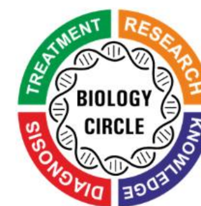
Biomedical Current Insights
DOI: https://doi.org/10.63626/v098eb13
ISSN Online: 3104-414X
ISSN Print: XXXX-XXXX



Research Article

CXCR4 blockage induces cytotoxic effects in liver cancer cells

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Abstract
Background: Hepatocellular carcinoma (HCC) is a malignant tumor which arises from the liver cells (hepatocytes). Chronic inflammation followed by fibroses and cirrhosis ultimately leads to the development of liver carcinoma. 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 (10-30%) especially in advanced stages of HCC. This, in turn, highlights the need to identify new therapeutic targets/compounds for HCC treatment. The purpose of this study was to evaluate the effects of blocking chemokine receptor (CXCR4) on cell proliferation and apoptosis related genes (FAS/FASL) in liver cancer cells (HepG2).
Methods: CXCR4 receptors were blocked by using FDA approved respective antagonist (AMD3100) in HepG2 cells and effects on proliferation 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 AMD3100 (50, 100, 200µM) in a separate experiment and expressional modulations in two apoptosis related genes (FAS and FASL) were identified by qRT-PCR methodology.
Results: Blocking CXCR4 by using AMD3100 induced minimal growth inhibitory effects on the cells. Blockage of CXCR4 induced marginal up-regulation of FAS and FASL genes in the cells.
Conclusion: Further investigations are needed to discover the effects of CXCR4 blockage on liver cancer cells.
Key Words: Chemokine, Receptor, Liver cancer, Cytotoxic, Drug

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the predominant and most lethal form of primary liver cancer, frequently associated with chronic hepatic conditions such viral infections, cirrhosis and exposure to aflatoxins. Principal risk factors for HCC include persistent infection with hepatitis B virus, hepatitis C virus, excessive alcohol consumption, liver cirrhosis, advanced age, male sex, diabetes mellitus, and aflatoxin exposure [1]. Males are disproportionately affected, potentially due to lifestyle factors and the influence of androgenic hormones [2]. Diagnosis of HCC is based on imaging techniques including ultrasound (US), magnetic resonance imaging (MRI), and computed tomography (CT), complemented by alpha-fetoprotein (AFP) as a diagnostic biomarker [3]. Treatment of HCC is stage-dependent, with surgical options such as liver resection or transplantation indicated for early-stage disease [4]. For advanced HCC, local ablative therapies like radiofrequency ablation (RFA) and systemic treatment with sorafenib, a multi-kinase inhibitor, are commonly utilized [5]. Emerging therapeutic modalities, including oncolytic virus therapy and immunotherapy, are currently being explored [6]. Effective management of HCC necessitates early detection, accurate staging, and personalized therapeutic approaches tailored to the disease's progression and the patient's clinical condition.
Chemokine are small chemo-attractive cytokines (8-14 kDa) and play a critical role in the directed migration of leukocytes, endothelial, and epithelial cells. The chemokine network consists of approximately 50 ligands and 20 receptors, which are G-protein-coupled and categorized based on type of ligands they bind. These receptors are integral to the pathophysiology of HCC mediating processes such as tumor progression, metastasis and angiogenesis [7]. Specific chemokine receptors are often up-regulated in HCC and facilitate key tumorigenic processes, including cell migration, invasion, angiogenesis, and lymphatic metastasis [8, 9]. CXC chemokine receptors, particularly CXCR2, CXCR3, CXCR4, and CXCR7, are critically involved in HCC metastasis [10]. The CXCL5/CXCR2 axis induces epithelial-mesenchymal transition (EMT) [11], while

CXCR4, upon binding with its ligand CXCL12, activates signaling pathways that promote cell migration, invasion, and angiogenesis, all contributing to an unfavorable prognosis [12]. CXCR7 mediates tumor progression through MAPK signaling pathways [13].
CXCR4 receptors are pivotal in the pathogenesis of HCC, enhancing cancer cell invasion and metastasis. These receptors represent promising therapeutic targets [14]. CXCR4 antagonist AMD3100 (Plerixafor) , a clinically FDA approved therapeutic agent, has been utilized in autologous stem cell transplantation across various oncological conditions [15]. Originally developed for HIV treatment, AMD3100 was subsequently identified as a CXCR4 antagonist with potential applications in other diseased conditions [16]. Inhibition of CXCR4 by AMD3100 has been demonstrated to attenuate the size and density of blood vessels, which are crucial for the sustained proliferation of malignant cells during the progression phase [17].
Since chemokine receptors and their ligands are essential to the development of HCC, they hold the potential to be significant therapeutic targets for the management of this cancer. The purpose of this study was to inhibit CXCR4 receptors on HCC cells using AMD3100 and evaluate its impact on cellular proliferation. Furthermore, modulations of apoptosis-related gene expression (FAS, FASL) in response to the blockage of CXCR4 receptors in HCC cells were investigated.

METHODS

Cell Culture

HepG2 cells (human hepatocellular carcinoma cell lines) obtained from Centre for Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore were cultured in DMEM (Gibco) supplemented with 10% FBS, 100µg/ml penicillin/streptomycin, and 2mM L-glutamine. Cells were maintained at 37°C in a humidified incubator with 5% CO_2 and passaged regularly to sustain optimal growth.

MTT Dye Reduction Assay

Cell viability was assessed by using the MTT dye reduction assay. HepG2 cells were seeded in 96-well plates (4000 cells/well) and treated with AMD3100 (7.5-500µM Merck, Cat#A5602) for 24, 48, and 72 hours. Following treatment, 10µl of MTT solution was added to each well, and after 4 hours incubation, the resulting formazan crystals were solubilized in DMSO. Absorbance was measured at 540 nm, with a 690 nm reference filter. Cell survival was expressed as a percentage of untreated controls.

CXCR4 Blockade and RNA Extraction

For gene expression analysis, the HepG2 cells were exposed to AMD3100 with three different concentrations (50, 100, and 200µM) for 48 hours in order to block CXCR4 receptors. After treatment, cell pellets were harvested and stored at -80°C. Untreated HepG2 cells were used as controls in these experiments.

RNA Extraction and cDNA synthesis

Total RNA was extracted using the Thermo Fisher RNA extraction kit (Cat#K0731), and RNA concentrations were determined by using a Nanodrop 2000 spectrophotometer. A total of 1000ng of RNA per sample was used for cDNA synthesis using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher, Cat#K1622).

Primer Designing

The expression of two apoptosis-related genes, FAS and FASL, was profiled using qRT-PCR. Primers targeting these genes were designed using Primer3 software based on sequences from NCBI GeneBank and are given in Table 1. The primers were optimized by gradient PCR with annealing temperatures of 56°C, 59°C, and 62°C to determine the most efficient conditions.

PCR for Genes

qRT-PCR was conducted by using SybrGreen fluorescence dye to quantify the expression of FAS and FASL. cDNA samples synthesized from HepG2 cells treated with varying concentrations of AMD3100 for 48 hours were used for FAS and FASL analysis, while untreated cells were included as controls. The relative expression levels of the target genes were determined using the $2^{-\Delta\Delta CT}$ method. HPRT1 was utilized as the reference gene for data normalization.

Genes	Primer Sequence (F.)	Primer Sequence (R.)
FAS	TCACCACTATTGCTGGAGTCA	GGTACTTAGCATGCCACTGC
FASL	CAGGCACCGAGAATGTTGTAT	TGGTAGCTGCTTTTTCATGCT
β-Actin	TCCACCTTCCAGCAGATGTG	GCATTTGCGGTGGACGAT
HPRT1	GACCAGTCAACAGGGGACAT	CTTGCGACCTTGACCATCTT

RESULTS

Blockage of CXCR4 induces toxic effects in liver cancer cells

HepG2 cells were exposed to various concentrations of AMD3100 (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 as explained in methodology section. It was observed that the effects were moderate for CXCR4 blockage via AMD3100 antagonists as shown by Figure 1. Initially, there was no inhibition of cellular proliferation after blocking the CXCR4 receptor with AMD3100 antagonist. Opposite to this, even some induction in proliferation (up to 20%) was observed for 24-hour period. For the next time interval (48 hours), there were no inhibitory effects on cell proliferation till 62μM concentration of AMD3100, while beyond this; there was some inhibition of cell viability (up to 20%) with highest applied concentration of the antagonist (500μM). For the longest exposure interval (72 hours), moderate inhibition of cell proliferation (up to 20%) was observed with 125-500μM concentration of AMD3100.

cDNA verification and Primer optimization

HepG2 cells were treated with different concentrations of AMD3100 (50, 100, 200 μM), followed by RNA extraction and cDNA synthesis. The successful synthesis of cDNA was confirmed by amplifying the β-actin gene using conventional PCR, and optimized working conditions for the designed primers were established using a gradient PCR approach. Annealing temperatures between 56ºC and 62ºC were tested with a cDNA sample from untreated control HepG2 cells. PCR amplification products were visualized via agarose gel electrophoresis. The primers exhibited gene-specific amplification at all tested annealing temperatures, with variable amplification levels for the FAS gene (Figure 2).

Expressional Analysis of FASL and FAS Genes after Blocking CXCR4

The expression of FAS and FASL genes in HepG2 cells was analyzed following treatment with various concentrations of AMD3100 (50, 100, and 200 μM). Total RNA was extracted, cDNA was synthesized, and real-time PCR was performed to assess gene expression, with normalization using HPRT1 as a reference gene. The fold changes in gene expression were calculated using the 2-ΔΔCT method.

Blocking CXCR4 induces the expression of FAS gene in HCC cells

CXCR4 blockade with AMD3100 showed minimal effects on FAS gene expression at 50μM (fold change 1.03) and 200μM (fold change 1.15). However, a significant induction of FAS gene expression (2.4-fold) was observed at 100 μM concentration of AMD3100, suggesting a specific concentration associated effect of CXCR4 blockade on FAS gene activation in HepG2 cells (Figure 3).

Blocking CXCR4 led to induction of FASL gene in HCC cells

CXCR4 blockade in HepG2 cells using AMD3100 resulted moderate concentration-dependent induction of the FASL gene, with fold changes of 1.1, 1.3, and 1.7 at 50, 100, and 200μM concentrations, respectively. The maximum induction observed was 1.7-fold. However, these gene expression changes did not correlate with cytotoxicity data, as no significant inhibition of cell proliferation was observed at the corresponding AMD3100 concentrations (Figure 4).

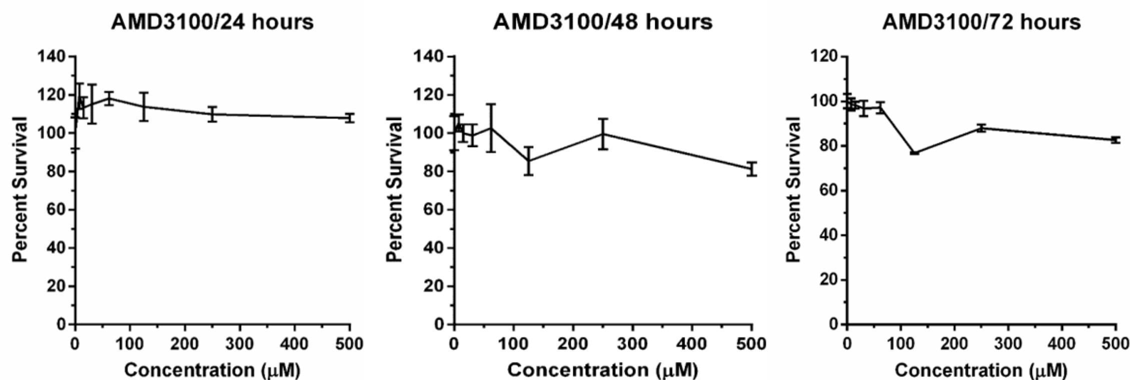

Figure 1: Cytotoxic effects after blocking CXCR4 in HepG2 cells. Following the blockage of CXCR4 by exposing the cells with AMD3100 for 24-72 hours, viable cell fractions were determined by MTT dye reduction assay. AMD3100 exposure induced moderate inhibition of cellular proliferation in HepG2 cells for later time intervals (48 and 72 hours).

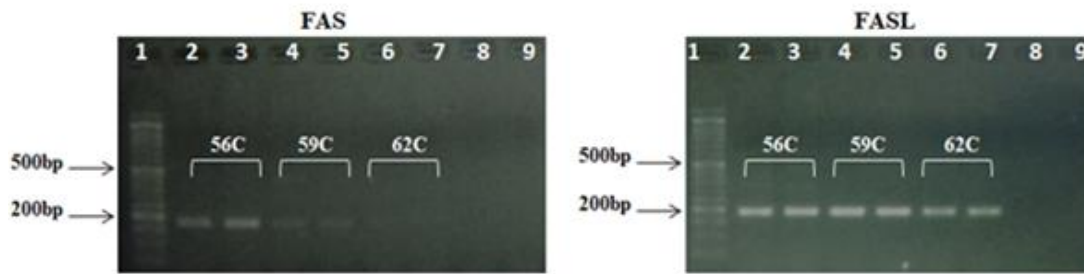

Figure 2: Electrophoresed cDNA samples of the genes**.** To optimize the conditions for amplification of the genes, a gradient PCR was performed. For this purpose, three different temperatures (56-62C) were checked. FAS (186bp) and FASL (185bp) showed good amplification outcomes. Sample distribution left to right Well 1: DNA marker (50bp). Well 2-7: untreated control. Well 8-9: PCR -ve control.

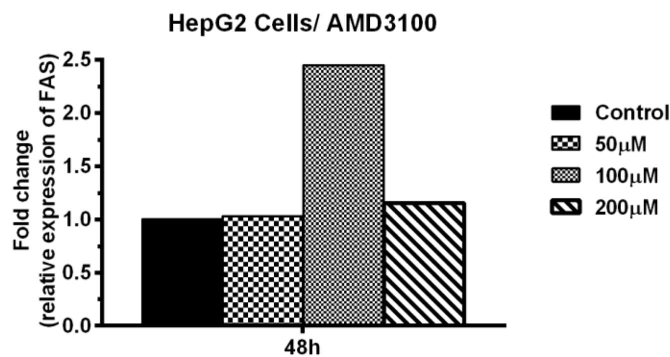

Figure 3: Blocking CXCR4 led to the induction of FAS gene in HCC cells. CXCR4 blockade in HepG2 cells using AMD3100 resulted in a moderate induction of the FAS gene, with fold changes of 1.1, 2.4, and 1.3 at 50, 100, and 200µM concentrations, respectively.

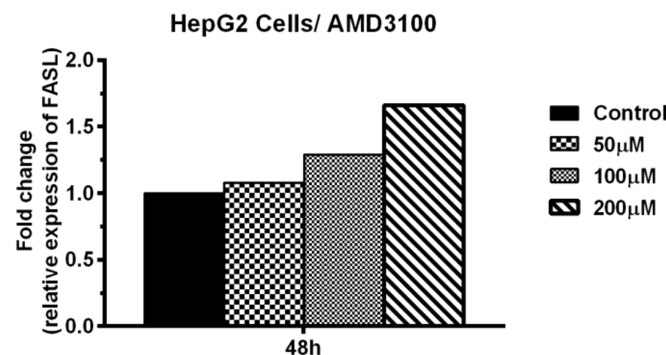

Figure 4: Blocking CXCR4 led to induction of FASL gene in HCC cells. CXCR4 blockade in HepG2 cells using AMD3100 resulted in a moderate, concentration-dependent induction of the FASL gene, with fold changes of 1.1, 1.3, and 1.7 at 50, 100, and 200 µM concentrations, respectively.

DISCUSSION

CXCR4 receptors are known for their role in HIV infections but also have important physiological functions, such as influencing stem cell movement, neovascularization, angiogenesis, and the migration of hematopoietic cells to the bone marrow [18, 19]. Due to their involvement in HIV entry, CXCR4 antagonists and monoclonal antibodies are being developed as HIV entry inhibitors. One such antagonist, AMD3100, is used during stem cell transplants for cancer treatment [15]. Additionally, CXCR4 is being studied for its role in various diseases, including cancer, and its inhibition is being explored for therapeutic purposes.

CXCR4 receptors, beyond their involvement in HIV, are being explored for their role in various diseases, including cancers. In HCC, CXCR4 expression is upregulated, correlating with poor prognosis, reduced survival, and metastasis. Elevated CXCR4 levels promote HCC cell migration and proliferation, while receptor inhibition reduces tumor growth [20]. The CXCR4-CXCL12 axis contributes to angiogenesis, and blocking CXCR4 with AMD3100 decreases tumor vascularity [21]. In a study, it was revealed that CXCR4 expression also impacts sorafenib treatment for viral-induced HCC, highlighting its potential as a prognostic marker and therapeutic target [22]. Additionally, CXCR4 polymorphisms may increase the risk for HCC [23].

In this study, the effect of CXCR4 inhibition using its antagonist (AMD3100) on HepG2 cells was investigated. The MTT assay revealed moderate effects on cell proliferation, with a slight decrease in proliferation at 24 hours and only ~20% inhibition at later time points (48-72 hours). This suggests that HepG2 cells do not rely solely on CXCR4 for proliferation. Furthermore, genetic redundancy in the chemokine network and negative feedback loop development by the cells may lead to the reduced effects of the antagonist. Particular attention is needed to see phenomenon of less responsiveness of the cells towards high concentration (250μM) of antagonist as compared to its lower concentration (125 μM). This shows that cells responded with the development of resistance mechanisms against the higher concentrations of AMD3100 antagonist.

As far as the expression of apoptosis-related genes FAS and FASL is concerned, CXCR4 blockage led to a marginal induction of FAS at 100μM of AMD3100 and a significant, concentration-dependent increase in FASL expression, indicating that CXCR4 inhibition may promote apoptosis in HepG2 cells by activating FASL-related apoptotic pathways. Nevertheless, detailed studies are needed to understand the impact of AMD3100 exposure on HCC cells, while including other representative cell lines, higher concentrations of the compound, longer exposure periods and other genetic networks.

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Ethics Approval: Not Applicable
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.

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