Novel Synthetic Mono-triazole Glycosides Induce G₀/G₁ Cell-cycle Arrest and Apoptosis in Cholangiocarcinoma Cells

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Abstract. *Background/Aim:* The treatment of cholangiocarcinoma (CCA) is still ineffective and the search for a novel treatment is needed. In this study, eight novel mono-triazole glycosides (W1-W8) were synthesized and tested for their anticancer activities in CCA cell lines. *Materials and Methods:* The anti-proliferation effect and the underlying mechanisms of the triazole glycosides were explored. Viable cells were determined using the MTT test. *Results:* Among glycosides tested, W4 and W5 exhibited the most potent anticancer activity in a dose- and time-dependent fashion. Flow cytometry and western blot analysis revealed that W4 and W5 induced G₀/G₁ phase cell-cycle arrest through down-regulation of cyclin D1, cyclin E and induction of cyclin-dependent kinase inhibitors, p27 and p21 protein expression. Annexin V/propidium iodide (PI) staining demonstrated that W4 and W5 also induced apoptotic cells in a dose-dependent manner via caspase signaling cascade. *Conclusion:* Together, these findings imply that the novel synthetic glycosides might be a promising anticancer agent for CCA.

Although there has been progress in the development of prevention and treatment of cancer, the successful treatment of cholangiocarcinoma (CCA) remains a challenge. Therefore, there is still an urgent need to search for some new and effective anticancer agents that have a broader spectrum of cytotoxicity to tumor cells.

Triazole compounds containing three nitrogen atoms in the five-membered aromatic azole ring are readily able to bind with a variety of enzymes and receptors in biological systems

via diverse non-covalent interactions and, thus, display versatile biological activities (1). The 1,2,3-triazole moiety has been a fruitful source of inspiration for medicinal chemists for many years. Their diverse inhibitory activities, including anti-fungal (2, 3), anti-bacterial (4, 5), anti-allergic (6), anti-inflammatory and others (7-9) are partially due to their synthetic accessibility by click chemistry. Besides existing compounds, triazoles can act as an important tool for medicinal chemists to develop newer compounds possessing the triazole moiety that could be better agents in terms of efficacy and safety. Molecular hybridization, which covalently combines two or more drug pharmacophores into a single molecule, is an effective tool to design highly active novel entities (10, 11). In addition, the hybrids may also minimize the unwanted side-effects and allow for synergistic action (12).

On the other hand, glycosides have existed extensively in the animals and plants and taken on important biological functions. Significant anti-bacterial and anticancer activities of glycosides have attracted many scientists to attempt to improve the biological activity of these compounds by glycosylation in order to increase their solubility in water and guidance qualities (13).

To combine the advantages of these two pharmacophores, 1,2,3-triazole and glycosides on the anti-tumor activity in CCA cells is challenging. In the present study, eight novel mono-triazole glycosides were developed using the O-glycosylation-click reactions resulting in high to excellent yields. The anticancer activities of these novel mono-triazole glycosides were investigated in CCA cell lines and the underlying cellular mechanisms were explored. These findings raise the possibility of using these triazole glycosides as candidate treatments for CCA.

Materials and Methods

Mono-triazole glycoside synthesis. 2,3-Unsaturated-glycosyl triazoles were synthesized in a simple one-pot process under mild conditions via tandem O-glycosylation using an iodine promoter and

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a mild copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction as previously described (14). Eight mono-triazole-glycoside candidates with distinct molecular structures were randomly selected for testing anti-tumor efficacy in CCA cell lines.

Cell lines, culture technique and treatments. KKK-055, KKK-100, KKK-213 and KKK-214 cell lines, established from primary tumors of CCA patients (15), were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan. The cell lines were maintained in HAM's F-12 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics/antimycotics, in a humidified atmosphere of 5% CO₂ at 37°C.

Antibodies and reagents. Antibodies against cyclin D1, cyclin E, p21, p27 and poly (ADP-ribose) polymerase-1 (PARP-1) were purchased from Santa Cruz Biotechnology (1:1,000; Santa Cruz, CA, USA). Caspase-3 and caspase-9 antibodies were obtained from Cell Signaling Technology (1:2,000; Beverly, MA, USA) and the β -actin antibody was from Sigma-Aldrich (1:40,000; St. Louis, MO, USA). MTT reagent and propidium iodide (PI) solutions were purchased from Invitrogen (Eugene, OR, USA). The Annexin V-FITC Apoptosis Detection Kit was from eBioscience (San Diego, CA, USA).

Cell viability assay. Cells (5×10³ cells/well), in a 96-well plate, were treated with various concentrations of mono-triazole glycosides. After 24 or 48 h of incubation, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells incubated with 0.1% dimethyl sulfoxide (DMSO) were used as controls. DMSO was added to resolve the formazan complex and the absorbance at 540 nm was recorded. The cytotoxicity of each glycoside was analyzed as relative viable cells compared to the vehicle control.

Cell cycle analysis. After treatment, cells were collected, washed with PBS and fixed in 70% cold ethanol for 1 h. Cells were then stained with 10 μ g/ml of PI for a few minutes and placed on ice until analysis. The analysis was performed on a BD FACSCanto II flow cytometer. The numbers of dead and live cells, as well as the numbers of cells distributed in G₀/G₁, S and G₂/M phases, were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA). Cell apoptosis was confirmed by annexin V staining. Cells were treated with vehicle or W4 or W5 for 48 h. After treatment, cells were washed and re-suspended in the staining buffer containing 1 μ g/ml PI and 0.025 μ g/ml annexin V-FITC at room temperature for 15 min in the dark. The stained cells were immediately determined using FACSCanto II and analyzed using FACSDiva software (BD Biosciences, San Jose, CA, USA).

Western blotting. Protein (20 μ g) was subjected to a 12% SDS-polyacrylamide gel electrophoresis and transferred to a Hybond™ - P PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membrane was then blocked with 3% BSA for 1 h at room temperature or overnight at 4°C, probed for 1 h at room temperature with each primary antibody and 1:20,000 horseradish peroxidase (HRP)-linked secondary antibody. The immunoactive bands were detected using an enhanced chemiluminescence prime Western blotting detection reagent kit (ECL; GE Healthcare). The same membrane was stripped-off and incubated with β -actin antibody as the loading control.

Table I. The half-maximal inhibitory concentrations of W4 and W5 on CCA cells at 48 h.

Cell line	IC ₅₀ at 48 h (μ M, MTT assay)	
	W4	W5
KKU-055	74.1	69.5
KKU-100	89.8	76.0
KKU-213	69.2	59.1
KKU-214	89.1	86.3

Statistical analysis. Results are shown as mean±SEM or mean±SD. The significant difference between mean values of the treated and untreated groups was determined by two-tailed Student *t*-tests. Statistical significance was established at *p*<0.05.

Results

W4 and W5 mono-triazole glycosides significantly reduced viability of CCA cells. The anti-proliferative effects of mono-triazole glycoside candidates (W1-W8, Figure 1A) were screened in the KKK-M213 cell line using the MTT assay. Cells were incubated with each candidate at 50, 100 and 200 μ M for 24 h. As shown in Figure 1B, W4 and W5 exhibited the most potent anti-proliferative effects compared to other candidates. These two glycosides were selected for the subsequent experiments. The anti-proliferative effects of W4 and W5 were demonstrated in 4 different CCA cell lines, KKK-055, KKK-100, KKK-M213 and KKK-M214, in concentration- (Figure 2A) and time- (Figure 2B) dependent manners. Treated CCA cells with W4 or W5 at 100 μ M for 48 h could inhibit 70-90% of cell viability in all cell lines tested.

The half-maximal inhibitory concentrations (IC₅₀) of each cell line for W4 and W5 are shown in Table I. The 48-h IC₅₀s of W4 and W5 against CCA cells were KKK-M213 <KKU-055 <KKU-100 and KKK-M214; however, there were no significant differences.

W4 and W5 glycosides induced G₀/G₁ cell-cycle arrest of CCA cells. To examine whether the growth inhibition induced by W4 and W5 glycosides was associated with regulation of the cell cycle, the cell-cycle distribution of CCA cells in the presence of W4 and W5 was analyzed by flow cytometry. As shown in Figure 3Aa-b, treatments of KKK-100 cells with W4 or W5 at 25 and 50 μ M for 48 h resulted in a significant accumulation of cells in the G₀/G₁ phase. At higher concentrations, 75 and 100 μ M, the subG₁ population was greatly induced with reduced numbers of G₀/G₁ cells. A similar observation was obtained for KKK-

A **B**

Figure 1. Anti-proliferative effects of 8 mono-triazole glycosides. (A) Chemical structure of W1-W8. (B) KKU-M213 cells were treated with 50, 100 and 200 μ M of W1-W8 glycosides for 24 h. The MTT assay was performed and the viable cells were calculated given the control as 100%. Data are expressed as the mean $\pm$ SEM of triplicate assays of three independent experiments. * p <0.01, ** p <0.001.

213 cells treated with W4 and W5 (Figure 3Ac-d). Accumulations of G₀/G₁ populations were observed with 25 and 50 μ M of both glycosides, while the subG₁ population was obviously increased at the 100 μ M treatment.

The effects of W4 and W5 on the regulation of the cell cycle were further determined. Cell lysates of KKU-100 and KKU-213 cells treated with W4 and W5 at 50 and 75 μ M for

48 h were analyzed for G₁ phase regulatory proteins, including cyclin D1, cyclin E, p21 and p27 using Western blot analysis. As shown in Figure 3B, W4 and W5 glycosides markedly down-regulated the protein levels of cyclin D1 and cyclin E but up-regulated the expressions of cell cycle inhibitors, p21 and p27, in a concentration-dependent manner in both cell lines.

Figure 2. Growth inhibitory effect of W4 and W5 glycosides on CCA cells. Four CCA cell lines, KKU-055, KKU-100, KKU-213 and KKU-214 were: (A) incubated in the presence of various concentrations of W4 or W5 for 48 h; (B) incubated in the presence of 100 μM W4 or W5 for 24 and 48 h. Cells treated with 0.1% DMSO were used as controls. The viable cells were determined using the MTT assay. Data are expressed as mean±SEM of triplicate assays of two independent experiments.

W4 and W5 induced cellular apoptosis of CCA cells. The capability of W4 and W5 glycosides on cell apoptosis was investigated next. Annexin V/PI double-labeling was used for the detection of phosphatidylserine externalization, a hallmark of apoptosis. As shown in Figure 4A, cells treated with W4 or W5 at 50 and 75 μM could induce apoptotic cells in both cell lines in a dose-dependent manner. The average proportions of apoptotic cells were significantly increased to approximately 45% in W4 and W5-treated KKU-100 cells (Figure 4Aa-b). Similar results were observed in KKU-213 cells with approximately 65% apoptotic cells induced when cells were treated with 75 μM of W4 or W5 (Figure 4Ac-d).

In order to investigate the mechanism by which W4 and W5 induces apoptosis, the apoptosis-related proteins were examined in KKU-100 and KKU-213 cells treated with 50 and 75 μM of W4 and W5 for 48 h. The western blot

analysis showed that W4 and W5 activated proteolytic cleavage of inactive pro-caspase-3 and -9 into active caspase-3 and -9 and induced cleaved PARP-1 protein expression in a concentration-dependent manner (Figure 4B).

Discussion

CCA is a cancer that is highly resistant to various anticancer drugs and, thereby, leads to poor prognosis (16-18). At present, there is no curative medical therapy recommended for CCA. The most studied chemotherapeutic drugs are 5-FU and gemcitabine that have been tested in combinations with a variety of anticancer drugs (19), some of which are highly toxic to normal tissues (20-23). A strategy to diminish the toxic effects of the chemotherapeutic agents is the co-treatment of the chemotherapeutic agents with anti-proliferation remedies. Numerous studies have shown that

Figure 3. Effect of W4 and W5 on cell cycle distribution and cell cycle regulatory proteins. (A) CCA cells, KKU-100 (a, b) and KKU-213 (c, d) were treated with 25, 50 and 100 μ M of W4 or W5 for 48 h. Cycle distribution was analyzed using flow cytometry. (B) CCA cells were treated with 50 and 75 μ M of W4 or W5 for 48 h and the expression of cell cycle regulatory proteins were determined using Western blotting. Cells treated with 0.1% DMSO were used as controls. Data are one representative of 2 independent experiments.

Figure 4. W4 and W5 induced cellular apoptosis. (A) CCA cells were treated with or without 50 and 75 μ M of W4 or W5 for 48 h and apoptotic cells were determined using flow cytometry with annexin V/PI staining. The quantitative analyses of apoptotic cells are shown in bar graphs. Data are expressed as mean $\pm$ SD of 3 independent experiments. (B) The expressions of apoptosis-associated proteins, total and cleaved forms of caspase-3, caspase-9 and PARP-1, were determined using western blot. Data are one representative from two independent experiments. * $p < 0.01$, ** $p < 0.001$.

naturally-occurring glycosides and their synthetic derivatives may serve as lead candidates in the development of cancer treatment, either as a single agent or in combination with the existing chemotherapeutic drugs (24-26).

In the current study, 8 novel-triazole glycosides were synthesized and examined for anti-tumor activity against CCA cells. The glycosides exhibited differential anti-proliferative activity on CCA cells, from mild to strong. The glycosides synthesized from long-chain aliphatic azides, W4, W5 and W6, effectively inhibited cell proliferation of CCA cells. On the other hand, the glycosides synthesized from phenyl ethyl azide (W7 and W8), benzyl azide (W3) and sulfonyl azide (W1, W2) showed only weak to no anti-proliferative activity against CCA cells. Increasing the length of the carbon chain should alter the polarity and lipophilicity of the compounds, thus allowing these synthetic compounds to pass through the cell membrane by passive diffusion and may, as a result, affect their cytotoxic activity. The presence of a long-chain aliphatic compounds in the structure of palladium complexes has previously been reported to enhance the higher efficiency of DNA-binding that resulted in significantly enhancing the cytotoxic activity and the apoptotic activity on HL-60 cells (27). Up to the present search, this is the first report on the effect of 1,2,3-triazole glycoside on cancer cell proliferation, cell cycle distribution and apoptosis.

The results showed that W4 and W5, the glycosides synthesized from a long-chain aliphatic azide, effectively inhibited cell proliferation of CCA cells. These 2 best glycosides were selected for further investigations. The IC_{50} value of W4 against 4 CCA cell lines was 69.2-89.8 μ M and of W5 was 59.1-86.3 μ M. It seems to be common for the synthetic glycosides to exhibit anti-tumor efficacy at the micromolar range but the exact values are largely due to the type of glycosides and the type of cancer cell line studied. For instance, a series of solasodine glycosides were synthesized and the 3 most potent compounds in the series significantly inhibited cancer cell lines of breast (MCF-7), prostate (PC-3) and a chronic myelogenous leukemia (K562), with the IC_{50} values ranging from 7.2 to 18.8 μ M (26). Paeoniflorin, a monoterpene glucoside, however, at a concentration of 200 μ M could achieve only about 25% inhibition against hepatoma cell lines, HepG2 and Bel-7402 (28). In addition, the study of synthetic phenylethanoid glycosides on cell proliferation of PC-3 cells showed that 30 μ M acteoside had 23%-30% inhibition, whereas echinacoside, calceolarioside-A and calceolarioside-B induced only 10-15% inhibition (29).

Cancer development is often due to perturbations in the cell cycle that leads to unlimited proliferation and confers apoptosis resistance (30). The progression of cells through the cell cycle is exerted by cyclin, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs). CDK4/6-Cyclin D and CDK2-Cyclin E work in concert to relieve inhibition of a dynamic transcription complex of the

retinoblastoma protein and E2 factor (E2F), whereas inhibition of the kinase activity of cyclin/CDK complex is mediated by several CKIs, including p21^{waf1/cip1} and p27^{kip1} (31). In the current study, treatment with 25 and 50 μ M of W4 and W5 arrested CCA cells at the G₁ phase and was accompanied by decreased S phase cell populations. These observations were further confirmed by Western blot analyses of cell cycle regulatory proteins. As expected, the crucial G1 cyclins, cyclin D and cyclin E proteins, were markedly suppressed, while the levels of cell cycle inhibitors, p21 and p27, were strongly enhanced by the glycoside treatments.

Defective apoptosis represents a major causative factor in the development and progression of cancer. Indeed, the majority of chemotherapeutic agents act through the apoptotic pathway to induce cancer cell death. Moreover, resistance to chemotherapeutic strategies seems to be due to alterations in the apoptotic pathway of cancer cells (32). Flow cytometry analysis of annexin V/PI staining demonstrated that W4 and W5 induced CCA cell apoptosis in a dose-dependent manner. Caspases are a family of endoproteases that provide critical links in cell regulatory networks controlling inflammation and cell death (33). Initiator caspases (-8 and -9) activate executioner caspases (-3, -6 and -7) that subsequently coordinate their activities to demolish key structural proteins and activate other enzymes contributing to apoptotic cell death. Another characteristic event of apoptosis is the proteolytic cleavage of PARP-1, a nuclear enzyme involved in DNA repair, DNA stability and transcriptional regulation (34). Treating CCA cells with W4 and W5 resulted in the activation of the caspase cascade as shown by an increase in the cleaved forms of caspase-3 and caspase-9 and, finally, facilitated the PARP-1 cleavage.

1,2,3-triazole-glycosides have been reported to possess an inhibitory effect on galectin-3 (35, 36), which was previously reported to be overexpressed in tumor tissues of CCA patients (37). Suppression of galectin-3 expression in CCA cell lines by siRNA enhanced apoptosis and sensitivity to chemotherapeutic drugs (38). In the present study, treatment of W4 and W5 effectively inhibited cell proliferation of CCA cells in a dose- and time-dependent fashion. These glycosides probably react with galectin-3 and enhanced apoptosis of CCA cells. This assumption, however, needs future investigation on the inhibition and binding of W4 and W5 on galectin-3. In addition, co-treatment of these glycosides and chemotherapeutic drugs in CCA cells should also be taken into consideration.

In conclusion, 8 novel mono-triazole glycosides were synthesized and evaluated for their anticancer activity against CCA cell lines. W4 and W5 exhibited the most potent anti-proliferative activity. These glycosides were further shown to inhibit the growth of CCA cells by arresting the cell cycle at G₁ phase, thus inducing apoptosis. Further studies on the

efficacy and safety in an animal model are needed to propose these compounds as candidates for a supplemental or alternative therapeutic approach of CCA.

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