Supporting Information to:

The Inhibition of Bone Resorption in Rats Treated with (−)-Menthol is Due to its Metabolites

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Materials and Methods

Collection of rat plasma and urine
To study \((\text{(-)}\)-menthol single-dose pharmacokinetics and metabolism, 11 male Wistar Hanlbm rats from RCC Ldt. (Füllinsdorf, Switzerland) received 100 mg of \((\text{(-)}\)-menthol (dissolved in 0.5 mL of almond oil) by gavage, after a fasting period of 20 h. After anaesthesia, 8-mL blood samples were collected by aortic puncture in glass vials containing 1000 units of heparin in 200 \(\mu\)L of 0.9% sodium chloride 0.5, 1, 2, 4, 8, and 24 h after \((\text{(-)}\)-menthol administration (1 or 2 rats per time-point). The blood samples were centrifuged immediately (10 min, 800 \(\times\) \(\text{g}\), 4 °C), and the plasma stored at \(-20\) °C until GC/MS analysis.

To study \((\text{(-)}\)-menthol multiple-doses pharmacokinetics and metabolism, 40 rats received 100 mg of \((\text{(-)}\)-menthol (dissolved in 0.5 mL of almond oil) by gavage on 4 consecutive days (at 24-h intervals). On the first 3 days, the rats had access to food (SoDi 2160; Kliba-Mühlen; Kaiseraugst, Switzerland; 23 g wet food = 13.1 g dry matter) right after the administration of \((\text{(-)}\)-menthol. On the fourth day no food was given and blood samples were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 h after \((\text{(-)}\)-menthol administration (4 rats per time-point).

Ten 10-week-old rats (each about 250 g BW) were transferred into individual metabolic cages. They received their food portion complemented with or without 100 mg of \((\text{(-)}\)-menthol (in 0.1 mL of almond oil) once a day for 10 days (\(n = 5\) in each group). All the rats ingested their food within 1 h after it was placed in the cage. The 24-h urine of each rat was collected in ice-cooled bottles. The 24-h urines from day 1, 2, 3, 4, 6, and 10 were collected to quantify \((\text{(-)}\)-menthol and its metabolites. For the extraction of metabolites for the \textit{in vitro} bone resorption assay, the daily urines of the control and treated animals, respectively, of the day 5, 7, 8, and 9 were pooled and the volume adjusted to 100.0 mL with bidistilled water. Urine samples were stored at \(-20\) °C until further processing.

Extraction of neutral urine metabolites
To test the effect of \((\text{(-)}\)-menthol metabolites \textit{in vitro}, urine extracts were prepared...
from urine of rats, which received (−)-menthol (= “metabolite extract”) and of control rats that did not receive (−)-menthol (= “blank extract”). An equivalent of a 24-h urine was diluted 1:1 with bidistilled water, then 10,000 units of β-glucuronidase were added, and the pH was adjusted to 6.8. The sample was filtered using a sterile syringe filter (pore size 0.2 µm) to reduce the risk of microbiological growth during the following incubation at 37 °C for 24 h. The completeness of the hydrolysis of the glucuronides was verified by GC/MS. The sample (at pH 6.8) was loaded onto a large volume extraction cartridge, Oasis® HLB (35 mL/6 g; Waters; Rupperswil, Switzerland) preconditioned with methanol and bidistilled water. The cartridge was washed with 2% ammonium hydroxide/5% methanol (120 mL) to remove acidic metabolites, and then with bidistilled water (20 mL). The neutral metabolites were eluted with methanol (90 mL). Methanol was evaporated at 40 °C/150 mbar. The extracts were determined by GC/MS and stored at -20°C until the in vitro testing.

**GC/MS analysis of (−)-menthol metabolites in plasma and urine**

Aliquots (500 µL) of plasma or urine (diluted 1:25 with water) were spiked with 10 µg of hexahydromandelic acid (100 µg/mL methanol; internal standard), and 0.4 mL of a 0.5 M phosphate buffer (pH 6.8) without β-glucuronidase (= unconjugated metabolites) or with 1000 units of β-glucuronidase (= total amount of unconjugated and conjugated metabolites). Enzymatic hydrolysis was performed at 37 °C for 16 h. The samples, acidified with 40 µL of phosphoric acid, were applied to Oasis® HLB extraction cartridges (3 mL/60 mg; Waters), preconditioned with methanol and bidistilled water. The cartridges were washed with 5% methanol (3 x 1 mL) before elution with methanol (3 x 0.5 mL). Methanol was evaporated under a stream of nitrogen at 20 °C. The residues were redissolved in 120 µL of acetonitrile, and then 30 µL of a mixture of BSTFA, TMCS, and TMSI (2:1:1) was added for silylation (1 h at 20 °C). A 1-µL aliquot of the sample was then injected into the GC/MS. The apparatus consisted of an HP 5890A series II GC, an HP 5972 mass selective detector (MSD), and HP 5895A ChemStation software. Separation was performed on a DB-1 capillary column (30 m x 0.25 mm ID, 0.25-µm film, J&W Scientific) with helium as carrier gas at 1.1 mL/min. Injector port and interface transfer line were maintained at 250 and 280 °C, respectively. The oven temperature was set as follows: 100 °C for 1.0 min, at 5 °C/min to 170 °C, 170 °C for 5.0 min, at 5 °C/min to
200 °C, at 20 °C/min to 280 °C, 280 °C for 5 min. The MS system was used in the total ion current mode ($m/z = 50$ to 550). Besides M-1, no other standards of (−)-menthol metabolites were commercially available. Therefore, M-1 was used as calibrator for all the metabolites, although this may lead to some overestimation mainly of the metabolites that showed oxidation at more than one position. Twenty-one metabolites were quantified, while 9 minor metabolites could not be quantified because of chromatographic interferences. The intra- and interday precision was ≤ 12.4% and 20.9%, respectively, measured at the limit of quantification (LOQ), and ≤ 8.3% and 11.3%, respectively, at higher concentrations. The LOQ was 0.05 and 1.25 µg/mL for plasma and urine, respectively.

**Calvaria assay**

Conditions for the experiments with (−)-menthol: Stock solutions of (−)-menthol were prepared in absolute ethanol. They were diluted 1,000-times with the culture medium resulting in 15.6, 52, and 156 µg/mL of (−)-menthol. Media of the corresponding control group were supplemented with an equivalent of absolute ethanol. (−)-Menthol, being volatile and thus likely to evaporate from the culture medium and distribute in all the wells of a dish [1], [2], required the following modifications of the original protocol to better maintain its concentration and to avoid the contamination of the control medium. Calvaria were incubated in 0.5 mL of medium in 24-well culture dishes. For the control and each concentration of (−)-menthol separate culture dishes were used. The bone explants were placed in the central wells and all wells of the culture dish were filled with the corresponding medium. Medium was changed daily.

**References**