Applicability of Different Soft Nucleophiles for Screening Reactive Metabolites Resulting from Microsomal Activation of Furan Mixtures and Cigarette Smoke

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Introduction

Background

The lung is the primary target organ for exposure to aerosol constituents delivered by cigarette smoke. Although the lung has a significant capacity for biotransforming xenobiotics with the aim of reducing any potential toxicity, in some instances biotransformation can result in the generation of more reactive, and often more toxic, metabolites[1]. Knowledge regarding these reactive (intermediate) metabolites will support efforts to establish a link between potentially harmful smoke constituents and disease relevant mechanisms[2] (i.e. oxidative stress, inflammation, apoptosis…).

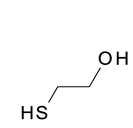
Due to the short lifespan of these highly reactive electrophilic intermediates, a widely accepted analytical approach is to trap them using a nucleophile, such as glutathione, and to generate more stable adducts that can be analyzed using liquid chromatography coupled to mass spectrometry (LC-MS)[3].

Aim

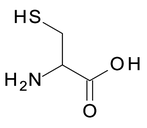
The use of glutathione has a significant disadvantage associated with the structural elucidation of conjugated electrophilic compounds, which limits its usefulness within a generic screening approach for phase I activation of xenobiotics in complex mixtures. The aim of this investigation was to compare the suitability of alternative nucleophiles for screening, which were assessed using reactive intermediates generated by the activation of simple furans and alkylfurans.

Strategy

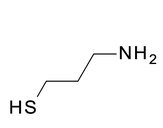
In order to define the most suitable approach for identifying and characterizing reactive intermediates generated by microsomal activation of cigarette smoke constituents, four different nucleophiles were evaluated for use within the test system. These were glutathione, cysteine, mercaptoethanol and 3-aminopropan-1-thiol.



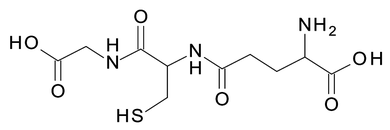
Mercaptoethanol



Cysteine



3-Aminopropan-1-thiol



Glutathione

Reaction kinetics with acrolein, application within microsomal incubations for phase I oxidation of furan and 2-methylfuran, and the ability to provide structural information using mass spectrometry were investigated for each nucleophile.

Materials and Methods

Reactivity experiments

Reaction kinetic experiments were performed by mixing together equimolar solutions of acrolein with each individual nucleophile (pH adjusted to 7.4). The nucleophile adduct formation rate was determined by direct on-line infusion into a high resolution LC-MS. Structural elucidation was performed by acquiring MS^n fragmentation spectra for each nucleophile adduct using an LTQ Orbitrap Elite mass spectrometer.

Microsomal incubations

Microsomal incubations were conducted at 37°C in 20mM phosphate buffer at pH7.4, which contained a mixture of furan compounds as substrate. Metabolic reactions were initiated by the addition of a cofactor mixture, and were terminated at specified timepoints by the addition of ice cold methanol and immediate freezing using dry ice. The samples were then thawed, centrifuged at 6800g for 30min to precipitate microsomal proteins and the supernatant analysed using high resolution LC-MS.

LC-MS analysis

LC system: Thermo Fisher Scientific Accela 1250™ LC
Injection volume: 10 µL
Eluent A: 0.1% formic acid in water / Eluent B : 0.1% formic acid in acetonitrile
Flow rate: 400 µL/min Column: Thermo Hypersil Gold aQ (150 x 2.1 mm, 1.9 µm)
HPLC gradient: 0-3min (0%B) ->15min (20%B) ->16-19min (100%B)

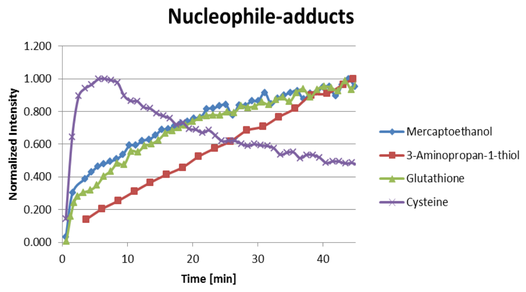
MS system: Thermo Fisher Scientific Q Exactive™ or Orbitrap Elite™
Ionisation mode: ESI positive
Scanning mode: full scan / MS^2 / MS^n
Scan range: 50 – 750 m/z
Resolution: 70000 / 120000

Q Exactive™

Results

Reaction kinetics

- Reactivities of the different nucleophiles with acrolein were compared by measuring the formation rate of their respective acrolein adducts
- Data processing was performed using Thermo Xcalibur™ version 2.2 (Thermo Scientific)
- Nucleophile adduct targets were extracted from the full scan data using a mass window tolerance of 5 ppm



Cysteine had the highest reactivity towards acrolein, with complete adduct formation achieved within 10 minutes. The observed reaction rates for mercaptoethanol and glutathione were comparable with each other, but were much lower than the reaction rate observed for cysteine. The lowest adduct formation rate was apparent for 3-aminopropan-1-thiol, where the reaction remained incomplete after the 45 minute experimental period.

Sensitivity

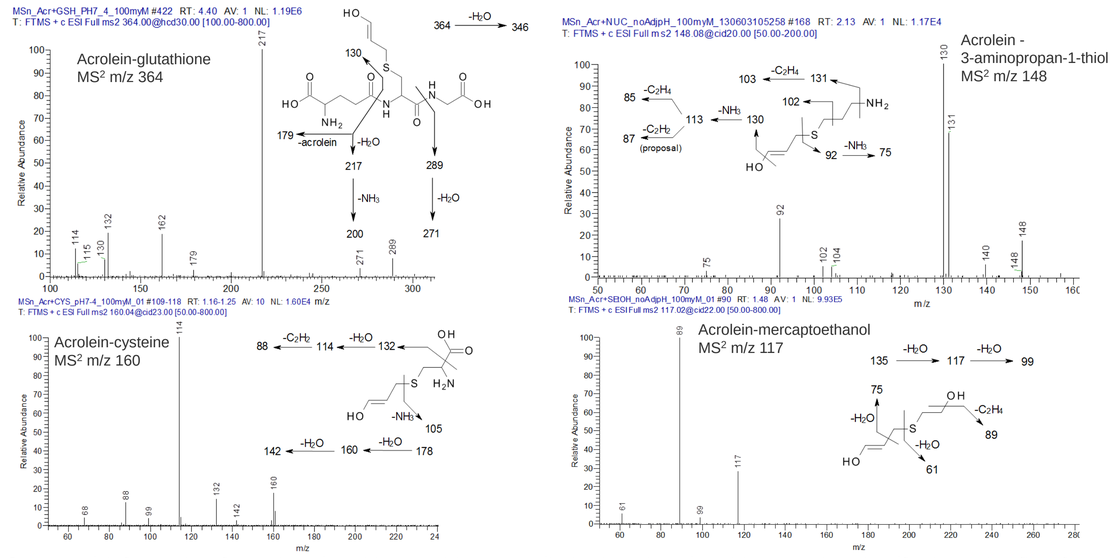
The sensitivity of nucleophilic adducts for mass spectrometry (ESI+) was evaluated by comparing the signal intensities of adduct specific ion traces during a direct infusion (~30pmol/second) of equimolar solutions of acrolein and the respective nucleophiles. All evaluated nucleophilic adducts displayed approximately equivalent sensitivity:

3-aminopropan-1-thiol-adduct ($3x10^6$) > mercaptoethanol-adduct ($2x10^6$) > glutathione-adduct / cysteine-adduct ($1x10^6$)

Results

Structural elucidation of nucleophilic adducts

The ability to provide structural information for the nucleophilic adducts using mass spectrometry was assessed by performing fragment assignment of the MS^2 spectra.

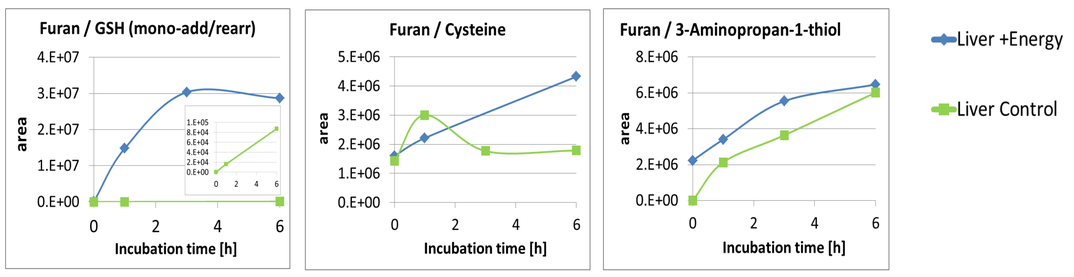


Structural information from the glutathione adduct was limited to the glutathione moiety itself, since the first fragmentation steps occurred on the backbone of the tripeptide structure alone. Cysteine, mercaptoethanol and 3-aminopropan-1-thiol were smaller nucleophiles and therefore provided more structural information related to the adducts.

In vitro metabolism of aerosol constituents

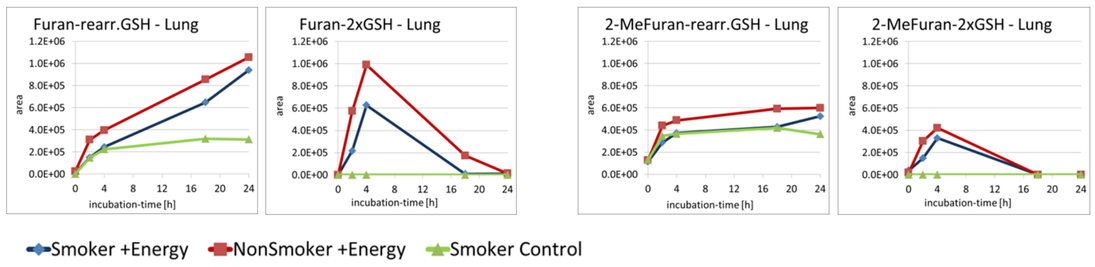
The suitability of each nucleophile for screening reactive metabolites resulting from in-vitro metabolism was evaluated by quantifying each nucleophile adduct formed during microsomal incubations with furan and 2-methylfuran (data for the latter not presented). Mercaptoethanol was found to be unsuitable due to inconsistency of results and was not considered further.

Human liver microsomes



The results for cysteine and 3-aminopropan-1-thiol demonstrated the generation of a significant amount of reactive intermediate metabolite (RIM) adducts in the control system (without NADPH regeneration system). This may be attributable to the presence of background levels of reactive furan metabolites bound within the microsomes themselves, which are released in the presence of highly nucleophilic moieties. This microsomal background could also be observed for glutathione, however much reduced compared to the smaller nucleophiles. For this reason, glutathione was considered to be the best suited nucleophile for furans RIM analysis.

Human lung microsomes (smoker versus non-smoker)



Glutathione was successfully applied for the assessment of human lung microsomal activation of furan and 2-methylfuran. The generation of corresponding RIMs, cis-2-butene-1,4-dial and 4-oxopent-2-enal, was demonstrated using human lung microsomes.

Conclusions

- Reactivity of the 4 tested nucleophiles with acrolein was evaluated, giving the order of reactivity:
  cysteine >> glutathione / mercaptoethanol > 3-aminopropan-1-thiol
- Sensitivities for the respective adducts using ESI(+) LC-MS were within the same order of magnitude
- Ability to provide structural information regarding the adducted moiety was clearly enhanced for the smaller nucleophiles 3-aminopropan-1-thiol and mercaptoethanol. Cysteine provided minimal information from MS-fragments for the adducted electrophile, while glutathione did not provide any information below MS^5
- Glutathione was the most appropriate nucleophile for use with microsomes, providing the lowest levels of background activity and the best discrimination between activation and control systems. All other nucleophiles generated significant background levels in the control experiments, and mercaptoethanol did not provide consistent results for the microsomal experiments performed
- The trapping of furan derived RIMs with glutathione was demonstrated for human lung microsomes

References

1) *Peterson L. A. Reactive metabolites in the biotransformation of molecules containing a furan ring. Chem. Res. Toxicol. 26:6 (2013).*
2) *Takakusa H. et al. Markers of Electrophilic Stress Caused by Chemically Reactive Metabolites in Human Hepatocytes . Drug Metabolism and Disposition. Vol. 36, No. 5 (2008).*
3) *Grillo, M. P. Strategies for the Detection of Reactive Intermediates in Drug Discovery and Development. Biotransformation and Metabolite Elucidation of Xenobiotics, John Wiley & Sons, Inc.: 245-294 (2010)*

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