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Intravasation of SW620 colon cancer cell spheroids through the blood endothelial barrier is inhibited by clinical drugs and flavonoids *in vitro*

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ABSTRACT

Mechanisms how colorectal cancer (CRC) cells penetrate blood micro-vessel endothelia and metastasise is poorly understood. To study blood endothelial cell (BEC) barrier breaching by CRC emboli, an *in vitro* assay measuring BEC-free areas underneath SW620 cell spheroids, so called "circular chemorepellent induced defects" (CCIDs, appearing in consequence of endothelial retraction), was adapted and supported by Western blotting, EIA-, EROD- and luciferase reporter assays. Inhibition of ALOX12 or NF-κB in SW620 cells or BECs, respectively, caused attenuation of CCIDs. The FDA approved drugs vinpocetine [inhibiting ALOX12-dependent 12(S)-HETE synthesis], ketotifen [inhibiting NF-κB], carbamazepine and fenofibrate [inhibiting 12(S)-HETE and NF-κB] significantly attenuated CCID formation at low μM concentrations. In the 5-FU-resistant SW620-R/BEC model guanfacine, nifedipine and proadifen inhibited CCIDs stronger than in the naïve SW620/BEC model. This indicated that in SW620-R cells formerly silent (yet unidentified) genes became expressed and targetable by these drugs in course of resistance acquisition. Fenofibrate, and the flavonoids hispidulin and apigenin, which are present in medicinal plants, spices, herbs and fruits, attenuated CCID formation in both, naïve- and resistant models. As FDA-approved drugs and food-flavonoids inhibited established and acquired intravasative pathways and attenuated BEC barrier-breaching *in vitro*, this warrants testing of these compounds in CRC models *in vivo*.

1. Introduction

A few cancer entities spread and colonise distant organs by intravasating lymphatic- and/or blood vessels first. Intravasation is achieved upon secretion of cancer-derived factors (Uchide et al., 2007; Nguyen et al., 2015), which subsequently trigger the retraction of the adjacent endothelial cell wall (Honn et al., 1994) thereby opening gaps ("circular chemically induced defects"; CCIDs) through which cancer bulks (or single cells) enter the vasculature (Uchide et al., 2007; Madlener et al., 2010) and metastasise (Kerjaschki et al., 2011). As breast cancer cells spread mainly through lymphatics (Karlsson et al., 2017), the lymph node status, which refers to axillary lymph nodes that are filled with metastatic cancer cells, is a prognostic marker (Sobin et al., 2009). Therefore, intravasation, which is resembled by the validated CCID *in vitro* assay, was studied in three-dimensional (3D) models consisting of breast cancer cell emboli and lymph endothelial cell (LEC) monolayers to elucidate its mechanisms (Vonach et al., 2011; Viola et al., 2013; Nguyen et al., 2016a,b). To this end, the role of ALOX12/

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Abbreviations

3D	3-dimensional
5-FU	5-Fluoruracil
12(S)-HETE	12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid, "S" stereoisomer
ALOX12/15	lipoxygenase 12/15
Bay11-7082	(E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile
BEC	blood endothelial cell
CAF	cancer associated fibroblast
CRC	colorectal cancer
CCID	circular chemorepellent induced defect
EC	endothelial cell
FCS	fetal calf serum
ICAM-1	intercellular adhesion molecule 1
LEC	lymph endothelial cell
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PS	penicillin/streptomycin
TCM	Traditional Chinese Medicine

15, its metabolite 12(S)-HETE and its receptor, which triggers the retraction of endothelial cells (Guo et al., 2011) as a prerequisite for breast cancer metastasis (Kerjaschki et al., 2011; Uchide et al., 2007), was established.

Colorectal cancer (CRC) is known to disseminate through lymphatics and promote lymph node metastasis, but also through other routes as lung and liver metastases are independent of the lymph node status. Therefore, it is likely that the blood vasculature is accountable for CRC distribution as well (Knijn et al., 2016). This is in accordance with a recent *in vitro* investigation reporting that colon cancer spheroids penetrate LEC- and blood endothelial cell (BEC) barriers alike (Holzner et al., 2016). Since mechanisms, of how colon cancer emboli breach the blood microvasculature are poorly defined, we studied a model consisting of SW620 cell spheroids and BECs and report on the correlation between tumour intravasation, 12(S)-HETE levels secreted by SW620 cells, and the NF-κB-dependent response in BECs. Once intravasative mechanisms were identified, tailored intervention measures were taken. For this, FDA approved drugs, which affected these mechanisms, were tested in the SW620/BEC model regarding their anti-intravasative properties. Furthermore, baicalein, which is a *bona fide* inhibitor of ALOX12 activity and 12(S)-HETE synthesis exhibiting anticancer activity *in vitro* and *in vivo* (Hsu et al., 2008), was investigated regarding its CCID-inhibitory property. Baicalein is a main compound contained in *Scuttelaria baicalensis* root (the major component in "Huang-Lian-Jie-Du-Tang") used in TCM and Kampo medicine to treat patients with severe inflammatory conditions (Ma et al., 2005). Therefore, also the structurally related flavonoids apigenin and hispidulin were selected and their effects on CCID formation was analysed. Apigenin is present in many spices, fruits, vegetables and medicinal plants such as *Thymus*, *Petroselinum*, *Punica*, *Vitis*, *Cynara, Allium, Chamaemelum*, *Solidago*, *Viola and Apium* (Sung et al., 2016) and hispidulin is contained in plants used in TCM and Ayurvedic medicine such *as Grindelia argentina, Arrabidaea chica, Saussurea involucrate, Crossostephium chinense, Scutellaria barbata*, and in traditional European medicine i.e. *Rosmarinus officinalis, Artemisia* and *Salvia* species (Patel and Patel, 2017).

Since 5-fluorouracil (5-FU), which is used as CRC standard therapy, induces chemo-resistance (Longley et al., 2003), the effectiveness of these compounds was, moreover, tested in a 5-FU-resistant SW620 (SW620-R) clone.

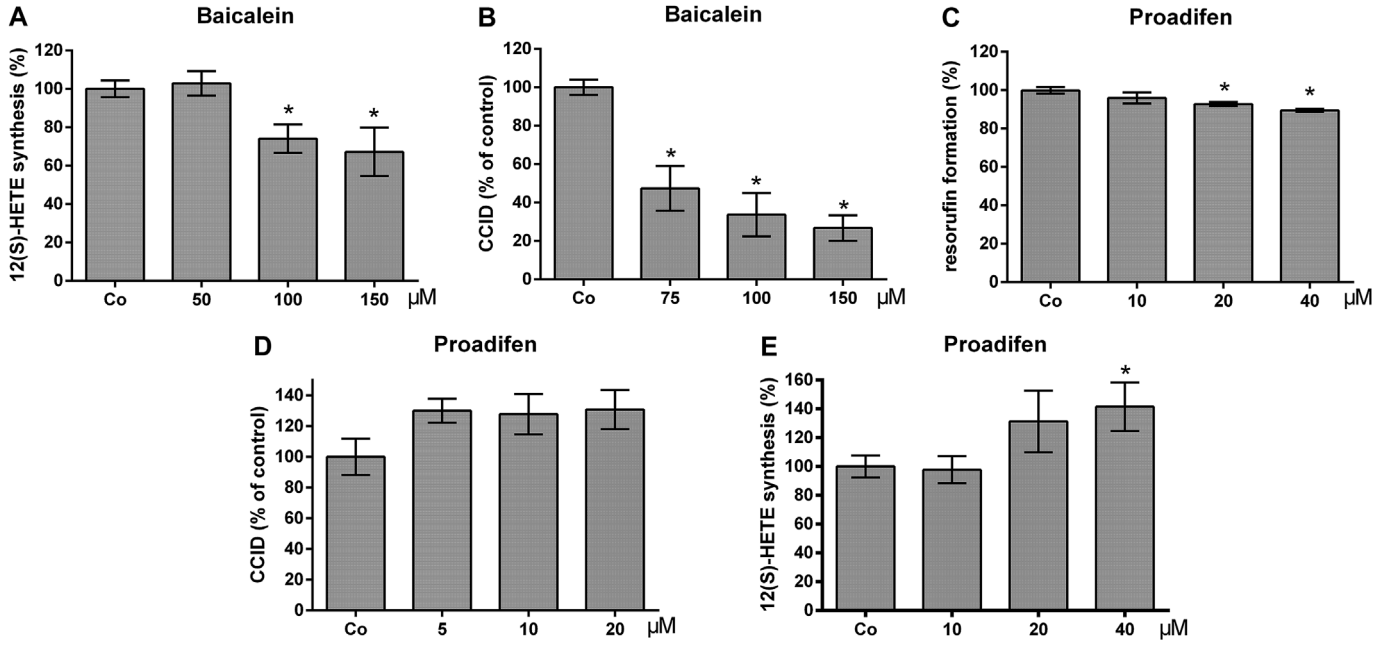

Fig. 1. Specific pathway inhibition. (a, e) SW620 cells were seeded in 3.5-cm dishes, grown to 80% confluence and treated with 10 μM arachidonic acid together with solvent (DMSO **(CAS-No. 67-68-5)**; Co) or the indicated concentrations of (a) baicalein **(CAS-No. 491-67-8)** and (e) proadifen **(CAS-No. 302-33-0)** for 4 h. Then, cell culture supernatants were aspirated and 12(S)-HETE was measured. Two independent experiments were performed and for each concentration three replicates were analysed. (b, d) SW620 spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of (b) baicalein and (d) proadifen. Then, spheroids and compounds were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. (c) SW620 cells were kept under steroid-free conditions and treated with the indicated concentrations of solvent (DMSO; Co) or proadifen. 5 μM ethoxyresorufin was added and after 3 h the formation of resorufin was analysed, which is specific for CYP1A1/1A2 activity. For each experimental point four replicates were analysed. Error bars indicate mean ± S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).