Abstract: We investigated the effect of 21 flavonoids in a three-dimensional in vitro system for their ability to inhibit gap formation by MCF-7 breast cancer spheroids in monolayers of lymphendothelial cells. Different representatives of the classes of flavones, flavonols, and flavanones were tested in the circular chemorepellent-induced defects (CCID)-assay. Bay11-7082, a known inhibitor of CCID formation served as the positive control. This study provides the first comparison of the potential of flavonoids to suppress features influencing the intravasation of MCF-7 breast cancer cells aggregates through the lymph endothelial barrier. The most significant effects were seen after incubation with the flavones luteolin, chrysin, and apigenin. Additional hydroxylation or methoxylation in positions 6 or 8, as expected, resulted in decreased activity. The tested flavanones remained without or low efficacy.

Keywords: CCID assay; inhibition of gap formation; flavonoids; anti-invasive; breast cancer; structure-activity relationship

1. Introduction

Intravasation through the lymphatic border is an early- and rate-limiting step in the metastatic cascade of breast cancer [1–3]. Yet, hardly any anti-metastatic drugs are available. As from 1981 to 2014 approximately 83% of the approved small molecule anticancer drugs were natural products, based on natural compounds or mimicking them in different ways [4]. We tested traditional medicinal plants regarding their anti-intravasative properties [5–8]. In a study on the effects of different Scrophularia extracts on breast cancer cells’ proliferation, cell death, and intravasation through lymph endothelial cell barriers, a methanolic extract of Scrophularia lucida L. (Scrophulariaceae) proved particularly active in inhibiting intravasation in vitro [8]. An activity-guided fractionation of this extract revealed hispidulin, a member of the class of flavonoids, as significantly contributing to this activity [9].

Because of the multitude of structures and amounts of flavonoids in human nutrition, these compounds have gained a lot of interest for the prevention, deceleration of development, or even alleviation of different “Western Diseases” such as cardiovascular or neurodegenerative disorders as well as various cancers. For flavonoids in cancer chemoprevention numerous modes of action, modulation of a multiplicity of proteins and inhibition of many enzymes involved in cancer
pathology have been shown [10]. Among those, different mechanisms involving many steps in the “metastatic cascade” influencing cell detachment, cell mobility, tissue barriers for intravasation and extravasation, angiogenesis etc., were studied in detail and many molecular targets involved in the anti-invasive and anti-metastatic properties of flavonoids such as luteolin, apigenin, kaempferol among others have been identified [11].

Nevertheless, for an activity-guided search for effective natural anticancer compounds (e.g., flavonoids) inhibiting mechanisms of metastasis, robust in vitro tools simulating the in vivo situation are necessary. For this purpose 3D multicellular tumor spheroids as used in the validated “circular chemorepellent-induced defect” (CCID) assay provide an excellent possibility [12]. Until now, data of the efficacy of flavonoids in this assay are scarce and only few flavonoids have been tested on breast cancer intravasation [1,3,9,13–16]. Yet, the IC50 of hispidulin was too high to be reached in vivo [9]. Therefore, to gain insight into structural features influencing the anti-intravasative potential of flavonoids, we compared hispidulin to 20 other structurally closely related flavonoids in this 3D assay consisting of MCF-7 cell spheroids and lymph endothelial cell (LEC) monolayers.

2. Materials and Methods

2.1. Materials

Apigenin, naringenin, hispidulin, scutellarein, baicalein, quercetin, wogonin, and diosmetin were purchased from Sigma-Aldrich (Vienna, Austria); nepetin, oroxylin A from Phytolab (Vestenbergsgreuth, Germany); kaempferol, acacetin, galangin, chrysin, homoeriodictyol from Carl Roth GmbH (Karlsruhe, Germany); herbcacetin, pinocembrin, luteolin, eriodictyol, norwogonin from Extrasynthese (Genay, France); and gossypetin from Indofine Chemical Company (Hillsborough, UK). The I-κBα phosphorylation inhibitor (E)-3-[4-methylphenylsulfonyl]-2-propenenitrile (Bay11-7082) was obtained from Biomol (Hamburg, Germany). Stock solutions were prepared in dimethylsulfoxide (DMSO; 99.7% cell culture grade, Sigma-Aldrich (Vienna, Austria)).

2.2. Cell Culture

Human microvessel endothelial cells were purchased from Clonetics (Basel, Switzerland), immortalized with human telomerase cDNA (TERT1) [17], and subsequently lymph endothelial cell population was isolated by single cell cloning [18]. For this work, LECs were authenticated by analyzing expression of the lymph endothelial cell markers PROX1, LYVE-1, and podoplanin by immunofluorescence and laser scanning microscopy (Supplemental Figure S1). LECs were cultivated in microvascular endothelial cell growth medium-2 (EGM2 MV) (Clonetics CC-4147; Lonza Group, Ltd., Basel, Switzerland) [1,8,18]. The human MCF-7 cell line was obtained from ATCC (Rockville, MD, USA). Cells were grown in minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% non-essential amino acids (all from Life Technologies, Lofer, Austria). All cells were grown at 37 °C in a humidified atmosphere containing 5% CO2.

2.3. 3D Co-Cultivation of MCF-7 Cancer Cells with LECs

MCF-7 cells were transferred to 30 mL MEM medium containing 6 mL of 1.6% methylcellulose solution (0.3% final concentration; cat. no. M-512, 4000 centipoises; Sigma, Munich, Germany). A total of 150 µL of the cell suspension, each, were then transferred to the wells of a 96-well plate (Greiner Bio-one, Cellstar 650185, Kremsmünster, Austria) for spheroid formation within the following two days. The developed MCF-7 spheroids were washed in phosphate buffered saline and transferred to cytotracker-stained (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) LEC monolayers that had been seeded into 24-well plates (Costar 3524, Sigma, Vienna, Austria) in 2 mL EGM2 MV medium (Kerjaschki et al., 2011; Vonach et al., 2011).
2.4. Circular Chemo-Repellent Induced Defect (CCID) Assay

After transfer of the MCF-7 cell spheroids (3000 cells/spheroid) to the 24-well plate containing LEC monolayers, four hours of incubation of the MCF-7 spheroids-LEC monolayer co-cultures followed. The CCID sizes in the LEC monolayer underneath the MCF-7 spheroids were photographed with an Axiovert (Zeiss, Jena, Germany) fluorescence microscope to visualize cytotracker (green)-stained LECs [1]. CCID areas were calculated with the Axiovision Re. 4.5 software (Carl Zeiss). MCF-7 spheroids were treated with solvent (DMSO) as negative control. The CCID sizes of at least 12 spheroids per experiment were measured.

2.5. Statistical Analyses

All experiments were performed in triplicate and analyzed by One-way ANOVA together with Tukeys post-hoc test with GraphPad Prism 5.0 program (San Diego, CA, USA).

3. Results

For the determination of the efficacy of flavonoids on the inhibition of lymph endothelial disintegration in the CCID assay in total 18 flavonoids were compared (Table 1).

Table 1. Compounds tested in the circular chemorepellent-induced defects (CCID) assay (MCF-7 spheroids on lymph endothelial cell (LECs)), the respective IC₅₀ (µM) and calculated logP (o/w) values.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Formula</th>
<th>IC₅₀ µM</th>
<th>logP(o/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pinocembrin</td>
<td><img src="image" alt="Pinocembrin" /></td>
<td>n.a.</td>
<td>2.69</td>
</tr>
<tr>
<td>2</td>
<td>Naringenin</td>
<td><img src="image" alt="Naringenin" /></td>
<td>n.a.</td>
<td>2.38</td>
</tr>
<tr>
<td>3</td>
<td>Eriodictyol</td>
<td><img src="image" alt="Eriodictyol" /></td>
<td>n.a.</td>
<td>2.11</td>
</tr>
</tbody>
</table>
Table 1. Cont.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Formula</th>
<th>IC$_{50}$ μM</th>
<th>logP(o/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Homoeriodictyol</td>
<td><img src="image" alt="Homoeriodictyol" /></td>
<td>99.46 μM</td>
<td>2.37</td>
</tr>
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</table>

Flavones and Flavonols

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Formula</th>
<th>IC$_{50}$ μM</th>
<th>logP(o/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Chrysin</td>
<td><img src="image" alt="Chrysin" /></td>
<td>24.76 μM</td>
<td>2.84</td>
</tr>
<tr>
<td>6</td>
<td>Galangin</td>
<td><img src="image" alt="Galangin" /></td>
<td>39.61 μM</td>
<td>2.61</td>
</tr>
<tr>
<td>7</td>
<td>Baicalein</td>
<td><img src="image" alt="Baicalein" /></td>
<td>&gt;100 μM</td>
<td>2.49</td>
</tr>
<tr>
<td>8</td>
<td>Oroxylin A</td>
<td><img src="image" alt="Oroxylin A" /></td>
<td>74.78 μM</td>
<td>2.76</td>
</tr>
<tr>
<td>9</td>
<td>Norwogonin</td>
<td><img src="image" alt="Norwogonin" /></td>
<td>n.a.</td>
<td>2.49</td>
</tr>
</tbody>
</table>
Table 1. Cont.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Formula</th>
<th>IC$_{50}$ µM</th>
<th>logP(o/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Wogonin</td>
<td><img src="image" alt="Structure of Wogonin" /></td>
<td>&gt;100 µM</td>
<td>2.76</td>
</tr>
<tr>
<td>11</td>
<td>Apigenin</td>
<td><img src="image" alt="Structure of Apigenin" /></td>
<td>34.11 µM</td>
<td>2.53</td>
</tr>
<tr>
<td>12</td>
<td>Kämpferol</td>
<td><img src="image" alt="Structure of Kämpferol" /></td>
<td>44.67 µM</td>
<td>2.31</td>
</tr>
<tr>
<td>13</td>
<td>Hispidulin</td>
<td><img src="image" alt="Structure of Hispidulin" /></td>
<td>88.65 µM</td>
<td>2.45</td>
</tr>
<tr>
<td>14</td>
<td>Luteolin</td>
<td><img src="image" alt="Structure of Luteolin" /></td>
<td>19.31 µM</td>
<td>2.26</td>
</tr>
</tbody>
</table>
Among those were four flavanones and ten flavones and four flavonols with different degrees of hydroxylation and/or methoxylation. All compounds possessed the typical 5,7-dihydroxy- pattern. The differences applied to hydroxylation in positions 3’, 4’, 6, and/or 8. One compound each with a methoxy group in positions 3’, 4’, or 8, as well as two compounds with a methoxy group in position 6 were included into the series. Three concentrations (10, 25 and 50 or 75 µM) of each compound were tested (Supplemental Figure S2a) and the effects were compared to Bay11-7082 (Supplemental Figure S2b), an irreversible inhibitor of I-κBα phosphorylation and of CCID formation [2], for which an IC₅₀ of 7.2 µM was determined.

The results showed that flavanones, lacking the double bond between positions 2 and 3, have obviously no or very little effect on the inhibition of CCID formation. This was independent of the

<table>
<thead>
<tr>
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<th>Formula</th>
<th>IC₅₀ µM</th>
<th>logP(o/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Diosmetin</td>
<td><img src="image" alt="Diosmetin" /></td>
<td>&gt;100 µM</td>
<td>2.53</td>
</tr>
<tr>
<td>16</td>
<td>Nepetin</td>
<td><img src="image" alt="Nepetin" /></td>
<td>78.97 µM</td>
<td>2.18</td>
</tr>
<tr>
<td>17</td>
<td>Quercetin</td>
<td><img src="image" alt="Quercetin" /></td>
<td>n.a.</td>
<td>2.03</td>
</tr>
<tr>
<td>18</td>
<td>Gossypetin</td>
<td><img src="image" alt="Gossypetin" /></td>
<td>n.a.</td>
<td>1.68</td>
</tr>
</tbody>
</table>

>100 µM = extrapolated IC₅₀ values between 100 and 200 µM; n.a. not active.
degree of hydroxylation in ring B (no OH (pinocembrin, 1), 4’-OH (naringenin, 2) or 3’,4’-diOH (eriodictyol, 3)). Only homoeriodictyol (4) with a 3’-methoxy-4’-hydroxy pattern resulted in a very high IC₅₀ value of 99.5 μM.

In contrast, the corresponding flavones chrysin (5, IC₅₀ 24.8 μM), apigenin (11, IC₅₀ 34.1 μM), and luteolin (14, IC₅₀ 19.3 μM) were the most active compounds. Unexpectedly, 14 with the lowest logP among these flavones showed the highest activity. Additional hydroxylation in position 3 in the flavonols galangin (6) and kaempferol (12) led to slightly higher IC₅₀ values as compared to 5 and 11. Interestingly, quercetin (17), the 3’,4’-diOH-flavonol corresponding to the 3’,4’-di-OH-flavone 14 remained without any activity. Gossypetin (18) with an additional 8-OH group as compared to 17 was inactive as well.

Methylation of the 4’-OH group in 14 to diosmetin (15) significantly decreased the activity to approximately one-tenth (IC₅₀ 183.2 μM).

Additional hydroxylation of the flavones in positions 6 or 8 also reduced the activity: only baicalein (7; 6-hydroxy-chrysin) showed a weak effect (IC₅₀ 130.2 μM) whereas norwogonin (9; 8-hydroxy-chrysin) was not active.

Methylation of the 6- or 8-OH groups in oroxylin A (8; 6-O-methyl-baicalein), wogonin (10; 8-O-methyl-norwogonin), and hispidulin (13; 6-O-methyl-scutellarein) resulted in an increase in the activity as compared to the respective unmethylated compounds with IC₅₀ values of 74.8 μM, 113.9 μM, and 88.6 μM, respectively.

In comparison to 14, the most active compound, and its 4’-O-methylderivative 15, with only one-tenth of its activity, the 6-methoxyderivative nepetin (16) resulted in an IC₅₀ value of 79 μM.

As proof of our hypothesis we included three further compounds, namely acacetin, scutellarein, and herbacetin (Table 2):

### Table 2. Compounds tested in the CCID assay (MCF-7 spheroids on LECs) as a proof of concept, the respective IC₅₀ (μM) and calculated logP (o/w) values.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Formula</th>
<th>IC₅₀ μM</th>
<th>logP(o/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>Acacetin</td>
<td><img src="" alt="Acacetin" /></td>
<td>36.92 μM</td>
<td>2.80</td>
</tr>
<tr>
<td>20</td>
<td>Scutellarein</td>
<td><img src="" alt="Scutellarein" /></td>
<td>n.a.</td>
<td>2.19</td>
</tr>
<tr>
<td>21</td>
<td>Herbacetin</td>
<td><img src="" alt="Herbacetin" /></td>
<td>n.a.</td>
<td>1.96</td>
</tr>
</tbody>
</table>

n.a. not active.
Acacetin (19), a 3′-OH methylated derivative of the highly active flavone apigenin (IC_{50} 34.1 μM), resulted in almost equivalent activity (IC_{50} 36.9 μM), underlining the impact of the 2,3-double bond and the free positions 6 and 8 on the effects of flavones in the CCID assay (Figure 1).

The importance of free positions 6 and 8 was additionally shown by the inclusion of scutellarein (20; 6-OH-apigenin) and herbacetin (21; 8-OH-kaempferol). As expected from the IC_{50} values of baicalein (IC_{50} 19.3 μM; logP 2.26), chrysin (IC_{50} 24.8 μM; logP 2.84), and apigenin (IC_{50} 34.1 μM; logP 2.53) in the CCID assay, no correlation between the IC_{50} and the logP values was seen. The only deducible trend was the lack of activity for compounds with logP values below 2.10. Compounds with similar logP values such as galangin (logP 2.61) and pinocembrin (logP 2.69) showed significant differences resulting in very good (IC_{50} 39.6 μM) and no activity, respectively. The activity of the most potent flavonoid luteolin was in a comparable range with the positive control Bay11-7082.

In summary based on the results of 21 flavonoids, flavones showed better effects in our experimental set-up in the CCID assay than the corresponding flavonols, whereas flavanones remained without effects. An additional hydroxy group at C-6 or C-8 or an additional methoxy group at C-8 in the flavonoid scaffold resulted in a significant loss of activity. Further substitution of C-6 with a methoxy group decreased the activity to a lesser extent. In contrast, the impact of the hydroxylation/methoxylation pattern in ring B at C-4′ or C-3′ and C-4′ was much less and did not follow a deducible order (luteolin (3′,4′-OH) > chrysin (no OH) > apigenin (4′ OH) > acacetin (4′ OCH₃)).

4. Discussion

The rationale to this study emerged from work on the anti-carcinogenic effects of a MeOH extract from the medicinal plant Scrophularia lucida which is endemic to the Eastern Mediterranean [8]. This extract strongly inhibited LEC barrier breaching because of the presence of hispidulin and unknown synergizing components [9]. Therefore, hispidulin was compared to other closely related flavonoids. Several flavonoids have been shown to disrupt the pathways and molecular mechanisms essential for cancer cell survival, also in breast cancer cells. Metastatic processes such as invasion...
and endothelial-to-mesenchymal-transition (EMT) were inhibited by such compounds as well [19,20]. Here, we did not focus on the tumor cells, but on the stabilizing effect of flavonoids on tumor-induced disintegration of the lymphendothelial barrier. In the clinical routine, the crossing of breast cancer cells into the lymphatic vasculature and subsequent colonization of the sentinel lymph node is a relevant prognostic marker. In our experimental set-up, anti-intravasative flavonoids with up to four-fold higher activity than hispidulin (IC$_{50}$: 89 µM) were identified, namely luteolin (IC$_{50}$: 19 µM) as the most potent one > chrysin (IC$_{50}$: 25 µM) > apigenin (IC$_{50}$: 34 µM) > acacetin (IC$_{50}$: 37 µM) > galangin (IC$_{50}$: 40 µM) > kaempferol (IC$_{50}$: 45 µM) (see Table 1). The tested flavonoids are present in fruits and vegetables, plants, mushrooms, bee pollen and propolis [21,22]. Yet, the bio-available concentrations are too low to achieve LEC barrier stabilization by dietary consumption [23]. Nevertheless, a significant inverse correlation with colorectal cancer risk and the intake of flavonoid subclasses (flavonols, flavan-3-ols, anthocyanidins) was reported [24]. However, a lower risk of breast cancer associated with flavonoids was not observed [25]. The most active flavonoids, luteolin, chrysin, and apigenin, are commercially available and particular formulations of luteolin and apigenin reached plasma concentrations in the range of those exhibiting significant in vitro anti-intravasative effects in our study. In detail, a single oral dose of luteolin or luteolin together with a peanut hull extract (14.3 mg/kg each) resulted in peak concentrations of 1.9 µg/mL and 8.3 µg/mL (respectively) of luteolin in rat plasma [26]. This corresponds to luteolin concentrations of ~6.9 µM and ~29.1 µM, respectively. Similarly, a single oral dose of apigenin or apigenin delivered with a carbon nanopowder solid dispersion carrier (60 mg/kg each) gave peak concentrations of 1.3 µg/mL and 3.2 µg/mL (respectively) of apigenin in rat plasma [27]. This corresponds to apigenin concentrations of ~4.8 µM and ~11.8 µM, respectively. Chrysanthemic acid at daily oral doses (50 mg/kg) in a mouse xenograft model with A549 non-small-cell lung cancer cells, reduced the tumor volume by 50% as compared to control. In breast cancer mouse models, oral administration of chrysin significantly reduced the growth of lung metastases of 4T1 breast cancer cells and tumor growth in a MDA-MB-231 model [28,29]. This proves the bio-availability of chrysin as well as its anti-carcinogenic potential [30].

However, it is still not known whether this effect was due to chrysin itself or due to a metabolite. For an anti-metastatic activity of chrysin, the inhibition of angiogenesis through reduced vascular endothelial growth factor (VEGF) expression might be responsible. Furthermore, chrysin induced E-cadherin, decreased vimentin, SNAI and SLUG levels thereby reverting EMT. Chrysin inhibited AKT-dependent matrix metalloprotease (MMP)-10 expression in triple negative breast cancer, cell migration and invasion [31], and MMP9 through JNK—ERK—AP1 [32]. Importantly, a daily dose of 3 g chrysin did not exhibit toxicity in a pilot clinical trial [33].

Luteolin (25 µM) did not compromise the viability of MDA-MB231- and MCF-7/6 cells. Luteolin concentrations as low as 1 µM inhibited the invasion of MDA-MB231 cells through a basement membrane extract [36]. This supported the notion that luteolin specifically targets migratory/metastatic processes but not proliferation/survival mechanisms in cancer cells. MMP2 and MMP9 contribute to the invrasavation in the MCF-7/LEC model [13] and this can explain the anti-intravasative properties of chrysin and luteolin. This is also in agreement with the observation that 20 µM luteolin inhibited the matrix metallo-protease 1 (MMP1) in MDA-MB231 cells [16]. MMP1, MMP2, and MMP9 are triggering the retraction of LECs [37] and the formation of CCIDs. Similar to chrysin, 10 µM luteolin induced the epithelial marker E-cadherin, down-regulated the mesenchymal markers vimentin, N-cadherin SNAI, and SLUG, and attenuated the migration of MDA-MB231 cells in a wound healing assay [38]. This evidenced the inhibition of EMT and of cell mobility. Apigenin as well inhibited SNAI, EMT, and metastasis of hepatocellular carcinoma [39]. Urokinase-plasminogen activator expression, migration, invasion and phorbol-12-myristate-13-acetate (PMA)-induced MMP-9 secretion were inhibited in MDA-MB231 cells [40] and in MCF-7 cells [41] upon treatment with apigenin.

In addition, apigenin, luteolin, and chrysin inhibit nuclear factor κB (NF-κB) activity [34,39,41–43]. MCF-7 cells secret 12-hydroxyeicosatetraenoic acid (12(S)-HETE), which triggers the activation of NF-κB in LECs as a prerequisite for CCID formation in the LEC monolayer [2,3]. Thus, luteolin,
chrysin, and apigenin stabilize the endothelial barrier function by inhibiting NF-κB in LECs despite the de-stabilizing effects of 12(S)-HETE. Another mechanism of luteolin and apigenin is the inhibition of FAK phosphorylation at Tyr397 and thereby attachment/detachment oscillations at the matrix, which is necessary for LEC migration and CCID formation [16].

The methylation of the hydroxy group at C-4’ in apigenin leads to acacetin, which was almost as active as apigenin. In MDA-MB231 cells acacetin (25 µM) significantly inhibited PMA-induced MMP9 mRNA expression after 48 h [34]. The absorption of methylated flavonoids in intact organisms is much higher as compared to the respective non-methylated compounds (i.e., apigenin). Therefore, these compounds show improved metabolic stability and bio-availability [44]. Acacetin is also commercially available and deserves further investigations for a potential pharmaceutical use. Diosmetin, the 4’-O-methyl-luteolin, was inactive in our assay. In another investigation, diosmetin (~30 µM) inhibited ~50% invasion and migration of SK-HEP1- and MHcc97H hepatocellular carcinoma cells after 24 h. However, these results did not correlate with an inhibition of MMP2 and MMP9 [45]. We suppose that in our experimental set-up treatment with diosmetin for more than 4 h may have resulted in a similar inhibition of LEC migration. Consequently, the lesser effect of diosmetin, compared to luteolin, must have been the consequence of an un-favorable structural alteration because of the hydroxyl group at 3’ in combination with the 4’O-methylation.

Galangin and kaempferol exhibited almost similar anti-intravasative activities. They inhibited MMP2, MMP9, migration and invasion, and suppressed EMT in MDA-MB-231- and MCF-7 breast cancer cells [34,46–48], as well as NF-κB activity in various experimental models [49,50]. The bioavailability of kaempferol after oral administration (100 mg/kg) in Spargue-Dawley rats was 2% [51]. After a single oral dose of galangin (10 mg/kg) only 220 ng/mL of the unmetabolized compound were detected in rat plasma [52].

Similar to the flavonoids above, oroxylin A, wogonin, and baicalein were also reported to inhibit EMT marker expression in MCF-7, A549, and MDA-MB231 cell lines, respectively [53–55] and to inhibit MMP2/9, migration and invasiveness of MDA-MB231 cells [56–58]. In a xenograft nude mouse model, baicalein inhibited tumor metastasis [59]. However, the IC₅₀ values of these flavonoids in our experiments were probably too high to achieve anti-intravasative effects in a patho-physiological setting.

Migration and invasion of MCF-7 cells were inhibited upon treatment with 80 µM quercetin and 20 µM blocked PMA-induced upregulation of MMP9 [60]. At a concentration of 50 µM this compound inhibited the migration of SAS oral squamous carcinoma cells which correlated with the suppression of MMP2 and MMP9 protein [61]. In contrast, suppression of MMP2 and MMP9 protein upon treatment with 25 µM quercetin did not show any correlation with the inhibition of cancer cell migration [62]. However, this concentration correlated with an inhibition of invasion of osteosarcoma cells in vitro and invasion of cancer cell xenografts into the lungs in a mouse model. This indicates that the anti-migratory property of quercetin was not necessarily connected to MMP2/9 expression in cancer cells. The migration of aorta blood endothelial cells was only weakly inhibited by 100 µM quercetin. This concentration had no effects on the tube formation of vascular endothelial cells, another cell migratory process [63]. In fact, the migration of blood endothelial cells during angiogenesis depends on VEGF/VEGFR2 instead of MMP2/9, and quercetin interferes with VEGFR2 expression [64,65]. However, in lymph endothelial cells, the VEGF/VEGFR2 signaling is not observed during intravasation of MCF-7 cells [13]. Also, the activation of NF-κB, on which the formation of CCIDs in the LEC monolayer is strictly dependent, was not inhibited by quercetin in the endothelial cell line ECV304 [66]. As CCID formation was not affected at 10–75 µM quercetin, this process might be devoid of targets addressed by quercetin.

The anti-intravasative activities of the other tested flavonoids were too low to consider targeting migratory and intravasation-supporting mechanisms in LECs, although herbacetin interferes with pro-metastatic HGF-cMET signaling in MDA-MB231 cells [67]. Hence, cMET obviously does not play a role in the migration of LECs.
The migration of LECs was dependent on the stimulation of NF-κB by MCF-7-secreted 12(S)-HETE and subsequent expression of ICAM-1 in LECs, which facilitates the adhesion of LECs to cancer cells as a prerequisite for CCID formation [3]. Furthermore, 12(S)-HETE induces LEC migration through the release of Ca^{2+} from intracellular stores, the induction of phospholipase C and generation of inositol 3-phosphate, as well as the activation of the RHO/ROCK pathway, both activating the mobility of protein MLC2 [37,68]. In breast cancer cells also matrix metalloproteases play a role in CCID formation such as MMP1 and MMP11 [37] and MMP2/9 and TIMP2 [13]. Whether MMP2/9 and TIMP2 activity is LEC- or cancer cell associated and whether and how these pathways are interconnected is still an open issue.

5. Conclusions

The comparison of 21 flavonoids for their potential to stabilize LEC barriers that were disintegrated by MCF-7 breast cancer spheroids clearly showed the highest activity for flavones with a moderate grade of hydroxylation. The vast majority of these compounds were studied in the CCID assay for the first time. Our results confirm studies which have shown the importance of the 2, 3 double bond in ring C of the flavonoid scaffold on different mechanisms in progression of cancer. Under consideration of the bio-availability of flavonoids, a sufficient provision of the most active compounds could be reached via special delivery systems.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-0532/87/3/23/s1, Figure S1: Lymph-endothelial marker expression in LEC. Figure S2: a Treatment of the MCF-7/LEC model with flavonoids; b Treatment of the MCF-7/LEC model with Bay11-7802. Figure S3: Correlation of logP values (o/w) and pIC50 values of 13 flavonoids which showed activity in the CCID assay.

Author Contributions: J.E., K.K., C.S. (Claudia Smöch), and C.S. (Christa Stadlbauer) performed the experiments, C.H.N. supervised the experiments, A.Ö. contributed to the initiation of the project, G.E. determined pK values, W.J., G.K., and L.K. planned the project and wrote the manuscript.

Funding: CHN was supported by a technology grant (TSA Doktorat) financed by the Austrian Federal Ministry of Science and Research (BMFW) in frame of Asea Uninet.

Conflicts of Interest: The authors declare no conflict of interest.

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