Prohapten activation and subsequent interaction with proteins: Mechanistic understanding and quantitative follow up...

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Metabolic studies

Liver metabolism...

Skin metabolism...
Identification of metabolites

Bio-distribution

Phase I metabolism

Phase II metabolism

GST/GSH

O-conjugate

Excretion

Toxicity

protein

SG
Metabolism and xenobiotics

Bio-distribution

Phase I metabolism

GST/GSH

Phase II metabolism

O-conjugate

Protein

Toxicity

Excretion
Two major pitfalls

Liver vs skin...

Highly reactive intermediates...
Non invasive approach...

- Direct observation...
- Highly non homogeneous environment...
HRMAS NMR...

- High-Resolution Magic Angle Spinning “HRMAS” Nuclear Magnetic Resonance...
- Bring to zero inhomogeneity associated with the sample...
HRMAS NMR...
Is it possible to follow the metabolism of chemicals in Reconstructed Human Epidermis?
Follow-up of the RIFM project...

Activation of cinnamyl alcohol...

Hydrolysis of iso/eugenyl acetates...
The cinnamyl alcohol story...

- Cinnamyl alcohol has been considered for many years as “the” model of pro-hapten, being activated by alcohol dehydrogenase to form the reactive cinnamaldehyde...

- However, about half of the patients sensitized to cinnamyl alcohol do not react when patch-tested with cinnamaldehyde...

- This suggests that at least one alternative metabolic pathway is taking place in Human epidermis, activating cinnamyl alcohol into at least another unknown reactive intermediate...
The cinnamyl alcohol story...

- The aim of this initial phase was to assess the potential of the HRMAS NMR / RHE model to investigate the metabolism of cinnamyl alcohol in a living 3D tissue...
- Cinnamyl alcohol was synthesized carbon-13 substituted either at position 1, 2 or 3...
The cinnamyl alcohol story...

1) LDA, -78°C
2) (EtO)₂P(=O)Cl

1) DIBAL-H, -78°C
2) NH₄Cl

46%

93%
The cinnamyl alcohol story...

1) LDA, -78°C
2) (EtO)₂P(O)Cl

1) DIBAL-H, -78°C
2) NH₄Cl

100% yield

46%
93%
The cinnamyl alcohol story...

- The behavior of cinnamyl alcohol and cinnamaldehyde in RHE was first assessed...

- Cinnamyl alcohol and cinnamaldehyde, substituted at position 1, were thus applied on RHE and post-incubated for 1, 8 and 24 h, respectively...

- Samples were processed and analyzed by HRMAS NMR to assess potential oxidation or reduction taking place at position 1...
The cinnamyl alcohol story...

- The behavior of cinnamyl alcohol and cinnamaldehyde in RHE is not symmetrical...
- A rapid (already after 1 h of incubation) and significant reduction of cinnamaldehyde into cinnamyl alcohol was observed...
- The opposite was not observed (no signal of cinnamaldehyde in cinnamyl alcohol treated RHE) even after 24 h...

\[
\begin{array}{c}
\text{OH} \\
\text{slow} \\
\text{FAST}
\end{array}
\]
The cinnamyl alcohol story...

1-(\(^{13}\)C)-Cinnamaldehyde (t = 8 h)

\(^1\)H-\(^{13}\)C Edited HSQC

64.7 ppm
1-(\(^{13}\)C)-Cinnamyl alcohol

200.6 ppm

1-(\(^{13}\)C)-Cinnamaldehyde

1-(\(^{13}\)C)-Cinnamyl alcohol (t = 8 h)

\(^1\)H-\(^{13}\)C Edited HSQC

64.7 ppm
1-(\(^{13}\)C)-Cinnamyl alcohol

?
The cinnamyl alcohol story...

- Samples treated with cinnamyl alcohol and cinnamaldehyde, respectively, were also analyzed for the formation of cinnamic acid...
- A significant amount of cinnamic acid was observed in cinnamaldehyde treated samples.
The cinnamyl alcohol story...

1-(\textsuperscript{13}C)-Cinnamaldehyde (t = 24 h)

- 200.6 ppm
- 178.2 ppm
- 64.7 ppm
The cinnamyl alcohol story...

- Samples treated with cinnamyl alcohol and cinnamaldehyde, respectively, were analyzed for the formation of cinnamic acid...
- Only a minute amount of cinnamic acid was detected in cinnamyl alcohol treated samples...

\[
\begin{align*}
\text{slow} & \quad \text{not observed} \\
\text{FAST} & \quad \text{OH} \\
\end{align*}
\]
The cinnamyl alcohol story...

1-(\textsuperscript{13}C)-Cinnamic alcohol (t = 1 h)
The cinnamyl alcohol story...

- Samples treated with cinnamyl alcohol and cinnamaldehyde, respectively, were also qualitatively analyzed for the formation of metabolites/adducts...

- A very clear difference was observed with the formation of new correlation signals but associated to very different patterns (no common signals associated to metabolites/adducts)...

- Signals associated to the reactivity of cinnamaldehyde were not observed in RHE treated with cinnamyl alcohol...
The cinnamyl alcohol story...

1-(13C)-Cinnamaldehyde (t = 8 h)

1H-13C Edited HSQC

1H-13C Edited HSQC

1-(13C)-Cinnamyl alcohol (t = 8 h)

1-(13C)-Cinnamyl alcohol Metabolites/adducts

1-(13C)-Cinnamyl alcohol Metabolites/adducts

64.7 ppm 91.1 ppm 172.4 ppm 178.9 ppm 200.6 ppm

45.6 ppm 64.7 ppm 71.6 ppm
The cinnamyl alcohol story...

The “dogma” of cinnamyl alcohol being a sensitizer through its oxidation into cinnamaldehyde can therefore be seriously questioned...

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OH

slow

FAST

X
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metabolites/adducts

X

metabolites/adducts
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The iso/eugenyl acetates story...

- Eugenol and isoeugenol are used as pure material but also as eugenyl and isoeugenyl esters (acetates or phenyl acetates)...

- According to LLNA data, acetate derivatives of eugenol and isoeugenol are classified as non-sensitizers while their parent materials are classified as weak and moderate skin sensitizers, respectively...

- However, clinical studies have shown that some individuals sensitized to isoeugenol also reacted when patch-tested to isoeugenyl acetate...
The iso/eugenyl acetates story...

- The mechanism underlying this observation is still not clear...

- It can be hypothesized that isoeugenyl esters are hydrolyzed either enzymatically (epidermal esterase) or chemically (hydrolysis)...

- The aim of this initial phase was to assess the potential of the “HRMAS NMR / RHE model” to investigate and characterize the behavior of eugenyl- and isoeugenyl acetates in a living 3D tissue...
The iso/eugenyl acetates story...

- Carbon-13 substituted iso/eugenyl acetates were synthesized
  - To increase the sensitivity...
  - To discriminate between acetates released by iso/eugenyl derivatives and other acetates...

[Chemical structures of iso/eugenyl acetates]
The iso/eugenyl acetates story...

- The stability of eugenyl and isoeugenyl acetates was first assessed in a 1:3 mixture of acetonitrile and phosphate buffer (PBS pH 7.4)...
- Reactions were followed by $^{13}$C NMR over a period of one month...
- Both eugenyl and isoeugenyl acetates were found to be rather stable toward chemical hydrolysis with only a slow release of free acetate over time...
The iso/eugenyl acetates story...

- Stability of iso/eugenyl acetates in a 1:3 mixture of acetonitrile and PBS pH 7.4
The iso/eugenyl acetates story...

- The stability of iso/eugenyl acetates was then tested on RHE...
- Eugenyl and isoeugenyl acetates, $^{13}$C-3 and $^{13}$C-4 were applied on three samples of RHE and incubated for 1, 8 and 24 h, respectively...
- Samples were then processed and analyzed by HRMAS NMR using a 1D-$^{13}$C sequence...
- With both eugenyl- and isoeugenyl acetates, only one signal at 183.4 ppm, corresponding to a free acetate ($\text{CH}_3\text{-COO}^-$), was detected...
The iso/eugenyl acetates story...

- Stability of eugenyl and isoeugenyl acetate on RHE

[Graphs showing stability measurements]
The iso/eugenyl acetates story...

- A second set of experiments was carried out with isoeugenyl acetate $^{13}$C-4 following incubation times of 5, 10, 15, 30 and 45 minutes, respectively...

- Spectra obtained indicate a very fast hydrolysis of isoeugenyl acetate $^{13}$C-4...

- Even after 5 min, the residual signal of isoeugenyl acetate ($\delta$ 170.8 ppm) was very small with a major signal at $\delta$ 183.4 ppm corresponding to the hydrolyzed acetate...
The iso/eugenyl acetates story...

- Stability of isoeugenyl acetate on RHE

RHE + isoeugenyl acetate $^{13}$C-4 (t = 45 min)

RHE + isoeugenyl acetate $^{13}$C-4 (t = 30 min)

RHE + isoeugenyl acetate $^{13}$C-4 (t = 15 min)

RHE + isoeugenyl acetate $^{13}$C-4 (t = 10 min)

RHE + isoeugenyl acetate $^{13}$C-4 (t = 5 min)

RHE blanc (t = 24 h)
The iso/eugenyl acetates story...

- Pre-treatment of RHE with inhibitors of esterase...
- Use of “fixed” RHE to confirm the role of enzymes in the hydrolysis of eugenyl and isoeugenyl acetates...
- Model the hydrolysis reaction of eugenyl and isoeugenyl acetates with porcine liver esterase...
- Perform the reaction at a lower temperature to slow down the enzymatic kinetic rate...
Conclusions/Perspectives...

- Results obtained so far clearly confirmed the high potential of HRMAS NMR, in association with RHE, to investigate activation mechanisms of prohaptens...

- Iso/eugenyl acetates appeared to be highly sensitive to hydrolysis...

- The formation of cinnamaldehyde by epidermal oxidation of cinnamyl alcohol, as a source of sensitization, appeared to be highly questionable...

- Further studies are needed...
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