Treatment with the C-C chemokine receptor type 5 (CCR5)-inhibitor maraviroc suppresses growth and induces apoptosis of acute lymphoblastic leukemia cells

Jie Zi1,2,3, Shushu Yuan1,2, Jianlin Qiao1,2,3, Kai Zhao1,2,3, Linyan Xu1,2,3, Kunming Qi3, Kailin Xu1,2,3, Lingyu Zeng1,2,3

1Blood Diseases Institute, Xuzhou Medical University, No. 99, West Huaihai Road, Xuzhou, Jiangsu Province, China; 2Key Laboratory of Bone Marrow Stem Cell, No. 99, West Huaihai Road, Xuzhou, Jiangsu Province, China; 3Department of Hematology, The Affiliated Hospital of Xuzhou Medical University, No. 99, West Huaihai Road, Xuzhou, Jiangsu Province, China

Received February 8, 2017; Accepted March 15, 2017; Epub April 1, 2017; Published April 15, 2017

Abstract: Acute lymphoblastic leukemia (ALL) is the most common hematological malignancy diagnosed in children and is a malignant disorder that originates from one single hematopoietic precursor committed to B- or T-cell lineage. C-C chemokine receptor type 5 (CCR5) is a chemokine and chemokine receptor pair playing critical roles in tumorigenesis. A highly potent competitive antagonist of CCR5, maraviroc, recently has been identified with suppression of cancer cells aggressive in a variety of cancers. However, the effects of maraviroc on ALL cells have not yet been defined. Here we report that CCR5 selective inhibitor significantly inhibited ALL cells SUP-B15 growth and induced SUP-B15 cells to undergo cell apoptosis. This cell apoptosis was associated with increased levels of cleavage of caspase-3 and caspase-9, and Poly (ADP-ribose) polymerase (PARP). Moreover, we demonstrated that maraviroc strongly inhibited SUP-B15 cells migration to C-X-C motif chemokine ligand 12 (CXCL12) and CXCL13, and adhesion to fibronectin and vascular cell adhesion molecule 1 (VCAM-1) in vitro. Importantly, CCR5-activated signaling proteins Janus Kinase 1 (JAK1), JAK2 and signal transducer and activator of transcription (STAT3) were inhibited by maraviroc. Finally, maraviroc suppressed the growth of SUP-B15 xenograft tumors in athymic mice. Collectively, this study demonstrated that CCR5 inhibition by maraviroc has the potential for the treatment of human ALL.

Keywords: ALL, CCR5, maraviroc, JAK, STAT3

Introduction

Acute lymphoblastic leukemia (ALL) is a predominantly childhood-based disease characterized by the presence of cell cycle-arrested leukemic cells circulating in blood, secondary lymphatic tissues, and bone marrow [1]. Proliferating recirculating ALL cells are typically found within microanatomical structures called ‘proliferation centers’ or ‘pseudofollicles’. From these proliferation centers, the ALL cells interact with accessory stromal or T cells, and thereby receive survival and growth signals [2]. Thus, key molecules such as chemokine receptors and integrins that facilitate the recirculating capacity of ALL cells via extravasation are likely to represent important ALL prognostic markers and therapeutic targets [3]. Furthermore, both chemokines and their G protein-coupled receptors have been identified as essential and selective mediators of leukocyte directional migration to inflammatory sites and/or secondary lymphoid organs. Furthermore, they have been shown to mediate numerous other processes essential to cancer progression, including cell survival, proliferation, and apoptosis, as well as angiogenesis and tumor growth [4].

C-C chemokine receptor type 5 (CCR5) belongs to the chemokine family of structurally related proteins that were initially recognized as mediators of chemotaxis and cellular homing [5].
Maraviroc suppresses ALL cells growth

Figure 1. CCR5 is up-regulated in ALL. A. Western blotting analysis of CCR5 protein expression in normal B lymphoblastoid cells HCC38 BL and three ALL cell lines; GAPDH was used as a loading control. Quantification of CCR5 blots relative to GAPDH was shown. B. The mRNA level of CCR5 was detected by real-time qPCR in indicated cells. GAPDH was used as the loading control. The data were presented as the mean ± SD. **P < 0.01 and ***P < 0.001 (versus HCC38 BL). C. HCC38 BL or SUP-B15 cells was fixed and incubated with primary antibodies against CCR5. Cells were immunostained with anti-rabbit FITC-conjugated secondary antibody and then stained with Hoechst 33258. The specimens were visualized and photographed using a fluorescence microscope (200×). Blue depicts the nucleus and green depicts CCR5. D. Exhibition of CCR5 expression of normal specimens and acute lymphoblastic leukemia from Coustan-Smith leukemia statistics in Oncomine dataset.

CCR5 exerts its activity via a G-protein receptor, and binds the chemokine (C-C motif) ligands 3, 4, and 5 (CCL3, CCL4, and CCL5, respectively). Elevated CCR5 expression has been observed in several cancer types including kidney, lung, brain, prostate, ovarian, breast, and pancreatic cancer, as well as in melanomas. Furthermore, elevated CCR5 expression has been shown to support tumor growth, angiogenesis, metastasis, and therapeutic resistance [6]. Several previous studies have also identified increased expression of CCR5 in cancer-associated fibroblasts (CAFs), which have been established to play an important role in tumorigenesis, and have been implicated in neoplastic progression [7]. Data from these studies suggest that soluble breast cancer factors initiate the trans-differentiation of normal human mammary fibroblasts to tumor-promoting CAFs via the induction of matrix metalloproteinase-1 (MMP-1) and CCR5 expression. In mouse models of human breast and prostate cancer (PCa), high intratumoral C-X-C motif chemokine 12 (CCL12) levels attract CCR5-positive inflammatory, vascular, and stromal cells into tumors. From this intratumoral position, the recruited cells support tumor growth by secreting growth factors, chemokines, cytokines, and pro-angiogenic factors. In addition, CCR5 is also expressed by cancer stem cells (CSCs), a recently identified, small subset of cancer cells that exhibit very similar characteristics to stem cells, which mediate tumor growth, metastasis, and recurrence, as well as therapeutic resistance. Taken together, these results demonstrate that CCR5 contributes to cancer recurrence.

CCR5 expression by cancer cells has also been observed to contribute to tumor progression in other hematological cancers, including chronic myeloid leukemia (CML), acute myelogenous leukemia (AML), and multiple myeloma (MM) [8]. In this context, CCR5 mediates the chemotaxis of leucocytes to inflammation sites, and promotes the recruitment of macrophages, monocytes, and T cells [9]. Notably, CCR5 only mediates immune function in response to specific stimuli; moreover, in some contexts, various mechanisms compensate for absent CCR5 expression and/or function. Notably, CCR5 may exert a far more important role in the immune response than in immune cell traffic regulation. The CCR5 expression in both stromal and hematopoietic cells is associated with tumor metastasis such as chondrosarcoma metastasis.
Maraviroc suppresses ALL cells growth

sis, and oral cancer cell migration [10]. Its expression correlates with multiple myeloma cell growth, bone marrow homing, and osteolysis. Additionally, CCR5 mediates the metastasis of aggressive NK-cell leukemia to the liver, leading to hepatosplenomegaly and hepatic failure. CCR5 inhibition has also been found to suppress the growth and survival of leukemia cells [11]. Studies investigating the underlying mechanism for this last function have focused

Figure 2. In vitro treatment of ALL cell lines with maraviroc inhibits the proliferation potential. A. ALL cells (MOLT-3, TALL-104 and SUP-B15) and macrophage cell line HCC38 BL cells were exposed to indicate concentrations of maraviroc for 24 h or 48 h. Cell viability was determined by MTT assay. The data were presented as mean ± SD. The values were expressed as percentage of viable cells normalized to percentage of viable cells in 0.5% DMSO-treated cells. The concentration of maraviroc resulting in 50% inhibition of control growth (IC50) was calculated by SPSS statistics software. B. Spheroid formation of SUP-B15 cells was determined after 20 days treatment with or without maraviroc. The diameters of spheroid colonies were measured and expressed as the mean ± SD. Data were representative of three independent experiments. *P < 0.05, **P < 0.01 vs. untreated control cells. C. Colony formation of SUP-B15 cells in the presence of maraviroc was measured during 14-day-incubation. The amount of representative colonies were measured and expressed as the mean ± SD. Data were representative of three independent experiments. *P < 0.05, **P < 0.01 vs. untreated control cells.
Maraviroc suppresses ALL cells growth

on the Janus kinases (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling pathways. JAKs are a family of receptor tyrosine kinases that are critical for a variety of cellular functions including growth, proliferation, differentiation, motility, survival, and intracellular trafficking [12]. JAK3, which is established as being highly expressed in the hematopoietic system, is hyper-activated in most B-cell lymphoblastic leukemias, and thus is a promising therapeutic target for this type of cancer. Similarly, the STAT3 pathway is frequently deregulated in a wide range of tumor types because of genetic and epigenetic mutations [13].

The results of the present study show that the CCR5 inhibitor maraviroc inhibits ALL cell growth, proliferation, motility, survival, and tumorigenic potential. Taken together, these results suggest that maraviroc is a novel ALL therapeutic target.

Results

CC chemokine receptor 5 (CCR5) is overexpressed in ALL

CCR5 plays an essential role in the development of malignant neoplasms, and has been previously demonstrated to be associated with cancer cell growth, survival, and apoptosis. To identify the role of CCR5 in ALL, western blot analysis was performed to determine the CCR5 protein production level exhibited by the ALL cell lines MOLT-3, TALL-104, and SUP-B15, and by the (control) B lymphoblastoid cell line, HCC38 BL. A significant increase in CCR5 production was observed in all ALL cell lines, (but most markedly in the SUP-B15 cell line), as compared with the HCC38 BL cells (Figure 1A). This was accompanied by a corresponding increase in the expression of CCR5 in ALL cells, as detected by qPCR (Figure 1B), and by a significant increase in CCR5 expression in SUP-B15 cells compared to HCC38 BL cells, as determined by immunofluorescence (Figure 1C). The expression pattern of CCR5 in primary tumor tissues was assessed using the Oncomine database (Coustan-Smith Leukemia dataset) [14], which revealed CCR5 expression to be significantly increased in acute lymphoblastic leukemia cells compared with related hematogones (Figure 1D). Taken together, these results demonstrate the overexpression of CCR5 both ALL cell lines and clinical samples.

Maraviroc inhibits the proliferation of ALL cells in vitro

To further investigate CCR5 expression and function in a clinically relevant setting, we evaluated the effect of the CCR5 inhibitor maraviroc on the analyzed ALL cell lines. As shown in Figure 2A, we observed that the proliferation of maraviroc-treated MOLT-3, TALL-104 and SUP-B15 cell to be inhibited in a dose-dependent manner. IC50 values calculated for each ALL cell line showed that 5 and 10 μM maraviroc exerted 50% inhibition after 24 h and 48 h of treatment, respectively. We also noted the inhibitory effect on HCC38 BL cells kept at high micromolar concentrations than the effect of equivalent doses and incubation time of maraviroc in ALL cells. Next, we analyzed spheroid formation in SUP-B15 ALL cells, and as expected, we found that SUP-B15 cells exhibited a reduced ability to form spheroids after maraviroc administration, as compared to control cells (Figure 2B). Finally, we examined whether maraviroc prevented ALL cell tumorigenicity. Malignant cancer cells resistant to chemotherapy-induced cell death are able to maintain anchorage-independent growth and survival to facilitate tumor development. As shown in Figure 2C, untreated SUP-B15 cells were observed to proliferate rapidly and to form sizable colonies from a single cell, whereas by comparison, maraviroc-treated SUP-B15 cells displayed both a significantly reduced number of colonies, and reduction in colony size in a dose-dependent manner. Collectively, these data demonstrate that maraviroc exerts universal anti-cancer activity in ALL.

Maraviroc induces apoptosis and inhibits chemotaxis in ALL cells

To validate the anti-cancer effect of maraviroc on ALL progression, the rate of maraviroc-induced apoptosis in SUP-B15 cells was evaluated via TUNEL staining. The results of this analysis showed the percentage of TUNEL-positive cells to increase from 46.6 ± 11.5 to 89.6 ± 6.8% in maraviroc-treated cells, whereas the proportion remained at 4.7 ± 1.58% in control cells (Figure 3A). Similarly, the rate of apoptosis in CRC cells was determined using an Annexin V-FITC-PI apoptosis assay, which
Maraviroc suppresses ALL cells growth

Figure 3. Effect of maraviroc on the apoptotic in SUP-B15 cells. A. Representative TUNEL immunofluorescent images of control cells and maraviroc treatment group, and mean apoptotic index in each group were shown. B. The number of apoptotic cells was significantly higher in maraviroc treatment group, as measured by Annexin V-FITC/PI staining and flow cytometry (**P < 0.01 for maraviroc treatment cells vs. control cells). FITC-A, fluorescein isothiocyanate-Annexin V; PI, propidium iodide. C. The cells were treated with specified dose of maraviroc and western blotting was performed. Dose-dependent increased in protein expression of cleaved caspase 3/9 and PARP cleav-
Maraviroc suppresses ALL cells growth

age was observed in SUP-B15 cells after maraviroc treatment. GAPDH was used as loading control. D. The cells were treated with specified dose of maraviroc and western blotting was performed. Dose-dependent decreased in XIAP, Survivin, c-IAP1, c-IAP2, Bcl2 and Bcl-XL expression and increased in pro-apoptotic Bad and Bax level was observed in SUP-B15 cells. GAPDH was used as loading control. E. SUP-B15 cells treated with different concentrations of maraviroc were allowed to migrate toward CXCL12 (upper graph) or CXCL13 (low graph) on Matrigel coated Transwells. Displayed was the mean ± SD migration of SUP-B15 cells on the vertical axes. Data were representative of three independent experiments. *P < 0.05, **P < 0.01 vs. untreated control cells. F. Inhibition of CCR5 by maraviroc impaired SUP-B15 cells adhesion to fibronectin and VCAM-1. SUP-B15 cells treated with different concentrations of maraviroc were allowed to adhere to surfaces coated with fibronectin (left graph) or VCAM-1 (right graph). Data were representative of three independent experiments. *P < 0.05, **P < 0.01 vs. untreated control cells.

showed SUP-B15 cells to exhibit an increased rate of apoptosis in response to maraviroc treatment, as compared to control cells (Figure 3B). Given that PARP-specific proteolytic cleavage by caspases is considered the characteristic of apoptosis, the caspase-9 and -3-cleavage activity in maraviroc-treated cells was measured. As shown in Figure 3C, treatment of SUP-B15 cells with maraviroc induced the activation of caspase 9 (as indicated by increased amounts of caspase 9-cleavage products) and caspase 3 (as shown by the presence of a double band representing the p19 proteolytic fragment and the active p17 subunit), in a dose-dependent manner. Members of the IAP protein family, including XIAP, survivin, c-IAP1, and c-IAP2, have recently emerged as critical regulators of apoptotic cell death, and hence, their potential effect on the regulation of maraviroc-induced apoptosis was evaluated. This analysis revealed that treatment of SUP-B15 cells with maraviroc results in dose-dependent downregulation of XIAP, survivin, c-IAP1, and c-IAP2 protein expression (Figure 3D). Bcl2 family proteins influence cellular apoptosis via the regulation of cytochrome C release, which in turn mediates caspase activation; thus, the effects of maraviroc on the expression of the pro-apoptotic Bcl2 proteins Bad and Bax were evaluated. Maraviroc treatment of SUP-B15 cells resulted in a dose-dependent inhibition of Bcl2 and Bcl-XL protein production. Furthermore, maraviroc treatment resulted in increased protein expression of Bad and Bax in a dose-dependent manner. ALL cell migration and adhesion are key mechanisms that enable the trafficking of cells between the peripheral blood and secondary lymphoid tissues. The secondary lymphoid tissue microenvironment contains a mix of various factors, including CXCL12 and CXCL13. These ligands are the major chemokines mediating the homing and retention of ALL cells in the lymph nodes (LN) and bone marrow (BM) microenvironments; thus, we investigated the effect of maraviroc treatment on the response of SUP-B15 cells to CXCL12 and CXCL13 treatment. The results of this analysis demonstrated that the propensity of SUP-B15 cells to undergo chemotaxis toward CXCL12 and CXCL13 was inhibited after treatment with maraviroc (Figure 3E). To determine the effect of maraviroc treatment on cell adhesion, we employed a functional assay utilizing fibronectin or VCAM-1 coated plates to model tumor-extracellular matrix interactions in the tissue microenvironment. As shown in Figure 3F, maraviroc treatment virtually abolished both SUP-B15 adhesion to the extracellular matrix component fibronectin, and the CCR5-mediated adhesion of SUP-B15 cells to the cellular adhesion molecule VCAM-1. Taken together, these data demonstrate that inhibition of CCR5 by maraviroc impairs chemokine-controlled adhesion and migration of ALL cells.

Maraviroc induces the downregulation of JAK/STAT signaling

The STRING database (http://string-db.org/) is a pre-computed global resource for the exploration and analysis of functional links between proteins. Based on the information contained therein, CCR5 was highly correlated with several stars molecules, such as JAK, chemokine (C-C motif) ligand 5 (CCL5), and CXCL12, etc. (Figure 4A). To elucidate the mechanisms by which maraviroc inhibits ALL cell growth and function, a microarray assay was performed in which SUP-B15 cells were treated with either maraviroc or vehicle. The results of the assay identified a number of genes whose expression was significantly altered in response to the administration of maraviroc (Figure 4B). Furthermore, a gene enrichment analysis indicated that JAK signaling was significantly enriched in maraviroc-treated cells (Figure 4C), and similarly, a western blot analysis demonstrated that the expression of p-JAK2 decreases in a dose-
Maraviroc suppresses ALL cells growth

Figure 4. Effect of maraviroc on JAK/STAT3 activation in SUP-B15 cells. A. Modules from the protein-protein interaction network of CCR5. Edges represent protein-protein associations. B. Clustering of genes differentially expressed after SUP-B15 cells treated with vehicle or maraviroc. C. Enrichment scores of differential gene expression in the maraviroc treatment SUP-B15 cells. D. Representative western blots shown the phosphorylation of JAK/STAT3 signaling in SUP-B15 cells after maraviroc treatment. E. SUP-B15 cells were incubated with vehicle or serial dilutions of maraviroc, then cells were centrifuged and incubated with Alexa Fluor® 488.
null
Figure 5. Treatment of maraviroc leads to tumor regression in mouse ALL model. A. Nude mice bearing SUP-B15 xenograft tumors were treated with vehicle or maraviroc twice per day when tumor volume reached between 80 and 100 mm³. The two-way ANOVA test was performed to compare between the maraviroc groups vs. the vehicle group. B. Treatment with maraviroc resulted in significantly tumor growth inhibition versus vehicle control. C. The body weight of maraviroc and vehicle treated mice was measured once every three days. Error bars represent mean ± SD (n = 6). D. Immunodeficient mice were subcutaneous implantation with SUP-B15 cells and were administered maraviroc, as shown in the schematic diagram. Most of the untreated mice died from cancer by day 39. The survival time in maraviroc treated group was evaluated. E. Mice bearing SUP-B15 tumor xenograft were treated with maraviroc at 10 and 30 mg/kg or vehicle and sacrificed at the end of the experiment. The tumor tissues were removed for further immunohistochemistry analysis using anti-Ki67 (left). Mice treatment with maraviroc induced apoptosis in SUP-B15 tumors (right). F. Representative images of mouse tumor samples subjected for immunohistochemistry detection with antibodies against p-JAK1, p-JAK2 and p-STAT3 were shown.
Cytokines are a large family of tumor and stromal cell-secreted cytokines whose main function is to mediate cancer cell directional migration and invasion abilities [16]. Most cancers overexpress both chemokines and chemokine receptors because of the activity of deregulated transcription factors and/or cancer-promoting genes [17]. For example, CCL5 and its cognate receptor CCR5 are thought to be critical to cancer progression in this context, and similarly, CXCL12 and CCR5 have been reported to be essential to multiple phases of tumorigenesis, including tumor cell proliferation, survival, invasion, and metastasis, as well as angiogenesis.

As a G-protein-coupled receptor (GPCR), CCR5 receptor activation is mediated by coupling to an intracellular heterotrimeric G-protein associated with the inner surface of the plasma membrane. This G-protein heterotrimer is composed of Gα, Gβ, and Gγ subunits, which in the basal state bind the guanine nucleotide GDP [18]. Upon activation by ligand binding, GDP is released and replaced by GTP, leading to the dissociation of the heterotrimer to form a βγ dimer, and a GTP-bound α monomer. GTP is subsequently rapidly hydrolyzed to GDP, resulting in the re-association of the receptor and the trimeric G-protein complex [19]. In addition to the above typical signal pathway, CCR5 signaling has been shown to intersect with the Ras-activated signaling pathway via several Src-related kinases including Src, Lyn, Fyn, and Lck. Moreover, CCR5 may promote tumor cell survival via both post-translational inactivation of the cell death machinery, and increased transcription of cell survival-related genes. Thus, extensive previous research promotes CCR5 as a promising therapeutic target for anti-cancer drug development [20]. The CCR5 inhibitor maraviroc has recently been shown to exert anti-cancer activity in colon, breast, and gastric cancer. In vitro, maraviroc disrupts pathways involved in tumor-microenvironment interactions, but induces only a moderate degree of apoptosis. To date, few studies have focused on the effect of maraviroc in ALL.

Thus, the present study initially aimed to determine the effect of maraviroc on ALL cell lines. The results of these in vitro analyses showed that maraviroc significantly inhibited the survival of SUP-B15 cells, induced cell apoptosis, and after longer incubation, significantly inhibited spheroid formation. In addition, the present study also examined the effects of maraviroc in various in vitro assays that modeled the interactions of ALL cells with their microenvironment. Maraviroc was observed to significantly inhibit SUP-B15 cell migration and adhesion in response to physiologically relevant stimuli. Furthermore, B-ALL-cell chemotaxis toward the tissue homing chemokines CXCL12 and CXCL13 was demonstrated to be inhibited by maraviroc for the first time. This finding may have significant clinical relevance to ALL progression, since along with the inhibition of adhesion molecule function and/or signaling, ALL cell-chemotaxis is likely to be fundamental to the early efflux of ALL cells from tumor tissues into the blood.

CCRF5-mediated chemotaxis is regulated by JAK/STAT3 signaling, and JAK activation can induce the phosphorylation of several focal...
Maraviroc suppresses ALL cells growth

adhesion components such as proline-rich kinase-2 (Pyk-2), Crk-associated substrate (p130Cas), focal adhesion kinase (FAK), Paxilin, Nck, Crk, and Crk-L. In addition, JAK activity may directly activate STAT3, thereby promoting tumor cell survival and/or proliferation. Thus, the JAK/STAT3-mediated signaling pathway has emerged as a central mechanism underlying the survival and expansion of various malignant tumors. In the present study, we found that maraviroc potently blocks JAK phosphorylation in B-cell leukemia SUP-B15 cells in vitro, at a concentration range similar to that required to inhibit the activity of STAT3. We also investigated the anti-tumor effect of maraviroc in vivo using a SUP-B15 xenografted athymic mouse model. Immunohistochemical and western blotting analyses revealed that the expression of Ki67, as well as that of CCR5 and its downstream signal molecules, were markedly decreased in xenografted tissues. In summary, the data generated by the present study demonstrate that maraviroc significantly inhibits ALL progression and tumorigenesis both in vitro and in vivo, via suppression of acute lymphoblastic leukemia cell proliferation and activity (Figure 6). We recommend that future studies focus on exploring the potential mechanisms underlying maraviroc-induced inhibition of ALL cells, as these may be vital to ALL screening and treatment in the clinical setting.

Disclosure of conflict of interest
None.

Address correspondence to: Kailin Xu and Lingyu Zeng, Department of Hematology, The Affiliated Hospital of Xuzhou Medical University, No. 99, West Huaihai Road, Xuzhou, Jiangsu Province, China. E-mail: lhmd@163.com (KLX); zengly2000@163.com (LYZ)

References


Maraviroc suppresses ALL cells growth


Maraviroc suppresses ALL cells growth

**Supplementary Figure 1.** mRNA levels of STAT3 target genes were determined by qRT-PCR analysis normalized against the level of GAPDH. Data are expressed as means ± SD of three independent experiments, each performed in triplicate. *P < 0.05, **P < 0.01 vs. vehicle control.

**Supplementary Figure 2.** Effect of maraviroc on cytotoxicity in normal cells and in mice. (A) Human normal liver cells LO2 were treated with maraviroc for 24 hours and cell viability was tested by MTT assay. Immunodeficient mice were subcutaneous implantation with SU-DHL-6 cells and were administered maraviroc, as shown in the schematic diagram. The AST (B), ALT (C) and creatinin (D) concentration in blood was determined. The data are presented as the mean ± SD.