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Research Article

Effects of chemokine receptor 5 (CCR5) blockages on cell survival and expression levels of apoptosis related genes (FAS, FASL) in hepatocellular carcinoma cells

Sobia Javeed[1,2], Afraz Numan[2], Muhammad Aiman Shahzad[2]

1. DHA Medical Laboratory, United Arab Emirates
2. Institute of Biomedical and Allied Health Sciences, University of Health Sciences, Lahore, Pakistan

Abstract

Background: Hepatocellular carcinoma (HCC) is a malignant tumor which arises from the liver cells (hepatocytes). HCC is the 3rd leading cause of cancer related mortalities worldwide. Various treatment strategies for HCC include surgery, radiotherapy, targeted agents and chemotherapy. Despite the availability of diverse therapeutic options, 5-year survival rates are low (20-30%) especially in advanced stages of HCC. This, in turn, highlights the need to identify new therapeutic targets/compounds for HCC treatment. Purpose of this study was to evaluate effects of blocking a chemokine receptor (CCR5) on cell proliferation and apoptosis related genes (FAS/FASL) in HCC cells (HepG2).

Methods: CCR5 receptor was blocked by using an FDA approved antagonist (Maraviroc) and effects on proliferation of HepG2 cells were identified. For this purpose, the cells were exposed to various concentrations (7.5-500µM) of the test compound and cell viability was monitored by MTT dye reduction assay for 24, 48 and 72 hours. Afterwards, HepG2 cells were exposed to three distinct concentrations of maraviroc (IC_{25}, IC_{50}, IC_{75}) in a separate experiment and expressional modulations in two apoptosis related genes (FAS and FASL) were identified by qRT-PCR methodology.

Results: The results indicated that blocking CCR5 via maraviroc induced substantial anti-proliferative effects in HepG2 cells. The effects were time and concentration dependent and were especially clearer following exposure with 100µM of maraviroc. Blockage of CCR5 induced marginal up-regulation of FAS gene in the cells. In contrast, blocking of CCR5 inhibited the expression of FASL gene in HepG2 cells in a concentration dependent format with a maximum inhibition of 5fold.

Conclusion: CCR5 blockage by maraviroc induces prominent cytotoxic effects in HCC cancer cells. Expressional modulations in apoptosis related genes are imposed in response to blockage of the chemokine receptor in the cells. Further studies are needed to understand the precise nature of growth inhibitory effects observed in response to blockage of CCR5 in HepG2 cells.

Key Words: Liver cancer, Chemokine, CCR5, Maraviroc, Cytotoxic, Genes expression

Corresponding Author: Muhammad Aiman Shahzad
Email: aimanshahzad@uhs.edu.pk

INTRODUCTION

Uncontrolled proliferation of cells leads to the formation of tumor mass and is known as cancer. There are more than 100 types of cancers and this division is based on either type of tissue or organ involved. Liver cancer is the sixth most frequent type of cancer and there are different types of liver cancer. HCC is the most common type of liver cancer and globally it is the well-known cause of deaths. HCC is different from "secondary" liver cancer which establishes because of spreading of tumor cells from other organs and migrating to the liver during a process called metastasis [1]. Some patients of liver cancer have no symptoms which becomes the reason of presenting at late stage of cancer. In liver cancer, currently standard staging system BCLC (Barcelona Clinical Liver Cancer) is used for treatment. There are also other treatment options to eliminate the growing tumor including microwave therapy, radiofrequency ablation, cryoablation, percutaneous injection of ethanol and electroporation treatment. Despite the availability of multiple therapeutic options, HCC is considered as a top ranked cancer and "silent killer". Major reasons are delayed diagnosis and tumor heterogeneity along with expressional modifications in genes and associated pathways.

Chemokines are small sized (8-14 kDa) chemo-attractive cytokines, which are mainly responsible for the directed migration of various kinds of leukocytes, endothelial and epithelial cells [2-4]. The chemokine network is comprised of about 50 ligands and 20 receptors. Chemokine receptors are found in the lipid bilayer with their seven trans-membrane loops. These receptors are G protein coupled receptors (GPCRs) and classified according to the type of their corresponding ligands. A typical chemokine receptor contains almost 350-370 amino acids in length with a short N-terminus, located in extracellular space. C-terminus of the receptor, located in intracellular space, contains serine and threonine residues, which act as phosphorylation sites for the receptor regulation and signaling [5, 6]. Receptors that couple to C-, CC-, CXC-, and CX3C-chemokines are named XCRn, CCRn, CXCRn, and CX3CRn, respectively.

Among the chemokine network, CC-chemokine receptor 5 (CCR5) is an important member and plays vital roles in inflammatory response of the body [7]. Macrophage-tropic strains of both types of human immunodeficiency virus type 1 and 2 (HIV-1, HIV-2) use CCR5 as cofactor, making it an integral part of pathogenesis of the infection and viral transmission. Inflammatory CC-chemokines of CCR5 which act as agonists are MIP-1f, MIP-1g, MCP-2, HCC-1 and RANETS. CCR5 is expressed in both innate immunity and cellular component of acquired immunity mainly in memory T-cells, macrophages, and immature dendritic cells, and is up-regulated by pro-inflammatory cytokines. CCR5 plays an important role in inhibition of cAMP production and activates P13-MAP kinase. It stimulates Ca^{+2} release and causes activation of tyrosine kinase cascades [8]. CCR5 and CCL5 levels increased in patients with chronic liver disease. Overexpression of CCR5 and its ligands (CCL3, CCL4, CCL5) have been correlated with increased disease burden and reduced overall survival [9-11]. HIV-1 strains transmit through co-receptor CCR5, so different pharmaceutical companies have synthesized antagonists against CCR5 which are being used as antiviral therapies. These specific antagonists are helpful for blockage of CCR5 signal transduction [12]. This CCR5 antagonist (maraviroc) has been approved by FDA for treatment of HIV infections. Blocking CCR5 with maraviroc has shown reduction in tumor burden during HCC development in *in vivo* studies [12-15]. The objectives of the current research were to block CCR5 receptors of HCC cells by maraviroc and investigating the corresponding effects on cell proliferation. Furthermore, it was aimed to measure the expressional modulations in apoptosis relevant genes (FAS, FASL) in response to blockage of CCR5 in HCC cells.

METHODS

Culturing of Cancer Cells

HCC cells (HepG2) were obtained from Centre for Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore. The cells were cultured in DMEM medium (Gibco, Cat#11965-092) supplemented with 10% FBS, 100µg/ml Penicillin/Streptomycin and 2mM L-glutamine. Standard cell culture incubation conditions (5% CO_2, 37˚C, 100% humidity) were provided for cultures throughout the experiments. The cells were passaged routinely to keep a well growing cell population.

Cytotoxicity Assay

Cytotoxic effects of blocking CCR5 receptor were investigated by MTT dye reduction assay. For this purpose, the cells were cultured in 96-well plates (4000 cells/well/100µl media) and treated next day with maraviroc (7.5-500µM, Selleck, Cat#S2003) antagonist for 24, 48 and 72 hours. Afterwards, surviving cell fractions from treated and untreated cells (control group) were examined by adding 10 µl/well MTT solutions (10 mg/mL in PBS) and dissolving newly formed formazan crystals with 50µL of DMSO. Optical density was measured by ELISA plate reader at wavelength 540nm with 690nm reference filter. Cell survival rates were calculated as the percentages of untreated control cells and inhibitory concentrations (IC) were determined by using software GraphPad Prism v5.

Gene Expression Analysis

HepG2 cells were cultured in 6-well plates at a density of 150,000 cells/well/2ml media and exposed to IC_{25} and IC_{50} and IC_{75} concentrations (identified from MTT dye reduction assay after 48 hours exposure time) of CCR5 antagonist (maraviroc). The cells were exposed to the antagonist for 48 hours, followed by collection of cell palettes and storage at -80^0C immediately. Total RNA content from collected cell pellets was extracted by using a commercially available kit (Thermo Fisher, Cat#K0731) following the manufacturer's protocol. A total of 1000ng extracted RNA/sample was used to synthesize cDNA (20µl) by using Revert Aid First Strand cDNA synthesis kit (Thermo Fisher, Cat#K1622).

Real Time PCR

Primers for selected FAS (*F: TCACCACTATTGCTGGAGTCA, R: GGTACTTAGCATGCCACTGC) and FASL (F: CAGGCACCGAGAATGTTGTAT, R: TGGTAGCTGCTTTTTCATGCT*) genes were designed by using Primer3 software after choosing the gene sequence from NCBI Gene bank. qRT-PCR was performed by using SybrGreen florescence dye (Thermo Fisher, Cat#K0221) for the selected genes

by using prepared cDNA samples from HepG2 cells treated with different concentrations of maraviroc for 48 hours. Following amplification procedures, $2^{-\Delta\Delta CT}$ method (Livak) was used to calculate expressional changes in the selected genes. Results were compared with untreated controls while expression levels of a reference gene (HPRT1) were used in these experiments for normalization of the data sets.

RESULTS

Blockade of CCR5 induces substantial toxic effects in liver cancer cells

HepG2 cells were exposed to for various concentrations of maraviroc (7.5-500µM) for 24, 48 and 72 hours. Following the exposure intervals, the viable fractions of the cells were identified by MTT dye reduction assay. Maraviroc exposure induced significant cytotoxic effects in HepG2 cancer cells as shown by Figure 1. Precisely, the effects were noticed for all three-time intervals. Considering the highest concentrations applied (500µM), the effects were time dependent as shown by inhibition of cell proliferation by 54, 76 and 88% after 24, 48 and 72 hours of exposure time respectively. For the three-time intervals, the effects were moderate till 250µM concentration of maraviroc, while there was a steep decline in viable cell fraction for next applied concentration (500µM). Overall, CCR5 blockage induced substantial cytotoxic effects in the liver cancer cells (Figure 1).

Expression Modulations in Genes

HepG2 cells were exposed to various concentrations of maraviroc (IC_{25}, IC_{50}, IC_{75}), total RNA was extracted followed by cDNA synthesis. Real time PCR based amplification of the two selected genes (FAS and FASL) was performed and data was normalized by amplifying a reference gene and fold changes were determined by $2^{-\Delta\Delta CT}$ method. CCR5 blockage by maraviroc induced the expression of FAS gene mildly as shown by an induction of 1.8 and 1.4fold with IC_{25} and IC_{75} concentrations of the test compound respectively (Figure 2). CCR5 blockage by using maraviroc inhibited FASL gene in HepG2 cells as shown below. The effects were concentration dependent as shown by inhibition of 1.5, 2.1 and 5.3fold in response to IC_{25}, IC_{50} and IC_{75} concentration of maraviroc. The results were almost in line with our cytotoxicity outcomes as there was also a consistent inhibition of proliferation in response to increasing concentration of maraviroc.

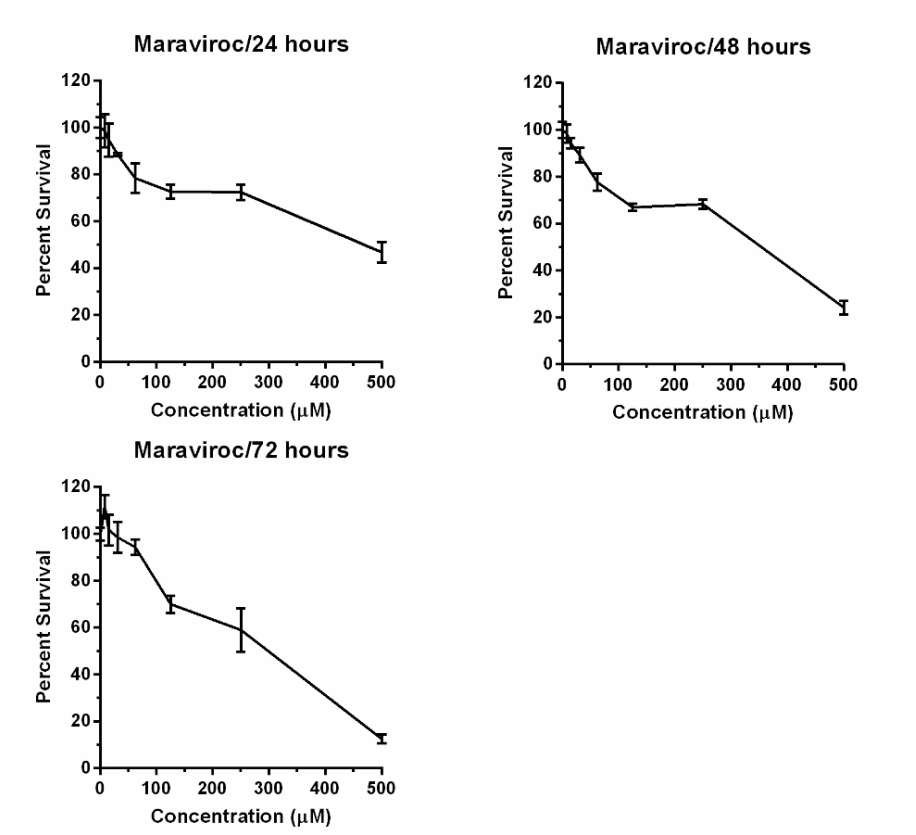

Figure 1: MTT result after CCR5 blockage for 24-72hours. Cells were exposed to different concentrations (7.5-500µM) of antagonist (maraviroc) and resulting effects on the cell proliferation were identified by MTT dye reduction assay. CCR5 blockage inhibited the liver cancer cell proliferation.

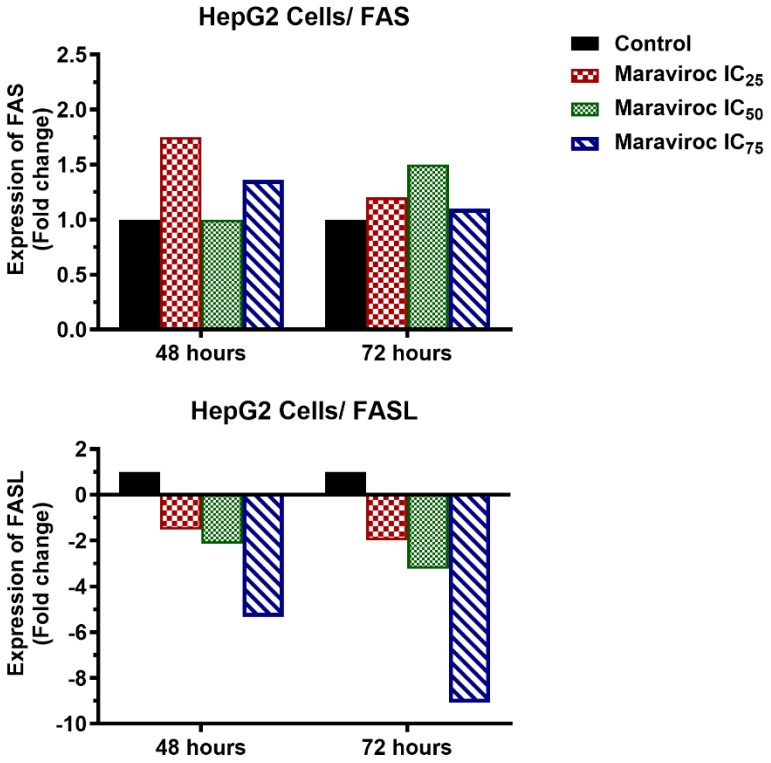

Figure 2: Expressional changes in FAS and FASL genes after CCR5 blockage. In response to exposure with maraviroc, expressional changes in the genes were identified by qRT-PCR. CCR5 blockage by maraviroc induced FAS marginally and inhibited FASL gene in HepG2 cells in a concentration dependent format.

DISCUSSION

CCR5 (CD195) chemokine receptor is mainly known for its association with HIV infections. Despite its role in HIV infections, a number of normal physiological roles are associated with this chemokine receptor. CCR5 along with its three cognate ligands (CCL3, CCL4, CCL5) is mainly responsible for recruitment of T cells, monocytes and macrophages to the site of inflammation [16]. Due to involvement of CCR5 receptor in the viral entry phase, antagonists and monoclonal antibodies are being developed to block this receptor, which, in turn, could serve as entry inhibitors for HIV infections. In this regard, a CCR5 antagonist "Maraviroc" has been approved by FDA for treatment of HIV infections. Apart from involvement in HIV infections, the CCR5 receptor is being studied for its proactive role in pathology of various other disease including cancers. In this respect, inhibition of this receptor via available antagonists is being exploited as therapeutic options.

HCC is a lethal condition and impose a significant morbidity and mortality burden. During the course of HCC development, changes in expressional profile of various genes including chemokine receptors have been noticed. In this context, changes in CCR5 profile along with their corresponding ligands have shown significant up-regulation during the course of HCC development. Higher expression of CCR5 has been found with poor prognosis, reduced overall survival and metastasis of HCC. Furthermore, these higher expressions of CCR5 have shown to promote the migratory and proliferative behavior of the HCC cells and abrogation of the receptors lead to the reduced tumor growth in *in vivo* conditions [12]. HCV/HBV mediated inflammation is hall mark for HCC development, where CCR5 along with its ligands play a pivotal role to attract various cell populations like macrophages, neutrophils and T-cells. Blocking CCR5 with maraviroc has shown reduction in tumor burden during HCC development in *in vivo* studies [13]. All in all, CCR5 could serve as independent prognostic markers and therapeutic targets in HCC.

In this study, we blocked the CCR5 by using corresponding antagonist (maraviroc) and studied the cytotoxic effects via MTT dye reduction assay. Furthermore, the impact of blocking the chemokine receptor on two important apoptosis related genes (FAS, FASL) was studied. As a first necessary step, it was important to validate the expression of CCR5 in selected HCC cells (HepG2) before blocking the receptors by using their corresponding antagonists. The real-time PCR analysis was performed to check expression of CCR5 in untreated HepG2 cells (data not shown). The receptor showed a good amplification as

shown by qRT-PCR with Ct value of 24.34 for CCR5 (Ct of HPRT1: 16.8).

Continuous proliferation is a major hallmark of cancer cells and drugs/compounds affecting this functional property are important entities to be exploited. Keeping in view of this phenomenon, potential cytotoxic effects of blocking CCR5 in HepG2 cells were worth investigating. To accomplish this task, HepG2 cells were exposed to various concentrations of CCR5 (maraviroc: 7.5-500μM) for 24-72 hours. Afterwards, the viable cell fractions were identified by MTT dye reduction assay. Blocking CCR5 induced substantial inhibition of cell proliferation for all time points as showed in Figure 1. The effects were induced almost in time and concentration dependent format as shown by continuous decrease in viable cell fractions with increasing concentrations of the antagonist and longer time interval. Precisely, it was not possible to attain 50% inhibition of cell viability even with highest concentration of maraviroc (500μM) after 24 hours exposure time, while more than 50% cell proliferation inhibition was noticed with 500μM concentrations of the test compound after 48- and 72-hours exposure. The results showed that selected HCC cells respond to longer exposure intervals and become sensitive over period of time in the presence of maraviroc. Our results indicated that blocking CCR5 via maraviroc induces substantial effects on cell proliferation of HepG2 cells.

Avoiding the apoptotic process is a necessary requisite for the cancer cells as it gives them a chance to survive for a longer time. For this purpose, cancer cells often up-regulate anti-apoptotic and down-regulate pro-apoptotic genes. FAS and FASL are two important apoptosis related genes and alterations in their expressional levels are often observed in cancer cells. Considering this, expressional profiling of FAS and FASL genes were compared between untreated HepG2 cells with the cells treated with various concentrations maraviroc by using real-time PCR methodology. Three different concentrations (IC_{25}, IC_{50}, IC_{75}) of maraviroc were selected for the real-time PCRs.

FAS are a well-known death receptor found in cell surface membrane and play a central role in regulation of programmed cell death by activation of caspases. Furthermore, it is reported to induce cell proliferation signaling in the cells like fibroblasts and T-cells. In this study, a fractional induction of FAS gene was noticed (maximum 1.8fold) in response to CCR5 blockage by exposing the cells to IC_{25} of maraviroc (Figure 2). For the next two higher concentrations of maraviroc (IC_{50} and IC_{75}), there was no or even lesser induction of the gene. In contrast, there was a concentration dependent inhibition of FASL gene as shown in Figure 2. A maximum of 5.3fold inhibition of FASL gene was observed when the cells were exposed to IC_{75} of maraviroc. Observing a fractional induction of the receptor (FAS) and inhibition of its corresponding ligand (FASL) indicate a moderate connection between levels of CCR5 blockage via maraviroc and expressional modulations in FAS-FASL axis. Furthermore, the changes in FAS-FASL axis are not in line with the cytotoxic effects (Figure 1), which indicates a potential involvement of other death mechanisms being initiated after CCR5 blockage in HepG2 cells.

Overall, the study revealed substantial cytotoxic effects on HepG2 cells in response to CCR5 blockage by using maraviroc. Expressional modulations in two selected apoptosis related genes (FAS and FASL) were not uniform in response to CCR5 blockage in HepG2 cells. All in all, cytotoxic effect on HepG2 cells by blocking CCR5 via maraviroc is an effective anti-proliferative option. Provided validation in larger cell line pool and *in vivo* investigations, CCR5 blockage can be instrumental in HCC treatment.

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Contributions: SJ perfomed experiments. AN analyzed the data. MAS drafted the manuscript.

Competing Interests: None

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.