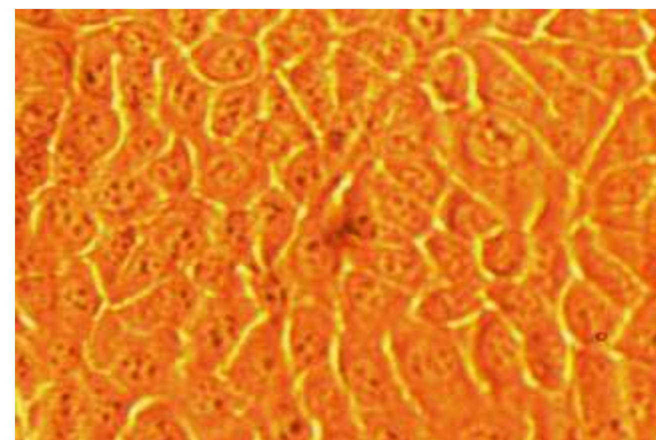
In vitro tools to assess skin metabolism

Methods

- 10 compounds identified as known substrates for specific phase I and II enzymes
- **Liver and skin S9 fraction**
  - 2h time course; 1mg/mL
  - Number of different co-factor conditions
    - NADPH only; UDPGA only; NADPH, UDPGA, acetyl CoA and GSH combined; Minus co-factor
- **HaCaT cell line**
  - Immortalised keratinocyte cell line
  - 24h time course
- LC-MS/MS methods used to monitor substrate depletion and metabolite formation
- Metabolites also identified using a metabolite identification approach utilising Xevo GS-2 Q-Tof UPLC MS/MS platform



HaCaT cells plated in a monolayer culture

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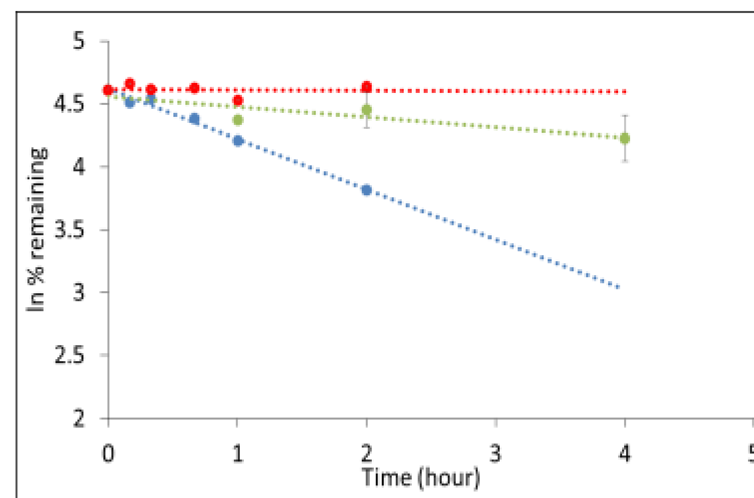
In vitro tools to assess skin metabolism

Substrate depletion approach

		$T_{1/2}$ (h)		
Enzyme	**Probe substrate**	**Liver S9**	**HaCaT**	**Skin S9**
CYP3A	Testosterone	0.697	> 8hr	> 8hr
CYP1A	Ethoxycoumarin	0.602	> 8hr	> 8hr
FMO	Benzydamine	3.11	> 8hr	> 8hr
AO	Phthalazine	0.237	> 8hr	> 8hr
hCE	Irinotecan	> 8hr	> 8hr	> 8hr
hCE	Procaine	2.41	1.65	2.36
UGT	7-Hydroxycoumarin	0.286	5.98	> 8hr
NAT	4-Aminobenzoic acid (PABA)	> 8hr	> 8hr	> 8hr
UGT/GST	2-Mercaptobenzothiazole (MBT)	2.41	> 8hr	> 8hr
GST	Aflatoxin B1	2.99	> 8hr	> 8hr



Benzydamine – HaCaT turnover limited to 4 hrs – toxicity observed.

● = Liver S9 ● = HaCaT ● = Skin S9

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In vitro tools to assess skin metabolism

Metabolite formation – Phase I metabolism

		Oxidation			Reduction			Desaturation			Hydrolysis		
Enzyme	**Probe substrate**	**LS9**	**HaCaT**	**SS9**	**LS9**	**HaCaT**	**SS9**	**LS9**	**HaCaT**	**SS9**	**LS9**	**HaCaT**	**SS9**
CYP3A	Testosterone	+	-	-	-	+	-	+	+	-	-	-	-
CYP1A	Ethoxycoumarin	+	-	-	-	-	-	-	-	-	-	-	-
FMO	Benzydamine	+	+	+	-	-	-	-	-	-	-	-	-
AO	Phthalazine	+	-	+	-	-	-	-	-	-	-	-	-
hCE	Irinotecan	-	-	-	-	-	-	-	-	-	+	+	+
UGT	7-Hydroxycoumarin	-	-	-	-	-	-	-	-	-	-	-	-
NAT	4-Aminobenzoic acid (PABA)	-	-	-	-	-	-	-	-	-	-	-	-
UGT/GST	2-Mercaptobenzothiazole (MBT)	+	-	-	-	-	-	-	-	-	-	-	-
GST	Aflatoxin B1	+	-	-	-	-	+	-	-	-	-	-	-

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In vitro tools to assess skin metabolism

Metabolite formation – Phase II metabolism

		Glucuronide conjugation			Acetylation			Glutathione conjugation		
Enzyme	**Probe substrate**	**LS9**	**HaCaT**	**SS9**	**LS9**	**HaCaT**	**SS9**	**LS9**	**HaCaT**	**SS9**
CYP3A	Testosterone	+	-	-	-	-	-	-	-	-
CYP1A	Ethoxycoumarin	+	-	-	-	-	-	+	-	-
FMO	Benzydamine	-	-	-	-	-	-	-	-	-
AO	Phthalazine	-	-	-	-	-	-	-	-	-
hCE	Irinotecan	-	-	-	-	-	-	-	-	-
UGT	7-Hydroxycoumarin	+	+	-	-	-	-	-	-	-
NAT	4-Aminobenzoic acid (PABA)	-	-	-	+	+	+	-	-	-
UGT/GST	2-Mercaptobenzothiazole (MBT)	+	+	-	-	-	-	+	-	-
GST	Aflatoxin B1	-	-	-	-	-	-	+	-	-

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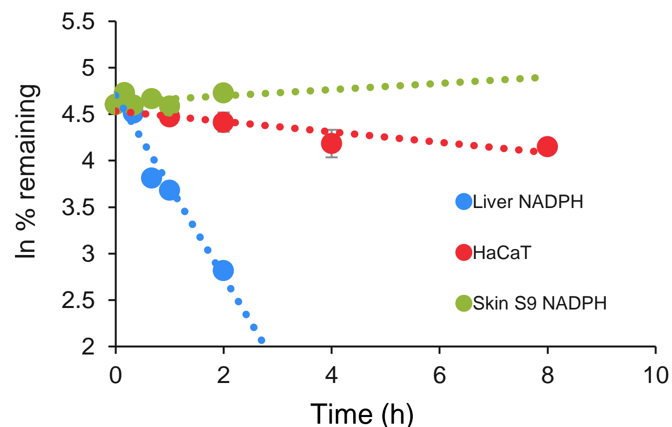
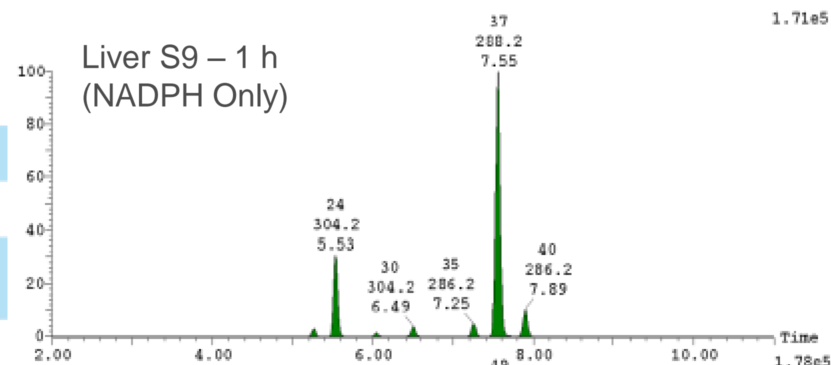
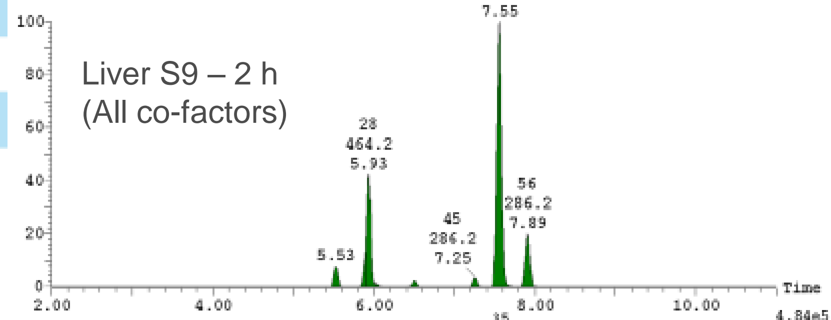
In vitro tools to assess skin metabolism

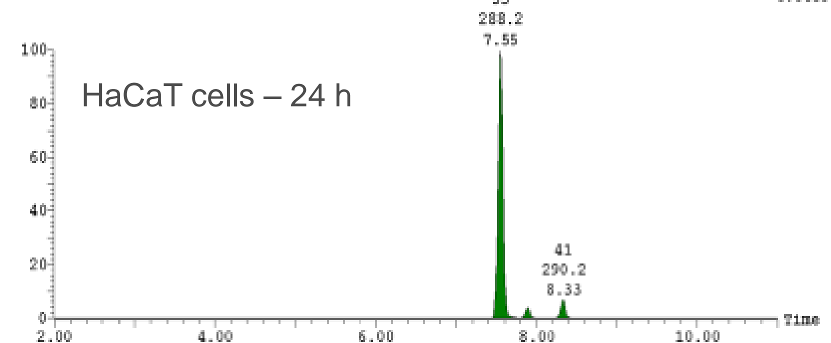
Testosterone metabolism in HaCaT cells

Metabolite Label	Retention Time	Metabolic Pathway	HaCaT cells	Liver S9
24	5.53	Oxidation	-	++
	5.89	Oxidation	-	+
28	5.93	Glucuronide Conjugation	-	++ (All co-factors present)
30	6.49	Oxidation	-	+
35, 45	7.25	Desaturation	-	+
35, 37, 49	7.55	Parent	N/A	N/A
40, 56	7.89	Desaturation	+	++
41	8.32	Reduction	++	-



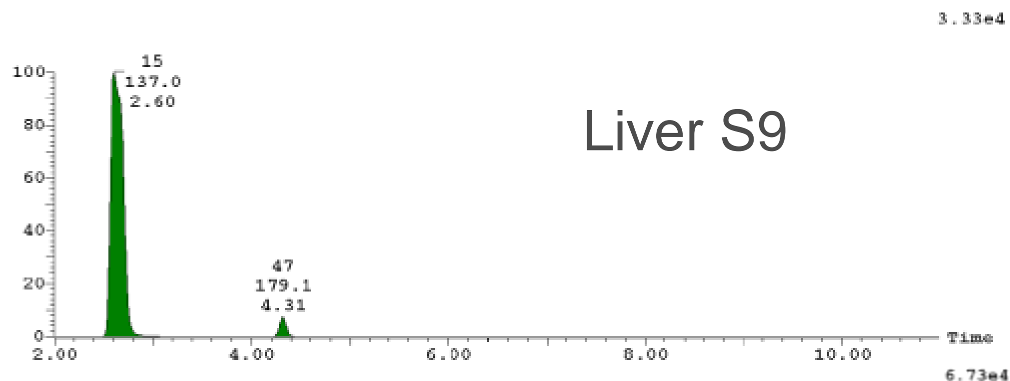


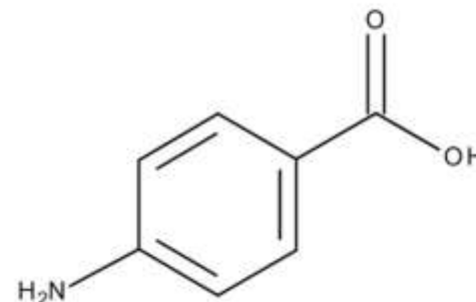


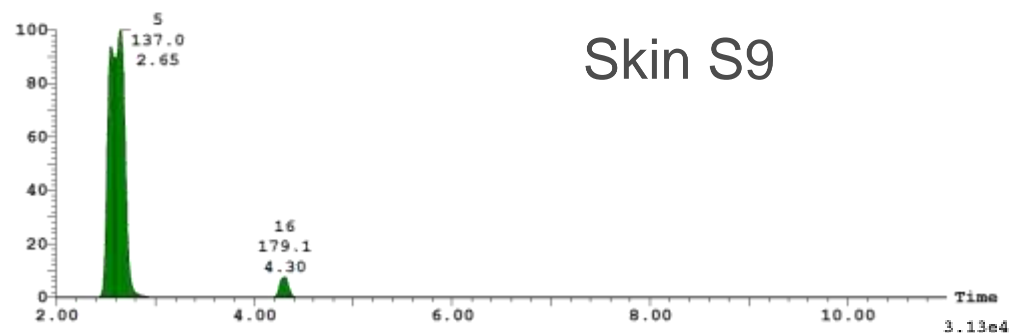


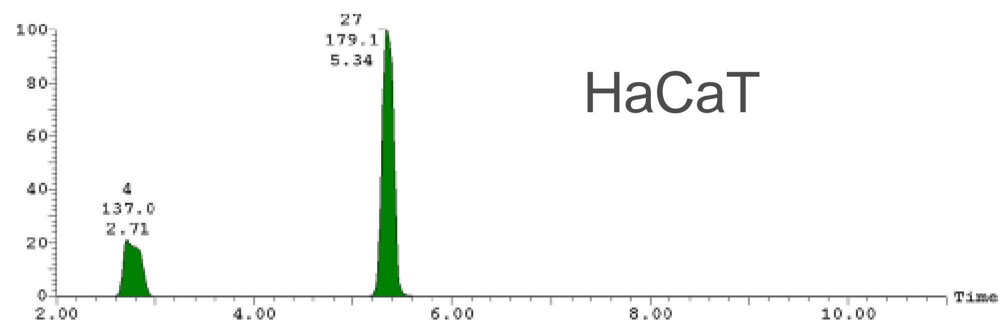
In vitro tools to assess skin metabolism

N-acetylation of PABA in skin









- N-acetylation observed in all systems with PABA
- Higher formation of 4-acetaminobenzoic acid was observed in HaCaT (77% of parent) compared to liver S9 (4% of parent)

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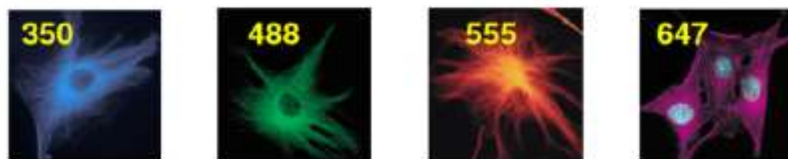
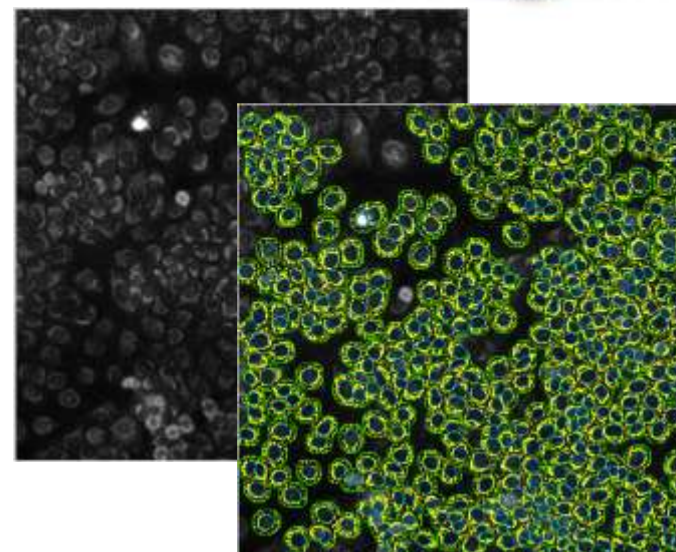
Alternative approaches to identifying evidence of bioactivation

High Content Screening (HCS) Technology for Cellular Toxicity Assessment

- Thermo ArrayScan VTI and XTI
- Automated fluorescence imaging and cellular analysis





Multi-parametric indicators of cell toxicity:

Mitochondrial potential

Glutathione (GSH) content

DNA damage

ATP content

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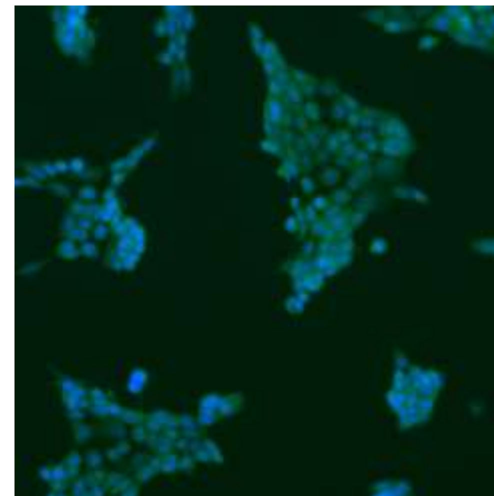
Alternative approaches to identifying evidence of bioactivation

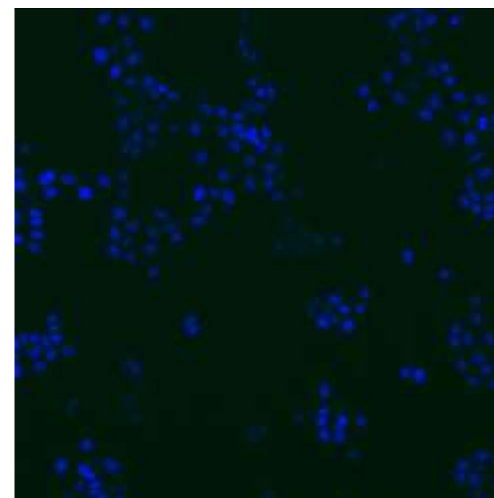
GSH content

- In GSH content assay (part of HCS approach) cells are treated with test compound for 4 and 24 h and cellular GSH is stained with monochlorobimane
- Assay utilises the thiol probe monochlorobimane (freely passes through membrane). Unbound probe shows very little fluorescence – but when bound to GSH it forms a strongly fluorescent adduct
- Allows for the identification of cellular GSH levels

Vehicle control 4 h



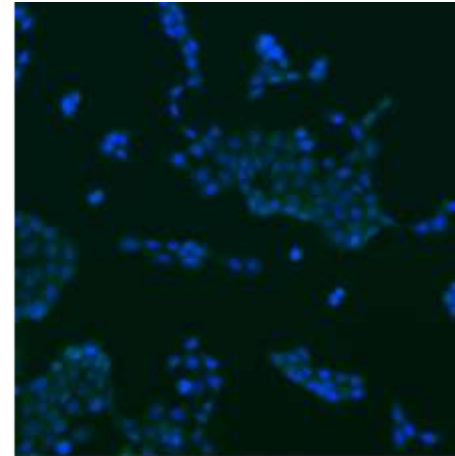
DNCB-treated 4 h



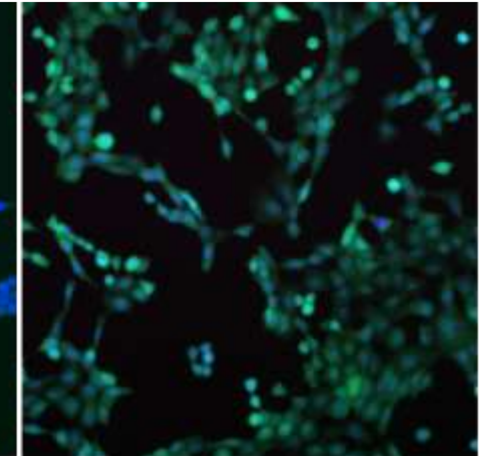
Alternative approaches to identifying evidence of bioactivation

GSH content

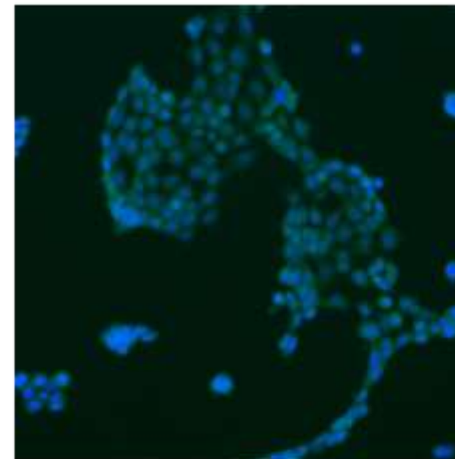
- No evidence of GSH conjugation in metabolic studies in HaCaT cells after 24 h
- Assessment of GSH content in HaCaT cells showed:
  - After 4 h - decreased levels of GSH at high concentrations
  - After 24 h – increased levels of GSH at lower exposure concentrations



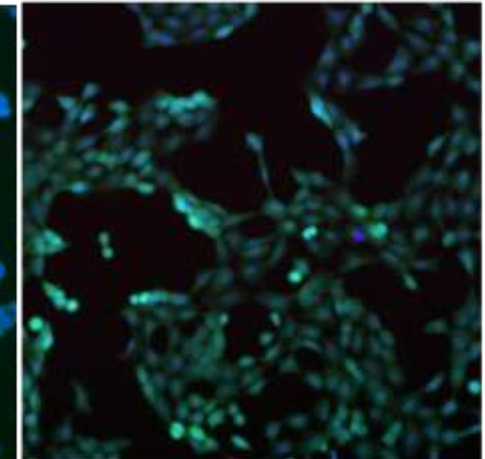
2-MBT 4 hr 2000 μM



2-MBT 24 hr 200 μM



Vehicle Control 4 hr



Vehicle Control 24 hr

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In vitro tools to assess skin metabolism

Summary

- Metabolic competence of the skin differs vastly from the hepatic system
- Overall HaCaT cells showed a higher metabolic capacity over skin S9 derived from epidermis and dermis. However, aldehyde oxidase was shown to have higher activity in skin S9
- FMO and esterase activity evident in all three systems
- Higher formation of the N-acetylated metabolite of PABA, was observed in HaCaTs, and the glucuronidation of multiple substrates was also observed in HaCaTs
- Specific reduction of testosterone to form dihydrotestosterone observed in HaCaTs
  - Suggested to be via 5α-reductase
  - Suggests potential steroid metabolic pathway in skin, that would otherwise undergo differential metabolism in liver

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Alternatives to 2D skin systems

- 2D systems not suitable for assessing lipophilic compounds which, due to lack of water solubility, can not be easily tested in monolayer cell cultures
- Human skin explants considered gold standard
  - Retains barrier properties
  - Challenges, ethically and practically, with sourcing and availability, and variability in quality
  - Non-viable skin (e.g. cadaver) not suitable for assessing skin metabolism
- 3D models developed to mirror in vivo situation for safety assessment of dermally applied compound regarding skin sensitisation, genotoxicity, irritation and corrosion
- Morphologically and functionality similar to native human skin
- Reconstructed human epidermis (RHE) models
  - Consist of stratum corneum and epidermis
  - Includes EpiSkin™, SkinEthic™, EpiDerm™
- Full thickness (FT) models
  - Consist of stratum corneum, epidermis and dermis
  - Includes T-Skin™, EpiDermFT™, Phenion®FT

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Metabolic competence of 3D skin models

- Broad assessment of enzyme expression and activity in 3D skin models
- Understanding of potential impact of metabolism in these systems

Use of Human *In Vitro* Skin Models for Accurate and Ethical Risk Assessment: Metabolic Considerations

Nicola J. Hewitt,*,[1] Robert J. Edwards,† Ellen Fritsche,‡ Carsten Goebel,§ Pierre Aeby,¶ Julia Scheel,‖ Kerstin Reisinger,‖ Gladys Ouédraogo,‖‖ Daniel Duche,‖‖ Joan Eilstein,‖‖ Alain Latil,‖‖‖ Julia Kenny,# Claire Moore,** Jochen Kuehnl,†† Joao Barroso,‡‡ Rolf Fautz,§§ and Stefan Pfuhler¶¶

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REVIEW ARTICLE

Review of the Availability of *In Vitro* and *In Silico* Methods for Assessing Dermal Bioavailability

Coralie Dumont, Pilar Prieto, David Asturiol, and Andrew Worth

Dermal Xenobiotic Metabolism: A Comparison between Native Human Skin, Four in vitro Skin Test Systems and a Liver System

Christian Wiegand[a] Nicola J. Hewitt[b] Hans F. Merk[c] Kerstin Reisinger[a]

[a]Henkel AG & Co. KGaA, Düsseldorf, [b]Erzhausen, and [c]Department of Dermatology, University Hospital, RWTH Aachen, Aachen, Germany

Xenobiotic-metabolizing enzymes in the skin of rat, mouse, pig, guinea pig, man, and in human skin models

F. Oesch · E. Fabian · K. Guth · R. Landsiedel

Hewitt 2013

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Overview of skin metabolism models

- Enzyme activities in skin are very low compared to liver
- Direct comparison of skin models is difficult
  - Difficulty in accurately measuring low enzyme activity
  - Comparison of different models in different labs
  - Unlikely, in all cases, that linearity with respect to time and protein has been ascertained
  - LOD/LOQ not defined/determined/reported
  - Unable to compare functional activity, simply confirm presence of activity
- Relevant morphology and functionality important
- FT models closer to human skin than RHE models
- Native human skin more complex than 3D skin models
- Absorption vs metabolism in culture
  - Does not reflect *in vivo* conditions
  - Reabsorption from medium

Dumont et al., 2015. App In Vitro Tox 1(2), 147-164; Hewitt et al., 2013. Toxicol Sci 133(2), 209-217

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Summary

- Increased appreciation for role of skin metabolism
- Ongoing challenges:
  - Dose
  - Low basal CYP activity
  - Analytical challenge
  - Enzymatic stability
  - Lack of in vivo data for comparison
  - Differential expression throughout epidermis and dermis
  - Potential impact of enzyme induction/inhibition
  - Standardised approach to assessing skin metabolism

Combination of skin metabolism, bioavailability, absorption and safety predictions for an integrated approach to safety assessment of cosmetic ingredients

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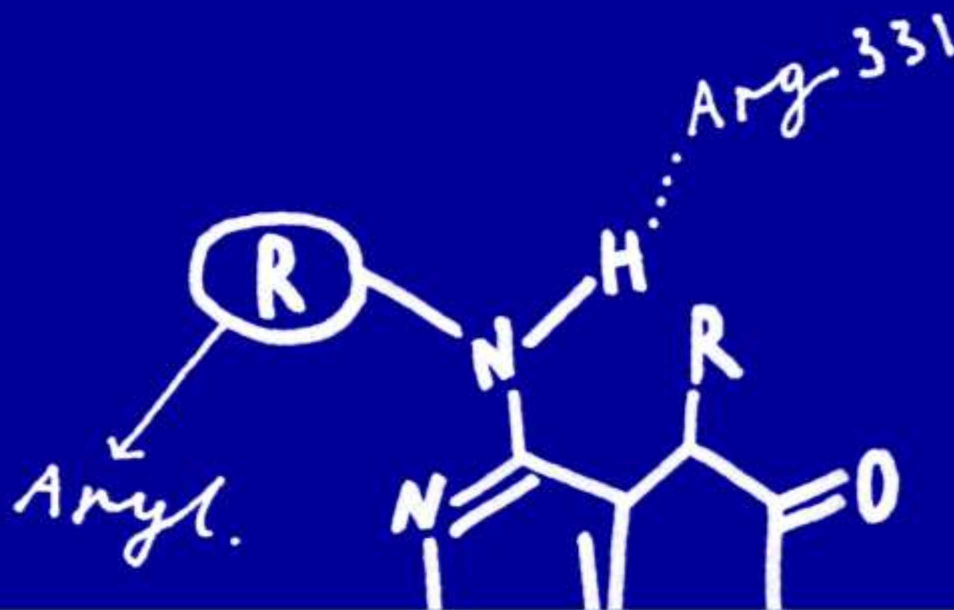

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Methods to evaluate *in vitro* models of skin metabolism

Cyprotex, in-cosmetics Global, April 2017