Cellular Oncology (2019) 42:93–106
https://doi.org/10.1007/s13402-018-0415-3

ORIGINAL PAPER

CCR5 blockage by maraviroc: a potential therapeutic option for metastatic breast cancer

Asim Pervaiz[1,2] · Michael Zepp[2] · Saqib Mahmood[1] · Doaa Mohamed Ali[2] · Martin R. Berger[2] · Hassan Adwan[2,3]

Accepted: 12 November 2018 / Published online: 19 November 2018

Abstract

Purpose Bone metastasis is observed in up to 70% of breast cancer patients. The currently available treatment options are palliative in nature. Chemokine receptor 5 (CCR5) has gained attention as therapeutic target in various malignancies. Here, we investigated the effects of targeting CCR5 by its antagonist maraviroc in metastatic breast cancer cells.

Methods In response to maraviroc exposure, cytotoxicity was assessed using an MTT proliferation assay, whereas the effects on colony formation and migration were assessed using colony formation, transwell chamber migration and scratch wound healing assays, respectively. Apoptosis-related activities were investigated using nuclear staining, annexin-V FITC staining and Western blotting. Cell cycle changes were analysed using flow cytometry and qRT-PCR for cell cycle relevant genes. A nude rat model for breast cancer bone metastasis was used to evaluate the in vivo efficacy of CCR5 targeting by maraviroc. Circulatory levels of the three cognate ligands for CCR5 (CCL3, CCL4, CCL5) were analysed in sera of breast cancer patients using ELISA.

Results We found that blockade of CCR5 attenuated the proliferation, colony formation and migration of metastatic breast cancer cells, and induced apoptosis and arrest in the G1 phase of the cell cycle. Expression profiling highlighted the involvement of cell cycle related signalling cascades. We also found that treatment with maraviroc significantly inhibited bone metastasis in nude rats implanted with MDA-MB-231 breast cancer cells. Finally, we found that the circulatory levels of three cognate ligands for the CCR5 receptor varied between breast cancer patients and healthy controls.

Conclusion Our findings indicate that targeting CCR5 may be an effective strategy to combat breast cancer bone metastasis.

Keywords Breast cancer · Bone metastasis · CCR5 · Maraviroc · Cell cycle arrest · Apoptosis

1 Introduction

Breast cancer is the most frequently diagnosed malignancy in women and the 2nd leading cause of cancer-related mortality worldwide [1, 2]. Early diagnosis and new surgical strategies have played major roles in improving the survival of primary breast cancer patients. The malignant nature of this disease is related to its tendency to metastasis to distant sites including bone, lung, liver and brain [3]. The available literature indicates that 5–10% of breast cancer patients are initially diagnosed with synchronous metastases, while 20–30% of tumour-resected patients develop metachronous metastases [4–6]. Bone, a rich source of growth factors, is the most favourable 'soil' for breast cancer cells ('seeds') and up to 70% of the patients experience osteolytic bone metastasis [7, 8]. Clinical features associated with metastasis are pain, hypercalcemia, fracture and paralysis resulting in significant morbidity and mortality. The 5-year survival rate of patients with bone metastasis is low (< 21%). In view of this dilemma, where available treatment options (i.e., surgery/radiotherapy/chemotherapy/immunotherapy/hormone therapy) are palliative in nature rather than curative, the need for exploring novel therapeutic targets for breast cancer bone metastasis is evident [9].

The chemokine network, comprising low molecular weight chemo-attractant cytokines, includes important players of

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13402-018-0415-3) contains supplementary material, which is available to authorized users.

✉ Martin R. Berger
m.berger@dkfz.de

[1] Institute of Biomedical and Allied Health Sciences, University of Health Sciences, Lahore, Pakistan

[2] Toxicology and Chemotherapy Unit, German Cancer Research Centre (DKFZ), Im Neuenheimer Feld 581, 69120 Heidelberg, Germany

[3] German University of Cairo, Cairo, Egypt

various homeostatic and inflammatory processes [10–13]. In the context of breast cancer metastasis, alterations in the chemokine network, either with anti-tumour or pro-tumour effects, have been witnessed [14–17]. The chemokine receptor 5 (CCR5 or CD195), which has originally been recognized as a co-receptor associated with HIV infections, is an important member of the chemokine network. Like many other chemokine receptors, CCR5 exhibits redundancy and interacts with multiple ligands of the chemokine network [18]. In addition, CCR5 is found on a wide range of cells including stromal cells, leukocytes and tumour cells. These characteristics reflect significance of the CCR5 receptor and its multidirectional role in both normal physiological and cancer-related processes. In breast cancer, higher expression levels of CCR5 and its cognate ligands, mainly CCL4 and CCL5, have been associated with their tumour-promoting functions. An established pro-tumour role of the CCR5-dependent chemokine axis has recently gained attention as a potential therapeutic target in advanced stage breast cancers [19–26].

Here, we investigated whether targeting CCR5 may contribute to the treatment of breast cancer bone metastasis. To this end, we selected two metastatic breast cancer-derived cell lines (MDA-MB-231 and MCF-7), blocked CCR5 using a FDA approved antagonist (maraviroc) and investigated its impact on several functional properties of the selected cells using various in vitro assays, followed by in vivo studies using a bone metastasis animal model. In addition, we measured the circulatory levels of three cognate ligands of the CCR5 receptor, i.e., CCL3, CCL4 and CCL5, in serum samples of breast cancer patients and healthy controls to assess potential morbidity-related changes in ligand levels.

2 Materials and methods

2.1 Cell cultures, maraviroc and antibodies

Human breast cancer-derived cell lines MDA-MB-231 and MCF-7 were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% foetal bovine serum (FBS), and maintained under standard conditions (5% CO_2, 37 °C, humidified incubator). Maraviroc was purchased either as powder (Selleck Chemical Co. China, UK-427857) or as tablets containing maraviroc for the treatment of patients (Viiv Healthcare GmbH, Germany). Antibodies directed against cleaved PARP, caspase-3 and -7 were purchased from Cell Signalling Technologies, while those directed against CCR5 and β-actin were purchased from Santa Cruz Biotechnology.

2.2 CCR5 expression in breast cancer cell lines

CCR5 expression was assessed using quantitative real-time PCR (qRT-PCR). To this end, total RNA was extracted from cell pellets using a RNeasy Mini kit (Qiagen, Hilden, Germany) after which cDNA synthesis, using Maxima reverse transcriptase (Thermo Scientific, Schwerte, Germany) and CCR5 amplification using gene specific primers (5'-AACCAGGCGAGAGACTTGTG-3′, 3'-GATCCAAC TCAAATTCCTTCTCA-5′), were carried out. The samples were processed in triplicate and the expression level of a reference gene (GAPDH) was used to normalize the data. CCR5 expression was also assessed at the protein level using Western blotting. For this purpose, protein lysates (30 μg) were subjected to electrophoresis, transferred to PVDF membranes and probed for CCR5 using a specific antibody (see above) as per manufacturer's protocol. β-actin was used to normalize the expression levels, and relative concentrations were analysed using the ImageJ software tool [26].

2.3 Cell proliferation and colony formation assays

To assess the effect of CCR5 blockade on proliferation, cells (4000 cells/well/100 μl medium) were treated with maraviroc (3.1–500 μM) dissolved in ethanol (100 mM stock) or vehicle only. The highest ethanol concentration used was ≤ 0.5% in any of the treated samples. Subsequently, proliferation was measured using an MTT dye reduction assay as described before [27]. Dose response curves and inhibitory concentrations (IC) were calculated using GraphPad Prism 6 software. For colony formation assessment after 48 h treatment with maraviroc (IC_{20}) or vehicle, cells ($0.5–1.0 \times 10^3$/2 ml semi-solid medium) were transferred to 6-well plates and incubated for 6–8 days under standard conditions, after which colony forming units (CFU) were determined as described before [27]. The data are presented as percentages of controls. In these and all following experiments, controls were treated with equal concentrations of the vehicle only.

2.4 Migration and scratch wound healing assays

To study chemo-attractant (FBS)-induced directional migration, cells (5×10^4 cells/filter) were transferred to hanging millicell filters (Millicell, Millipore) and treated with low concentrations of maraviroc (IC_{20}) or vehicle only, after which migrating cells were counted as described before [27]. With a doubling time of 22–25 h for MDA-MB-231 and 35–40 h for MCF-7, the cells were allowed to migrate towards FBS for 24 and 48 h. Scratch wound healing was assessed by seeding 1×10^5 cells/well in a 12-well plate. The next day cell monolayers were scratched using a 200 μl sterile plastic pipette tip, exposed to vehicle or maraviroc (IC_{20}) and photographed at 0 and 24 h using an Axio Observer Z1 microscope (Carl Zeiss) to monitor the "wound healing" responses of the cells.

2.5 Apoptosis and cell cycle analyses

Apoptotic effects in response to maraviroc exposure were assessed using an annexin V-FITC labelling assay [28]. In brief, cells were treated with maraviroc (IC_{25}, IC_{50} or IC_{75}) for 48 h after which the percentages of apoptotic cells were determined using an annexin V-FITC apoptosis detection kit (eBioscience, Germany) in conjunction with flow cytometry (BD Biosciences, USA). Following exposure to maraviroc (IC_{25}, IC_{50} or IC_{75}) for 48 h, effects on the cell cycle were assessed using a propidium iodide (PI)-based DNA staining method [27]. The relative percentages of cells in different cell cycle phases were determined using the ModFit LT 4.1 software tool.

2.6 Nuclear staining and Western blotting

Following maraviroc exposure for 48 h, the effects on apoptosis-induced nuclear condensation/fragmentation were studied by Hoechst 33342 staining [27]. In addition, Western blot analyses were performed to assess cleaved PARP and cleaved caspase-3 and -7 expression levels according to a procedure described before [28]. Briefly, cells were exposed to maraviroc (IC_{25}, IC_{50} or IC_{75}) for 48 h followed by harvesting and lysis. After measurement of protein concentrations, samples were subjected to electrophoresis and analysed using pre-selected antibodies. β-actin levels were used to normalize the data. Relative protein concentrations from treated and control groups were assessed by densitometric analysis of digitized autographic images using the ImageJ software tool.

2.7 Cell cycle panel analysis

Maraviroc mediated cytostatic changes were studied in detail using a Human Cell Cycle Regulation Panel (Cat. 05339359001, Roche). Briefly, cells were exposed to maraviroc (IC_{25} and IC_{75}) for 48 h followed by total RNA extraction using a RNeasy Mini kit (Qiagen, Hilden, Germany) and cDNA synthesis using Maxima reverse transcriptase (Thermo Scientific, Schwerte, Germany). The expression profiles of 84 cell cycle relevant genes were assessed using the above mentioned panel in conjunction with qRT-PCR. Based on the results obtained, affected cell cycle signalling pathways were predicted using the Ingenuity Pathway Analysis tool (Redwood, USA).

2.8 In vivo tumorigenesis, treatment and tumour growth monitoring

In vivo experiments were performed according to relevant ethical standards, international guidelines and the Declaration of Helsinki. Male nude rats (RNU strain, 5-6 week old) were obtained from the Charles River Laboratory (Germany) and kept under controlled conditions (temperature: 22 ± 1 °C, humidity: 50 ± 10%, dark-light-rhythm: 12 h) in a specific pathogen free (SPF) environment. After an adaptation period of one week, the rats were transplanted with 1×10^5 MDA-MB-231 cells/rat (transfected with $RFP^{mStrawberry}$/Luciferase markers) through the saphenous artery for inducing bone metastasis as described before [29–31]. Rats, transplanted with tumour cells were divided into three random groups: (a) vehicle treated control (6 rats), (b) treatment starting from the 2nd day after transplantation (6 rats) and (c) treatment starting from the 7th day after transplantation (4 rats). For treatment, maraviroc was extracted from commercially available tablets using ethanol as solvent. Daily intra-peritoneal injections of 25 mg maraviroc/kg/rat were continued for 4 weeks for group b and 3 weeks for group c. Tumour growth was monitored in treated and control rats once per week using bioluminescence imaging (BLI).

2.9 Circulatory ligand level assessment in clinical samples

During the period 2016–2017, 32 blood samples were collected from naïve breast cancer patients at the Institute of Nuclear Medicine and Oncology Lahore (INMOL), Pakistan. Sera were separated from the blood samples using a serum separator tube after allowing the samples to clot for 30 min and centrifugation for 15 min (1000x g). The resulting sera were stored at ≤ −20 °C. Circulatory levels of the three CCR5 receptor ligands [CCL3 (SEH00566A), CCL4 (SEH00563A), CCL5 (SEH00703A)] were assessed by enzyme-linked immunosorbent assay (ELISA) using single-analyte ELISArray kits (Qiagen). The results were compared with those from 16 volunteer healthy controls.

2.10 Statistical analysis

GraphPad Prism 6 software was used for statistical analysis of the data. Student *t*-test and one-way ANOVA were used for comparisons between two or more groups, respectively. Data are presented as mean ± SD and a p value < 0.05 is considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3 Results

3.1 CCR5 expression and its blockage by maraviroc negatively regulates the proliferation, colony formation and migration of breast cancer cells

Initially, the expression of CCR5 was determined at both the mRNA and protein levels in MDA-MB-231 and MCF-7 breast cancer-derived cells. Although clearly detectable in both cell lines, we found that the relative expression level of CCR5 was higher in MCF-7 cells (Fig. 1a, b). Migration of cancer cells to distant sites followed by colonization and

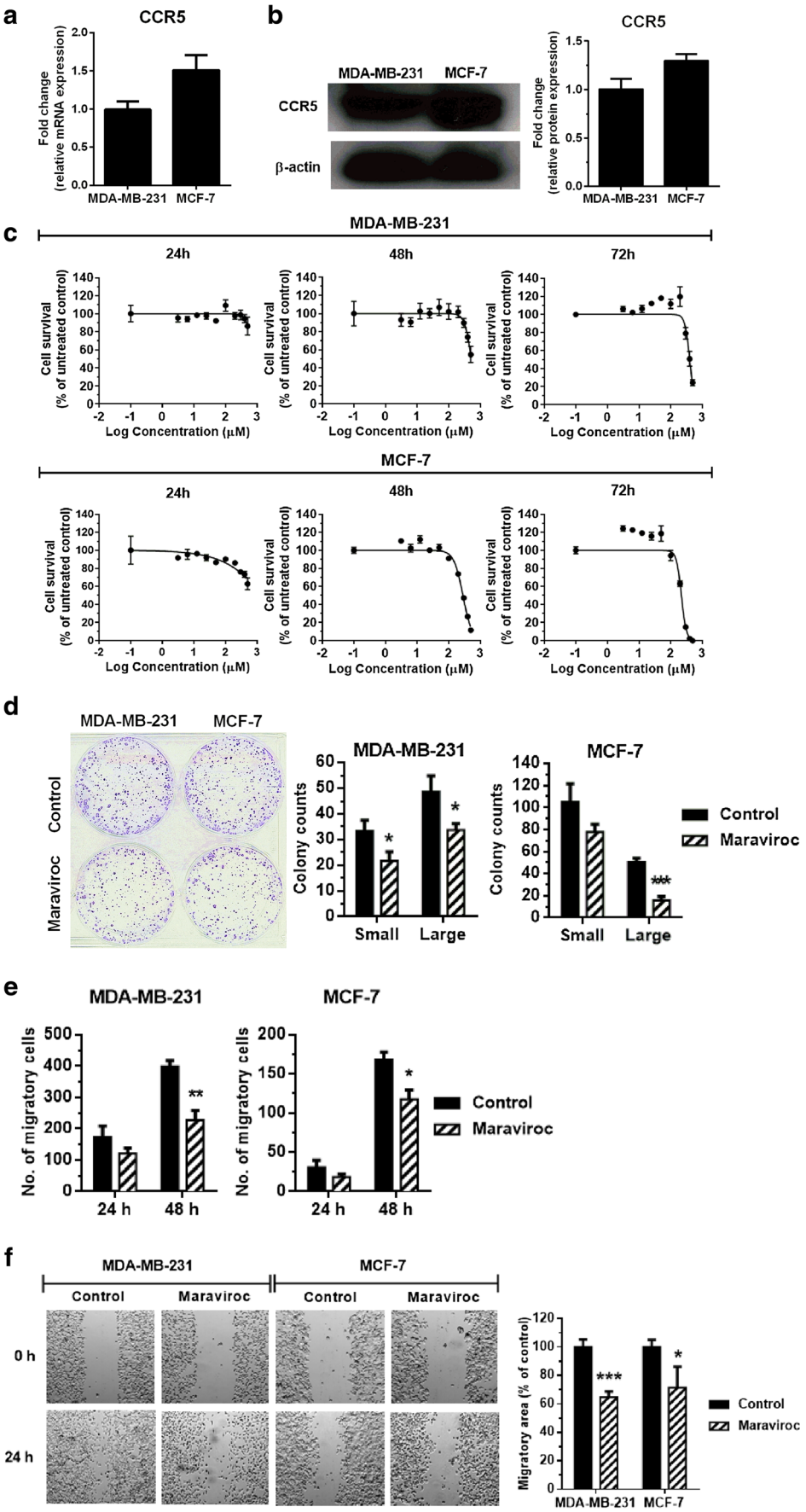


Fig. 1 CCR5 expression and maraviroc-mediated inhibition of proliferation, colony formation and migration of breast cancer cells. **a, b** CCR5 expression was analysed in breast cancer cells by qRT-PCR and Western blotting. The expression level of CCR5 was 1.51- and 1.29-fold higher in MCF-7 cells than in MDA-MB-231 cells at the mRNA and protein levels, respectively. Experiments were repeated at least twice to validate the expression levels in the two cell lines. **c** MDA-MB-231 and MCF-7 cells were treated with increasing concentrations of maraviroc (3.1–500 μM) and cytotoxic effects were investigated by MTT assay. Inhibitory concentrations (IC) were analysed using GraphPad Prism 6 software. **d** Exposure to low concentrations of maraviroc (IC_{20}) for 48 h induced significant anti-clonogenic effects in MDA-MB-231 and MCF-7 cells. The colonies were stained with Giemsa for photographic purposes. **e, f** Blockage of CCR5 by maraviroc (IC_{20}) inhibited the migration of MDA-MB-231 and MCF-7 cells in transwell migration and scratch wound healing assays. The asterisks denote statistically significant differences between control and treated groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Error bars denote standard deviations

Table 1 Cell growth inhibition after 48 and 72 h of maraviroc exposure

MDA-MB-231[a), b)]	MCF-7 [a), b)]
IC_{50} (48 h) = 521 μM (476-569 μM, 95% CI)	IC_{50} (48 h) = 282 μM (268-297 μM, 95% CI)
IC_{50} (72 h) = 403 μM (379-429 μM, 95% CI)	IC_{50} (72 h) = 220 μM (199-242 μM, 95% CI)

[a)] IC_{50}: Concentration inhibiting cellular growth by 50%

[b)] 95% CI: 95% Confidence interval

proliferation are basic features of the metastatic process. Considering these features, the importance of the CCR5-related chemokine axis for breast cancer cells was tested using MTT cell proliferation, colony formation, migration and

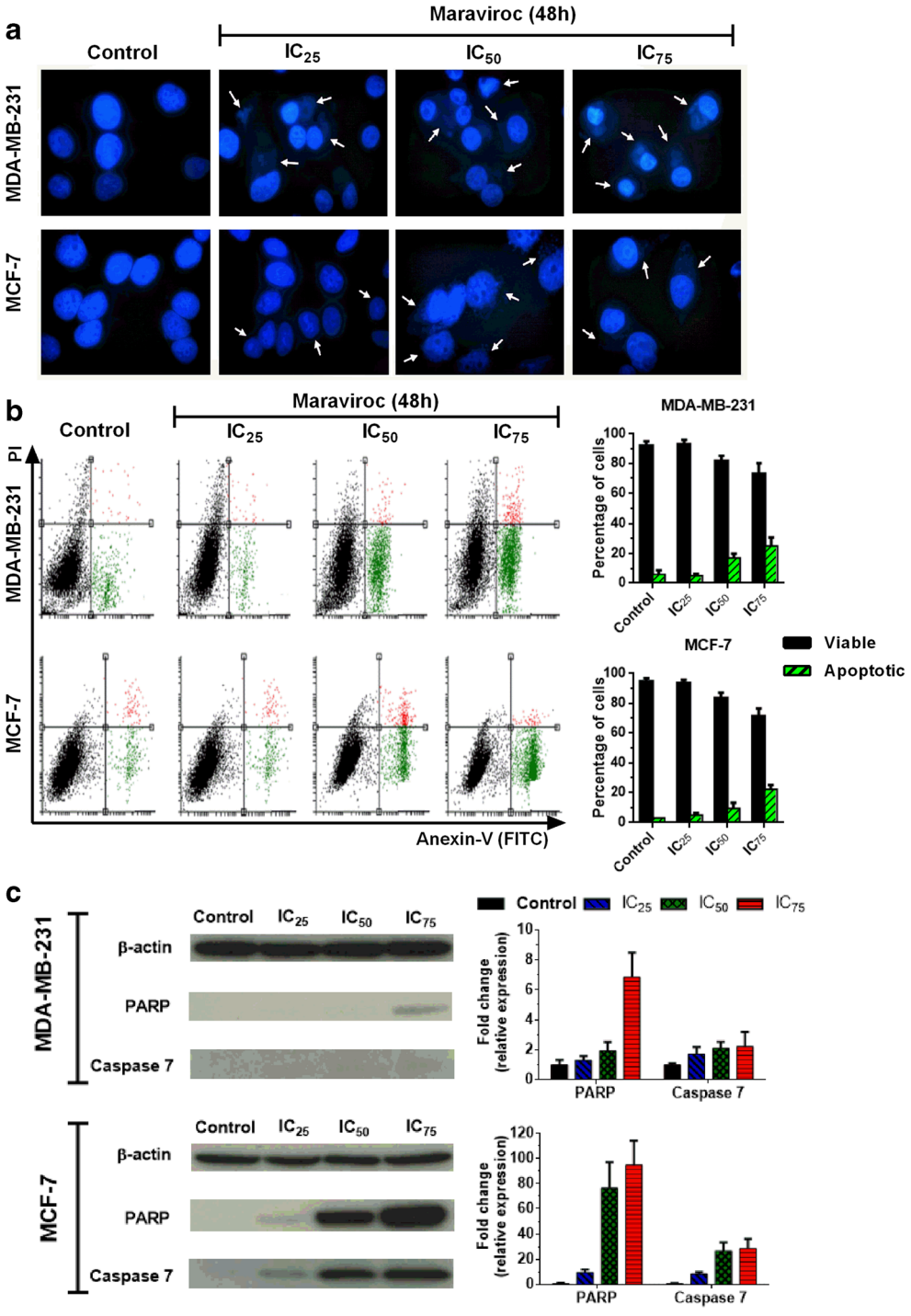


Fig. 2 Apoptotic effects induced by maraviroc in breast cancer cells. **a** Maraviroc exposure for 48 h induced significant apoptotic changes, including nuclear shrinkage and fragmentation (indicated by white arrows), in breast cancer cells as examined by Hoechst 33342 staining. **b** Annexin-V/PI staining to explore apoptosis induced by blockage of CCR5 with maraviroc. Breast cancer cells were exposed to increasing concentrations of maraviroc and percentages of apoptotic cells, stained with annexin-V (shown in lower right quadrants), were determined by flow cytometry. Experiments were repeated at least twice to validate the apoptotic effects. **c** Following treatment of cells with maraviroc for 48 h, Western blot analyses were performed to determine the expression levels of cleaved caspase-3 (data not shown here as no expression was found), cleaved caspase-7 and cleaved PARP. β-actin was used as loading control and fold changes were calculated using the *ImageJ* tool. Experiments were repeated at least twice to validate the expression profile of caspase-7 and PARP in the two cell lines. Error bars denote standard deviations

scratch wound healing assays. Following exposure to increasing maraviroc concentrations (3.12–500 μM), the MTT assay revealed inhibitory effects of CCR5 blockage on cell proliferation. Clear cytotoxic effects were manifest at higher maraviroc concentrations (≥ 100 μM) and caused a steep decline in the percentage of viable cells after 48 and 72 h, as revealed by dose-response curves (Fig. 1c). The corresponding concentrations inhibiting 50% cell growth (IC_{50}), calculated using the GraphPad Prism 6 software tool, are listed in Table 1. By comparing the IC_{50} values, we found that MCF-7 cells were ~2-fold more sensitive to maraviroc than MDA-MB-231 cells. A colony formation assay was carried out to explore CCR5-dependent clonogenic abilities of the respective breast cancer cells. Blockage of CCR5 by low concentrations of maraviroc (IC_{20}) for 48 h and subsequent seeding of the cells (single cell suspensions) in semi-solid growth media revealed a significant inhibition of their colony forming capacities compared to the respective controls (Fig. 1d). The migratory behaviour of breast cancer cells, either towards increasing concentrations of FBS (migration assay) or towards other cells (scratch wound healing assay), was studied after exposing the cells to low concentrations of maraviroc (IC_{20}) for 24–48 h. Both types of migration assays revealed that blocking CCR5 by maraviroc significantly inhibited the directional migration of the breast cancer cells (Fig. 1e, f). Although the effects observed after 48 h could be partially due to the anti-proliferative activity of maraviroc, the observed anti-migratory effects were distinctively stronger (> 40%, MDA-MB-231, > 30%, MCF-7) than would have been expected from the applied concentrations (IC_{20}).

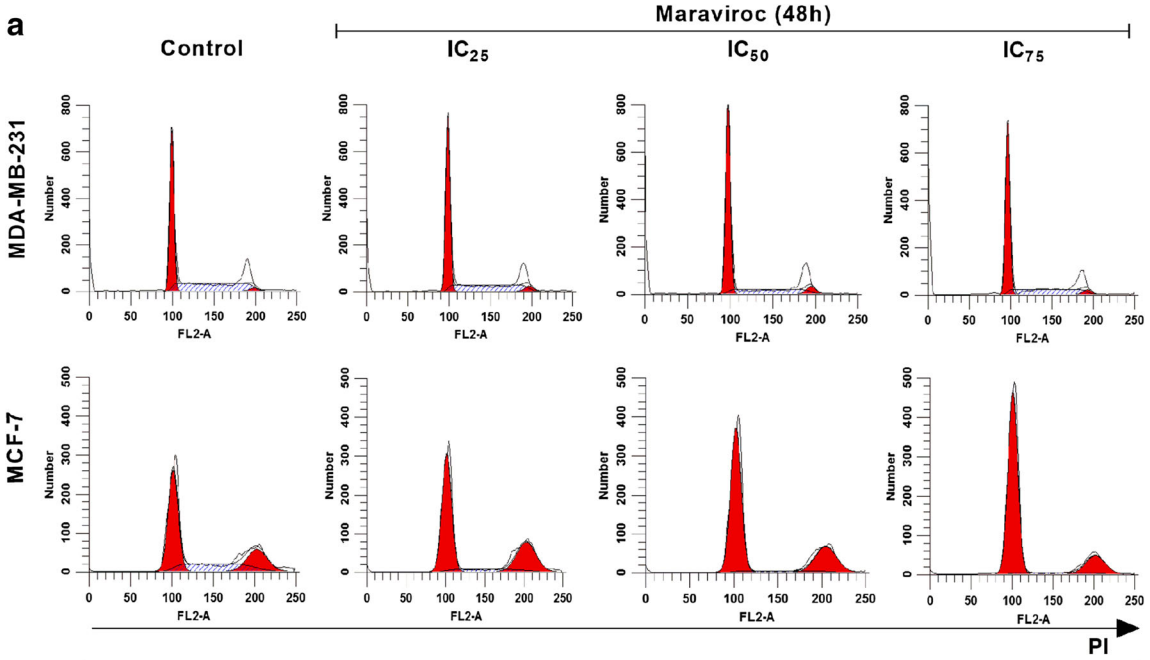
3.2 CCR5 blockage by maraviroc induces apoptosis in breast cancer cells

In pursue of long-term survival, cancer cells try to avoid apoptosis, a natural phenomenon for the neat and clean elimination of dying cells. Here, we found that blockage of CCR5 by maraviroc leads to apoptosis at morphological, functional and molecular levels in breast cancer cells as assessed by Hoechst 33342 staining, annexin-V labelling/FACS flow cytometry and Western blotting, respectively. In response to maraviroc exposure, we observed significant signs of apoptosis such as nuclear condensation and initiation of fragmentation in the respective breast cancer cells in a concentration-dependent manner, while almost no such effects were observed in control cells treated with the vehicle (Fig. 2a). These maraviroc-mediated apoptotic effects were substantiated by positive annexin-V labelling of the cells going through apoptosis and by exposing membrane-bound phosphatidylserine molecules as targets for the fluorescent dye molecules (Fig. 2b). The concentration-dependent apoptotic effects observed after annexin-V labelling were in line with their counter-morphological effects observed after Hoechst 33342 staining. The molecular mechanisms underlying these apoptotic effects were subsequently assessed through Western blot analyses of cleaved caspase-3, cleaved caspase-7 and cleaved PARP, the final players in the apoptotic process. We found that exposure to maraviroc induced the expression of cleaved caspase-7 and cleaved PARP in both breast cancer cell lines tested in a concentration-dependent manner. The caspase-3 expression level was found to be below the detection limit (data not shown). Exposure to maraviroc (IC_{50}) induced more effectively cleaved caspase-7 and cleaved PARP levels (13- and 41-fold, respectively) in MCF-7 cells than in MDA-MB-231 cells (Fig. 2c).

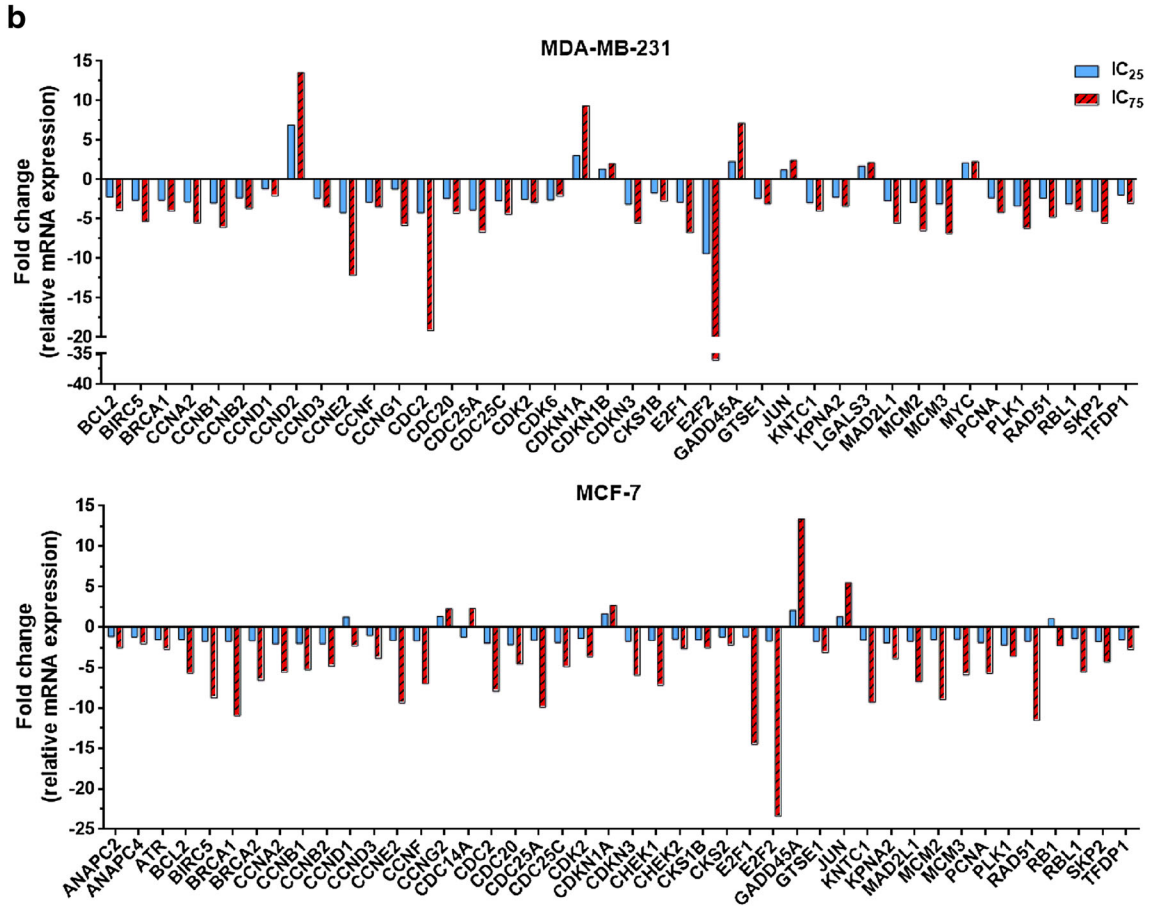
3.3 Maraviroc mediated blockage of CCR5 modulates expression changes in cell cycle relevant genes leading to G1 arrest

Evasion from normal cell cycle checkpoints, which leads to uncontrolled proliferation, is a key feature of cancerous cells. Here, we explored whether blocking of CCR5 by maraviroc may induce cell cycle arrest through gene expression alterations in breast cancer cells. We found that exposure to maraviroc for 48 h, followed by PI labelling and flow cytometry analysis, resulted in a G1 cell cycle phase arrest in the breast cancer cells tested. The effects were more pronounced and concentration-dependent in MCF-7 cells than in MDA-MB-231 cells (Fig. 3a). To uncover the molecular mechanisms underlying these cell cycle alterations, expression changes in 84 known cell cycle-relevant genes were assessed in both cell lines using a ready-made qRT-PCR based panel. This panel includes key players such as cyclins, cyclin-dependent kinases (CDKs), checkpoint

Fig. 3 Blockade of CCR5 by maraviroc interferes with the cell cycle of breast cancer cells. **a** Cell cycle distributions of cells treated with maraviroc were determined by propidium iodide (PI)-based staining. Quantitative fractions (%) from three different phases of the cell cycle, provided in the table below the figure, were determined by flow cytometry. Experiments were repeated at least twice to validate cytostatic effects imposed by maraviroc in the breast cancer cells. **b** Alterations in multiple cell cycle-related genes in response to maraviroc exposure for 48 h were evaluated by qRT-PCR using the *Human Cell Cycle Panel, 96*. CP (crossing point) values as indicators of linear amplification of transcribed cDNAs were calculated using the *Second Derivative Maximum Method*, while the ΔΔ*CT* method was used to analyze relative expressional levels of the genes from the treated samples. The expression levels from control samples were set to unity while amplification of seven reference genes, incorporated in the panel, was used to normalize the data. **c** Significant changes in cell cycle gene expression (≥ 2-fold) were compared between MDA-MB-231 and MCF-7 cells. A total of 34 genes (68%) was altered in both cells in a similar fashion, whereas 6 (12%) and 10 (20%) genes were altered only in MDA-MB-231 or MCF-7 cells, respectively. **d** The data sets from **b** were used to generate schematic signalling models for cell cycle relevant pathways using *Ingenuity Pathway Software Analysis*. The working models revealed major alterations in the expression levels of genes involved in estrogen-dependent G1 to S phase entry and G1-S phase checkpoint regulation of the cell cycle



Cell Line	MDA-MB-231				MCF-7			
Maraviroc	0	IC_{25}	IC_{50}	IC_{75}	0	IC_{25}	IC_{50}	IC_{75}
Phase G0/G1 (%)	56	63	72	68	53	61	71	80
Phase S (%)	42	33	22	28	24	8	4	4
Phase G2/M (%)	2	4	6	4	23	31	25	16



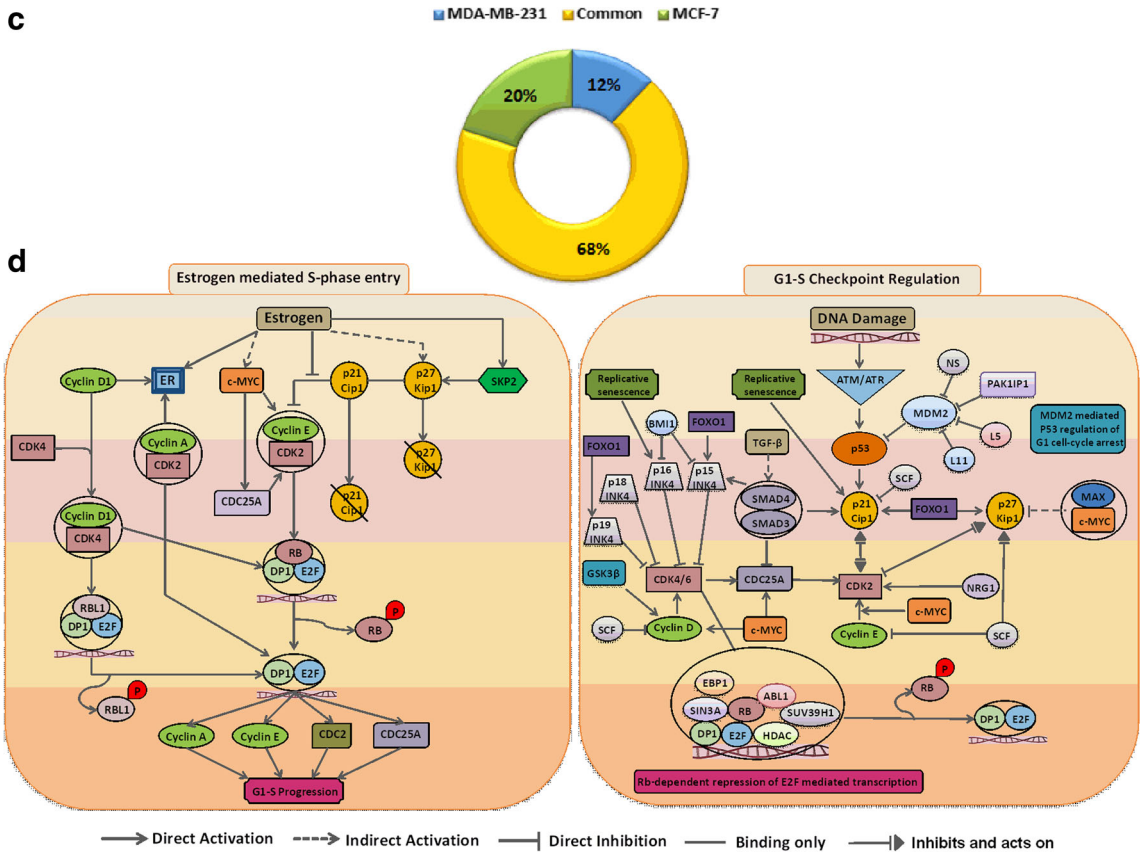


Fig. 3 (continued)

components and cell cycle inhibitors, facilitators and inducers (see supplementary data for details). We found that blockage of CCR5 by maraviroc (IC_{75}) altered the expression (≥ 2fold) of 40 and 44 genes of the panel in MDA-MB-231 (40/84 = 48%) and MCF-7 (44/84 = 52%) cells, respectively (Fig. 3b). Furthermore, by comparing the expression changes, we found that the two cell lines exhibited a significant similarity (68%) in the gene expression alteration patterns (Fig. 3c). In addition to this, based on the identified expression alterations after maraviroc exposure (IC_{75}), we were able to establish working models describing the signalling cascades using *Ingenuity Pathway Software Analysis*. This analysis primarily highlighted the involvement of estrogen-mediated G1 to S phase entry of the cell cycle as indicated by the highest overlap (21/26 = 81%, p = 8.51E-47) of involved altered genes compared to all genes included in the software protocol/core analysis (Fig. 3d). G1-S checkpoint regulatory signalling was found to be the second potential cascade involved in maraviroc-induced cell cycle arrest, as indicated by an overlap of 42% (27/65, p = 1.43E-48) of its involved genes. Major up-stream regulators proposed by the analysis for these canonical pathways include cell cycle inhibitors (CDKN1A/2A), TP53 and cell cycle activators (E2F family of transcription factors).

3.4 CCR5 blockage by maraviroc suppresses bone metastasis in an animal model

The observed cytotoxic and cytostatic effects of maraviroc in the in vitro experiments above, and supportive evidence from the literature on CCR5 axis involvement in breast cancer progression, prompted us to evaluate the efficacy of maraviroc on bone metastasis inhibition in an animal model. Following transplantation of MDA-MB-231 cells to the left hind leg of rats via the saphenous artery, they were treated with maraviroc (25 mg/kg/day). To discriminate a concomitant versus a delayed tumour onset after treatment, the rats were dosed starting after either 2 or 7 days following tumour cell transplantation (groups B and C, respectively). Tumour growth, measured by bioluminescence imaging (BLI), was found to be continuous in the vehicle treated control group A till the 3rd week after transplantation. In week 4, a non-significant reduction in signal intensity was observed, probably due to necrotic tissue areas, which are unable to metabolise the substrate (luciferin). Following treatment with maraviroc, a substantial and steady reduction in tumour growth was observed in group B (p = 0.0364, week 4), whereas only a marginal inhibition was observed in group C compared to the vehicle treated control group A (Fig. 4).

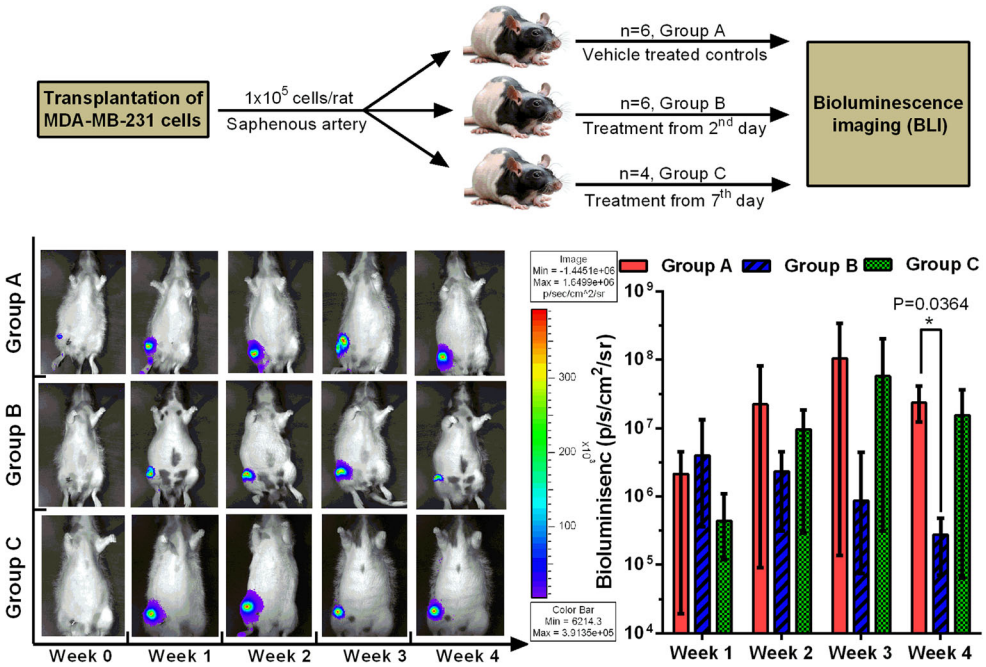


Fig. 4 Treatment with maraviroc inhibits breast cancer bone metastasis. Following transplantation of MDA-MB-231 cells into the hind leg through the saphenous artery, rats were treated with maraviroc (25 mg/kg/rat/day) through intra-peritoneal injections. Treatment started after 2 or 7 days following transplantation for groups B and C, respectively, while control rats (group A) were treated with vehicle only. Tumour growth was monitored once per week using bioluminescence imaging. Continuous tumour growth was observed in the control group A till the 3rd week, while there was some reduction in signal intensity in week 4. When compared with the control group A, treatment with maraviroc considerably reduced the tumour growth in group B during the whole test period, while minimal inhibition was observed in group C. Error bars denote standard deviations

3.5 Circulatory CCR5 ligand levels in breast cancer patients are distinct from those in healthy controls

After having uncovered a putative role of CCR5 as a therapeutic target in our pre-clinical experiments, we expanded the work to clinical grounds by assessing the circulatory levels of the three cognate CCR5 ligands in serum samples of breast cancer patients compared to healthy controls. We restricted ourselves to patients with stage II and III disease as they cover the major proportion of diagnosed cases in Pakistan (≥ 85%) and are more in focus of pharmacological treatment [32]. By comparing the mean ligand levels in healthy controls, we observed large differences in relative ratios, ranging from 3- to 55-fold, when relating CCL3 to CCL4 and CCL5, respectively. The abundance of CCL5 was also highlighted by a factor 19-fold relative to that of CCL4 (Table 2). Although the three ligands showed varied profiles, their respective calculated average concentrations revealed differences between the healthy controls and the breast cancer patients tested (Fig. 5a). The average concentration of CCL3, compared to healthy controls (17.2 pg/ml), was slightly higher in stage II patients (21.3 pg/ml) and almost 1.5-fold lower in stage III patients (11.4 pg/ml). The average concentrations of CCL4 were significantly higher in stage II patients (107.9 pg/ml) compared to healthy controls (70.6 pg/ml, p = 0.041) and stage III patients (63.6 pg/ml, p = 0.001). The latter group did not differ, however, from the control group. The average concentrations of CCL5 were almost similar in healthy controls (1347.6 pg/ml) and stage II patients (1348.7 pg/ml). However, there were significant differences when comparing the CCL5 levels of stage III patients (1297.2 pg/ml) with those of healthy controls (p = 0.0011) and stage II patients (p = 0.0006) (Table 2). When comparing the three ligand levels between age groups, we found that the CCL3 and CCL4 levels exhibited decreases with increasing ages in the healthy controls, while those in the stage II and III patients almost remained constant. In contrast, we found that the CCL5 levels did not fluctuate distinctly in healthy controls and patients in different age groups (Fig. 5b).

4 Discussion

It has been reported that chemokines play crucial roles in the organ-specific homing of breast cancer cells during metastasis [33, 34]. CCR5 and its cognate ligands (CCL3, CCL4, CCL5) have been found to comprise an important mechanistic axis for breast cancer progression [24–26, 35], and the widely accepted pro-tumour role of CCR5 in various malignancies was compelling enough to evaluate its therapeutic relevance [36–38]. Recently, we contributed to this notion by successfully targeting CCR5 with a FDA approved antagonist (maraviroc)

Table 2 Demographic and clinical data of patients and clinical samples

	Healthy Controls	Stage II [a)]	Stage III [a)]
Numbers	16	16	16
Gender	Female	Female	Female
Age (years)	Range 26–54	Range 33–55	Range 32–60
	Average 38.2	Average 42.1	Average 45.4
CCL3 (pg/ml)	Range: 5.0–128.7	Range: 6.0–133.4	Range: 5.0–45.2
	Average: 17.2	Average: 21.3	Average: 11.4
CCL4 (pg/ml)	Range: 16.2–178	Range: 50.3–327	Range: 16.0–224.9
	Average: 70.6	Average: 107.9	Average: 63.6
CCL5 (pg/ml)	Range: 1286.2–1396.8	Range: 1252.1–1396.8	Range: 1264.5–1367.7
	Average: 1347.6	Average: 1348.7	Average: 1297.2

[a)] Breast cancer patient stage

in colorectal cancer cells [28]. In addition to these pre-clinical studies, a first proof of concept for its clinical efficacy became available after the treatment of patients with colorectal cancer liver metastasis with maraviroc in a phase I study [39]. Maraviroc is a competitive (non-allosteric) antagonist of the CCR5 receptor, which was initially designed as an entry inhibitor to treat HIV infections [40]. Due to its favourable pharmacological profile with minimal liver toxicity, the primary organ for its metabolism [41], maraviroc is now in focus as a potential therapeutic compound for treating cancer.

Well-established multiple functions governed by the CCR5 axis include chemotaxis, cytoskeletal changes, cellular adhesion, survival/proliferation signalling, transcription regulation and contributions to cytostatic and apoptotic events [18, 28, 42, 43]. Here, we found that targeting CCR5 in breast cancer cells with maraviroc induces significant anti-neoplastic effects at the functional level (i.e., proliferation, migration and colony formation), as well as apoptosis and cell cycle arrest at the sub-cellular level. We found that MCF-7 cells were more responsive than MDA-MB-231 cells as indicated by its lower IC_{50} values, higher levels of cleaved PARP/caspase-7, as well as higher cell cycle arrest ratios in the G1 phase. These observations may be explained, at least in part, from the observed CCR5 expression level differences, i.e., MCF-7 cells showed higher expression levels than MDA-MB-231 cells. The differences in expression levels point at a greater functional dependency of MCF-7 cells on CCR5 and, concomitantly, a higher sensitivity to blockade by maraviroc. Additionally, these differences may, at least partly, be explained by disturbances in cell cycle-related pathways, with a primary involvement of the estrogen receptor and TP53. Estrogens are known to induce the proliferation of ER-positive cells by stimulating G1/S transition, while TP53 is known to act as a major up-stream regulator of cytostatic and apoptotic processes [44, 45]. Considering these facts, ER-positive MCF-7 cells with wild-type TP53 (luminal-like features) were expectedly more responsive to maraviroc, whereas MDA-MB-231 cells with an ER-negative status and mutated TP53 (basal-like features) showed a lower sensitivity to CCR5 blockage. In addition, the presence of a dominant-negative form of CCR5 (CCR5Δ32) in MDA-MB-231 cells [46] may contribute to the reduced sensitivity observed. We nevertheless used MDA-MB-231 cells for our in vivo experiments, because we reasoned that regression of lesions in this breast cancer skeletal metastasis model, representing the difficult to treat triple negative or basal breast cancer subtype, would have a greater translational relevance than regression of lesions of a hormone responsive cell line, representing the luminal A subtype [47]. This clinical description is corroborated for MDA-MB-231 cells by their highly invasive and metastatic nature due to the expression of EMT markers, a high expression of proteases, a low expression of claudins, and the formation of loosely cohesive grape-like or stellate structures. In addition, MDA-MB-231 cells are more tumorigenic in nature, harbour pathogenic mutations (TP53, BRAF, CDN2A, KRAS and NF2), exhibit stem cell-like properties (i.e., $CD44^{+}CD24^{-/low}$ phenotype) and grow aggressively in vivo. In contrast, MCF-7 cells exhibit non-invasive and non-metastatic properties, form tightly cohesive structures and often need hormone-based assistance (e.g. estrogen) to grow under in vivo conditions. Based on these characteristics, MDA-MB-231 cells were considered more suited for an animal model that mimics breast cancer metastasis. In this context, we previously established and characterized a breast cancer bone metastasis animal model [30, 31] in which implanted MDA-MB-231 cells cause osteolytic lesions in the femur, tibia and fibula as identified by X-rays, computed tomography and immunohistochemistry. Despite its therapy-resistant nature, we found that this model was useful to assess the efficacy of maraviroc for treating breast cancer skeletal metastases. We found that targeting CCR5 by maraviroc significantly reduced the tumour burden when the treatment was started during early stage metastasis, which in turn indicates a significant dependency on the CCR5 axis during

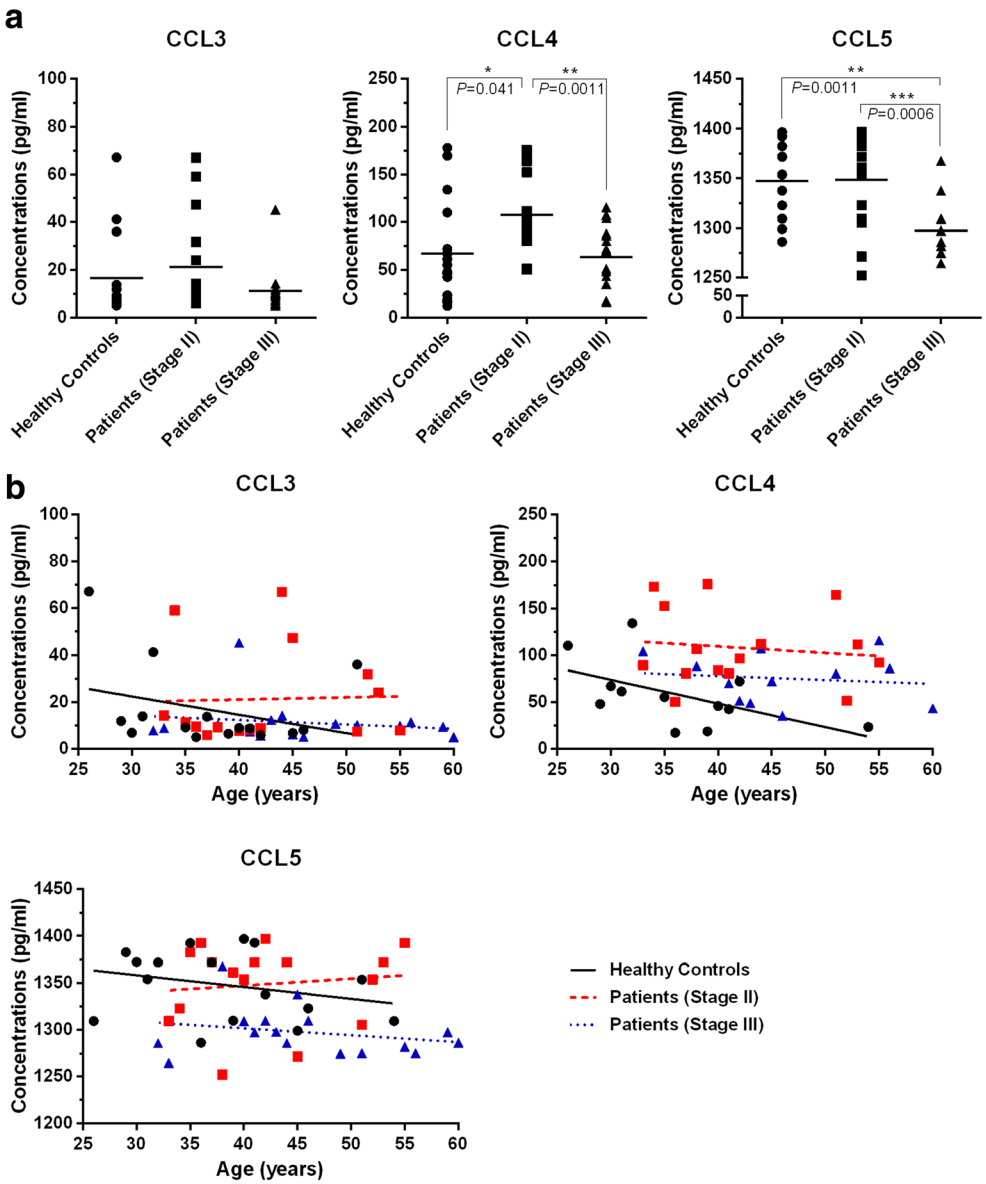


Fig. 5 Circulatory levels of three CCR5 receptor ligands (CCL3, CCL4 and CCL5) in breast cancer patients and healthy controls. **a** Circulatory levels of the three CCR5 receptor ligands were analysed by ELISA in serum samples of healthy controls, as well as stage II and III breast cancer patients (16 female individuals/group). Compared to the average concentrations of CCL3 from healthy controls (17.2 pg/ml), an almost 1.5-fold reduction in stage III patients (11.4 pg/ml) was observed, while stage II patients showed comparable levels (21.3 pg/ml). Conversely, average concentrations of CCL4 were significantly higher in stage II patients (107.9 pg/ml) compared to healthy controls (70.6 pg/ml, $p = 0.041$) and stage III patients (63.6 pg/ml, $p = 0.0011$). Average concentrations of CCL5 did not differ significantly between healthy controls (1347.6 pg/ml) and stage II patients (1348.7 pg/ml). Differences were statistically significant between stage III patients (1297.2 pg/ml) compared to stage II patients ($p = 0.0006$) and healthy controls ($p = 0.0011$). **b** CCL5 ligand levels with increasing ages did not show distinct changes in healthy controls and patients with stage II and III disease. The other two ligands (CCL3 and CCL4) showed a reduction with old age in healthy controls, while patients with stage II and III breast cancer almost maintained constant levels

the initial phase of bone colonization. In addition, we found that targeting CCR5 may be an efficient approach to treat the luminal type of breast cancer in a laboratory setting. Interestingly, Velasco-Velazquez et al. [36] reported that patients with basal and HER2-positive breast cancer subtypes may exhibit increased CCR5/CCL5 expression levels and, as such, be highly sensitive to maraviroc treatment.

Recently, several studies have highlighted the importance of CCR5 in various biological processes including the regulation of metabolic activities and angiogenic switching to neovascularization followed by invasion and progression of breast cancers [48–50]. CCR5 has also been shown to play a significant role in immune modulation under normal physiological conditions and during cancer progression [51]. Halama

et al. [39] reported the occurrence of macrophage repolarization in response to maraviroc treatment with subsequent anti-tumour effects in colorectal cancer patients with liver metastases. In breast cancer patients, tumour-associated macrophages and other immune cells have also been shown to play influential roles in progression towards metastasis [52–56]. Thus, CCR5 targeting by maraviroc could serve as a double-edged sword for breast cancer bone metastasis with anti-neoplastic effects on breast cancer cells and concomitant anti-tumour immune modulation effects. CCR5 ligands (CCL3, CCL4, CCL5) originating from cancer cells or their microenvironment, may perform various functions including cross-talk with surrounding cells, immune modulation and growth promotion in metastatic niches. Abrogation of these ligands has been shown to result in a better prognosis and a reduction in the overall disease burden [34, 57]. Increased ligand levels have been found in advanced stage breast cancer (II and III) and to correlate with tumour stage [22, 25]. One important aspect may be that the circulatory ligand levels may not represent the actual levels at the tumour sites. Other than that, ligand levels may also fluctuate depending on breast cancer sub-type and menstrual cycle [26]. Significant induction of CCR5 has been reported in tissues of stage II and III breast cancer patients and to positively correlate with metastatic behaviour [25]. An important phenomenon of the chemokine network is redundancy, i.e., multiple ligands can interact with one receptor and vice versa. So, targeting CCR5 will ideally abrogate signal activation of three cognate ligands.

In summary, we found that CCR5 blockage in breast cancer cells significantly attenuates properties required for progressive growth. Our findings support the clinical use of CCR5 antagonists for the treatment of breast cancer bone metastasis. In this respect, maraviroc is a readily available candidate compound for therapeutic use. Further (pre-)clinical trials are required to authenticate the present findings and to validate CCR5 as a therapeutic target for breast cancer bone metastasis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval For clinical investigations, informed consent was obtained from all patients and the study was approved by the relevant authorities from University of Health Sciences and INMOL hospital, Lahore, Pakistan. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Animal experiments were approved by the author's institutional review board and governmental animal ethics committee (Regierungspräsidium, Karlsruhe, Germany). All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

References

1. L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal, Global cancer statistics, 2012. CA Cancer J Clin **65**, 87–108 (2015). https://doi.org/10.3322/caac.21262
2. C. DeSantis, R. Siegel, P. Bandi, A. Jemal, Breast cancer statistics, 2011. CA Cancer J Clin **61**, 409–418 (2011). https://doi.org/10.3322/caac.20134
3. M. Yousefi, R. Nosrati, A. Salmaninejad, S. Dehghani, A. Shahryari, A. Saberi, Organ-specific metastasis of breast cancer: Molecular and cellular mechanisms underlying lung metastasis. Cell Oncol **41**, 123–140 (2018). https://doi.org/10.1007/s13402-018-0376-6
4. C. Fontanella, V. Fanotto, K. Rihawi, G. Aprile, F. Puglisi, Skeletal metastases from breast cancer: Pathogenesis of bone tropism and treatment strategy. Clin Exp Metastasis **32**, 819–833 (2015). https://doi.org/10.1007/s10585-015-9743-0
5. H. Kennecke, R. Yerushalmi, R. Woods, M.C. Cheang, D. Voduc, C.H. Speers, T.O. Nielsen, K. Gelmon, Metastatic behavior of breast cancer subtypes. J Clin Oncol **28**, 3271–3277 (2010). https://doi.org/10.1200/JCO.2009.25.9820
6. L. Gerratana, V. Fanotto, M. Bonotto, S. Bolzonello, A.M. Minisini, G. Fasola, F. Puglisi, Pattern of metastasis and outcome in patients with breast cancer. Clin Exp Metastasis **32**, 125–133 (2015). https://doi.org/10.1007/s10585-015-9697-2
7. G.R. Mundy, Metastasis to bone: Causes, consequences and therapeutic opportunities. Nat Rev Cancer **2**, 584–593 (2002). https://doi.org/10.1038/nrc867
8. L.A. Liotta, E.C. Kohn, The microenvironment of the tumour-host interface. Nature **411**, 375–379 (2001). https://doi.org/10.1038/35077241
9. P. Samadi, S. Saki, F.K. Dermani, M. Pourjafar, M. Saidijam, Emerging ways to treat breast cancer: Will promises be met? Cell Oncol (2018). https://doi.org/10.1007/s13402-018-0409-1
10. M. Stanisic, S.P. Lyngstadaas, A.H. Pripp, A.O. Aasen, K.F. Lindegaard, J. Ivanovic, E. Ilstad, A. Konglund, T. Sandell, O. Ellingsen, T. Saehle, Chemokines as markers of local inflammation and angiogenesis in patients with chronic subdural hematoma: A prospective study. Acta Neurochir **154**, 113–120; discussion 120 (2012). https://doi.org/10.1007/s00701-011-1203-2
11. F.R. Balkwill, The chemokine system and cancer. J Pathol **226**, 148–157 (2012). https://doi.org/10.1002/path.3029
12. F. Sallusto, M. Baggiolini, Chemokines and leukocyte traffic. Nat Immunol **9**, 949–952 (2008). https://doi.org/10.1038/ni.f.214
13. A. Mantovani, R. Bonecchi, M. Locati, Tuning inflammation and immunity by chemokine sequestration: Decoys and more. Nat Rev Immunol **6**, 907–918 (2006). https://doi.org/10.1038/nri1964
14. D. Mukherjee, J. Zhao, The role of chemokine receptor CXCR4 in breast cancer metastasis. Am J Cancer Res **3**, 46–57 (2013)
15. A. Muller, B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verastegui, A. Zlotnik, Involvement of chemokine receptors in breast cancer metastasis. Nature **410**, 50–56 (2001). https://doi.org/10.1038/35065016
16. B.Z. Qian, J. Li, H. Zhang, T. Kitamura, J. Zhang, L.R. Campion, E.A. Kaiser, L.A. Snyder, J.W. Pollard, CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature **475**, 222–225 (2011). https://doi.org/10.1038/nature10138
17. B. Sharma, K.C. Nannuru, M.L. Varney, R.K. Singh, Host Cxcr2-dependent regulation of mammary tumor growth and metastasis. Clin Exp Metastasis **32**, 65–72 (2015). https://doi.org/10.1007/s10585-014-9691-0

18. M. Oppermann, Chemokine receptor CCR5: Insights into structure, function, and regulation. Cell Signal **16**, 1201–1210 (2004). https://doi.org/10.1016/j.cellsig.2004.04.007
19. P. Weitzenfeld, A. Ben-Baruch, The chemokine system, and its CCR5 and CXCR4 receptors, as potential targets for personalized therapy in cancer. Cancer Lett **352**, 36–53 (2014). https://doi.org/10.1016/j.canlet.2013.10.006
20. M. Velasco-Velazquez, R.G. Pestell, The CCL5/CCR5 axis promotes metastasis in basal breast cancer. Oncoimmunology **2**, e23660 (2013). https://doi.org/10.4161/onci.23660
21. T.T. Murooka, R. Rahbar, E.N. Fish, CCL5 promotes proliferation of MCF-7 cells through mTOR-dependent mRNA translation. Biochem Biophys Res Commun **387**, 381–386 (2009). https://doi.org/10.1016/j.bbrc.2009.07.035
22. S. Ali, G. Lazennec, Chemokines: Novel targets for breast cancer metastasis. Cancer Metastasis Rev **26**, 401–420 (2007). https://doi.org/10.1007/s10555-007-9073-z
23. Y. Niwa, H. Akamatsu, H. Niwa, H. Sumi, Y. Ozaki, A. Abe, Correlation of tissue and plasma RANTES levels with disease course in patients with breast or cervical cancer. Clin Cancer Res **7**, 285–289 (2001)
24. S. Sasaki, T. Baba, T. Nishimura, Y. Hayakawa, S. Hashimoto, N. Gotoh, N. Mukaida, Essential roles of the interaction between cancer cell-derived chemokine, CCL4, and intra-bone CCR5-expressing fibroblasts in breast cancer bone metastasis. Cancer Lett **378**, 23–32 (2016). https://doi.org/10.1016/j.canlet.2016.05.005
25. Y. Zhang, F.Y. Meng, W.L. Li, C.X. Zhou, Z. Guan, H.Y. Fan, Association of chemotactic factor receptor 5 gene with breast cancer. Genet Mol Res **12**, 5289–5300 (2013). https://doi.org/10.4238/2013.November.7.4
26. A. Khalid, J. Wolfram, I. Ferrari, C. Mu, J. Mai, Z. Yang, Y. Zhao, M. Ferrari, X. Ma, H. Shen, Recent advances in discovering the role of CCL5 in metastatic breast Cancer. Mini Rev Med Chem **15**, 1063–1072 (2015)
27. A. Pervaiz, M. Zepp, H. Adwan, M.R. Berger, Riproximin modulates multiple signaling cascades leading to cytostatic and apoptotic effects in human breast cancer cells. J Cancer Res Clin Oncol **142**, 135–147 (2016). https://doi.org/10.1007/s00432-015-2013-3
28. A. Pervaiz, S. Ansari, M.R. Berger, H. Adwan, CCR5 blockage by maraviroc induces cytotoxic and apoptotic effects in colorectal cancer cells. Med Oncol **32**(158), 158 (2015). https://doi.org/10.1007/s12032-015-0607-x
29. T. Bauerle, J. Peterschmitt, H. Hilbig, F. Kiessling, F.P. Armbruster, M.R. Berger, Treatment of bone metastasis induced by MDA-MB-231 breast cancer cells with an antibody against bone sialoprotein. Int J Oncol **28**, 573–583 (2006)
30. M. Zepp, Bäuerle, T.J., Elazar, V., Peterschmidt, J., Lifshitz-Shovali, R., Adwan, H., Armbruster, F.P., Golomb, G., Berger, M.R., in Breast Cancer, Current and Alternative Therapeutic Modalities, ed. by Gunduz M, Gunduz E. (InTech, Rijeka, 2011), p. 453–488
31. T. Bauerle, H. Adwan, F. Kiessling, H. Hilbig, F.P. Armbruster, M.R. Berger, Characterization of a rat model with site-specific bone metastasis induced by MDA-MB-231 breast cancer cells and its application to the effects of an antibody against bone sialoprotein. Int J Cancer **115**, 177–186 (2005). https://doi.org/10.1002/ijc.20840
32. S. Khokher, M.U. Qureshi, S. Mahmood, S. Sadiq, Determinants of advanced stage at initial diagnosis of breast Cancer in Pakistan: Adverse tumor biology vs delay in diagnosis. Asian Pac J Cancer Prev **17**, 759–765 (2016)
33. M.I. Palacios-Arreola, K.E. Nava-Castro, J.I. Castro, E. Garcia-Zepeda, J.C. Carrero, J. Morales-Montor, The role of chemokines in breast cancer pathology and its possible use as therapeutic targets. J Immunol Res **2014**, 849720 (2014). https://doi.org/10.1155/2014/849720
34. T. Kitamura, J.W. Pollard, Therapeutic potential of chemokine signal inhibition for metastatic breast cancer. Pharmacol Res **100**, 266–270 (2015). https://doi.org/10.1016/j.phrs.2015.08.004
35. M. Velasco-Velazquez, W. Xolalpa, R.G. Pestell, The potential to target CCL5/CCR5 in breast cancer. Expert Opin Ther Targets **18**, 1265–1275 (2014). https://doi.org/10.1517/14728222.2014.949238
36. M. Velasco-Velazquez, X. Jiao, M. De La Fuente, T.G. Pestell, A. Ertel, M.P. Lisanti, R.G. Pestell, CCR5 antagonist blocks metastasis of basal breast cancer cells. Cancer Res **72**, 3839–3850 (2012). https://doi.org/10.1158/0008-5472.CAN-11-3917
37. A. Mencarelli, L. Graziosi, B. Renga, S. Cipriani, C. D'Amore, D. Francisci, A. Bruno, F. Baldelli, A. Donini, S. Fiorucci, CCR5 antagonism by Maraviroc reduces the potential for gastric Cancer cell dissemination. Transl Oncol **6**, 784–793 (2013)
38. Y. Tanabe, S. Sasaki, N. Mukaida, T. Baba, Blockade of the chemokine receptor, CCR5, reduces the growth of orthotopically injected colon cancer cells via limiting cancer-associated fibroblast accumulation. Oncotarget **7**, 48335–48345 (2016). https://doi.org/10.18632/oncotarget.10227
39. N. Halama, I. Zoernig, A. Berthel, C. Kahlert, F. Klupp, M. Suarez-Carmona, T. Suetterlin, K. Brand, J. Krauss, F. Lasitschka, T. Lerchl, C. Luckner-Minden, A. Ulrich, M. Koch, J. Weitz, M. Schneider, M.W. Buechler, L. Zitvogel, T. Herrmann, A. Benner, C. Kunz, S. Luecke, C. Springfeld, N. Grabe, C.S. Falk, D. Jaeger, Tumoral immune cell exploitation in colorectal cancer metastases can be targeted effectively by anti-CCR5 therapy in Cancer patients. Cancer Cell **29**, 587–601 (2016). https://doi.org/10.1016/j.ccell.2016.03.005
40. R. Yost, T.R. Pasquale, E.G. Sahloff, Maraviroc: A coreceptor CCR5 antagonist for management of HIV infection. Am J Health Syst Pharm **66**, 715–726 (2009). https://doi.org/10.2146/ajhp080206
41. D.K. Walker, S. Abel, P. Comby, G.J. Muirhead, A.N. Nedderman, D.A. Smith, Species differences in the disposition of the CCR5 antagonist, UK-427,857, a new potential treatment for HIV. Drug Metab Dispos **33**, 587–595 (2005). https://doi.org/10.1124/dmd.104.002626
42. Y. Wu, A. Yoder, Chemokine coreceptor signaling in HIV-1 infection and pathogenesis. PLoS Pathog **5**, e1000520 (2009). https://doi.org/10.1371/journal.ppat.1000520
43. X. Jiao, M.A. Velasco-Velazquez, M. Wang, Z. Li, H. Rui, A.R. Peck, J.E. Korkola, X. Chen, S. Xu, J.B. DuHadaway, S. Guerrero-Rodriguez, S. Addya, D. Sicoli, Z. Mu, G. Zhang, A. Stucky, X. Zhang, M. Cristofanilli, A. Fatatis, J.W. Gray, J.F. Zhong, G.C. Prendergast, R.G. Pestell, CCR5 governs DNA damage and breast cancer stem cell expansion. Cancer Res **78**, 1657–1671 (2018). https://doi.org/10.1158/0008-5472.CAN-17-0915
44. S.F. Doisneau-Sixou, C.M. Sergio, J.S. Carroll, R. Hui, E.A. Musgrove, R.L. Sutherland, Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocr Relat Cancer **10**, 179–186 (2003)
45. J. Chen, The cell-cycle arrest and apoptotic functions of p53 in tumor initiation and progression. Cold Spring Harb Perspect Med **6**, a026104 (2016). https://doi.org/10.1101/cshperspect.a026104
46. S. Manes, E. Mira, R. Colomer, S. Montero, L.M. Real, C. Gomez-Mouton, S. Jimenez-Baranda, A. Garzon, R.A. Lacalle, K. Harshman, A. Ruiz, A.C. Martinez, CCR5 expression influences the progression of human breast cancer in a p53-dependent manner. J Exp Med **198**, 1381–1389 (2003). https://doi.org/10.1084/jem.20030580
47. X.F. Dai, H.Y. Cheng, Z.H. Bai, J. Li, Breast Cancer cell line classification and its relevance with breast tumor subtyping. J Cancer **8**, 3131–3141 (2017). https://doi.org/10.7150/jca.18457

48. D. Gao, R. Rahbar, E.N. Fish, CCL5 activation of CCR5 regulates cell metabolism to enhance proliferation of breast cancer cells. Open Biol **6** (2016). https://doi.org/10.1098/rsob.160122
49. D. Gao, L.H. Cazares, E.N. Fish, CCL5-CCR5 interactions modulate metabolic events during tumor onset to promote tumorigenesis. BMC Cancer **17**, 834 (2017). https://doi.org/10.1186/s12885-017-3817-0
50. M.J. Sax, C. Gasch, V.R. Athota, R. Freeman, P. Rasighaemi, D.E. Westcott, C.J. Day, I. Nikolic, B. Elsworth, M. Wei, K. Rogers, A. Swarbrick, V. Mittal, N. Pouliot, A.S. Mellick, Cancer cell CCL5 mediates bone marrow independent angiogenesis in breast cancer. Oncotarget **7**, 85437–85449 (2016). https://doi.org/10.18632/oncotarget.13387
51. C.E. de Oliveira, J.M. Oda, R. Losi Guembarovski, K.B. de Oliveira, C.B. Ariza, J.S. Neto, B.K. Banin Hirata, M.A. Watanabe, CC chemokine receptor 5: The interface of host immunity and cancer. Dis Markers **126954**, 2014–2018 (2014). https://doi.org/10.1155/2014/126954
52. I. Vasiliadou, I. Holen, The role of macrophages in bone metastasis. J Bone Oncol **2**, 158–166 (2013). https://doi.org/10.1016/j.jbo.2013.07.002
53. E. Obeid, R. Nanda, Y.X. Fu, O.I. Olopade, The role of tumor-associated macrophages in breast cancer progression (review). Int J Oncol **43**, 5–12 (2013). https://doi.org/10.3892/ijo.2013.1938
54. Q. Zhang, J. Qin, L. Zhong, L. Gong, B. Zhang, Y. Zhang, W.Q. Gao, CCL5-mediated Th2 immune polarization promotes metastasis in luminal breast Cancer. Cancer Res **75**, 4312–4321 (2015). https://doi.org/10.1158/0008-5472.CAN-14-3590
55. Y. Vahidi, Z. Faghih, A.R. Talei, M. Doroudchi, A. Ghaderi, Memory CD4(+) T cell subsets in tumor draining lymph nodes of breast cancer patients: A focus on T stem cell memory cells. Cell Oncol **41**, 1–11 (2018). https://doi.org/10.1007/s13402-017-0352-6
56. I.A. Voutsadakis, Expression and function of immune ligand-receptor pairs in NK cells and cancer stem cells: Therapeutic implications. Cell Oncol **41**, 107–121 (2018). https://doi.org/10.1007/s13402-018-0373-9
57. E. Lee, E.J. Fertig, K. Jin, S. Sukumar, N.B. Pandey, A.S. Popel, Breast cancer cells condition lymphatic endothelial cells within pre-metastatic niches to promote metastasis. Nat Commun **5**, 4715 (2014). https://doi.org/10.1038/ncomms5715