Myrtucommulone from *Myrtus communis*: Metabolism, Permeability, and Systemic Exposure in Rats

### Abstract

Nonsteroidal anti-inflammatory drug intake is associated with a high prevalence of gastrointestinal side effects, and severe cardiovascular adverse reactions challenged the initial enthusiasm in cyclooxygenase-2 inhibitors. Recently, it was shown that myrtucommulone, the active ingredient of the Mediterranean shrub *Myrtus communis*, dually and potently inhibits microsomal prostaglandin E$_2$ synthase-1 and 5-lipoxygenase, suggesting a substantial anti-inflammatory potential. However, one of the most important prerequisites for the anti-inflammatory effects *in vivo* is sufficient bioavailability of myrtucommulone. Therefore, the present study was aimed to determine the permeability and metabolic stability *in vitro* as well as the systemic exposure of myrtucommulone. In vivo, myrtucommulone undergoes extensive phase I metabolism in human and rat liver microsomes, yielding hydroxylated and bihydroxylated as well as demethylated metabolites. Physiologically-based pharmacokinetic modeling of myrtucommulone in the rat revealed rapid and extensive distribution of myrtucommulone in target tissues including plasma, skin, muscle, and brain. As the development of selective microsomal prostaglandin E$_2$ synthase-1 inhibitors represents an interesting alternative strategy to traditional nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors for the treatment of chronic inflammation, the present study encourages further detailed pharmacokinetic investigations on myrtucommulone.

### Abbreviations

- COXIBs: cyclooxygenase-2 inhibitors
- HBSS: Hanks buffered salt solution
- HLM: human liver microsomes
- LC: liquid chromatography
- 5-LO: 5-lipoxygenase
- mPGES: microsomal prostaglandin E$_2$ synthase
- MC: myrtucommulone
- MRM: multiple-reaction monitoring
- NSAIDs: nonsteroidal anti-inflammatory drugs
- P$_{app}$: apparent permeability coefficient
- PBPK: physiologically-based pharmacokinetic modeling
- Pgp: P-glycoprotein
- RLM: rat liver microsomes
- TEER: transepithelial electrical resistance
- UGT: uridine glucuronosyltransferase
- DMEM: Dulbecco’s modified Eagle’s medium

### Introduction

Being far from a real breakthrough in the discovery of safe NSAIDs [1], interest in alternative well-tolerated anti-inflammatory herbal remedies is growing.

Myrtle (*Myrtus communis*, Myrtaceae) is a Mediterranean shrub applied as a culinary spice and flavoring agent for alcoholic beverages [2, 3]. In traditional folk medicine it has been used as an antiseptic, antidiabetic, and anti-inflammatory remedy. Scientific studies support the antibacterial [4], antihyperglycemic [5, 6], analgesic [7], and antioxidant [8–10] properties. Recently, anti-proliferative, antibacterial, and anti-inflammatory effects of the ethanolic myrtle extract Myrta-
cine in keratinocytes were reported, suggesting a therapeutic value in the treatment of acne [11]. It was shown that MC (© Fig. 1) suppresses the biosynthesis of eicosanoids by direct inhibition of key enzymes like COX-1 and 5-LO in cell-free and cell-based models [3]. Moreover, MC inhibits the mPGES-1-mediated conversion of PGH2 to PGE2 in cell-free assays [12] as well as in intact A549 cells and in human whole blood [12]. In vivo, i.p. administration of MC (0.5, 1.5, and 4.5 mg/kg) reduced the development of mouse carrageenan-induced paw edema in a dose-dependent manner and exerted anti-inflammatory efficacy in the pleurisy model in mice [13]. However, no availability studies have been performed yet in animals or humans. Since poor absorption and/or extensive metabolism may play a crucial role in limiting systemic availability, the present study aimed to determine the permeability of MC in Caco-2 cells and its metabolic stability in rat and human liver microsomes as well as its oral availability in a pilot rat study. Moreover, PBPK was applied to estimate the distribution of MC in potential target tissues.

Material and Methods

Standards and reagents
MC with a purity of 99% was synthesized by Prof. Johann Jauch, University of Saarland, Dept. of Organic Chemistry, Saarbruecken, Germany [14]. Hyperforin dicyclohexyl ammonium salt (purity 99%), used as an internal standard, was supplied by Dr. W. Schwabe Arzneimittel GmbH. Other reagents included tetrahydrofuran (gradient grade; Acros Organics SA), acetonitrile for LC-MS (Sigma-Aldrich), distilled water, tert-butyl methyl ether, ethyl acetate (Merck), and ammonium formate (p.a.; VWR). Blank human plasma was obtained from Deutsches Rotes Kreuz-Blutspendedienst, Mannheim, Germany. Pooled HLM, RLM, NADPH-regenerating solutions A and B, and UGT reaction mix solutions were purchased from BD Biosciences.

Caco-2 cells were seeded on Transwell® polycarbonate inserts with a mean pore size of 0.4 µm (Corning Incorporated) and were grown in DMEM, containing 25 mM glucose supplemented with 10% FCS, 1% nonessential amino acids, and gentamycin (0.1 mg/mL). All media and HBSS were purchased from Biochrom AG.

Permeation of MC was investigated in both directions at 37°C with and without verapamil (50 µM) and additionally at 4°C in the apical-to-basolateral (AB) direction (n = 6 each).

Prior to each experiment, Caco-2 monolayers were washed with HBSS. TEER was measured before and after the transport experiments. FITC-Dextran (MW 4400 g/mol) was used to gauge the integrity of the monolayers. Transports with propranolol hydrochloride (n = 3) served as a positive control for the functionality of every cell passage. During incubation, the plates were agitated at 37°C on a shaker at 120 rpm. Receiver fluid was withdrawn after 15, 30, 60, and 90 min and replaced by equal volumes of fresh buffer solution. At the end of the experiment, samples were collected from both sides, and the plates were washed three times with pure HBSS followed by methanol. Then, each transwell filter was cut off, placed in 0.5 mL methanol for 15 min and vortexed in order to lyse the cells. All samples were stored at −20°C. For analysis, an aliquot of 150 µL was mixed with equal amounts of acetonitrile containing the I.S. to yield a final concentration of 10 µg/mL MC and 1% DMSO. This MC concentration was chosen to allow its detection at the basolateral side even in case of poor permeability. For testing the interaction of MC with P-glycoprotein (Pgp), the Pgp substrate verapamil (50 µM) was added to the working solutions. Permeation of MC was investigated in both directions at 37°C with and without verapamil (50 µM) and additionally at 4°C in the apical-to-basolateral (AB) direction (n = 6 each).

Calculation of permeability coefficients
The permeability coefficient (cm/s) was calculated by the following equation:

\[ P_{\text{app}} = \frac{\Delta C}{\Delta t} \times \frac{V}{A} \]
where \( \frac{dc}{dt} \) is the flux rate (\( \mu g/(mL \times s) \)) through the monolayer; \( V_r \) is the volume of the receiver chamber (mL); \( A \) is the surface area of the cell monolayer; and \( C_0 \) is the initial concentration of the donor fluid (\( \mu g/mL \)).

Animal study

The rat study was approved by the local ethical committee on the 2nd November 2009 and given the approval number 2009/0017055. Male Wistar Han rats (250 g; Harlan) were provided with standard rodent chow and water in a controlled environment. Animal care complied with Italian regulations on protection of animals used for experimental and other scientific purposes (Ministerial Decree 116192) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986).

Following an overnight fast, 4 mg/kg MC (0.5 mL of a MC-solution 2 mg/mL) were orally administered to three rats. The control group (n = 3) received vehicle (0.5 mL) consisting of 0.5% carboxymethylcellulose and 10% Tween-20. Food was allowed 4 h after MC administration. Blood samples were taken from untreated rats 1, 4, 8, 14, 24, and 48 h after MC administration and from vehicle-treated rats 4 h after vehicle administration. Approximately 4 mL blood were collected by intracardiac puncture into citrate tubes (0.1 M). Blood samples were centrifuged, and the supernatants (plasma fraction) were stored at \( -20 \)°C until analysis.

Sample preparation of rat plasma samples

Based on the LC-MS/MS method described elsewhere [15] demonstrating cross-validation between rat and human plasma, calibration samples were prepared by spiking 1 mL of human plasma with 50 \( \mu L \) of the particular spike solution to achieve concentrations of 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 75.0, and 100.0 ng/mL of MC. Accordingly, 1 mL of thawed plasma samples was spiked with 50 \( \mu L \) acetonitrile. Furthermore, 50 \( \mu L \) of the U.S. solution containing 100 ng internal standard were added to all samples.

Following extraction for 25 min with 5 mL 20% ethyl acetate in tert-butyl methyl ether in a horizontal position on a flatbed mixer and centrifugation for 15 min (2500 g, 10°C), the organic layer was evaporated to dryness, and the residue was dissolved in 100 \( \mu L \) mobile phase. Then, the samples were sonicated for another minute and centrifuged for 5 min (2500 g, 10°C). Finally, 50 \( \mu L \) of the supernatant were injected into the LC-MS/MS system. Samples showing a concentration > 100 ng/mL were diluted 1 : 10 in order to fit the validated concentration range.

LC-MS/MS

Liquid chromatography was performed on an Agilent 1200 series with a Gemini C6 phenyl column (Phenomenex), 250 × 4.6 mm.i.d.; 5 \( \mu m \) and a Gemini C6 phenyl security guard cartridge (Phenomenex) 4 × 3 mm at a flow rate of 0.6 mL/min and column temperature of 40°C in a run time of 15 min. The mobile phase consisted of acetonitrile:water (85:15 v/v) containing 6 mM ammonium phosphate solution pH 3.0, (60:40, v/v). Samples were detected by a photo diode array detector (Agilent Technologies) at 230 nm. Sample concentration was calculated using the Mass Hunter software.

Pharmacokinetics and PBPK modeling

Basic pharmacokinetic parameters (peak concentrations, \( C_{max} \); concentration peak time, \( t_{max} \); area-under-the-curve, AUC\(_{0-\infty} \); and elimination half-life, \( t_{1/2} \)) for MC were determined by the statistical software package BiAS (v10.0). The PKSim (version 4.1; Bayer Technology Services GmbH) algorithm was utilized for the present PBPK model in the rat [17, 18]. Based on the lipophilicity and solubility of MC blood flow, limited partitioning was assumed. Initial inputs to the model for MC included molecular weight (668.4 D), calculated LogP of 4.5 (based on structure), measured a pKa of 5, measured aqueous solubility of 90 \( \mu M \), measured protein binding of 99.9% (using ultracentrifugation), and an estimated oral clearance of 4.7 L/h/kg based on the mean concentration-time data obtained in this study.

Results and Discussion

MC undergoes extensive phase I metabolism in human liver microsomes. Thus after 15 min of incubation with HLM, more than 50% of the initial MC was metabolized, and around 10% remained after 120 min. In RLM, phase I metabolism also occurred at an extensive but overall slower rate with nearly 100% of the initial MC concentration being detectable after 15 min, decreasing to 70% after 30 min, ending up with 45% after 120 min incubation time. Just as the control incubations without cofactors, the glucuronidation experiments showed no decrease in MC concentration, suggesting that MC is not susceptible to glucuronidation (data not shown).

Screening of the incubation solutions in the full-scan modus revealed various metabolites with mass shifts of +16, +32, and...
−14 u compared to MC that could not be detected in the control samples (Fig. 2). They were identified on the basis of their comparable fragmentation pattern with MC (Fig. 3). Product ion scans were performed to confirm the full scan results (Fig. 4). Ion masses of m/z 698.4 (MC + 2OH), 683.2 (MC + OH), 667.5 (MC), 653.4 (MC − CH$_3$), 623.4 (MC − C$_3$H$_7$) were chosen as precursor ions. Control samples revealed only the peaks at t = 4.1 min and 8.1 min (MC). Both the peak at 4.1 min and the peak at 5.6 min in the incubation solution did not yield the characteristic MC fragmentation pattern, indicating no relation to MC (peaks No. 1 and 2 in Table 1). The other peaks showed one or more characteristic fragments of MC indicating a link to the parent compound (Table 1). Peaks No. 3 and 5 yielded the parent compound, suggesting the formation of some polar metabolites that are obviously unstable in the mass spectrometer fragmenting immediately to MC. Hence, it may be concluded that phase I metabolism results in hydroxylated, bihydroxylated, and demethylated metabolites and some other minor polar compounds. Screening of plasma samples from MC-treated rats confirmed the presence of these metabolites and the absence of glucuronidated conjugates. Moreover no sulphated conjugates could be identified in rats, suggesting that MC is mainly subject to phase I but not to phase II metabolism.

An almost linear transport of MC was observed across the Caco-2 monolayer (Fig. 5), the validity of which was proven by TEER values > 250 Ω cm$^2$, the impermeability of FITC-dextran, and Papp values of propranolol (34.66·10$^{-6}$ cm/s) corresponding to the literature [19, 20]. In the AB direction, MC was highly permeable with Papp values of 35.9·10$^{-6}$ cm/s at 37°C and 14.1·10$^{-6}$ cm/s at 4°C. The addition of verapamil at 37°C resulted in a Papp value of 14.0·10$^{-6}$ cm/s, indicating that MC is not a Pgp substrate as the MC concentrations in the receiver compartment were not increased. The lower Papp value at 4°C suggests that active transport (sensitive to temperature) may play a role in the absorption of MC. As expected, Papp values were lower in the basolateral-to-apical BA direction than in the absorptive direction. At 37°C, the BA Papp values were determined to be 3.1·10$^{-6}$ cm/s and 4.4·10$^{-6}$ cm/s upon the addition of verapamil, indicating that MC is not subject to secretion processes. Around 29.8–53.9% of the initial MC concentration was detected in the cells in the absorptive and 5.3–9.2% in the secretory direction. However, because of the high Papp value in the absorptive direction, accumulation in the cells and/or in the filter membrane was not paid further attention. The observed high MC permeability, in spite of its relatively high MW (667 Da) and low aqueous solubility of 90 µM, may be thus attributed to its lipophilicity and possible participa-
tion of active transport carriers in absorption. According to Yee, $P_{\text{app}}$ values > $10^{-6}$ cm/s reflect high absorption in vivo [20, 21]. Hence it may be assumed that MC, not being subject to active efflux mechanisms, might be well absorbed in humans. After oral administration of 4 mg/kg MC to rats, maximum plasma concentrations of 190.0, 182.7, and 403.3 ng/mL were consistently attained at 1 hour post-dose. The areas under the plasma concentration curve AUC$_{0-48}$ (calculated using the trapezoidal rule in SigmaPlot v11.0) were 658.3, 548.1, and 1646.9 ng/mL*h, respectively. Parameter estimates obtained from the mean concentration-time curve as depicted in Fig. 6 (top) are C$_{\text{max}}$ of 258.6 ng/mL, t$_{\text{max}}$ of 1 h, and a t½ of 10.3 h. The achieved plasma levels exceeded the expectations regarding the availability of a non-soluble, highly lipophilic, metabolically unstable drug and may be attributed to the high permeability of MC observed in the Caco-2 model. Based on the applied dose and the resulting mean plasma concentration-time curve, a clearance (CL/F) of 4.7 L/h/kg, and a volume of distribution (Vd/F) of 70.1 L/kg may be assumed. The calculated clearance is in line with the extensive metabolism observed for MC in vitro. Furthermore, the high vol-

### Table 1 Overview of MC metabolites (product ion scan).

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Retention time (min)</th>
<th>Product ion</th>
<th>Characteristic fragment</th>
<th>Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.1</td>
<td>483.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>5.6</td>
<td>667.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
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<td>667.5</td>
<td>431.5</td>
<td>unknown</td>
</tr>
<tr>
<td>4</td>
<td>5.8</td>
<td>653.4</td>
<td>415.4</td>
<td>demethylation</td>
</tr>
<tr>
<td>5</td>
<td>5.9</td>
<td>667.5</td>
<td>429.0</td>
<td>unknown</td>
</tr>
<tr>
<td>6</td>
<td>6.1</td>
<td>653.4</td>
<td>416.9</td>
<td>demethylation</td>
</tr>
<tr>
<td>7</td>
<td>6.25</td>
<td>623.4</td>
<td>428.6</td>
<td>demethylation</td>
</tr>
<tr>
<td>8</td>
<td>6.4</td>
<td>623.4</td>
<td>420.0</td>
<td>demethylation</td>
</tr>
<tr>
<td>9</td>
<td>6.6</td>
<td>683.2</td>
<td>446.6; 265.3</td>
<td>hydroxylation</td>
</tr>
<tr>
<td>10</td>
<td>6.7</td>
<td>652.1</td>
<td>249.2; 431.1</td>
<td>demethylation</td>
</tr>
<tr>
<td>11</td>
<td>6.9</td>
<td>626.2</td>
<td>195.1; 249.2; 431.1; 485.2</td>
<td>demethylation</td>
</tr>
<tr>
<td>12</td>
<td>7.0</td>
<td>625.9; 626.7</td>
<td>195.1; 431.1</td>
<td>demethylation</td>
</tr>
<tr>
<td>13</td>
<td>7.1</td>
<td>653.4</td>
<td>416.9</td>
<td>demethylation</td>
</tr>
<tr>
<td>14</td>
<td>7.2</td>
<td>698.1</td>
<td>195.1; 249.2; 431.1</td>
<td>dihydroxylation</td>
</tr>
<tr>
<td>15</td>
<td>7.3</td>
<td>697.6</td>
<td>431.1</td>
<td>dihydroxylation</td>
</tr>
<tr>
<td>MC</td>
<td>8.1</td>
<td>667.5</td>
<td>195.1; 249.2; 431.3; 485.2</td>
<td>parent compound</td>
</tr>
</tbody>
</table>

Fig. 4 Product ion scan of MC incubation solution (black line) in HLM containing NADPH-regenerating system in comparison to MC control sample (gray line) lacking cofactor solutions.

Fig. 5 Amount of MC (% of the initial concentration) transported over the time (0–90 min) under different conditions in the Caco-2 model.

Gerbeth K et al. Myrtucummulone from Myrtus communis... Planta Med 2012; 78: 1932–1938
to this model, a rapid and extensive drug distribution is suggested, which is underlined by the high volume of distribution determined for MC (Fig. 6, bottom). Notably, highly perfused tissues (e.g., liver) mimic the plasma concentration time course. Tissue exposures predicted for skin, muscle, and brain indicate that multicompartment kinetic behavior is likely and that some degree of accumulation in these organs is possible. It may be assumed that MC reaches a plateau in the skin and brain after single oral dosing that exceeds the plasma concentrations at the end of the dosing interval. This underlines the pharmacological effects of MC proposed in a keratinocyte model [11]. In contrast to skin, data on potential effects of MC on the central nervous system do not exist yet, but the observed accumulation of MC in brain tissue encourages further studies evaluating possible anti-inflammatory actions in the CNS. Of course, these simulations represent a preliminary projection of MC disposition in rats and should be confirmed by future biodistribution studies.

Against the background that selective mPGES-1 inhibitors represent an interesting alternative strategy to traditional NSAIDs and COXIBs for the treatment of chronic inflammation [22], the present study encourages further detailed pharmacokinetic investigations on MC.

**Conflict of Interest**

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Gerbeth K et al. Myrtucommulone from Myrtus communis... Planta Med 2012; 78: 1932–1938
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