

Cytotoxic effects of chemokine receptor 4 inhibition by AMD3100 in biliary tract cancer cells: Potential drug synergism with gemcitabine

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Abstract. Biliary tract cancer (BTC) remains one of the most life-threatening types of cancer due to the lack of efficient therapies. Advanced tumour stages at the point of diagnosis and high chemoresistance are two of the predominant reasons for a 5-year survival rate of only ~5%. The present study investigated the effect of the chemokine receptor 4 (CXCR4) inhibitor AMD3100 (Plerixafor), alone and in combination with standard gemcitabine chemotherapy, on the proliferation of BTC cells. The expression of CXCR4 was analysed by reverse transcription-quantitative polymerase chain reaction in eight heterogeneously differentiated BTC cell lines. The effects of treatment with the CXCR4 antagonist, AMD3100, on cell viability and anchorage-independent growth, and the possible synergistic cytotoxic effects of AMD3100 with standard chemotherapeutics were assessed. The expression of CXCR4 was observed to a variable extent in all eight BTC cell lines, with SkChA-1 cells exhibiting the highest expression levels. Treatment with AMD3100 led to a marginal decrease in cell viability in the cell lines, with the exception of the CCSW-1 cells, and a significant reduction in the GBC, MzChA-1, SkChA-1 and TFK-1 cell lines. The combined treatment of the SkChA-1 cells with varying concentrations of AMD3100 and standard gemcitabine chemotherapy revealed a more marked overall cytotoxicity, indicating a potential

synergistic effect. In addition, AMD3100 significantly reduced anchorage-independent growth in the SkChA-1 cells. Overall, the results of the present study suggest that the inhibition of CXCR4 by AMD3100, in combination with gemcitabine, may be a suitable strategy for the future therapy of BTC.

Introduction

Biliary tract cancer (BTC) is a highly aggressive and heterogeneous type of cancer, originating in the bile ducts, with high rates of relapse and poor clinical outcome (1). Despite current treatment strategies, including surgery, liver transplantation, chemotherapy and photodynamic therapy, the 5-year survival rate of only ~5% remains low (2,3). For advanced, inoperable BTC, palliative chemotherapy with cisplatin and gemcitabine results in a median survival rate of ~1 year (4,5).

Chemokines are chemoattracting proteins, which bind to their respective receptors and thereby activate them. Chemokine receptor 4 (CXCR4) is a chemokine receptor, which specifically binds to chemokine ligand 12 (CXCL12). First identified on leukocytes, CXCR4 is expressed by several different cell types (6). It is important in organogenesis, and in tissue repair and regeneration in adults. Additionally, the expression of CXCR4 was identified in hematopoietic and non-hematopoietic tissue-committed stem cells (6-8).

The CXCR4/CXCL12 axis is important in adhesion, invasion, metastasis, migration and proliferation in various types of cancer, including breast cancer (9-13), small-cell lung cancer (14), head and neck squamous cell carcinoma (15), neuroblastoma (16), hepatocellular carcinoma (17) and colon cancer (18), and is generally associated with high aggressiveness and a poor prognosis. Several previous studies have suggested a role for the CXCR4/CXCL12 axis in BTC (19-23). Lee *et al* demonstrated that CXCL12 is associated with an advanced histological grade and nodal metastasis in gallbladder cancer, and observed increased anchorage-dependent, and -independent growth of gallbladder cancer cells *in vitro*, in a CXCR4-dependent manner (21). Leelawat *et al* compared the

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gene expression levels of cluster of differentiation (CD)24⁺ and CD24⁺ BTC cells, and revealed an upregulation of CXCR4 in the CD24⁺ subpopulation (19). AMD3100 (Plerixafor) is a non-peptide antagonist of CXCR4, which inhibits CXCL12 binding and is currently used for hematopoietic stem cell mobilization and transplantation following chemotherapy of hematological malignancies (24,25). The expression of CD24 is accompanied by poor clinical outcome in BTC, and AMD3100 inhibits the invasiveness of the CD24⁺ subpopulation (19), therefore suggesting CXCR4 as a potential target for the treatment of BTC.

Taken together, CXCR4/CXCL12 are important in several types of cancer, potentially including BTC. Therefore, the aim of the present study was to examine the expression levels of CXCR4 in a larger panel of BTC cell lines (n=8). Following confirmation of the expression levels, the effects of the AMD3100 CXCR4 antagonist, alone and in combination with the standard chemotherapeutic gemcitabine, on cell viability and anchorage-independent growth pattern were investigated.

Materials and methods

Reagents and cell culture. AMD3100 (MozobilTM) was supplied by Sanofi-Aventis (Paris, France) as a 20 mg/ml subcutaneous injection solution, and was stored at room temperature. Gemcitabine was obtained from the hospital pharmacy (Landesapothek, Salzburger Landeskliniken), as a stock solution of 152 mM in H₂O, and stored in aliquots at room temperature. Resazurin was purchased from Sigma-Aldrich (Vienna, Austria), dissolved in Dulbecco's phosphate-buffered saline (Sigma-Aldrich) and sterile filtered using a 0.2 µm filter (Sarstedt, Nüxsmbrecht, Germany). A total of five bile duct carcinoma cell lines: CCSW-1 (G2), BDC (G4), EGI-1 (G3), SkChA-1 (G3), TFK-1 (G2); and three gallbladder cancer cell lines: MzChA-1 (G1), MzChA-2 (G2) and GBC (G1), were cultured in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Pasching, Austria), supplemented with 10% (v/v) fetal bovine serum (FBS; PAA Laboratories) - see (26,27) for original references. Together, these were referred to as BTC cell lines (1). Subsequent experiments were performed using cells within 10 passages at seeding densities of 3.95x10⁴ for BDC and MzChA-2, 4.74x10⁴ for CCSW-1 and GBC, 5.53x10⁴ for SkChA-1, 6.32x10⁴ for EGI-1 and TFK-1, and 7.11x10⁴ for MzChA-1 (per cm² of the culture dishes), in 10% FBS-DMEM. Treatment with AMD3100 was performed in serum-free (sf)DMEM to avoid interaction of the drug with the serum ingredients.

Drug cytotoxicity. The dose- and cell line-dependent cytotoxicity of AMD3100 was investigated on the cells in 96-well microplates (Greiner Bio-One, Frickenhausen, Germany), using a resazurin assay (Sigma-Aldrich) and an Infinite M200 microplate reader (Tecan, Grödig, Austria) to assess the metabolic activity, as described previously (27,28). For the cell line-dependent analysis, 400 µg/ml AMD3100 was added to the cells in sfDMEM for 72 h, and the cell viability was normalized against untreated (sfDMEM only) samples. For dose-dependent analysis, a 1:2 dilution series ranging between 0.8 and 400.0 µg/ml of AMD3100 were

added to SkChA-1 cells in sfDMEM for 72 h, and the viability was normalized against the untreated samples.

Anchorage-independent growth. The anchorage-independent growth of the SkChA-1 cells was assessed using the CytoSelect 96-well Cell Transformation assay (Cell Biolabs, Inc., San Diego, CA, USA) and an Infinite M200 microplate reader. The SkChA-1 cells were seeded at a density of 1x10⁵ cells/ml in a semi-solid agar, according to the manufacturer's instructions, and incubated with 100 µg/ml AMD3100 in DMEM for 14 days at 37°C. Anchorage-independent growth was monitored using a light microscope (Motic AE31; Nikon Instruments, Melville, NY, USA) equipped with a CCD-I300B digital camera (Allied Vision Technologies/VDS, Vosskuhler, Stadtroda, Germany), quantified using CyQuant GR dye and the growth was normalized against the untreated (DMEM only) samples.

Analysis of the mRNA expression of CXCR4. For mRNA expression analysis, the cells were grown in 35 mm cell culture dishes in DMEM for 72 h. The total RNA was isolated using the Direct-zolTM RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) with TRIzol Reagent (Ambion, Life Technologies, Vienna, Austria). The cDNA synthesis was performed through reverse transcription (RT), using 1 µg isolated RNA and an ImProm-IITM Reverse Transcriptase system (Promega, Madison, WI, USA), according to the manufacturer's instructions. Quantification of the cDNA was determined through quantitative polymerase chain reaction (qPCR), using a GoTaq qPCR Master mix (Promega) and a ViiA7 real time PCR system (Applied Biosystems, Invitrogen Life Technologies, Carlsbad, CA, USA). cDNA (0.3 ng) was used for PCR and the following cycling conditions were used: 95°C for 2 min and 45 cycles of 95°C for 3 sec and 60°C for 30 sec. The samples were measured at least three times, and subsequent melting curve analysis was performed for all the primers, to confirm the specificity of the PCR products. The samples were normalized against β-actin and the data were analyzed, according to the ΔΔCt method (29). The primer sequences used for RT-qPCR were as follows: β-actin, forward GCACTCTTCCAGCCTTCCTTCC and reverse TCTTTGCGGATGTCCACGTCAC and CXCR4 forward GTCATCTACACAGTCAACCTCTACAGCAGT and reverse AAGATGAAGTCGGGAATAGTCAGCAG.

Double treatments. The SkChA-1 cells were seeded into 96-well microplates and incubated with different concentrations of either AMD3100 (200, 100, 50, 25 or 12.5 µg/ml) or gemcitabine (1000, 500, 250, 125 or 62.5 µM), or with combinations of these concentrations (constant 25 µg/ml AMD3100 combined with 1000, 500, 250, 125 and 62.5 µM gemcitabine or constant gemcitabine (500 µM) combined with 200, 100, 50, 25 and 12.5 µg/ml AMD3100). Cell viability was measured using a resazurin assay and an Infinite M200 microplate reader. The obtained data were entered into CompuSyn software (version 1.0) to calculate the combination index (CI), in which drug combinations leading to a CI >1.1 were considered to be antagonistic, and CI values <0.9 were considered to be synergistic (30).

Statistical analyses. Unless otherwise indicated, all data are presented as the mean ± standard error of the mean, of at

least three biological replicates. An unpaired Student's t-test was used for calculation of the significance of differences in anchorage-independent growth. A paired Student's t-test was performed for calculation of the significance of the effect of AMD3100 on cell viability. All calculations were performed using OriginPro 9.1 software (OriginLab, Northampton, MA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of CXCR4 in the BTC cell lines. The present study confirmed the mRNA expression levels of CXCR4 in eight BTC cell lines using RT-qPCR. The expression values differed between the cell lines, with SkChA-1 exhibiting the highest expression levels of CXCR4 (Fig. 1A).

Effect of treatment with AMD3100 on overall cell viability. The present study subsequently investigated whether the addition of the CXCR4 antagonist, AMD3100, had an effect on the cell viability of the CXCR4-expressing cell lines. The cells were treated with 400 $\mu\text{g/ml}$ AMD3100 for 72 h. The results revealed that the addition of AMD3100 led to a reduction in cell viability in seven of the cell lines, with no effect for CCSW-1. Significant decreases of 20-30% were observed in the GBC, MzChA-1, SkChA-1 and TFK-1 cell lines (Fig. 1B). Although no correlation was observed between the expression levels of CXCR4 and the reduction in cell viability by AMD3100 (data not shown), the most marked effect of AMD3100 on cell viability was observed in the SkChA-1 cells which also exhibited the highest mRNA expression levels of CXCR4.

To investigate the dose-dependent effects of AMD3100 on SkChA-1 cells, a dilution series ranging between 0.8 and 400.0 $\mu\text{g/ml}$ was used, with cells incubated with AMD3100 for 72 h at 37°C. As shown in Fig. 1C, treatment with AMD3100 reduced the overall cell viability in a dose-dependent manner, with a significant reduction of ~20% at concentrations >100 $\mu\text{g/ml}$ (Fig. 1C).

Taken together, treatment with AMD3100 reduced the cell viability in seven of the eight BTC cell lines assessed. As the most marked decline in cell viability was observed in the SkChA-1 cells, this cell line was selected for use in the subsequent experiments.

Combined treatment of AMD3100 and gemcitabine. The present study subsequently investigated whether the combination of AMD3100 with gemcitabine, a standard chemotherapeutic drug, further reduced the viability of the SkChA-1 cells, and whether these drugs acted synergistically. The results suggested that at least two combinations: 50 $\mu\text{g/ml}$ AMD3100 with 500 μM gemcitabine, and 12.5 $\mu\text{g/ml}$ AMD3100 with 500 μM gemcitabine, demonstrated a synergistic cytotoxic effect on the SkChA-1 cells (Fig. 2). Experiments using a combination of AMD3100 and cisplatin revealed no synergistic effect (data not shown).

Effect of AMD3100 on anchorage-independent growth. Anchorage-independent growth is a hallmark of increased tumor aggressiveness, metastatic potential and cancer

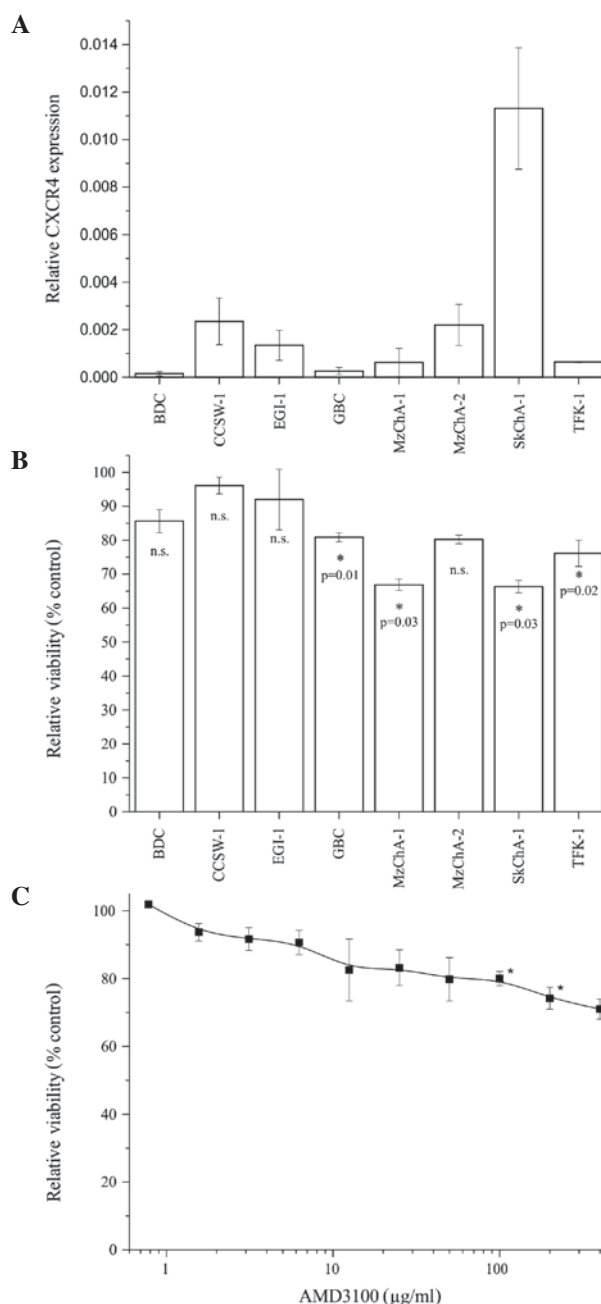


Figure 1. Expression levels of CXCR4 and the cytotoxic effects of AMD3100 on BTC cell lines. (A) BTC cells were analyzed for the mRNA expression of CXCR4 using reverse transcription-quantitative polymerase chain reaction. The data were normalized against β -actin and are expressed as the mean \pm standard error of the mean. (B) Effect of treatment with AMD3100 (400 $\mu\text{g/ml}$) on cell viability in the BTC cell lines following incubation for 72 h. The data are expressed as the percentage of untreated control wells, as the mean \pm standard error of the mean. (C) Dose-dependent effect of AMD3100 on SkChA-1 cell viability following incubation for 72 h. The data are expressed as the percentage of untreated controls, as the mean \pm standard error of the mean. CXCR4, chemokine receptor 4; BTC, biliary tract cancer; ns, not significant.

stem cell (CSC) characteristics (31). In order to assess the effect of AMD3100 on anchorage-independent growth, the SkChA-1 cells were seeded into semisolid agar and incubated for 14 days at 37°C, in the presence or absence of AMD3100. Treatment with AMD3100 significantly inhibited anchorage-independent growth in the SkChA-1 cells, leading to a reduction of ~50% (Fig. 3A). Representative images of

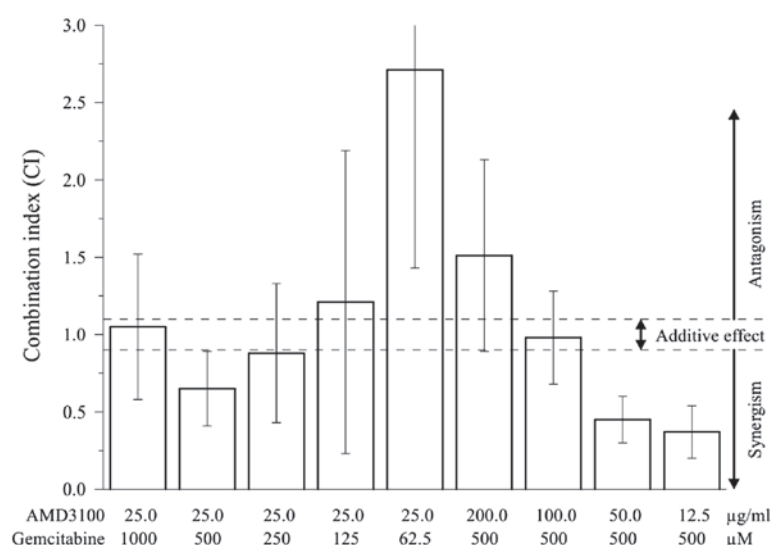


Figure 2. Combined treatment with AMD3100 and gemcitabine on SkChA-1 cells. The SkChA-1 cells were incubated for 72 h with different concentrations and combinations of AMD3100 and gemcitabine. CompuSyn software was used to calculate the CI. CI values <0.9 indicated synergistic effects and CI values between 0.9 and 1.1, or >1.1 indicated additive or antagonistic effects (30). The data are expressed as the mean \pm standard error of the mean. CI, combination index.

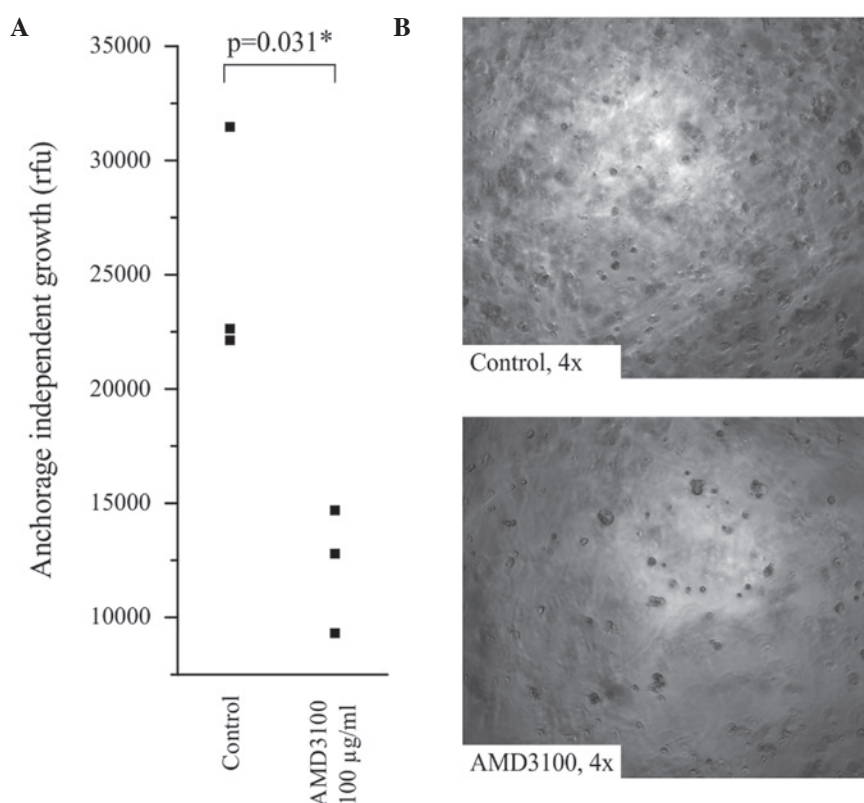


Figure 3. Effect of AMD3100 on anchorage-independent growth of SkChA-1 cells. (A) SkChA-1 cells were incubated in semisolid agar for 14 days in the absence or presence of 100 μ g/ml AMD3100. Anchorage-independent growth was quantified using CyQuant GR dye. (B) Representative images of the untreated (control) SkChA-1 cells and the SkChA-1 cells treated with AMD3100. rfu, relative fluorescent unit.

SkChA-1 cells with and without AMD3100 are shown in Fig. 3B and indicated a reduction in cell aggregates.

Discussion

The high mortality rates and poor prognosis of BTC underlines the requirement for novel therapeutic approaches. The CXCR4

chemokine receptor and its ligand, CXCL12, are involved in the regulation of tissue-committed stem cells (6-8). Furthermore, these proteins are involved in cancer cell invasion, metastasis, migration and proliferation (9-18). CXCR4 is expressed in several types of cancer (7,8), including BTC (19,22,23), and its expression correlates with high levels of aggressiveness and a poor prognosis (7). Previous studies have demonstrated the

involvement of CXCR4 signaling in the migration and invasion of BTC cells, which can be reduced by the pharmacological inhibition of CXCR4 using AMD3100 (19,22,23).

In accordance with these results, the present study detected the mRNA expression of CXCR4 in eight BTC cell lines, to a variable extent, indicating that this signaling axis exhibits different activities in different cell lines. In the BDC and GBC cell lines, the mRNA expression levels of CXCR4 were almost undetectable, whereas those of CXCR4 were highest in the SkChA-1 cells, compared with the other cell lines.

Treatment of the cell lines with a high concentration (400 $\mu\text{g/ml}$) of the AMD3100 CXCR4 antagonist for 72 h reduced cell viability in seven cell lines, with no effect in the CCSW-1 cells, of which significant reductions were observed in the GBC, MzChA-1, SkChA-1 and TFK-1 cells. Since SkChA-1 not only demonstrated the highest expression levels of CXCR4, but also the most marked decline in cell viability following treatment with AMD3100, the present study assessed the dose-dependent (0.78-400 $\mu\text{g/ml}$) effect of AMD3100 on the cell viability of the SkChA-1 cell line. A dose-dependent reduction in viability following 72 h AMD3100 treatment was confirmed, with significant reductions observed at concentrations >100 $\mu\text{g/ml}$. These effects were possibly caused by a slowdown of proliferation, rather than actual cytotoxicity, as no previous reports have provided evidence to suggest the induction of cell death following the inhibition of CXCR4. For example, treatment of a cholangiocarcinoma cell line with AMD3100 revealed no effect on cell viability (19). Although the present study used a higher concentration of AMD3100 compared with (19), it is also possible that, due to the heterogeneity of BTC, cell lines respond differentially to the inhibition of CXCR4. Previous studies using BTC cell lines demonstrated an inhibitory effect on cell migration following the inhibition of CXCR4 receptors, while the addition of the CXCL12, SDF-1 α ligand increases the proliferative capacity of BTC cells (22,23). In the present study, no correlation was observed between the expression of CXCR4 and the effect of AMD3100 on cell viability.

A significant reduction of 50% was observed in the anchorage-independent growth of the SkChA-1 cells following treatment with AMD3100. This is particularly interesting, as this assay is considered a functional assessment of stemness characteristics in the CSC theory (31). Therefore, subsequent investigations into the CSC-specific effects of the pharmacological inhibition of CXCR4 are required. In line with this theory, CXCR4 is upregulated in the CD24⁺ subpopulation, and the expression of this surface molecule is associated with a poor clinical outcome (19) and has been suggested as a marker for putative CSCs in BTC (32).

Gemcitabine combined with cisplatin is the current standard chemotherapy for patients with advanced BTC (4,5). The present study examined the possible synergistic effects of each of these drugs combined with AMD3100 on SkChA-1 cell viability. Combination with cisplatin revealed no synergistic effect (data not shown), whereas several combinations of gemcitabine with AMD3100 indicated a clear synergistic effect of those two drugs (30). Notably, at a constant gemcitabine concentration (500 μM) the synergistic effect was more pronounced as the concentrations of AMD3100 decreased, with the lowest CI value being 12.5 $\mu\text{g/ml}$. This

result suggested that inhibition of CXCR4 combined with gemcitabine may be a suitable treatment strategy, and is in line with a report by Singh *et al*, which demonstrated that the inhibition of CXCR4 using AMD3100 eliminated gemcitabine resistance in pancreatic cancer cells (33).

In conclusion, the present study demonstrated that CXCR4 was expressed in BTC cell lines and that the inhibition of this receptor affected anchorage-dependent and anchorage-independent growth. Furthermore, it was revealed for the first time, to the best of our knowledge, that the combination of AMD3100 with standard chemotherapeutic gemcitabine may be a promising optional therapeutic approach for BTC.

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