BJC

In vitro inhibition of breast cancer spheroid-induced lymphendothelial defects resembling intravasation into the lymphatic vasculature by acetohexamide, isoxsuprine, nifedipin and proadifen

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Background: As metastasis is the prime cause of death from malignancies, there is vibrant interest to discover options for the management of the different mechanistic steps of tumour spreading. Some approved pharmaceuticals exhibit activities against diseases they have not been developed for. In order to discover such activities that might attenuate lymph node metastasis, we investigated 225 drugs, which are approved by the US Food and Drug Administration.

Methods: A three-dimensional cell co-culture assay was utilised measuring tumour cell-induced disintegrations of the lymphendothelial wall through which tumour emboli can intravasate as a limiting step in lymph node metastasis of ductal breast cancer. The disintegrated areas in the lymphendothelial cell (LEC) monolayers were induced by 12(S)-HETE, which is secreted by MCF-7 tumour cell spheroids, and are called 'circular chemorepellent induced defects' (CCIDs). The putative mechanisms by which active drugs prevented the formation of entry gates were investigated by western blotting, NF-κB activity assay and by the determination of 12(S)-HETE synthesis.

Results: Acetohexamide, nifedipin, isoxsuprine and proadifen dose dependently inhibited the formation of CCIDs in LEC monolayers and inhibited markers of epithelial-to-mesenchymal-transition and migration. The migration of LECs is a prerequisite of CCID formation, and these drugs either repressed paxillin levels or the activities of myosin light chain 2, or myosin-binding subunit of myosin phosphatase. Isoxsuprine inhibited all three migration markers, and isoxsuprine and acetohexamide suppressed

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the synthesis of 12(S)-HETE, whereas proadifen and nifedipin inhibited NF-κB activation. Both the signalling pathways independently cause CCID formation.

Conclusion: The targeting of different mechanisms was most likely the reason for synergistic effects of different drug combinations on the inhibition of CCID formation. Furthermore, the treatment with drug combinations allowed also a several-fold reduction in drug concentrations. These results encourage further screening of approved drugs and their *in vivo* testing.

INTRODUCTION

Lipoxygenases were shown to have a significant role in tumour metastasis. MCF-7 breast cancer cells secrete 12(S)-HETE (Uchide *et al*, 2007), a metabolite of lipoxygenases 12 and 15-1 (ALOX12 or ALOX15-1), which repels different types of endothelial cells (Honn *et al*, 1994; Madlener *et al*, 2010). When placing MCF-7 cell spheroids on lymphendothelial cell (LEC) monolayers, the secreted 12(S)-HETE causes the retraction of underneath growing LECs, giving way to cell-free zones called 'circular chemorepellent induced defects' (CCIDs). The CCIDs are formed by the directional migration of LECs, and this phenomenon faithfully resembles bulky tumour intravasation through the lymphatic cell wall. Therefore, CCIDs, which are caused by 12(S)-HETE, are considered as entry gates for intravasating tumour emboli (Kerjaschki *et al*, 2011). 12(S)-HETE production correlates with lymph node metastasis of ductal breast cancer in human patients and inhibiting 12(S)-HETE synthesis by experimental drugs (i.e., the ALOX12/15 inhibitors baicalein or NDGA) and genetic manipulations (knock down of ALOX15) reduce the size of CCIDs and the metastatic spread into lymph nodes in a breast cancer xenograft mouse model (Kerjaschki *et al*, 2011). NDGA and baicalein are preclinical drugs which have no approval for clinical use. Hence, we screened 225 drugs, which are approved by the US Food and Drug Administration (FDA), regarding their CCID-inhibitory properties to evaluate their potential in anti-metastatic therapy.

MATERIALS AND METHODS

Reagents and antibodies. The NF-κB inhibitor parthenolide, the I-κBα phosphorylation inhibitor (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile (Bay11-7082) and the ALOX12/15 inhibitor baicalein (EI-106) were purchased from Biomol (Hamburg, Germany), and 12(S)-HETE was purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetohexamide (no. A178), dimethyl sulfoxide (DMSO, minimum 99.5% GC, no. D4540), isoxsuprine hydrochloride (no. I0880), nifedipin (no. N7634) and proadifen hydrochloride (no. P1061) were purchased from Sigma-Aldrich (Munich, Germany). Mouse monoclonal anti-CD54 (ICAM-1) antibody was from Immunotech (Marseille, France), and polyclonal rabbit anti-paxillin (H-114) (sc-5574) and β-tubulin (H-235, sc-9104) were from Santa Cruz Biotechnology (Heidelberg, Germany). Monoclonal mouse anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10), monoclonal rabbit anti-p44/42 MAPK (Erk1/2) (137F5), polyclonal rabbit anti-phospho-myosin light chain 2 (MLC2) (Ser19), polyclonal rabbit anti-MLC2 and polyclonal rabbit anti-myosin-binding subunit of myosin phosphatase (MYPT1) were from Cell Signaling (Danvers, MA, USA). Monoclonal mouse anti-β-actin (clone AC-15) was from Sigma-Aldrich, polyclonal rabbit anti-phospho-MYPT1 (Thr696) was from Upstate (Lake Placid, NY, USA), and monoclonal mouse anti-CD31 (JC70A), polyclonal rabbit anti-mouse and anti-rabbit IgGs were from Dako (Glostrup, Denmark).

Cell culture. Human MCF-7 breast cancer cells were purchased from the American Type Culture Collection (Rockville, MD, USA)

and grown in MEM medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS) (Invitrogen, Karlsruhe, Germany). Telomerase-immortalised human LECs were grown in EGM2 MV (CC-4147; Clonetics, Allendale, NJ, USA), all at 37 $^{\circ}$C in a humidified atmosphere containing 5% CO$_2$. For CCID formation assays, LECs were stained with cytotracker green purchased from Invitrogen. Three-dimensional co-cultivation of breast cancer spheroids with LEC monolayers: MCF-7 cells (input of 3000 cells for one spheroid) were transferred to 30 ml MEM medium containing 6 ml of a 1.6% methylcellulose solution (0.3% final concentration; cat. no.: M-512, 4000 centipoises; Sigma-Aldrich). A total of 150 μl of this cell suspension (containing 3000 cells) was transferred to each well of a 96-well plate (Cellstar 650185; Greiner Bio-one, Kremsmünster, Austria) to allow spheroid formation within 48 h.

The CCID assay. In this model, the sizes of the cell-free areas (CCIDs) are measured that are formed in the endothelial monolayer directly underneath 12(S)-HETE-secreting tumour spheroids, which is a mechanistic cause for this phenomenon (Madlener *et al*, 2010; Kerjaschki *et al*, 2011; Vonach *et al*, 2011; Viola *et al*, 2012). MCF-7 spheroids were washed in PBS and transferred to cytotracker-stained LEC monolayers that were seeded into 24-well plates (Costar 3524; Sigma-Aldrich) in 2 ml EGM2 MV medium. After 4 h of incubation, the CCID areas in the LEC monolayers underneath the MCF-7 spheroids were photographed using an Axiovert (Zeiss, Jena, Germany) fluorescence microscope to visualise cytotracker (green)-stained LECs underneath the spheroids. The CCID areas were calculated with the Axiovision Re.4.5 software (Zeiss). DMSO-treated co-cultures served as negative control. For each condition, the CCID size of 12 or more spheroids (unless otherwise specified) was measured. During the experiments, which were mostly short term, we did not observe toxic effects of the used drugs (monitored by HOPI staining; Grusch *et al*, 2002).

Sodium dodecyl sulphate (SDS) gel electrophoresis and western blotting. LECs were grown in petri dishes (6-cm diameter) to 80% confluence and treated with 1 μM 12(S)-HETE and/or the indicated drug concentrations. Then, cells were washed twice with cold PBS and lysed in a buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride and 1 mM protease inhibitor cocktail (Sigma, Schnelldorf, Germany). The lysates were centrifuged at 12 000 r.p.m. for 20 min at 4 $^{\circ}$C, and the supernatants were transferred to 1.5-ml tubes and stored at $-$20 $^{\circ}$C until further analysis. Equal amounts of protein lysate were mixed with ($\times$2) SDS sample buffer and loaded onto a 10% polyacrylamide gel. SDS polyacrylamide gel electrophoresis and western blotting were according to the protocol described by Vonach *et al* (2011).

NF-κB luciferase assay. 10$\times$10^6 HEK293-NF-κB-Luc cells (Panomics, Fremont, CA, USA) were seeded in 20 ml full growth DMEM medium in a 15-cm dish. Next day, cells were transfected with an expression plasmid for green fluorescent protein (GFP) (pEGFP-N1; Clontech, Mountain View, CA, USA). A total of 30 μl Lipofectamin 2000 (Invitrogen) and 7.5 μg DNA were mixed in 2 ml transfection medium and incubated for 20 min at room temperature, followed by adding this mixture to the cells. After

incubation for 6 h in humidified atmosphere containing 5% CO_2, 4×10^4 cells per well were seeded in serum- and phenol red-free DMEM in a transparent 96-well plate. On the next day, cells were treated with the indicated drugs and 2.5 μM parthenolide as a specific inhibitor of NF-κB (control). One hour after treatment, cells were stimulated with 2 ng ml^{-1} human recombinant TNFα for additional 4 h. Luminescence of the firefly luciferase and fluorescence of the GFP were quantified on a GeniusPro plate reader (Tecan, Grödig, Austria). The luciferase signal derived from the NF-κB reporter was normalised by the GFP-derived fluorescence to account for differences in cell number (Giessrigl *et al*, 2012).

12(S)-HETE assay. 12(S)-HETE, which is secreted by MCF-7 spheroids, is a trigger factor of directional LEC migration and, thus, CCID formation. Hence, we studied the influence of candidate drugs on 12(S)-HETE synthesis in a MCF-7 clone. A commercially available 12(S)-HETE EIA (enzyme immunoassay) kit was not sensitive enough to detect 12(S)-HETE produced by normal MCF-7 cells. Therefore, ALOX12 cDNA was overexpressed to detect 12(S)-HETE baseline levels and its alterations after drug treatment.

Cell culture. ALOX15 of MCF-7 cells were knocked down by lentiviral-packaged shRNA, and 12(S)-HETE synthesis was reconstituted by stable transfection of ALOX12 cDNA as described (Kerjaschki *et al*, 2011), resulting in MCF-7^{ALOX12} cells. MCF-7^{ALOX12} cells were cultivated in MEM medium (Gibco no. 10370-047, Karlsruhe, Germany) supplemented with 10% FCS, 1% GlutaMAX-I (Gibco no. 35050-038), 1% PS (Gibco no. 15140-148), 1 mg ml^{-1} G148-sulphate (PAA, no. P25-011), 150 μg ml^{-1} hygromycin B (Sigma-Aldrich, no. H3274) and 1 μg ml^{-1} puromycin. The cells were grown at 37 $^{\circ}$C in a humidified atmosphere containing 5% CO_2.

Treatment. MCF-7^{ALOX12} cells were seeded in 3.5-cm dishes and grown in 2.5 ml complete MEM medium without selection pressure. The next day, the medium was changed to serum-free medium and cells were kept at 37 $^{\circ}$C for 24 h. Then, cells were treated with 10 μM arachidonic acid (no. A3555, Sigma-Aldrich) and simultaneously with different concentrations of the indicated compounds for 4 h when the supernatants were aspirated, centrifuged at 2000 r.p.m. at 4 $^{\circ}$C for 5 min, collected in cryotubes, flash frozen and stored at -80 $^{\circ}$C until analysis.

12(S)-HETE extraction. Medium samples of MCF-7^{ALOX12} cells were slowly thawed at room temperature and centrifuged at 3000 r.p.m. for 2 min. Extraction cartridges (Oasis HLB 1cc; Waters, Milford, MA, USA) were equilibrated with 2×1 ml methanol (highest purity) and 2×1 ml distilled H_2O, and 1.25 ml of medium samples were passed through the cartridges. Afterwards, the cartridges were washed with 3×1 ml distilled H_2O, and 12(S)-HETE was eluted with 500 μl methanol. The methanolic solution was flash frozen and stored at -80 $^{\circ}$C until further analysis.

Analysis by 12(S)-HETE EIA. The 12(S)-HETE EIA (enzyme immunoassay) kit (no. ADI-900-050) was purchased from Enzo Life Sciences (Lausen, Switzerland), and the samples were prepared and the assay was performed according to the instructions of the manufacturer. In detail, the methanolic solution was evaporated with a speed-vac concentrator, 250 μl of assay buffer were added to the dried samples and a 1 : 54 dilution was prepared. The standards (a solution of 500 ng ml^{-1} 12(S)-HETE, no. 80-0607) for the 12(S)-HETE standard curve were prepared according to the instruction manual. Then, 100 μl of the samples and standards were added to a 96-well plate coated with goat anti-rabbit IgG

antibody (no. 80-0060). Furthermore, 50 μl of 12(S)-HETE conjugate (no. 80-0610; blue solution) and 50 μl of rabbit polyclonal antibody (no. 80-0611; yellow solution) were added into the appropriate wells. Afterwards, the plate was incubated at room temperature for 2 h on a shaker (226 r.p.m.). Then, wells were emptied and washed 3 $\times$ with 400 μl wash buffer (1 : 20 dilution of wash buffer concentrate, no. 80-1286), and after the final wash, the plate was tapped on a flint-free paper towel to completely remove the wash buffer. A total of 200 μl of the pNPP substrate (no. 80-0075) solution was pipetted into each well and incubated for 3 h at 37 $^{\circ}$C in a humidified atmosphere containing 5% CO_2. Then, 50 μl of the stop solution (no. 80-0247) was added into each well, and absorbance was measured with a Wallac 1420 Victor 2 multilabel plate reader (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA).

Statistical analysis. For statistical analyses, Excel 2003 software and Prism 5 software package (GraphPad, San Diego, CA, USA) were used. The values were expressed as mean ± s.e.m., and the Student's *t*-test was used to compare differences between controls and individual samples, whereas analyses of variance (ANOVAs; one-way ANOVA together with Dunnett's post test) was used to analyse treatment groups. Statistical significance level was set at $P < 0.05$. The calculations of drug-combination effects were performed using the 'Calcusyn' software (Biosoft, Ferguson, MO, USA) designed by Chou and Talalay (1981, 1984). This method analyses the interaction among drugs in a given combination and calculates a predictive value at which the effect of a drug combination is additive or above it is synergistic, or below it is antagonistic.

RESULTS

Acetohexamide, isoxsuprine, nifedipin and proadifen inhibit CCID formation. The screening of 225 FDA-approved drugs and four natural products (Figure 1A–J) showed that acetohexamide, nifedipin, proadifen and isoxsuprine inhibited CCID formation dose dependently (5–60 μM; Figure 2A–D). The strongest inhibitory effect was observed with isoxsuprine followed by proadifen > nifedipin > acetohexamide.

Acetohexamide and isoxsuprine reduce the 12(S)-HETE level. 12(S)-HETE, which is secreted by MCF-7 spheroids, is a major trigger factor of CCID formation in LEC monolayers. 12(S)-HETE is the product of ALOX12/15. Therefore, we analysed whether acetohexamide, isoxsuprine, nifedipin and proadifen inhibited 12(S)-HETE synthesis in an ALOX12-expressing MCF-7 clone (MCF-7^{ALOX12} cells) using an adapted EIA assay, which measures the 12(S)-HETE levels in cell-culture supernatants. Acetohexamide and isoxsuprine inhibited 12(S)-HETE synthesis, whereas nifedipin did not alter 12(S)-HETE levels significantly, and proadifen even induced the 12(S)-HETE level in MCF-7^{ALOX12} cells (Figure 3).

Proadifen and nifedipin inhibit activation of NF-κB. Recent studies showed that the activity of NF-κB (in addition to the activity of ALOX12/15 of MCF-7 cells) contributes to CCID formation (Vonach *et al*, 2011; Viola *et al*, 2012). Therefore, we assesed the effect of acetohexamide, isoxsuprine, nifedipin and proadifen on NF-κB activity using a luciferase reporter fused to a NF-κB-responsive promoter. Nifedipin and proadifen inhibited the activation of the NF-κB reporter construct, whereas acetohexamide and isoxsuprine did not alter the read out (Figure 4).

Inhibition of mobility markers. The formation of CCIDs, which are triggered by MCF-7 spheroids, requires LEC mobility and, therefore, the induction of proteins facilitating cell migration. Hence, the expression of the mobility proteins MYPT, MLC2 and
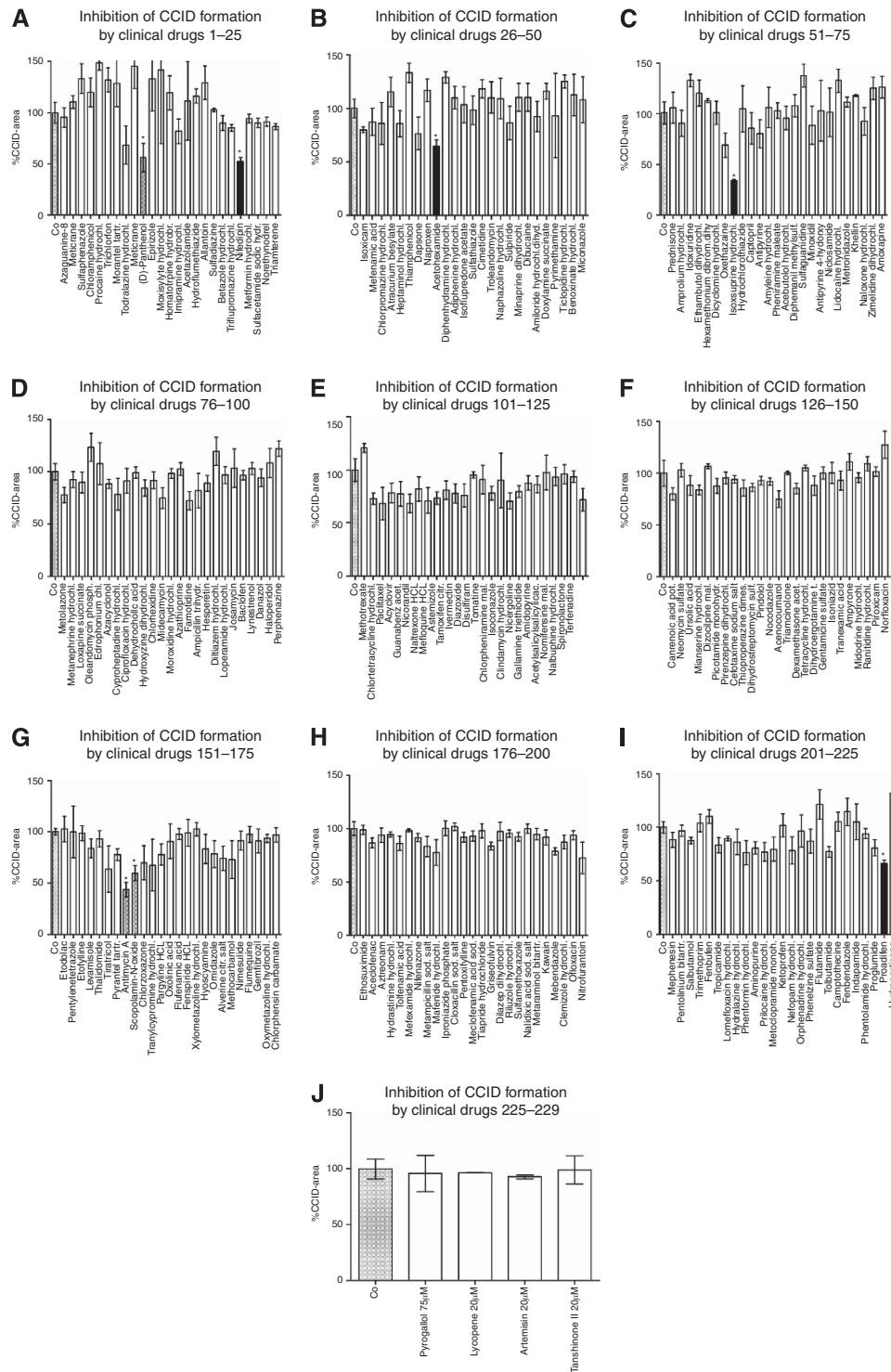
Figure 1. (A–J) Effect of FDA-approved drugs (A–I) and natural compounds (J) on CCID formation. MCF-7 spheroids were placed on LEC monolayers and co-cultivated for 4 h either with medium alone and solvent (DMSO; Co) or with 40 μM of the indicated drugs, and then the areas of CCIDs were measured. Asterisks indicate significance (*t*-test compared with untreated controls; $P < 0.05$) and error bars indicate mean ± s.e.m. Black columns indicate the drugs whose activities were confirmed in subsequent dose response experiments (see Figure 2A–D). Grey columns indicate those drugs that turned out to be false positives.

paxillin was studied and also that of Erk1/2 because it was shown to mediate migratory signals (Webb *et al*, 2004). We simplified the three-dimensional co-culture cell model and replaced MCF-7 spheroids (as source of 12(S)-HETE stimulation) by direct 12(S)-HETE treatment, which allowed to perform western blot analyses. Interestingly, 12(S)-HETE transiently downregulated Erk1/2 phosphorylation (Vonach *et al*, 2011; Viola *et al*, 2012). Acetohexamide,

isoxsuprine, nifedipin and proadifen inhibited one or more mobility markers (MLC2, MYPT and paxillin) in LECs (Figure 5A–D), thereby providing a molecular explanation for attenuated LEC migration. Proadifen downregulated paxillin expression and reversed 12(S)-HETE-mediated Erk1/2 de-phosphorylation. Acetohexamide strongly inhibited 12(S)-HETE-induced phosphorylation of MLC2, and also MYPT
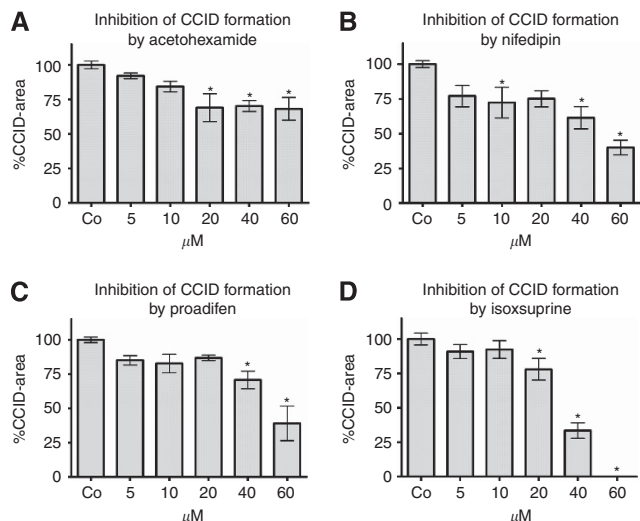
Figure 2. (**A–D**) Inhibitory effect of acetohexamide, nifedipin, proadifen and isoxsuprine on CCID formation. MCF-7 spheroids were placed on LEC monolayers and co-cultivated for 4 h either with medium alone and solvent (DMSO; Co) or with increasing concentrations of acetohexamide, nifedipin, proadifen and isoxsuprine, and then the areas of CCIDs were measured. Per experimental condition, the areas underneath of at least 12 spheroids were analysed. Asterisks indicate significance (one-way ANOVA and Dunnett's post test compared with untreated control; $P < 0.05$) and error bars indicate mean ± s.e.m.
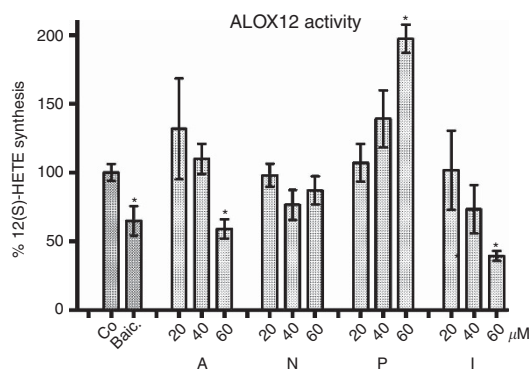

Figure 3. Effect of acetohexamide, nifedipin, proadifen and isoxsuprine on ALOX12 activity. MCF-7^{ALOX12} cells were seeded in 3.5-cm dishes and grown to 70% confluence and treated with 10 μM arachidonic acid together with the indicated concentrations of acetohexamide (A), nifedipin (N), proadifen (P) and isoxsuprine (I) and 50 μM baicalein (Baic.) for 4 h. DMSO was used as control (Co). Then, 12(S)-HETE was determined by EIA. Experiments were performed in triplicate. Asterisks indicate significance (one-way ANOVA and Dunnett's post test compared with untreated control; $P < 0.05$) and error bars indicate mean ± s.e.m.

phosphorylation was weakly inhibited. Nifedipin strongly suppressed the phosphorylation of MYPT, whereas phosphorylation of MLC2 became induced. Isoxsuprine was the only drug inhibiting the induction of all three mobility markers. Therefore, all four CCID-inhibitory drugs affected the expression/activation of one or more mobility proteins.

Combinatorial drug treatment further reduces CCID areas. The drug concentrations that substantially inhibited CCID formation were rather high and physiologically not relevant (60 μM).
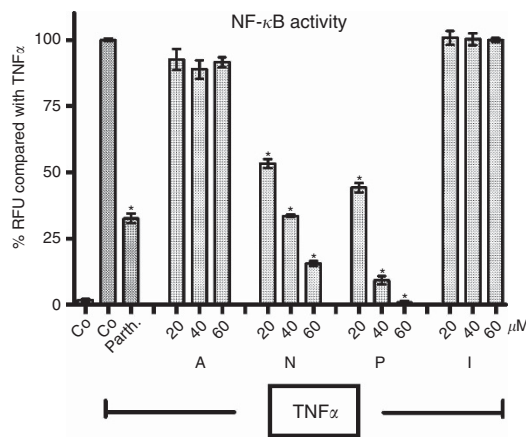

Figure 4. HEK293-NFκB-Luc cells transfected with the cDNA of GFP were treated with the indicated drug concentrations of acetohexamide (A), nifedipin (N), proadifen (P), isoxsuprine (I) or 2.5 μM parthenolide (Parth.) as a specific inhibitor of NF-κB, or solvent (Co; DMSO) control. After stimulation with TNFα (2 ng ml^{-1}) for 4 h, luminescence of the firefly luciferase and fluorescence of GFP were quantified, and the luciferase signal was normalised by the GFP-derived fluorescence. Experiments were performed in triplicate. Asterisks indicate significance (one-way ANOVA and Dunnett's post test compared with TNFα control; $P < 0.05$) and error bars indicate mean ± s.d.
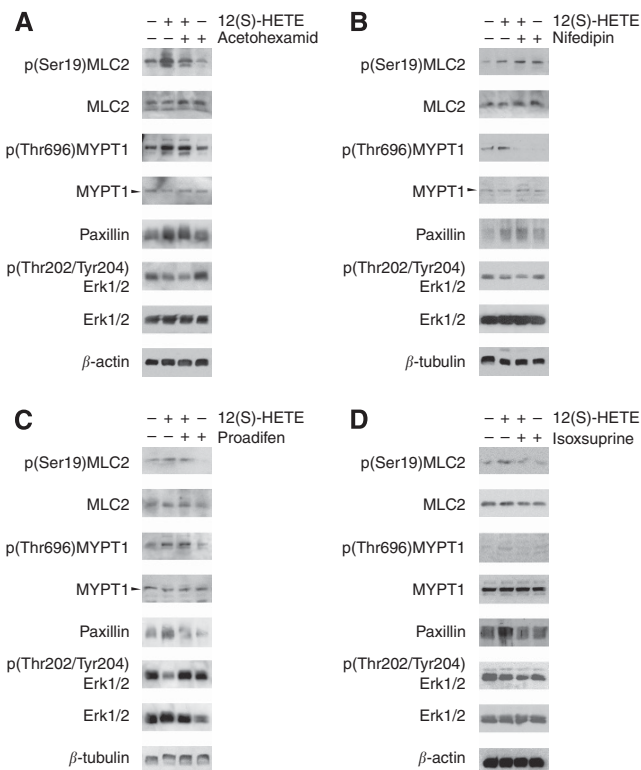

Figure 5. (**A–D**) Analysis of protein expression. LECs were pretreated with 60 μM acetohexamide (**A**), nifedipin (**B**), proadifen (**C**), isoxsuprine (**D**) and solvent (negative control; − , −) for 20 min and/or stimulated afterwards with 1 μM 12(S)-HETE for 60 min. Then, cells were harvested and protein lysates were analysed by western blotting. β-actin and β-tubulin analyses served as loading control.

Therefore, we performed combination experiments aiming towards the reduction of the individual drug concentrations. Together with the other three drugs (acetohexamide, nifedipin and proadifen,

20 μM each), the effect of 20 μM isoxsuprine was significantly improved (Table 1). Two of the drug combinations (isoxsuprine together with nifedipin, or proadifen) synergised in inhibiting CCID formation when compared with single-drug treatment, whereas isoxsuprine in combination with acetohexamide was additive (Figure 6A, Table 2). This was in agreement with the observation that isoxsuprine was the most potent CCID inhibitor and with its property to downregulate all three of the analysed LEC mobility markers.

The weakest drug and also a weak cooperation partner was acetohexamide, which inhibited ALOX12 less efficiently than isoxsuprine. Acetohexamide, which downregulated two mobility markers (MYPT and MLC2), synergised just with nifedipin. Nifedipin synergised with two drugs (isoxsuprine and acetohexamide) and suppressed MYPT phosphorylation, whereas that of MLC2 became even induced. Therefore, the inhibition of MYPT activity may have been crucial for the synergistic and additive inhibition of CCID formation (by nifidipin and acetohexamide, nifidipin and isoxsuprine and acetohexamide and isoxsuprine, respectively), whereas MLC2 may have been negligible. From this perspective, the reason why nifidipin and proadifen was not efficient remains obscure. From another perspective, both proadifen and nifedipin inhibited NF-κB but not ALOX12, which could explain why this combination did not further reduce CCID formation (Figure 6B). Proadifen synergised only with isoxsuprine, and this combination inhibited CCID formation most effectively and was only topped by the combination of all four drugs. Furthermore, also nifedipin & isoxsuprine, and nifedipin & acetohexamide synergistically reduced CCID formation. In these combinations, the first drug inhibited NF-κB and the other one inhibited ALOX12 (respectively). The antagonistic effect (determined by Calcusyn software) of proadifen and acetohexamide co-treatment may have been due to the opposing effects of the two drugs on 12(S)-HETE synthesis. Interestingly, the combination treatment with the two drugs that inhibit ALOX12 (acetohexamide and isoxsuprine) inhibited CCID formation additively. In contrast, the combination treatment with the two NF-κB-inhibitory drugs (proadifen and nifedipin) was antagonistic, which may have been because of the 12(S)-HETE-increasing property of proadifen.

DISCUSSION

Metastasising ductal breast cancer emboli secrete 12(S)-HETE, which causes the subsequent retraction of beneath LECs, thereby forming entry ports for the tumour bulk into the lymphatic vasculature (Kerjaschki et al, 2011). This way the tumour passes on from lymph node to lymph node. Normally, 12(S)-HETE is produced by ALOX12 and ALOX15-1 (Funk, 1996) of platelets,

leukocytes, smooth muscle, epithelial, neuronal and fibroblast cells (Spector et al, 1988), induces retraction of microvascular endothelial cells (MECs) and increases tumour cell adhesion to exposed ECM (Tang et al, 1993; Honn et al, 1994). Also, MCF-7 breast cancer cells secrete 12(S)-HETE (Uchide et al, 2007), inducing a mobile phenotype and CCIDs in adjacent LECs and causing lymph node metastasis in scid mice (Madlener et al, 2010; Kerjaschki et al, 2011; Vonach et al, 2011). Hence, CCID formation in the three-dimensional co-culture model consisting of MCF-7 spheroids and LEC monolayers faithfully resembles the *in vivo* situation. Previous experiments could associate phosphorylated MYPT1 with LEC migration (Kerjaschki et al, 2011; Vonach et al, 2011). MYPT1 is the regulatory/targeting subunit of the myosin phosphatase, which regulates the interaction of actin and myosin in response to signalling through the GTPase Rho (Feng et al, 1999). Here we demonstrate an increase of phosphorylation and hence activation of both proteins – MYPT1 and MLC2 – in 12(S)-HETE-treated LECs. Also, paxillin expression was induced by 12(S)-HETE. Paxillin is essential for labile adhesions facilitating rapid cell migration (Huang et al, 2003; Deakin and Turner, 2008) and cell polarity (Digman et al, 2008) as prerequisites for directional LEC movement (West et al, 2001; Jianxin et al, 2009) and, hence, CCID formation. Paxillin expression is indicative for cell plasticity and a marker of an undifferentiated phenotype and hallmark protein of EMT (Lu et al, 2006; Zeisberg and Neilson, 2009; Paulitschke et al, 2010; van Zijl et al, 2011). Endothelial paxillin facilitates the transmigration of neutrophils through the endothelial barrier and, thus, influences the behaviour of other cell types that are in direct contact with endothelial cells (Parsons et al, 2012). Because 12(S)-HETE induced MYPT, MLC2 and paxillin in LECs, these proteins can serve as biochemical mobility markers in the CCID-forming process and, moreover, provide a mechanistic explanation for the inhibitory effects of the candidate drugs.

Acetohexamide, isoxsuprine, nifedipin and proadifen not only exhibited an impact on LEC motility markers but also targeted distinct cellular mechanisms relevant for CCID formation, such as the activities of ALOX12 and NF-κB. The chemical structures of these drugs are not related to each other and they are used against distinct diseases. Isoxsuprine is used as a vasodilator in humans and equines (Knoll et al, 1996; Erkert and Macallister, 2002), is applied in humans for the treatment of premature labour (Giorgino and Egan, 2010), and in the horse, isoxsuprine is used to treat hoof-related problems and to increase circulation within the hoof (Erkert and Macallister, 2002). Isoxsuprine is a beta-adrenergic agonist that causes direct relaxation of uterine (Kato et al, 1971) and vascular smooth muscle (Belloli et al, 2000). In smooth muscle, MLC kinase phosphorylates MLC2 (Ikebe and Hartshorne, 1985), and this is correlated with myosin ATPase activity and smooth muscle contraction (Tan et al, 1992). 12(S)-HETE induces the phosphorylation of MLC in melanoma cells (Rice et al, 1998), and also in MECs, 12(S)-HETE-induced mobility is mediated through enhanced phosphorylation (activation) of proteins co-migrating with MLC (Tang et al, 1993). Conversely, de-phosphorylation of MLC would suspend cell contraction and movement. Therefore, the smooth muscle-relaxing effect of isoxsuprine could be because of the as yet unreported property to inhibit the phosphorylation and activity of MLC2, which itself correlates directly with LEC mobility (Vonach et al, 2011). Furthermore, isoxsuprine exhibited a suppressive effect on 12(S)-HETE levels in MCF-7^{ALOX12} cells, which was not observed so far. Isoxsuprine strongly affected LEC migration in the co-culture model when applied in combination with the NF-κB-inhibitory drugs, because NF-κB is involved in a separate CCID-promoting mechanism. It is widely accepted that NF-κB is associated with cancer development (Folmer et al, 2009), promoting oncogenesis through the transcriptional activation of genes associated with

Table 1. Significance of improvement of CCID inhibition by treatment with drug combinations

	A	N	P	I
A and I	0.0066	—	—	0.0090
N and I	—	0.0016	—	0.0016
P and I	—	—	<0.0001	0.0005
P and A	n.s.	—	n.s.	—
P and N	—	n.s.	n.s.	—
N and A	0.0052	0.0021	—	—
I, A, N and P	<0.0001	<0.0001	<0.0001	<0.0001

Abbreviations: A = acetohexamide; I = isoxsuprine; N = nifedipin; P = proadifen. The effect of combination treatment versus single-drug treatment on CCID inhibition was analysed by t-test, and the P-values are shown (P<0.05 – significant).
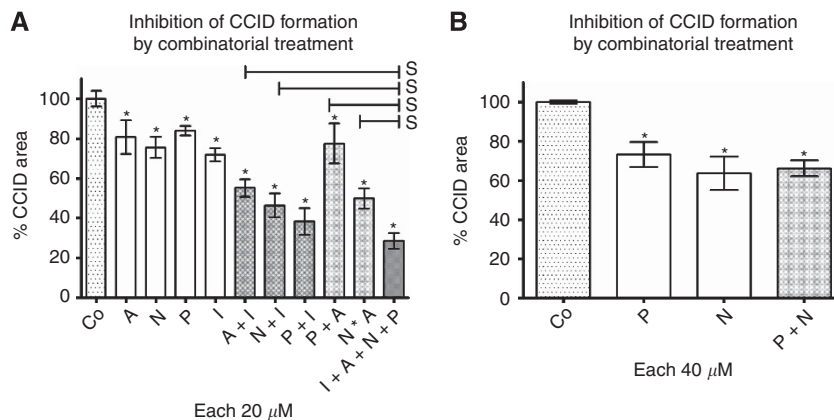
Figure 6. (**A**, **B**) Analysis of drug combinations on CCID formation. MCF-7 spheroids were placed on LEC monolayers and co-cultivated for 4 h either with solvent (DMSO; Co) or (**A**) with the indicated single drugs (20 μM) or drug combinations: acetohexamide (A), nifedipin (N), proadifen (P), isoxsuprine (I) (20 μM each; solvent was kept constant). (**B**) The combination of P together with N (40 μM) had no additional effect. Per experimental condition, the areas underneath of at least 12 spheroids were analysed. Error bars indicate mean ± s.e.m., asterisks significance (*t*-test) between control and treatments and 's' between double treatments and quadruple treatments (s; $P < 0.05$). The significant differences between single treatments and double (and quadruple) treatments are shown in Table 1.

Table 2. Synergistic, additive or antagonistic effects on CCID formation by drug combinations

Compound(s)	Concentration (μM)	CCID formation (% of control) ± s.d.	Predicted value[a]
A	20	80.73; ± 29.63	
N	20	75.48; ± 18.42	
P	20	83.87; ± 10.85	
I	20	71.87; ± 11.57	
A and I	20/20	**55.12; ± 18.33** [a]	58.02
N and I	20/20	**46.43; ± 21.89** [b]	54.25
P and I	20/20	**38.35; ± 27.80** [b]	60.28
P and A	20/20	**77.53; ± 34.95** [c]	67.71
N and A	20/20	**49.93; ± 17.43** [b]	60.93
I, A, N and P	20/20/20/20	**28.59; ± 13.91** [b]	36.73
N	40	63.67; ± 29.16	
P	40	73.20; ± 21.77	
N and P	40/40	**66.15; ± 14.11** [c]	46.60

Abbreviations: A = acetohexamide; I = isoxsuprine; N = nifedipin; P = proadifen. Co-cultivated MCF-7 spheroids and LECs were treated with individual drugs or with the indicated drug combinations for 4 h. Then, CCID formation (% of control) was measured and analysed by Calcusyn software. The CCID data are means of at least 12 determinations; s.d. [a]Predicted value indicating additive effect: (% of dug '1' × % of drug '2')/100. [b]Synergistic combination effect according to the equation of Chou and Talalay (1984). [c]Antagonistic combination effect according to the equation of Chou and Talalay (1984). Bold values are the data derived from drug combinations.

cell proliferation, angiogenesis and metastasis (Orlowski and Baldwin, 2002).

Nifedipin interacts with the binding site of the receptors associated with calcium channels (Swanson and Green, 1986) and inhibits the influx of exogenic calcium in the smooth muscle cells (Wiemer *et al*, 1992). This causes their relaxation (Kanmura *et al*, 1983), and therefore, it is administered to patients with coronary heart diseases and hypertonia (Lundy *et al*, 2009). The smooth muscle-relaxing property of nifedipin is reminiscent to that of isoxsuprine and could be because of the inactivation of the mobility marker MYPT. This would mechanistically link the therapeutic

effect of nifedipin to the inhibition of LEC mobility and CCID formation. In addition, nifedipin inhibited NF-κB (but not ALOX12), and neither this nor its MYPT-inhibitory property have been observed so far.

Proadifen blocks glibenclamide-sensitive K + channels (Sakuta and Yoneda, 1994), stimulates prostocylin through the inhibition of platelet thromboxane synthesis (Boeynaems *et al*, 1987) and inhibits neuronal nitric oxide synthase (Metea and Newman, 2006) and hepatic drug metabolism through the inhibition of the cytochrome P-450 (CYP) system (Ueno *et al*, 1997). The CYP was shown to promote metastases in humans (Jiang *et al*, 2007). Recently, it was demonstrated that proadifen inhibits CYP also in MCF-7 cells (Seelinger *et al*, 2012), and hence, CYP activity of MCF-7 cells might contribute to CCID formation of LECs, that is, by disturbing arachidonic acid metabolism for which ALOX is also competing. In fact, proadifen treatment perturbed (increased) the 12(S)-HETE level in MCF-7^{ALOX12} cells. However, proadifen inhibited also NF-κB, and paxillin expression in LECs and thus, counteracted intercellular communication and directional LEC migration.

Acetohexamide is used for the treatment of diabetes mellitus type 2 because it has a positive influence on the release of insulin from the active beta cells in the pancreas. Further, it improves the binding between insulin to insulin receptors (Sheldon *et al*, 1966; Nakamura, 1984; Imamura *et al*, 1989). We could not link the therapeutic use or known mechanism of acetohexamide to its CCID-inhibitory property. It was the drug with the weakest effect and correlated with the inhibition of ALOX12 and MLC2 phosphorylation.

Summing up, screening of 229 drugs could identify four drugs that inhibited CCID formation and detailed investigations elucidated their potential mechanisms responsible for this inhibitory property. For three drugs, we could, in part, link the mechanism to their therapeutic effects.

Recent reports demonstrate that inhibition of ALOX12/15 is a prime target for the attenuation of lymph node metastasis (Kerjaschki *et al*, 2011; Vonach *et al*, 2011), and together with inhibition of NF-κB attenuates CCID formation additively (Viola *et al*, 2012). Some drug combinations with different mechanistic profiles exhibited synergistic effects, and this allowed reducing the drug concentrations. This supports the notion that the individual drugs targeted more than just one CCID-forming mechanism. Hence, as a consequence from our preliminary data, these and

other drug combinations could be considered for adjuvant anti-metastatic therapy.

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