3'-Azidothymidine may potently inhibit the biosynthesis of polylactosamine chains on highly glycosylated-CD147 and reduce matrix metalloproteinase-2 expression in SGC-7901 and U251 cells

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Abstract. Alterations to N-linked glycans are closely associated with cancer progression. Of particular importance in tumor growth and invasion, is the synthesis of complex N-linked oligosaccharides containing poly-N-acetyllactosamine (polylactosamine) chains, which have previously been reported to inhibit 3'-azidothymidine (AZT). Cluster of differentiation 147 (CD147) is a glycoprotein that carries β1,6-branched polylactosamine sugars on its N-glycans. The present study aimed to explore the mechanism by which AZT may affect matrix metalloproteinase-2 (MMP2) expression and the cell cycle via regulation of the N-glycans on CD147 in SGC-7901 and U251 cell lines. Subsequent to treatment with various concentrations of AZT, the N-glycans of highly glycosylated (HG)-CD147 were observed to decrease in the two cell lines, and the expression of MMP2 was also significantly decreased. In addition, cell cycle analysis demonstrated that the percentage of the cells in the G_1 phase increased in a dose-dependent manner with AZT treatment, indicating that AZT may inhibit cell proliferation in SGC-7901 cells. It was suggested that AZT may reduce the biosynthesis of polylactosamine chains on CD147 and reduce MMP2 expression to inhibit cell proliferation in SGC-7901 and U251 cells. Thus, AZT is suggested to be an antineoplastic drug, which may be effective therapeutically for certain types of cancer through acting on the N-glycans of HG-CD147.

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Introduction

Glycosylation is one of the most frequently occurring post-translational modifications of proteins and is suggested to be involved in the regulation of a variety of biological and physical processes, including the secretion, stability, folding and solubility of proteins (1). It has also been observed that the majority of proteins in the serum and the plasma membrane are glycosylated (2). In addition, several previous studies have suggested that aberrant glycosylation of cell surface glycoproteins results in significant alterations in the invasive and metastatic abilities of cancer cells (3,4). For example, the synthesis of polylactosamine chains in colon cancer cells has been demonstrated to be associated with metastasis (5).

Polylactosamine is a unique glycan composed of tandem repeating units of Galβ1-4GlcNAc at the nonreducing terminal (Fig. 1). A previous study observed that polylactosamine-type N-glycans were highly expressed in certain types of cancer cells, including U937 (histiocytic lymphoma), ACHN (human kidney glandular cancer), MKN45 (human gastric cancer), A549 (human lung cancer) and Jurkat cells (acute T-cell leukemia) cell lines (6). Togayachi *et al* (7) reported that the polylactosamine residues on glycoproteins influenced basal levels of lymphocyte and macrophage activation. Additionally, 24 glycoproteins that possess polylactosamine-type N-glycans have been demonstrated to be present in malignant cells (8).

Cluster of differentiation 147 (CD147), which is also termed Basigin or extracellular matrix metalloproteinase inducer (EMMPRIN), is a glycoprotein that carries polylactosamine on its N-glycosylation sites (9). CD147 is a transmembrane glycoprotein and its extracellular region contains three N-linked glycosylation sites (Asn44, Asn152 and Asn186), which make similar contributions to form the highly and lowly glycosylated forms (HG- and LG-CD147, respectively). The present study demonstrated that LG-CD147 contains a series of high-mannose structures and HG-CD147 contains complex-type N-linked glycans, including β1,6-branched polylactosamine (9). LG-CD147 does not self-aggregate and is not

able to induce the production of MMPs, whereas HG-CD147 molecules have been reported to self-aggregate and activate MMP production in tumor cells (9,10). MMP2 has been demonstrated to degrade and destroy the extracellular matrix and basement membrane close to the tumor surface, which aids in the infiltration of tumor cells into surrounding tissues, and promotes tumor cell invasion and metastasis. CD147 is present in tissues with increased MMP expression levels, which suggests that CD147-mediated MMP induction may be involved in the physiological or pathological mechanisms of cancer progression (11,12).

Previous studies have demonstrated that 3'-azidothymidine (AZT; Fig. 2) inhibits the biosynthesis of β1,6-branched N-linked oligosaccharides and polylactosamine chains in cells (13,14). Additional studies have observed that glycosyltransferase β3GnT8 was involved in the synthesis of the polylactosamine chains on the β1,6-branched N-glycans, and influenced the invasion and growth of gastric cancer cells by regulating the expression levels of MMP2 (4,15). However, it remains unclear whether the β1,6-branched polylactosamine chains are critical in this mechanism. Therefore, in the present study, two different tumor cell lines were treated with various concentrations of AZT, the expression levels of HG-CD147 and MMP2 were measured, and the cell cycle was analyzed to investigate cell proliferation in order to elucidate how the β1,6-branched polylactosamine on HG-CD147 may affect MMP2 expression levels and cell proliferation.

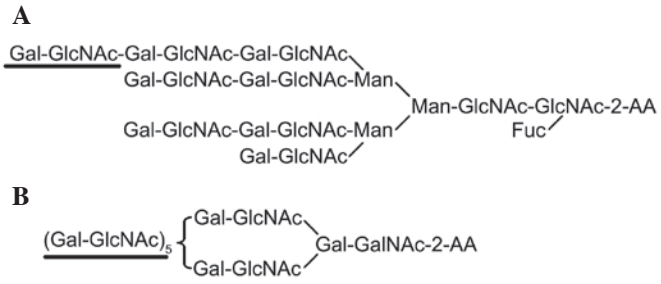

Figure 1. Typical structures of polylactosamine-type glycans. (A) N-glycans and (B) O-glycans.

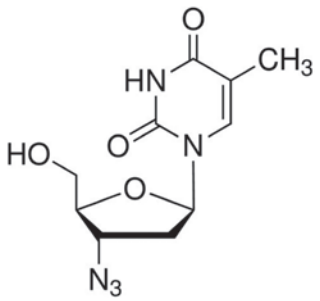

Figure 2. Structural formula of 3'-azidothymidine.

Materials and methods

Materials. The U251 human glioma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The SGC-7901 human gastric cancer cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Goat polyclonal anti-CD147 antibody (cat. no. sc-9753) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and the mouse polyclonal anti-β-actin antibody (cat. no. AA-128-1), and anti-rabbit-horseradish peroxidase (HRP), anti-goat-HRP and anti-mouse-HRP secondary antibodies were purchased from Beyotime Institute of Biotechnology (Haimen, China). AZT was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were commercially available in China.

Cell culture. The U251 cells were cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). The SGC-7901 cells were cultured in RPMI-1640 (Gibco Life Technologies) medium supplemented with 10% FBS (Gibco Life Technologies). The two cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37˚C.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the two cancer cell lines using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Complementary DNA (cDNA) was generated from total RNA using M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). The PCR reaction mix consisted of 12.5 μl Easy Taq PCR Supermix (Beijing Transgen Biotech Co., Ltd., Beijing, China), 0.5 μl forward primer, 0.5 μl reverse primer, 1 μl cDNA and 0.5 μl ddH_2O. The PCR cycling (Veriti® 96-well Thermal cycler; Applied Biosystems, Foster City, CA, USA) conditions were as follows: Initial denaturing at 95˚C for 5 min, 30 cycles of denaturing at 95˚C for 30 sec, annealing at 60˚C for 45 sec, elongation at 72˚C for 1 min and at 72˚C for 10 min. The annealing temperature of MMP2 was 55˚C and of β-actin was 53˚C. Specific primers (Invitrogen Life Technologies) used for the genes and expected product sizes were as follows: Forward: 5'-AACCCTCAGAGCCAC CCCTA-3' and reverse: 5'-GTGCATACAAAGCAAACT GC-3' (286 bp) for MMP-2; and forward: 5'-GAGCTACGA GCTGCCTGACG-3' and reverse: 5'-CCTAGAAGCATT TGCGGTGG-3' (416 bp) for β-actin. The PCR products were separated using electrophoresis on 10 g/l agarose gels and visualized using ethidium bromide staining.

Western blot analysis. Protein was extracted from cell lysates using ice-cold radio immunoprecipitation assay lysis buffer (50 mmol/l Tris (pH 7.4), 150 mmol/l sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium or thovanadate, 10 mM sodium fluoride, 5 mM EDTA, 10 μg/ml leupeptin; Beyotime Institute of Biotechnology) supplemented with 1 mmol/l phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology). The protein concentration in cell lysates was determined using a protein assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). An equal quantity of protein from each sample was mixed with 4X loading buffer (containing 250 mmmol/l Tris-hydrochloric acid, 40% glycerol, 5% SDS, 0.005% bromophenol blue and 100 mmol/l dithiothreitol; Beyotime Institute of Biotechnology) and was denatured for 5 min at 100˚C. Total proteins were then separated by SDS-PAGE (10% acrylamide

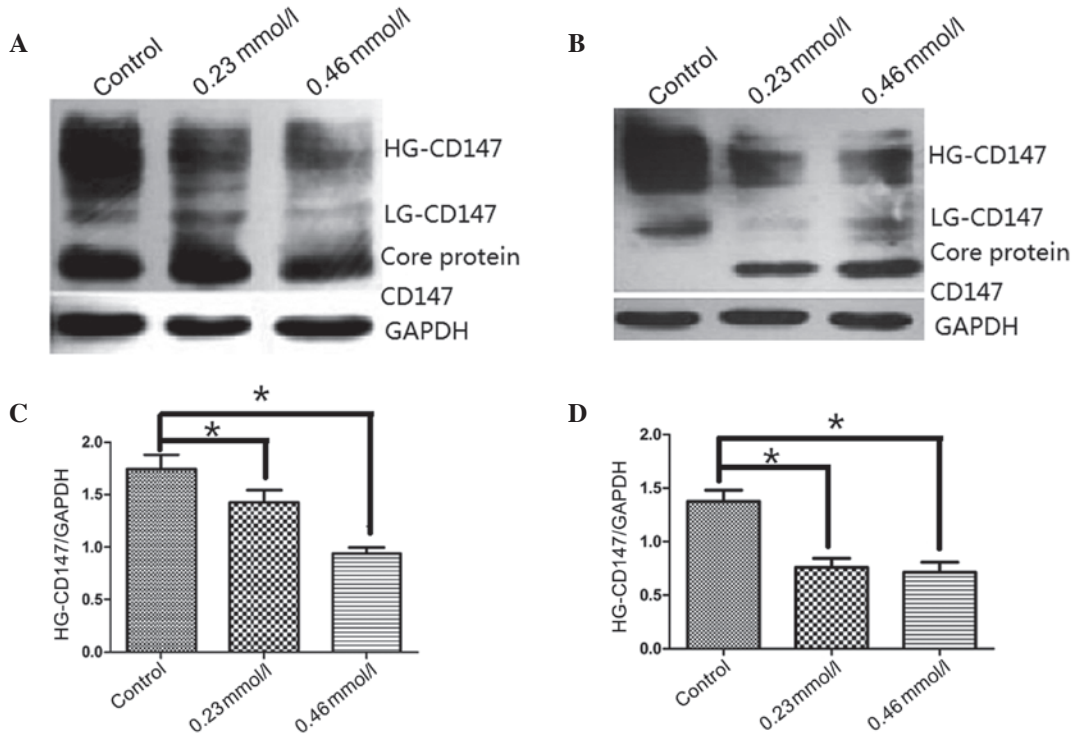

Figure 3. Western blotting to examine the effect of AZT on CD147 N-glycosylation in SGC-7901 and U251 cells. The cells were treated with various concentrations of AZT for 48 h. The blots were stripped and reprobed with a human β-actin probe to confirm equal loading. In total, 30 μg total protein was loaded in each lane. Western blot analysis of (A) SGC-7901 cells and (B) U251 cells. The relative protein expression of CD147 in (C) SGC-7901 cells and (D) U251 cells. The experiments were representative of three independent experiments and error bars represent the mean ± standard deviation; *P<0.05 vs. control. AZT, 3'-azidothymidine; CD147, cluster of differentiation 147.

gel) and transferred onto polyvinylidene fluoride membranes (Pall Corporation, Beijing, China) that had been pretreated with methanol. The membranes were then blocked for 1 h at room temperature in PBST [phosphate-buffered saline (PBS) with 0.05% Tween-20] (Gibco Life Technologies) containing 5% skimmed milk. The proteins were analyzed using the specific antibodies mentioned. The blots were incubated overnight at 4˚C with the primary antibodies against CD147 (1:300) and β-actin (1:1,000). Subsequent to removal of the primary antibodies, the blots were then incubated for 1 h at room temperature with goat anti-rabbit, donkey anti-goat or rabbit anti-mouse HRP-conjugated secondary antibodies (1:1,000). ECL Plus Detection system (Beyotime Institute of Biotechnology) was used according to the manufacturer's instructions to detect chemiluminescence.

Regulation of CD147 and MMP2 expression by various concentrations of AZT. The U251 and SGC-7901 cells were seeded into a 6-well plate and incubated overnight. Subsequently, the cells were washed once with PBS and cultured for 48 h in fresh culture medium in the absence or presence of various concentrations of AZT (0, 0.23 and 0.46 mmol/l). The cells were then harvested, and western blotting and RT-PCR analysis were conducted to measure CD147 and MMP2 expression.

Analysis of the cell cycle by flow cytometry. The SGC-7901 cells were plated at a density of $5x10^5$ cells/well on 6-well plates and incubated in serum-free medium for 24 h in order to arrest the cells at the same stage of the cell cycle. Subsequent to exposure to various concentrations of AZT for 24 h, the cells were harvested and fixed with cold 70% ethanol overnight. The fixed cells were incubated in PBS containing 50 μg/ml RNase A (Sigma-Aldrich), 0.25% Triton X-100 (Sigma-Aldrich) and 0.1 mmol/l EDTA (Thermo Fisher Scientific) for 30 min at 37˚C. Propidium iodide (PI) was added to the cell suspension at a concentration of 100 μg/ml and incubated for 15 min at room temperature in the dark. The cell cycle was analyzed using flow cytometry with Cell Quest Pro software (FACScan; BD Biosciences, San Jose, CA, USA).

Statistical analysis. All results are expressed as the mean ± standard deviation. Statistical significance was evaluated with data from three independent experiments using Student's t-test. Statistical analyses were conducted using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Regulation of N-glycosylation on CD147 by AZT in SGC-7901 and U251 cells. To examine the effect of AZT on CD147 N-glycosylation, the two cell lines were treated with various AZT concentrations for 48 h. Proteins from these treated cell lines were assessed using western blot analysis. As demonstrated in Fig. 3, CD147 was clearly expressed in SGC-7901 and U251 cells; however, subsequent to treatment with AZT (0.23 and 0.46 mmol/l) the glycosylation level of HG-CD147

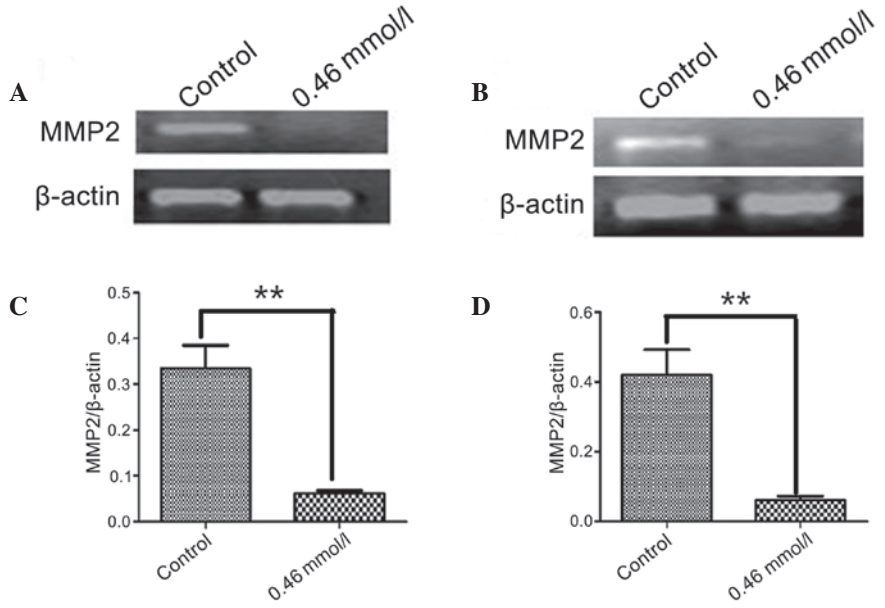

Figure 4. RT-PCR to examine the effect of AZT on MMP2 expression in SGC-7901 and U251 cells. Total RNA were isolated from the AZT-treated cells and β-actin was used as the control. Western blot analysis of (A) SGC-7901 and (B) U251 cells. The relative mRNA expression levels of MMP2 in (C) SGC-7901 and (D) U251 cells. The experiments are representative of three independent experiments and error bars represent the mean ± standard deviation; **P<0.01. RT-PCR, semiquantitative reverse transcription-polymerase chain reaction; AZT, 3'-azidothymidine; MMP2, matrix metalloproteinase-2.

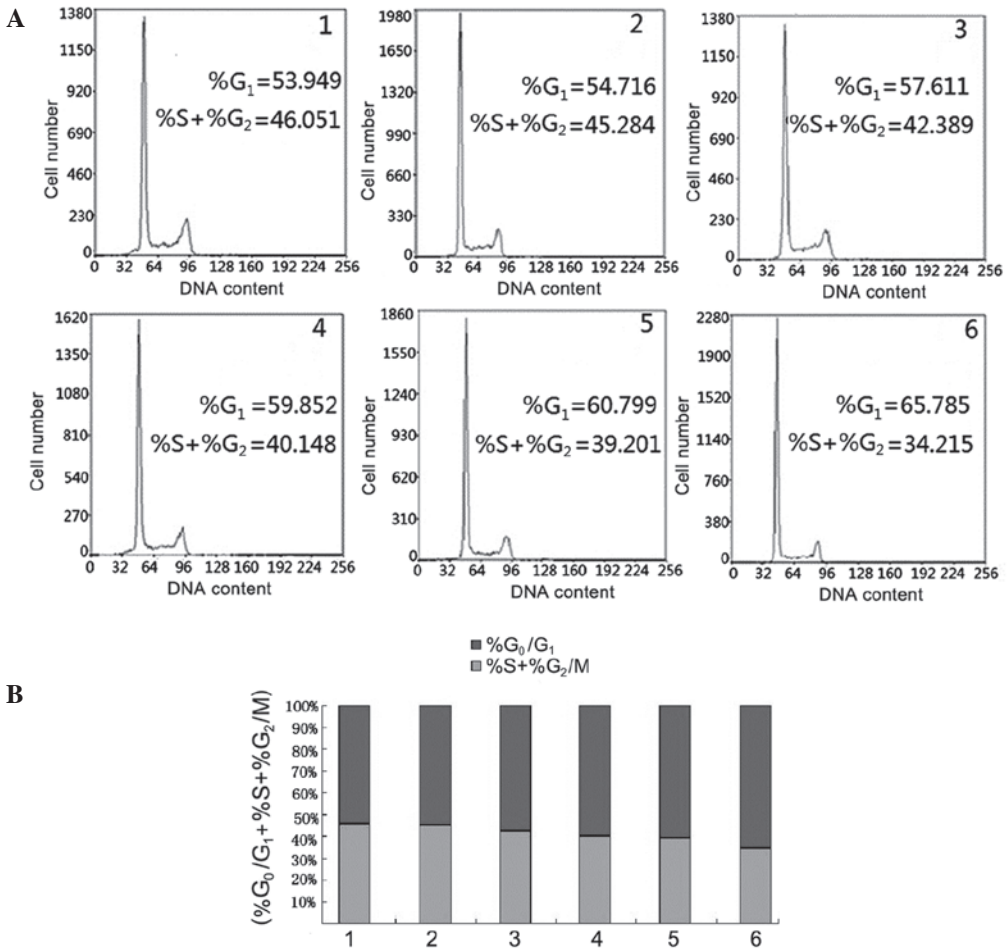

Figure 5. Flow cytometric analysis to examine the cell cycle distribution of SGC-7901 cells undergoing AZT treatment. (A) The results of the cell cycle distribution with various concentrations of AZT. 1, no treatment; 2, 0.23; 3, 0.46; 4, 0.69; 5, 0.92; and 6, 1.15 mmol/l. The percentages of AZT-treated cells in the G_1 phase were 53.949, 54.716, 57.611, 59.852, 60.799 and 65.785%, respectively for the concentrations of AZT listed above. (B) The relative percentages of cells in different stages of the cell cycle with various AZT concentrations. The experiments were representative of three independent experiments. AZT, 3'-azidothymidine.

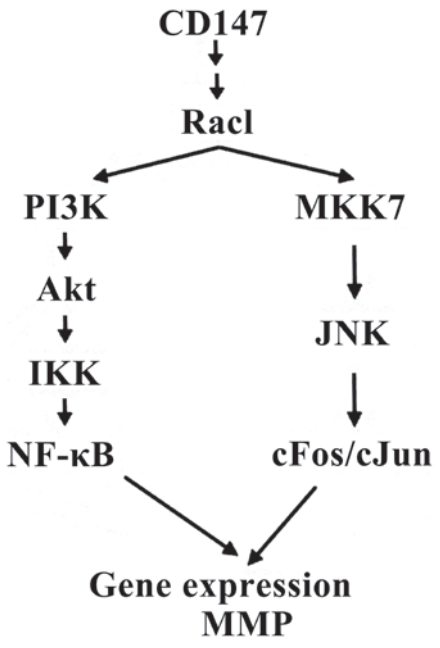

Figure 6. CD147 as an upstream modulator that induces MMP production through different signal transduction pathways. Adapted from reference (24). CD147, cluster of differentation 147; MMP2, matrix metalloproteinase-2; PI3K, phoinositide 3-kinase; MKK7, mitogen-activated protein kinase 7; Akt, protein kinase B; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; NF-κB; nuclear factor-κB.

located at 45-65 kDa was significantly reduced compared with the controls in the two cell lines ($P<0.05$). Furthermore, the glycosylation level of LG-CD147 was also significantly reduced in the treated cells, as compared with the two control cell lines. The results suggest that AZT may inhibit the biosynthesis of polylactosamine chains on N-glycans of CD147.

Regulation of MMP2 expression by AZT in SGC-7901 and U251 cells. To examine the effect of AZT on the mRNA levels of MMP2, the two cell lines were treated with 0.46 mmol/l AZT for 48 h and RT-PCR analysis was conducted. As demonstrated by agarose gel electrophoresis in Fig. 4, following AZT treatment in SGC-7901 and U251 cells, MMP2 expression was almost eradicated, compared with the control group ($P<0.01$). These results indicate that AZT may also inhibit the expression of MMP2.

Cell cycle analysis. To investigate the effect of AZT treatment on cell cycle distribution in SGC-7901 cells, the cells were treated with various concentrations of AZT (0.23, 0.46, 0.69, 0.92 and 1.15 mmol/l) for 24 h and subjected to flow cytometric analysis subsequent to staining of the chromosomal DNA with PI. As demonstrated in Fig. 5, AZT treatment altered the cell cycle distribution of SGC-7901 cells. The proliferation index $[\%S+\%G_2/M)/(\%G_0/G_1+\%S+G_2/M)]$ values for G_1 phase in the AZT-treated cells was 53.949, 54.716, 57.611, 59.852, 60.799 and 65.785% for the different AZT concentrations, respectively. These results indicated that the percentage of AZT-treated cells in the G_1 phase increased and the number of cells in the S, G_2 and M phases reduced in a dose-dependent manner. This suggests that AZT may arrest the cell cycle in the G_1 phase to inhibit cell proliferation.

Discussion

It has been reported that AZT may markedly alter the profile of N-linked oligosaccharides in the K562 erythroleukemia cell line and the SKSSK-MEL-30 melanoma cell line (13). A previous study demonstrated that in these cell lines, AZT treatment markedly reduced the synthesis of highly branched complex oligosaccharides and polylactosamine chains (poly-LacNAc) (14). Poly-LacNAc contains linear LacNAc (Galβ1-4GlcNAcβ1-3) N repeats, which are formed by the addition of galactose (Gal) to terminal GlcNAc residues in the Golgi complex (16). However, it has been reported that the mechanism of AZT-mediated inhibition of poly-LacNAc synthesis is via the inhibition of UDP-GalNAc movement into the Golgi complex and blocking the addition of LacNAc repeats. In addition, AZT is also reported to block the formation of the GlcNAc-β-1,6-Man branch, a preferred initiation site for poly-LacNAc synthesis (17,18).

Alterations in N-linked glycans are associated with the progression of cancer. Of particular importance in tumor growth and invasion is the synthesis of complex N-linked oligosaccharides containing long polylactosamine chains (19). These outer branch modifications are suggested to be critical in cell-cell recognition and communication events, due to the modulation of the structural and the functional properties of carrier glycoproteins associated with cancer progression (20). Highly branched complex glycan chains, for example the polylactosamine extension, appear to be important for the oncogenic phenotype of tumor cells (21). In addition, polylactosamine side chains have been suggested to serve critical roles in the adhesive properties of tumorigenic cells (22). Saitoh *et al* (4) demonstrated that lysosomal associated membrane proteins isolated from a highly metastatic cancer cell line contained markedly more polylactosamine side chains than one with low metastatic activity. From this, it was concluded that increased quantities of these side chains are characteristic of the metastatic phenotype.

The glycoprotein HG-CD147 is a key carrier of β1,6-branched polylactosamine sugars on N-glycans in tumor cells (9). β1,3-N-acetylglucosaminyltransferases (β3GnTs) may catalyze the initiation and elongation of polylactosamine chains on O-glycans, N-glycans and glycolipids (17,23,24). A previous study identified that β3GnT8 was involved in the biosynthesis of β1,6-branched polylactosamine sugars on N-glycans *in vitro* and it may alter the glycosylation of HG-CD147 and further regulate the metastatic potential of colorectal cancer cells (25).

CD147 is composed of two domains in the extracellular region, a single transmembrane domain and a short cytoplasmic domain containing 39 amino acids. The extracellular region contains three Asn glycosylation sites; however, the glycan portion of the molecule differs depending on the source of CD147. CD147 glycosylation has been previously demonstrated to determine its ability to activate MMPs. Purified deglycosylated CD147 and LG-CD147 failed to induce MMP activity, whereas HG-CD147 effectively induced MMP activity. Since the glycosylated regions of CD147 serve an important role in MMP expression and HG-CD147 contains multiple β1,6-branched polylactosamine sugars, which are catalyzed by β3GnT8 and may regulate the metastatic potential

of colorectal cancer cells. Therefore, AZT was used in the present study to examine whether the β1,6-branched polylactosamine sugars on CD147 are important in SGC-7901 and U251 cells.

Subsequent to treatment of SGC-7901 and U251 cells with AZT, it was observed using western blot analysis that the quantity of N-glycans of HG-CD147 was reduced, compared with that in the control group ($P<0.05$; Fig. 3). This result indicated that the N-glycans of CD147 contain β1,6-branched polylactosamine sugars and may be inhibited by AZT. As HG-CD147 regulates MMP expression, AZT may additionally affect MMP expression in SGC-7901 and U251 cells. MMPs are a class of molecules that have been demonstrated to be integral to tumor progression, facilitating cell migration and invasion through their ability to degrade the extracellular matrix. As shown in Fig. 4, MMP2 expression was almost eradicated compared with the control group subsequent to treatment with AZT ($P<0.01$), it is thus suggested that AZT may prevent the expression of MMP2. In addition, the cell cycle distribution of SGC-7901 cells was altered by AZT, with a dose-dependent increase in the percentage of cells in the G_1 phase, indicating that AZT may arrest the cell cycle in G_1 phase to inhibit cell proliferation (Fig. 5). These results suggest that AZT may inhibit the biosynthesis of polylactosamine chains on CD147 and thus affect MMP2 expression and cell cycle progression in SGC-7901 and U251 tumor cells.

It has been reported that CD147 may activate several transcription factors, including activator protein 1 (AP-1) and nuclear factor-κB (NF-κB), which leads to the MMP-inducing activity of CD147 (25). For example, MMP3, 7, 9, 10, 12 and 13 contain a minimum of one AP-1 binding site in their proximal promoter region (17). MMP2, 3, 9 and 14 are also NF-κB responsive in a cell- and stimulus-specific manner (26-28). In addition, CD147 may also induce IκB kinase (IKK) activation in the PI3K/Akt-dependent signaling pathway and induce c-Jun N-terminal kinase-dependent c-Jun activation via the mitogen-activated protein kinase 7 (MKK7) signaling pathway. Thus, a previous study demonstrated that CD147 induced MMP expression predominantly via PI3K/Akt/IKK-dependent NF-κB and MKK7/JNK-dependent AP-1 signal transduction pathways (25) (Fig. 6). Therefore, it was hypothesized that the AZT-mediated inhibition of MMP2 expression by reducing CD147 N-glycans in the present study, may be via the above two pathways.

In conclusion, AZT may inhibit the biosynthesis of polylactosamine chains on CD147 in tumor cells. The glycoprotein CD147 is hypothesized to be an upstream modulator inducing MMP production in tumor cells and AZT may be effective therapeutically as an antineoplastic drug in certain types of cancer by altering the glycosylation levels of HG-CD147.

Acknowledgements

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