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# In vitro metabolism of naphthalene and its alkylated congeners by human and rat liver microsomes via alkyl side chain or aromatic oxidation

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ABSTRACT

Mineral oils are widely applied in food production and processing and may contain polycyclic aromatic hydrocarbons (PAHs). The PAHs that may be present in mineral oils are typically alkylated, and have been barely studied. Metabolic oxidation of the aromatic ring is a key step to form DNA-reactive PAH metabolites, but may be less prominent for alkylated PAHs since alkyl substituents would facilitate side chain oxidation as an alternative. The current study investigates this hypothesis of preferential side chain oxidation at the cost of aromatic oxidation using naphthalene and a series of its alkyl substituted analogues as model compounds. The metabolism was assessed by measuring metabolite formation in rat and human liver microsomal incubations using UPLC and GC-MS/MS. The presence of an alkyl side chain markedly reduced aromatic oxidation for all alkyl-substituted naphthalenes that were converted. 1-n-Dodecyl-naphthalene was not metabolized under the experimental conditions applied. With rat liver microsomes for 1-methyl-, 2-methyl-, 1-ethyl-, and 2-ethyl- naphthalene, alkyl side chain oxidation was preferred over aromatic oxidation. With human liver microsomes this was the case for 2-methyl-, and 2-ethyl-naphthalene. It is concluded that addition of an alkyl substituent in naphthalene shifts metabolism in favor of alkyl side chain oxidation at the cost of aromatic ring oxidation. Furthermore, alkyl side chains of 6 or more carbon atoms appeared to seriously hamper and reduce overall metabolism, metabolic conversion being no longer observed with the C12 alkyl side chain. In summary, alkylation of PAHs likely reduces their chances of aromatic oxidation and bioactivation.

## 1. Introduction

Mineral oils are applied in food production processes and applications, such as food packaging material, food additives, processing aids and machine lubricants. Hydrocarbons that may be present in mineral oils have been reported as an emerging food safety issue by the European Food Safety Authority (EFSA) [1]. The presence of mineral oil derived aromatic hydrocarbons is considered a potential concern because of the mutagenicity and carcinogenicity of some PAHs with three to seven, non- or simple-alkylated, aromatic rings [1]. A key step in formation of the DNA reactive genotoxic metabolites of PAHs is metabolic bioactivation of the aromatic hydrocarbons via aromatic ring oxidation by cytochrome P450 enzymes [2]. However, this aromatic oxidation and bioactivation has been primarily studied for unsubstituted ('naked') PAHs, also referred to as pyrogenic PAHs because they are typical for coal-derived products and combustion products. In contrast, the PAHs present in petroleum-derived substances, also referred to as petrogenic PAHs, are primarily alkylated PAHs [3], for which the knowledge on metabolism is limited, although it may be hypothesized that alkylation may influence metabolism of the aromatic hydrocarbons.

To get more insight into cytochrome P450-mediated oxidative metabolism of alkylated PAHs that may be present in mineral oils, the aim of the present study was to characterize the relative aromatic and alkyl side chain oxidation of a series of alkylated naphthalene congeners. It has been reported that naphthalene is metabolized to trans-1,2-dihydro-1,2-naphthalenediol, 1-naphthol and 2-naphthol, all metabolites resulting from aromatic oxidation, by cytochromes P450 in incubations with pooled human liver microsomes [4]. The P450 isoform CYP1A2 was identified as the most efficient enzyme in production of 1,2-dihydro-1,2-naphthalenediol and 1-naphthol, and CYP3A4 was the most effective enzyme for 2-naphthol production [4]. However, data on metabolic kinetics of alkylated naphthalenes are limited and the oxidation pattern of naphthalene substituted with longer alkyl chains is

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still unclear.

Based on the available literature [5] and thermodynamic grounds, it can be hypothesized that alkylated PAHs will undergo oxidative metabolism on the alkyl side chain more easily than on the condensed aromatic rings, facilitating their excretion and detoxification over their potential bioactivation. The present study investigates the hypothesis on preferential side chain oxidation at the cost of aromatic oxidation using naphthalene and a series of its alkyl substituted analogues as the model compounds. To this end, the oxidative metabolism of 1-methylnaphthalene, 2-methylnaphthalene, 1-ethylnaphthalene, 2-ethylnaphthalene, 1-n-hexylnaphthalene and 1-n-dodecylnaphthalene in human and rat hepatic microsomal incubations was characterized, and the kinetics and metabolic efficiencies for formation of the different metabolites were quantified.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Naphthalene (≥99%), and 1-methylnaphthalene (≥94%) were purchased from Merck (Darmstadt, Germany). 2-Methylnaphthalene (≥97%), 1-ethylnaphthalene (≥97%), 2-ethylnaphthalene (≥99%), and the reference standards 1-naphthol (≥99%), 1-(hydroxymethyl) naphthalene (≥98%), 2-(hydroxymethyl) naphthalene (≥98%), 1-(1-hydroxylethyl) naphthalene (≥99%), 2-(1-hydroxyethyl) naphthalene (≥98%), and 2-(hydroxyethyl) naphthalene (≥98%), and also tetrahydrofuran (≥99.9%), and trifluoroacetic acid (≥99%), were purchased from Sigma-Aldrich (St.Louis, USA). 1-n-Hexylnaphthalene (≥99.3%) was synthesized by the Biochemical Institute for Environmental Carcinogens (Groβhansdorf, Germany). 1-n-Dodecylnaphthalene (≥95%) was purchased from Combi-Blocks (San Diego, USA). Acetonitrile was bought from Biosolve (Dieuze, France). α-Naphthaflavone was obtained from Acros (New Jersey, USA). Dimethyl sulfoxide (DMSO) and $K_2HPO_4 \cdot 3H_2O$ were supplied by Merck (Darmstadt, Germany). NADPH was obtained from Carbosynth (Berkshire, UK). Gentest™ pooled male Sprague Dawley rat liver microsomes (RLM) and Ultrapool™ human liver microsomes (HLM) 150 with a protein concentration of 20 mg/ml were supplied by Corning (New York, USA), and the latter contained cytochrome P450 liver enzymes of 150 individuals.

### 2.2. In vitro incubations of naphthalene and its alkylated congeners with rat and human liver microsomes

Microsomal oxidation of naphthalene and its alkylated congeners by RLM and HLM was investigated. An overall 200 μl incubation system consisted of potassium phosphate buffer (0.1 M, pH 7.4), 5 mM $MgCl_2$, RLM/HLM at a final microsomal protein concentration of 0.5 mg/ml, 1 mM NADPH, and each of the individual test compounds at concentrations ranging from 0 to 600 μM. Test compounds were naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, 1-ethylnaphthalene, 2-ethylnaphthalene, 1-n-hexylnaphthalene and 1-n-dodecylnaphthalene. The final concentration of substrate solvent, either DMSO or tetrahydrofuran (the latter used for 1-n-hexylnaphthalene and 1-n-dodecylnaphthalene due to their low solubility in DMSO), in the incubation mixture was 1% (v/v), which did not affect the enzymatic activity of liver microsomes [6]. The incubation mixtures were prepared and incubated in glass vials to avoid plastic binding of the substrates. The glass vials were capped to prevent substrate loss due to volatility. After pre-incubation of the incubation mixture at 37 °C for 1 min, the enzymatic reaction was initiated by adding microsomes to the incubation mixture which was subsequently incubated at 37 °C for 10 min. The reaction was terminated by adding 100 μl ice-cold acetonitrile followed by vortexing. After 5 min centrifugation at 5000 rpm, 4 °C, the supernatant was collected for ultra-performance liquid chromatography (UPLC) analysis. However, concentrations of the metabolites of two of the test substrates, 1-n-hexylnaphthalene and 1-n-dodecylnaphthalene, in the supernatant appeared too low to detect metabolism due to binding to microsomal protein. Therefore, a diisopropylether (DIPE) extraction of the metabolites was performed after the reaction was stopped by the addition of 20 μl 10% $HClO_4$. The incubation mixture (total volume of 220 μl) was extracted three times with 1 ml DIPE. Each time, the upper layer was collected and the combined DIPE fractions were subsequently vaporized under a nitrogen stream. The residues were dissolved in 100 μl methanol and analyzed by UPLC.

To investigate the species differences between HLM and RLM, an inhibition study of CYP1A in incubations of naphthalene, 1-methylnaphthalene and 1-ethylnaphthalene was performed. An overall 200 μl incubation system consisted of potassium phosphate buffer (0.1 M, pH 7.4), 5 mM $MgCl_2$, RLM/HLM at a final microsomal protein concentration of 0.5 mg/ml, 1 mM NADPH, 1 μM α-naphthoflavone (an inhibitor of CYP1A), and each of the individual test compounds at a concentration of approximately the same value as $K_M$. The test concentration of each substrate was 60 μM for HLM and 200 μM for RLM. The incubation was performed by the same procedure as described above, with an incubation time of 10 min at 37 °C. The reaction was stopped by addition of 100 μl ice-cold acetonitrile followed by vortexing and a 5-min centrifugation at 5000 rpm at 4 °C, the supernatant was collected for ultra-performance liquid chromatography (UPLC) analysis.

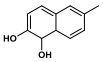
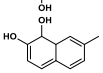
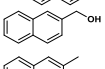
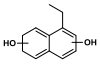
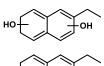
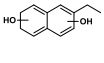
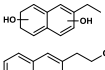
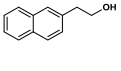
### 2.3. UPLC analysis

The metabolites formed were analyzed and quantified using an Acquity UPLC system equipped with a photodiode array (PDA) detector (Waters, Milford, MA). The metabolites and their parent compound were separated on a reverse phase Acquity UPLC® BEH C18 column (21 × 50 mm, 1.7 μm, Waters, Milford, MA) and detected at wavelength ranging from 190 nm to 400 nm. Eluent A was nano-pure water containing 0.1% trifluoroacetic acid (v/v), and eluent B was acetonitrile containing 0.1% trifluoroacetic acid (v/v). The gradient elution started from 90% A to 10% B applied from 0.0 min to 0.5 min, which was changed to 10% A and 90% B from 0.5 to 12.5 min and then kept at 10% A and 90% B from 12.5 min to 13.5 min, changed back to 90% A and 10% B from 13.5 to 14.5 min and then maintained at the starting conditions from 14.5 min until 17 min. The total run time was 17 min using a flow rate of 0.6 ml/min. The temperature of the column was set at 40 °C and the autosampler at 10 °C during the UPLC analysis. The injection volume was 3.5 μl. Metabolites were quantified using their peak area at the wavelength specified in Table 1, using calibration curves of available reference compounds. Metabolites were identified by comparing the retention time (RT) and UV spectra to those of reference standard chemicals on UPLC. When reference standard chemicals were not available commercially, metabolite identification by GC-MS/MS was performed. The unknown minor metabolites were categorized based on elution time and mass spectra both on UPLC and GC-MS/MS.

### 2.4. Metabolite identification by GC-MS/MS

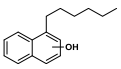
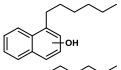
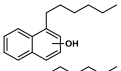
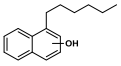
To prepare samples for metabolite identification by GC-MS/MS, each individual substrate (300 μM final concentration) was incubated in a volume of 400 μl in potassium phosphate buffer (0.1 M, pH 7.4) containing 5 mM $MgCl_2$, RLM/HLM at a final microsomal protein concentration of 1 mg/ml, and 1 mM NADPH at 37 °C for 10 min. After incubation, the incubation mixture was centrifuged for 5 min at 5000 rpm and 4 °C. The supernatant of the incubation mixture was transferred to a fresh vial and extracted 3 times with 100 μl dichloromethane (DCM) followed by vortexing. DCM phase containing the substrate and metabolites was separated from the aqueous mixture. The organic (lower) phase was collected from each extraction, combined and used for GC-MS/MS measurement. The spectra of each

**Table 1**
The Michalis Menten parameters including $K_{M,\ app}$, $V_{max,\ app}$ and intrinsic clearance ($Cl_{int}$) calculated as $V_{max,\ app}/K_{M,\ app}$, of the metabolites produced from alkyl substituted naphthalenes and naphthalene in human and rat microsomal incubations (Fig. 1). The retention time (RT) and wavelength (λ) used to identify and quantify the metabolites by UPLC-UV analysis are also presented. Results are shown as mean ± standard error (SE) from three independent microsomal incubations. Abbreviations. ND = not detected. NM = no metabolism. NA = not applicable. app = apparent.

| **Parent compound** Metabolites | Structure | Species | $K_{M,\ app}$ (μM) | $V_{max,\ app}$ (pmol/min/mg microsomal protein) | $Cl_{int}$ ($V_{max}/K_M$) μl/min/mg protein |
|---|---|---|---|---|---|
| **Naphthalene RT = 5.59min, λ = 220.7 nm** | | | | | |
| 1,2-dihydro-1,2 naphthalenediol |  | Human | 28.1 ± 7.3 | 1517.0 ± 78.8 | 54.0 |
| RT = 1.43min, λ = 215.8 nm | | Rat | 562.2 ± 238.2 | 634.4 ± 133.3 | 1.1 |
| 1-naphthol |  | Human | 47.0 ± 7.7 | 507.4 ± 20.4 | 10.8 |
| RT = 3.91min, λ = 211.6 nm | | Rat | 436.3 ± 95.2 | 4578.0 ± 444.0 | 10.5 |
| **1-methylnaphthalene RT = 6.33min, λ = 223.7 nm** | | | | | |
| Dihydro-1-methyl naphthalenediol |  | Human | 5.3 ± 1.3 | 107.3 ± 2.0 | 20.1 |
| RT = 2.14min, λ = 217.6 nm | | Rat | ND | ND | - |
| Dihydro-1-methyl naphthalenediol |  | Human | 11.5 ± 4.7 | 163.1 ± 8.4 | 14.2 |
| RT = 2.18 min, λ = 220.7 nm | | Rat | 683.3 ± 335.8 | 56.0 ± 14.8 | 0.1 |
| 1-(hydroxymethyl) naphthalene |  | Human | 63.5 ± 12.3 | 989.6 ± 52.7 | 15.6 |
| RT = 3.29min, λ = 223.7 nm | | Rat | 278.9 ± 49.5 | 6823.0 ± 437.9 | 24.5 |
| 1-methylnaphthol RT = 4.49min, λ = 228.5 nm |  | Human | 190.7 ± 18.6 | 141.0 ± 5.5 | 0.7 |
| | | Rat | 408.7 ± 55.0 | 310.5 ± 18.0 | 0.8 |
| 1-methylnaphthol |  | Human | 61.8 ± 8.0 | 325.1 ± 11.4 | 5.3 |
| RT = 4.59min, λ = 210.4 nm | | Rat | 1008.0 ± 315.0 | 1291.0 ± 249.8 | 1.3 |
| 1-methylnaphthol |  | Human | ND | ND | - |
| RT = 4.95min, λ = 219.4 nm | | Rat | 295.1 ± 160.5 | 76.5 ± 15.4 | 0.3 |
| **2-methylnaphthalene RT = 6.43min, λ = 223.7 nm** | | | | | |
| 3,4-dihydro-2-methylnaphthalenediol |  | Human | ND | ND | - |
| RT = 1.70min, λ = 224.3 nm | | Rat | 505.8 ± 183.3 | 56.4 ± 9.7 | 0.1 |
| 5,6-dihydro-2-methylnaphthalendiol |  | Human | 139.8 ± 59.2 | 72.0 ± 10.9 | 0.5 |
| RT = 1.96min, λ = 217 nm | | Rat | ND | ND | - |
| 7,8-dihydro-2-methylnaphthalenediol |  | Human | 31.4 ± 4.3 | 266.8 ± 7.7 | 8.5 |
| RT = 2.25min, λ = 219.4 nm | | Rat | 2542.0 ± 1419.0 | 268.2 ± 118.5 | 0.1 |
| 2-(hydroxymethyl) naphthalene |  | Human | 50.4 ± 5.9 | 1760.0 ± 51.9 | 34.9 |
| RT = 3.39min, λ = 224.9 nm | | Rat | 194.5 ± 36.5 | 6769.0 ± 386.6 | 34.8 |
| 2-methylnaphthol |  | Human | 93.6 ± 28.7 | 23.5 ± 2.2 | 0.3 |
| RT = 4.48min, λ = 227.3 nm | | Rat | 464.2 ± 78.6 | 111.5 ± 8.6 | 0.2 |
| 2-methylnaphthol |  | Human | 88.4 ± 17.5 | 35.1 ± 2.1 | 0.4 |
| RT = 4.58min, λ = 227.9 nm | | Rat | 650.8 ± 235.2 | 72.8 ± 13.8 | 0.1 |
| 2-methylnaphthol |  | Human | 226.5 ± 67.5 | 47.68 ± 6.0 | 0.2 |
| RT = 4.66min, λ = 215.8 nm | | Rat | 218.1 ± 50.3 | 166.5 ± 12.4 | 0.8 |
| 2-methylnaphthol |  | Human | 66.2 ± 17.2 | 25.4 ± 1.8 | 0.4 |
| RT = 4.72min, λ = 215.8 nm | | Rat | 483.8 ± 91.6 | 166.0 ± 14.6 | 0.3 |
| **1-ethylnaphthalene RT = 7.00min, Ab = 224.3 nm** | | | | | |
| Dihydro-1-ethyl naphthalenediol |  | Human | 14.9 ± 2.7 | 386.8 ± 10.4 | 25.9 |
| RT = 2.96min, λ = 218.2 nm | | rat | 140.9 ± 62.9 | 186.3 ± 21.7 | 1.3 |
| 1-(1-hydroxyethyl) naphthalene |  | Human | 73.6 ± 7.7 | 1001.0 ± 30.0 | 13.6 |
| RT = 3.92min, λ = 223.7 nm | | Rat | 221.4 ± 34.4 | 4831.0 ± 243.2 | 21.8 |
| 1-naphthyl methylketone |  | Human | 86.0 ± 19.4 | 119.3 ± 8.2 | 1.4 |
| RT = 3.97min, λ = 224.3 nm | | Rat | 209.7 ± 76.5 | 280.3 ± 32.3 | 1.3 |
| 1-ethylnaphthol |  | Human | 67.3 ± 9.5 | 239.7 ± 9.5 | 3.6 |
| RT = 5.29min, λ = 211 nm | | Rat | 391.0 ± 97.6 | 338.2 ± 35.7 | 0.9 |
| 1-ethylnaphthol |  | Human | ND | ND | - |
| RT = 5.34min, λ = 224.3 nm | | Rat | 72.1 ± 19.1 | 349.8 ± 17.6 | 4.9 |
| RT = 6.65min, λ = 227.3 nm | Unknown | Human | 99.1 ± 16.3 | 72.5 ± 3.8 | 0.7 |
| | | Rat | 226.1 ± 83.6 | 291.7 ± 35.3 | 1.3 |
| **2-ethylnaphthalene RT = 7.11min, λ = 224.9 nm** | | | | | |
| Dihydro-2-ethyl naphthalenediol |  | Human | ND | ND | - |
| RT = 2.64min, λ = 224.9 nm | | Rat | 23.7 ± 23.6 | 27.0 ± 2.8 | 1.1 |
| Dihydro-2-ethyl naphthalenediol |  | Human | 21.9 ± 7.2 | 34.4 ± 2.0 | 1.6 |
| RT = 3.04min, λ = 220 nm | | Rat | ND | ND | ND |
| Dihydro-2-ethyl naphthalenediol |  | Human | 24.1 ± 3.6 | 110.1 ± 3.1 | 4.6 |
| RT = 3.08min, λ = 220 nm | | Rat | Ambiguous | Ambiguous | - |
| 2-(2-hydroxyethyl) naphthalene |  | Human | 13.0 ± 4.8 | 89.0 ± 4.5 | 6.9 |
| RT = 3.91min, λ = 224.3 nm | | Rat | 99.8 ± 21.6 | 26.5 ± 1.3 | 0.3 |

(*continued on next page*)

**Table 1** (*continued*)

| **Parent compound**<br>Metabolites | Structure | Species | $K_{M,\ app}$ (μM) | $V_{max,\ app}$ (pmol/min/mg microsomal protein) | $Cl_{int}$ ($V_{max}/K_M$) μl/min/mg protein |
|---|---|---|---|---|---|
| 2-(1-hydroxyethyl) naphthalene |  | Human | 21.9 ± 3.2 | 2540.0 ± 65.1 | 115.9 |
| RT = 3.96min, λ = 224.3 nm | | Rat | 100.2 ± 23.3 | 5200.0 ± 268.2 | 51.9 |
| RT = 6.66min, λ = 243.8 nm | Unknown | Human | 64.6 ± 7.4 | 76.0 ± 2.4 | 1.2 |
| | | Rat | 65.7 ± 18.7 | 280.9 ± 14.5 | 4.3 |
| **1-n-hexylnaphthalene RT = 9.58min, λ = 224.9 nm** | | | | | |
| 1-hexylnaphthol |  | Human | 38.4 ± 11.7 | 119.4 ± 12.8 | 3.1 |
| RT = 5.95min, λ = 219.4 nm | | Rat | 119.2 ± 55.8 | 48.1 ± 12.1 | 0.4 |
| 1-hexylnaphthol |  | Human | 44.7 ± 14.4 | 52.3 ± 6.3 | 1.2 |
| RT = 6.1min, λ = 224.9 nm | | Rat | 110.0 ± 30.4 | 178.5 ± 25.8 | 1.6 |
| 1-hexylnaphthol |  | Human | 139.3 ± 87.6 | 40.3 ± 14.4 | 0.3 |
| RT = 6.21min, λ = 223.7 nm | | Rat | 364.9 ± 259.0 | 55.4 ± 28.9 | 0.2 |
| 1-hexylnaphthol |  | Human | 58.2 ± 20.9 | 31.6 ± 4.7 | 0.5 |
| RT = 6.38min, λ = 223.7 nm | | Rat | 97.3 ± 35.4 | 59.7 ± 10.9 | 0.6 |
| **1-n-dodecylnaphthalene RT = 12.3min, λ = 224.9 nm** | | | | | |
| NM | NA | Human | - | - | - |
| NM | | Rat | - | - | - |

metabolite obtained from measurement were compared with the mass spectra data center NIST library (14, 14s, 17-1, 17-2, 17s) installed in GC-MS solution software Version 4.45 (Shimadzu, Japan).

The metabolites formed from naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, 1-ethylnaphthalene, 2-ethylnaphthalene and 1-n-hexylnaphthalene were analyzed using a Shimadzu GC-MS/MS system consisting of GC-2010 Plus coupled with Mass spectrometer TQ8040 (Shimadzu, Japan). A 30 m capillary column with 0.25 mm diameter (SH-Rxi-5ms, Shimadzu, Japan) was used to separate the metabolites upon injection of 1 μl of the extract with split ratio of 20:1, using a constant flow of helium gas (1 ml/min). The column oven temperature started at 80 °C and 1-min hold, increased to 280 °C at a rate of 40 °C/min from 1 min to 6 min, followed by 2 min hold at 280 °C. The total run time was 8 min and electron ionization (70eV) was used to generate the ions of metabolites for mass spectrometric detection.

### *2.5. Data analysis*

The metabolite concentrations were quantified by UPLC and used to calculate velocity of the enzymatic reaction in pmol/min/mg microsomal protein. The kinetic parameters $K_M$ and $V_{max}$ were obtained using a nonlinear regression (curve fit) applying the Michaelis Menten equation in GraphPad Prism 5 (San Diego, USA). Some kinetic curves seemed to be better fitted by other modelling approaches, such as substrate inhibition and sigmoidal models, however the outcome parameters from fits were not realistic. Michalis Menten approach gave basically an equally good fit, with more realistic parameters and was therefore applied as a first approximation to describe the metabolism in terms of $V_{max,\ app}$ and $K_{M,\ app}$ The intrinsic clearance ($Cl_{int}$) was calculated as $V_{max}$ divided by $K_M$ to compare the metabolic efficiency of formation of the different metabolites.

## 3. Results

### *3.1. Microsomal metabolism of naphthalene and its alkyl substituted analogues*

The substrate concentration-dependent metabolite formation of naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, 1-ethylnaphthalene, 2-ethylnaphthalene and 1-n-hexylnaphthalene by both RLM and HLM is presented in Fig. 1. Since inhibition was apparent at higher concentrations in the microsomal incubations with 1-hexylnaphthalene, these concentrations were excluded from the Michalis-Menten analysis and for the calculation of $V_{max,\ app}$ and $K_{M,\ app}$. 1-n-Dodecylnaphthalene was not metabolized under the experimental conditions applied. The kinetic parameters including $K_{M,\ app}$, $V_{max,\ app}$ and $Cl_{int}$, derived from modelling the data are presented in Table 1.

Two metabolites were detected in metabolic incubations of naphthalene with both HLM and RLM (Fig. 1) and were identified as 1-naphthol and 1,2-dihydro-1,2-naphthalenediol. The dihydrodiol was the most abundant metabolite formed by HLM, while 1-naphthol was the primary metabolite formed by RLM. The $Cl_{int}$ for 1,2-dihydro-1,2-naphthalenediol formation by HLM was significantly higher than that by RLM, but for 1-naphthol formation the $Cl_{int}$ by HLM and RLM were similar (Table 1).

The primary metabolites formed by both RLM and HLM from the investigated alkylated naphthalenes were alcohols, which are more polar than the parent compounds. The most abundant metabolites formed by RLM and HLM from 1-methylnaphthalene, 2-methylnaphthalene, 1-ethylnaphthalene, and 2-ethylnaphthalene were identified as metabolites resulting from side-chain oxidation being 1-(hydroxymethyl)naphthalene, 2-(hydroxymethyl)naphthalene, 1-(1-hydroxylethyl)naphthalene, and 2-(1-hydroxyethyl)naphthalene, respectively (Fig. 1), as demonstrated by co-elution and similar absorbance spectra as the reference standards in the UPLC analysis.

Minor metabolites of 1-methylnaphthalene, 2-methylnaphthalene, 1-ethylnaphthalene, 2-ethylnaphthalene, and 1-n-hexylnaphthalene were characterized by comparing mass spectra of these metabolites obtained using GC-MS/MS to those of reference spectra in the NIST library. Two metabolites of 1-methylnaphthalene eluting at 2.14 min and 2.18 min (Table 1) were characterized as dihydro-1-methylnaphthalenediols based on their molecular ion peak of *m/z* 176 and a similar fragmentation pattern in the mass spectra as observed for 1,2-dihydro-naphthalenediol. Three minor 1-methylnaphthalene metabolites with retention time of 4.30 min, 4.59 min and 4.95 min (Table 1) were tentatively identified as 1-methylnaphthols based on their molecular ion peak of *m/z* 158 and MS spectra comparable to reference spectra in the NIST library.

The minor metabolites formed from 2-methylnaphthalene (Table 1) eluting at 1.70 min, 1.96 min and 2.25 min were characterized as dihydro-2-methylnaphthalenediols with a molecular ion peak of *m/z* 176, and three 2-methylnaphthalene dihydrodiols with *m/z* 176, including

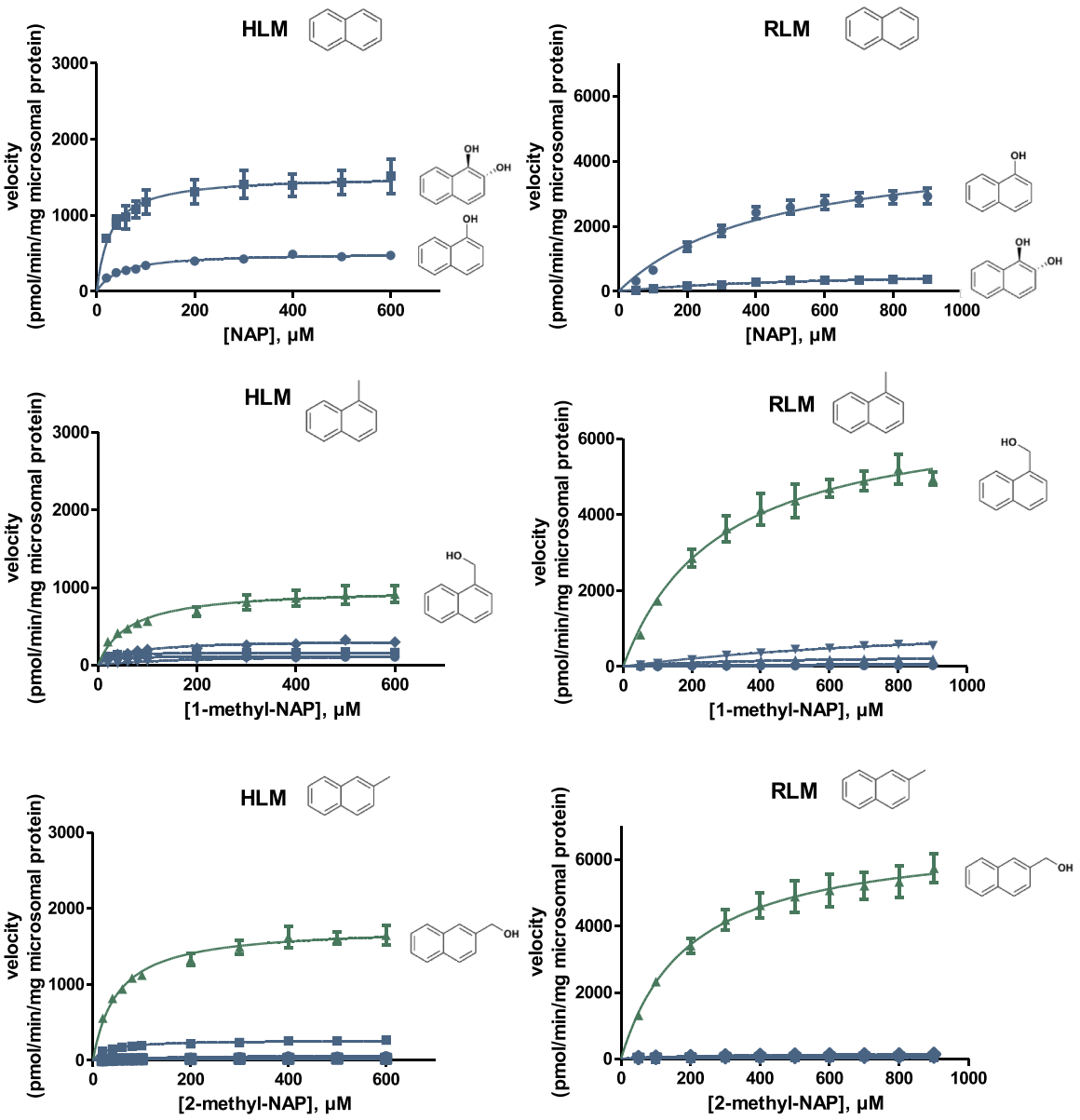



**Fig. 1.** Substrate-concentration dependent metabolism of alkyl substituted naphthalenes and naphthalene itself by human and rat liver microsomes. Green lines represent alkyl chain oxidation and blue lines stand for aromatic ring oxidation. The figures with enlarged scale of minor metabolites are attached as Fig. 1 to the supplementary materials. Abbreviation. NAP = naphthalene. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

the 3,4-dihydrodiol, the 5,6-dihydrodiol and the 7,8-dihydrodiol, in line with what was reported previously for conversion of 2-methylnaphthalene in rat liver microsomal incubations [7]. In the present study, formation of 5,6-dihydro-2-methylnaphthalenediol was observed only at high concentrations of 600 μM 2-methylnaphthalene incubated with RLM.

Among the five minor metabolites of 1-ethylnaphthalene, the metabolite eluting at 2.96 min was identified as dihydro-1-ethylnaphthalenediol based on its *m/z* 190 and its fragmentation pattern being similar to those of an available reference compound. Furthermore, the mass spectra of the metabolite with retention time 3.97 min and a base peak of *m/z* 155 and a molecular ion peak of *m/z* 170, suggested it to be 1-naphthylmethylketone because these mass spectra characteristics were similar to those of 2-naphthylmethylketone (a metabolite of 2-ethylnaphthalene, vide infra) in the NIST library. Two additional metabolites with retention time of 5.29 min and 5.34 min and a base peak of *m/z* 157 were assigned as 1-ethylnaphthols. The metabolite with retention time of 6.65 min was not found in GC-MS analysis so assigned as unknown.

The 3 metabolites of 2-ethylnaphthalene eluting at 2.64 min, 3.04 min and 3.08 min were characterized as dihydro-2-ethylnaphthalenediols with a molecular ion peak of *m/z* 190. The metabolite of 2-ethylnaphthalene with retention time of 3.91 min showed a base peak of *m/z* 127 and a molecular ion peak of *m/z* 172 in the mass spectra, while the metabolite co-eluted with the reference compound 2-hydroxyethylnaphthalene on UPLC and its MS spectrum also matched the spectrum of 2-hydroxyethylnaphthalene in the NIST library. An additional metabolite of 2-ethylnaphthalene was detected at the GC with retention time of 5.5 min. This metabolite was not observed in the UPLC chromatogram. A molecular peak of *m/z* 170 and a base peak of *m/z* 127 of this additional metabolite observed in the mass spectrum, matched the mass spectrum of 2-naphthylmethylketone in the NIST library.

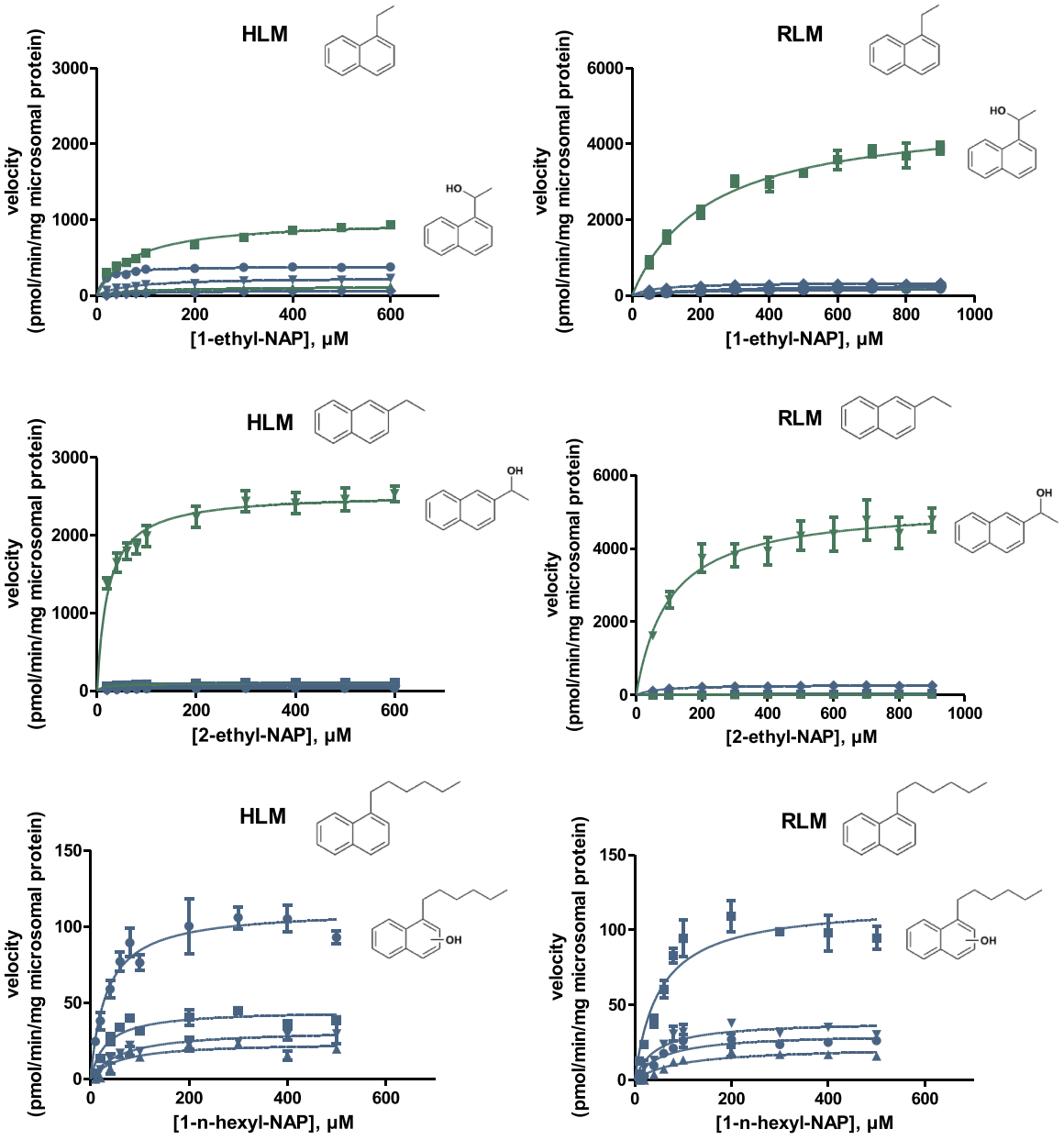



**Fig. 1.** (*continued*)

The metabolite with retention time of 6.66 min was not found in GC-MS analysis so assigned as unknown.

Four minor metabolites of 1-n-hexylnaphthalene with retention times ranging from 5.95 min to 6.38 min on UPLC were tentatively ascribed to different forms of 1-hexylnaphthol because of their molecular ion peak at *m/z* 228, and a fragmentation pattern similar to other naphthols with high intensity peaks at *m/z* 157.

### 3.2. Intrinsic clearance by aromatic and alkyl side chain oxidation

Based on the kinetic data obtained, the intrinsic clearance by formation of aromatic ring versus alkyl side chain oxidation metabolites of each test compound was calculated by adding up the $Cl_{int}$ values of the respective metabolites, and the data thus obtained are presented in Fig. 2. The intrinsic clearance of naphthalene via aromatic ring oxidation metabolites, including 1-naphthol and dihydrodiol with HLM was 64.74 μl/min/mg protein. In comparison, the intrinsic clearance via aromatic ring oxidation of 1-methylnaphthalene, 2-methylnaphthalene, 1-ethylnaphthalene, 2-ethylnaphthalene and 1-n-hexylnaphthalene by HLM were 1.6–12.7 fold lower than that for naphthalene, indicating that metabolites resulting from aromatic ring oxidation were formed relatively less efficient for alkyl substituted naphthalenes. For 1-methylnaphthalene, 1-ethylnaphthalene, 2-methylnaphthalene and 2-ethylnaphthalene this decreased intrinsic clearance via formation of metabolites resulting from aromatic ring oxidation was accompanied by a significant increase in intrinsic clearance via alkyl chain oxidation metabolites with HLM. For 2-methylnaphthalene and 2-ethylnaphthalene the intrinsic clearance via side chain oxidation even substantially exceeded that via aromatic oxidation by 3.4 and 16.8 fold, respectively.

In RLM incubations, the intrinsic clearance of naphthalene by metabolite formation from naphthalene was 11.62 μl/min/mg protein, which is 5.6 fold lower than the intrinsic clearance observed with HLM. Also, with RLM the intrinsic clearance via aromatic ring oxidation of 1-methylnaphthalene, 2-methylnaphthalene, 1-ethylnaphthalene, 2-ethylnaphthalene and 1-n-hexylnaphthalene was reduced, being 1.4–7.0 fold lower than that for naphthalene. In incubations with RLM,

alkyl chain oxidation dominated over aromatic ring oxidation for all alkyl substituted naphthalenes with the exception of 1-n-hexylnaphthalene.

### 3.3. Inhibition studies on the oxidative metabolism of naphthalene, 1-methylnaphthalene and 1-ethylnaphthalene

To study the remarkable difference in metabolism between rat and human metabolic activity the influence of CYP1A inhibition was determined in microsomal incubations of naphthalene, 1-methylnaphthalene and 1-ethylnaphthalene by coincubation with α-naphthoflavone, a specific inhibitor of CYP1A. It was shown that the formation of dihydrodiols in incubations with HLM decreased 11%–39% for the three test compounds inhibition of CYP1A (Fig. 2 and Table 1 both in supplementary materials), however no differences in dihydrodiol formation by RLM was observed. Alkyl side chain oxidation on 1-methylnaphthalene and 1-ethylnaphthalene was also inhibited 14%–28% by α-naphthoflavone in incubations with HLM, while little difference in alkyl side chain oxidation upon CYP1A inhibition by rat was observed with 0–3% increase compared to the control (see Table 2).

### 3.4. Effect of alkyl chain length on total intrinsic clearance

Fig. 3 shows the relationship between intrinsic clearance of the alkylated naphthalenes and the number of carbon atoms in their alkyl side chain. The total intrinsic clearance was calculated by adding up the $Cl_{int}$ values of all metabolites of the respective substrate.

The results thus obtained reveal that the total $Cl_{int}$ of the alkylated naphthalenes by both HLM and RLM becomes less efficient with increasing side chain carbon length especially when the number of carbons in the side chain is 6 or higher (Fig. 3). The $Cl_{int}$ of 1-n-hexylnaphthalene (C6) was substantially lower than that of unsubstituted, methyl (C1)- or ethyl (C2)- substituted naphthalene, while 1-n-dodecylnaphthalene (C12) was not converted to a detectable level.

## 4. Discussion

In the present study the biotransformation of the model compounds naphthalene and six of its alkylated analogues was studied in human and rat liver microsomal incubations to better characterize the effect of alkyl substitution on the metabolism of these aromatic hydrocarbons. This is of interest because of safety concerns regarding the presence of substituted aromatic hydrocarbons in mineral oils, and the lack of data on consequences of alkyl substitution on their metabolic fate. It was expected that alkylation would result in substantial side chain oxidation at the cost of aromatic ring oxidation. For PAHs such a metabolic switch from aromatic to side chain oxidation would shift metabolic patterns in favor of detoxification at the cost of potential bioactivation. In line with this hypothesis, alkyl chain substitution resulted in a marked reduction in the intrinsic clearance via aromatic ring oxidation as shown by the 3- to 17- fold reduction in $Cl_{int}$ via aromatic ring oxidation as compared to this $Cl_{int}$ for the naked parent compound naphthalene. This reduction in $Cl_{int}$ values for aromatic oxidation was accompanied by a substantial increase in intrinsic clearance via side chain oxidation (Fig. 2). This increase in alkyl side chain oxidation at the cost of aromatic ring oxidation by cytochromes P450 is in line with thermodynamic considerations which predict that alkyl side chains attached to aromatic rings are more easily oxidized than the aromatic ring itself. This holds especially for the carbon atom at the benzylic position, since the radical resulting after hydrogen abstraction by the reactive high valency iron oxo intermediate of the cytochrome P450 enzyme catalyzing the reaction will be resonance stabilized by the aromatic ring. This consideration is in line with the observation that for ethyl substituted naphthalenes alkyl oxidation occurred preferentially at the benzylic carbon of the side chain. For 1-n-hexylnaphthalene side chain oxidation was not observed, which is most likely due to steric hindrance by the C6 alkyl

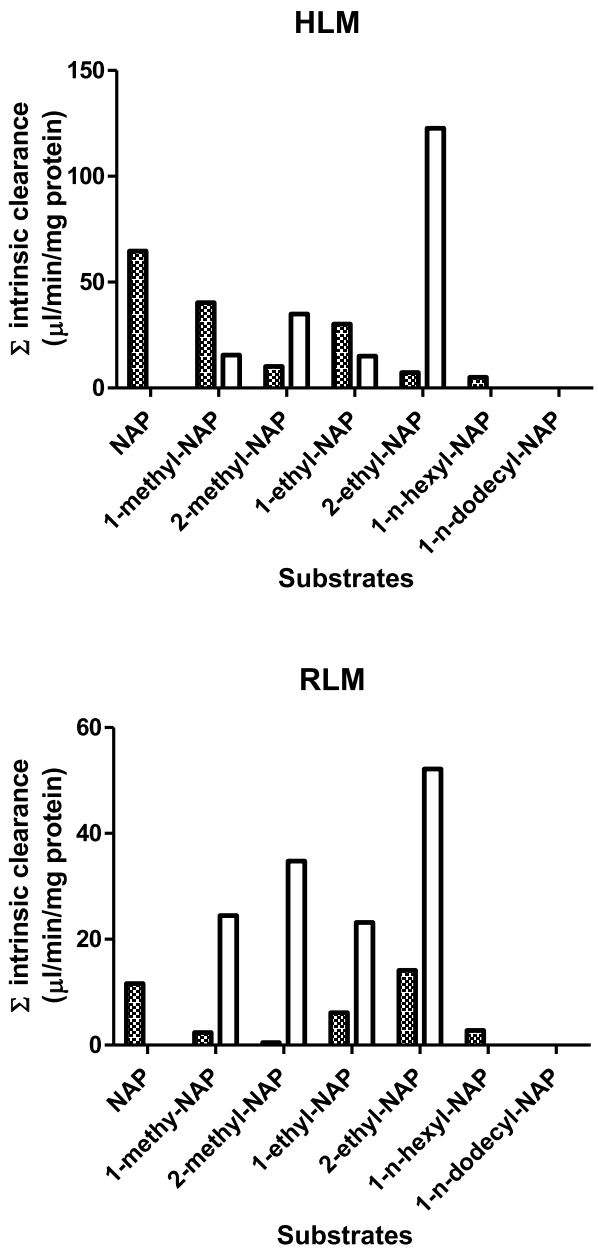



**Fig. 2.** Intrinsic clearance via aromatic ring oxidation and alkyl chain oxidation by HLM and RLM for the different model compounds. ▩ Aromatic ring oxidation; ▭ alkyl chain oxidation. Abbreviation. NAP = naphthalene.

**Table 2**
Inhibition of metabolic activity of aromatic ring and alkyl chain oxidative metabolites of naked and substituted naphthalenes by α-naphthoflavone. The results are expressed by percentage of control activity ± SD. Detailed results of each metabolite can be found in Table 1 and Fig. 2 both in the supplementary material.

| Substrate | Species | Aromatic ring oxidation (%) | Alkyl chain oxidation (%) |
|---|---|---|---|
| naphthalene | Human | 75 ± 3 | 0 |
| | Rat | 114 ± 20 | 0 |
| 1-methyl-naphthalene | Human | 87 ± 5 | 86 ± 6 |
| | Rat | 94 ± 2 | 100 ± 11 |
| 1-ethyl-naphthalene | Human | 86 ± 1 | 72 ± 6 |
| | Rat | 99 ± 3 | 103 ± 13 |

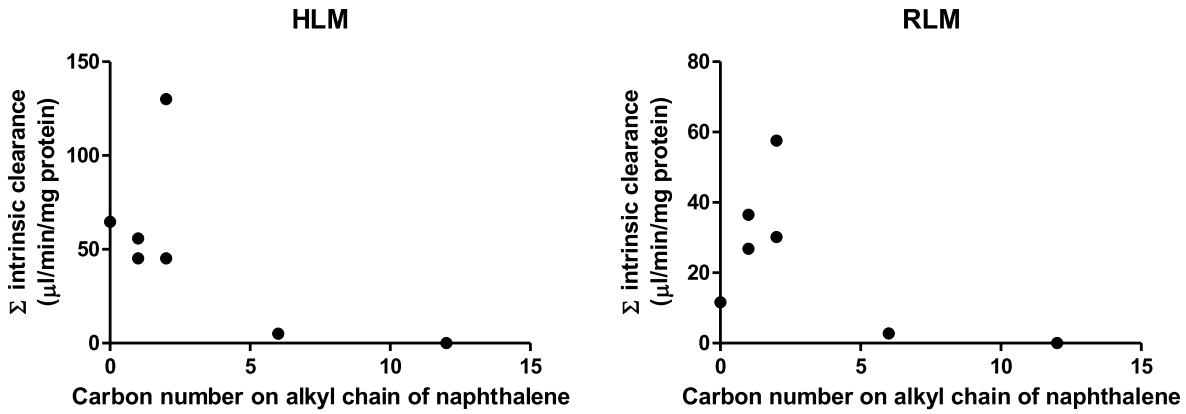



**Fig. 3.** Relationship between total $Cl_{int}$ of alkylated naphthalenes and the number of carbon atoms in the alkyl side chain. The intrinsic clearance was calculated by adding up the $Cl_{int}$ values of all metabolites for the respective model compound (Table 1).

side chain positioning the substrate within the active site of the respective P450s in such a way that the position can no longer be attacked by the activated heme cofactor. This steric hindrance may also explain the substantial reduction in total $C_{lint}$ with increasing alkyl chain length, resulting in substantial reduction and even no detectable substrate conversion for 1-n-hexylnaphthalene and 1-n-dodecylnaphthalene respectively.

This substantial reduction in intrinsic clearance when the length of the alkyl chain amounted to 6 or more carbon atoms is in line with data reported in a study on rat hepatic metabolism of n-alkanes which also showed that metabolic conversion decreased with increasing carbon number [8]. This study on n-alkanes reported that metabolism in rat liver microsomal incubations was no longer observed with tetradecane (C14), which is in line with the results obtained in this study for 1-n-dodecylnaphthalene (C12), especially when considering that also the carbon atoms in the naphthalene moiety would contribute to the size of the substrate dimensions. Based on these results it is tempting to speculate that alkyl substituents of substantial chain length will also prevent metabolic conversion and thus bioactivation of alkylated substituted PAHs like phenanthrene and benzo[a]pyrene, when present in mineral oils.

The observations of the present study also match the limited data available in the literature on metabolism of alkylated naphthalenes. In rat studies, it was found that the metabolic oxidation of 2,6-diisopropylnaphthalene happens on the isopropyl side chain [9,10]. 1-Methylnaphthalene, 2-methylnaphthalene, 1-ethylnaphthalene, 2-ethylnaphthalene, 2,7-dimethylnaphthalene were oxidized on the alkyl chain to alcohol metabolites in vitro by P450 CYP101B1 obtained from the bacteria *Nocardioides aromaticivorans* [5]. Furthermore, the metabolic oxidation of 2-methylnaphthalene and 1,6-dimethylnaphthalene by liver P450 enzymes from mice and rats respectively resulted in the formation of both aromatic ring oxidation dihydrodiols and side chain oxidation hydroxyl metabolites [11,12]. In general, upon microsomal conversion, naphthalene and its alkylated congeners are oxidized to dihydrodiols or alcohols. These metabolites are expected to be subsequently modified by phase II enzymes to glucuronides, sulfates and UDP glucuronic acid, followed by their excretion from the body [13].

The results obtained in the present study also reveal that the metabolic conversion of the alkylated naphthalenes is not fully dominated by chemical reactivity alone, since some substrate and species-specific differences were observed pointing at an additional influence of positioning of the substrate within the active site of the P450 involved. Thus, results obtained with HLM were different from those obtained with RLM for 1-methylnaphthalene and 1-ethylnaphthalene for which the preferential side chain over aromatic ring oxidation was only observed with RLM. The outcome of the inhibition study also suggests that isoforms play an important role in dihydrodiols formation in humans but less so in rats. For the series of alkyl substituted naphthalenes as a whole, however, alkyl oxidation could easily compete with aromatic hydroxylation and shown by $Cl_{int}$ values that were 0.4- to 16.8-fold and 2.8- to 20.8-fold higher than the $Cl_{int}$ values for aromatic hydroxylation for HLM and RLM, respectively.

Together the results of the present study support the conclusion that alkyl substitution of naphthalene results in a shift in metabolite clearance in favor of side chain oxidation at the cost of aromatic hydroxylation, while alkyl substituents with 6 or more carbon atoms may seriously reduce metabolic conversion. These conclusions are based on results obtained with a series of naphthalene model compounds. It remains to be established whether similar results would be obtained when studying the metabolism of more mineral oil relevant naked PAHs and their alkyl substituted analogues, especially including aromatic hydrocarbons with more than 3 aromatic rings. This provides an interesting topic for further research. It is expected, but remains to be demonstrated, that alkyl substituents on more “mineral oil relevant PAHs” will show similar metabolic shifts in their oxidation pattern. However, in case of PAHs with 3 or more rings, also the position of the aromatic rings with respect to one another will become a factor that may affect metabolism and toxicity. For example the toxicity of some PAHs may relate to the presence of a so-called “bay region” [14]. For alkylated PAHs this could mean that they may become more and not less toxic than their naked analogues especially when the alkyl chain would form extra (fake) bay regions thus providing extra options for formation of DNA reactive bay-region diol epoxide metabolites. Also, the species differences in metabolic clearance via aromatic ring or side chain oxidation between human and rats remains to be explored to a further extent. To evaluate the consequences of the structure of the substrates for the vivo biotransformation patterns taking also subsequent conjugation reactions into account, future studies may use cellular in vitro models and/or develop physiologically based kinetic models that describe in vivo kinetics, elucidating the consequences of our in vitro findings for the in vivo metabolite patterns in both rat and human. Finally, the Michaelis Menten parameters obtained in the present study provide a basis for development of physiologically based kinetic (PBK) models for studies on the consequences of alkylation of aromatic hydrocarbons on the metabolic clearance and bioactivation of this important group of constituents present in mineral oils. The present study and further research will also serve as a basis for better risk assessment of mineral oil.

Nevertheless, based on the results of the present study, the main conclusion is that alkylation of PAHs likely reduces their chances on aromatic oxidation and potential bioactivation.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: P.J.B. is employed by Shell International, a member company of Concawe, and chairman of the toxicology group of Concawe. Prof. P.J.B. is totally free (by contract) to freely design and conduct research and express his own scientific opinion without any obligation towards either Shell or Concawe. The current findings are not intended to constitute any product endorsement.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2019.108905.

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