Abstract. Background/Aim: Polycomb repressive complex 2 (PRC2), an epigenetic master regulator, contributes to progression and development of biliary tract cancer (BTC). The present study investigated the effects of the PRC2 inhibitor 3-deazaneplanocin A (DZNep) on BTC cell lines. Materials and Methods: In vitro effects of DZNep treatment were analyzed for cell viability, gene expression and functional characteristics of cancer stem cell (CSC). Results: DZNep treatment caused a cell line- and dose-dependent decrease in viability. In the EGI-1 cell line, a direct cytotoxic effect was accompanied by mRNA down-regulation of the PRC2 core components, cyclins as well as of CSC-related genes. Furthermore, DZNep affected putative CSCs by reduction of sphere formation and aldehyde dehydrogenase-1-positive cells. The stem cell characteristics of these sub-populations were verified by real-time polymerase chain reaction analysis. Conclusion: Taken together, our results show that DZNep might be a promising pharmacological agent for future therapies regarding BTC.

Biliary tract cancer (BTC) is a heterogeneous malignancy originating from transformed epithelial cells of the bile duct system. BTC is frequently incurable due to advanced stage at the time of diagnosis, high recurrence rates and limited therapeutic options (1-3); for patients with advanced BTC, the median overall survival is about 12 months under standard chemotherapy with cisplatin and gemcitabine (4, 5).

Epigenetic alterations have been associated with the development of BTC (6). The polycomb repressive complex 2 (PRC2) mediates gene silencing via tri-methylation of histone 3 on lysine 27 (H3K27me3). It consists of the three core components embryonic ectoderm development (EED), SUZ12 polycomb repressive complex 2 subunit (SUZ12) and enhancer of zeste homolog 2 (EZH2), the latter harboring methyltransferase activity of PRC2 (7). Abnormal PRC2 activity contributes to development and progression of BTC and is associated with poor clinical outcome (8). Furthermore, EZH2 activity was related to maintenance of a stem cell-like phenotype (9, 10). Cancer stem cells (CSCs) play a major role in cancer recurrence and chemoresistance, the latter probably caused by high expression of drug efflux pumps (11, 12). For BTC, there is strong evidence for involvement of CSCs as shown in several publications (13-15). Various studies looked at the effect of the PRC2 inhibitor 3-deazaneplanocin A (DZNep) in different types of cancer (9, 16-18). However, only one publication exists that discusses the impact of DZNep on BTC cells (19) and to our knowledge, no data are available describing the effect of DZNep on CSCs in BTC.

The aim of the present study was to investigate the effect of DZNep on cell viability using eight different BTC cell lines. Additionally, we studied changes in gene expression...
associated with DZNep treatment and furthermore analyzed the effect of DZNep on reducing subpopulations of BTC cells with functional stem cell characteristics.

Materials and Methods

Substances and cell culture. DZNep was obtained from Sigma Aldrich (Vienna, Austria) dissolved in water (stock concentration 5 mM) and stored in aliquots at −20°C. Resazurin was purchased from Sigma Aldrich, dissolved in Dulbecco’s Phosphate Buffered Saline (DPBS: Sigma Aldrich) and sterilely filtrated. Five bile duct carcinoma cell lines CCSW-1 [grade (G)2 (20)], BDC [G4 (21)], EGI-1 [G3 (22)], SkChA-1 [G3 (23)], TFK-1 [G2 (24)] and three gallbladder cancer cell lines MzChA-1 [G1 (23)], MzChA-2 [G2 (23)] and GBC [G1 (25)] were cultured using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria) as described elsewhere (26, 27) and are together termed as BTC cell lines (28). Cells were seeded at the following densities: BDC, MzChA-2: 3.95×10⁴/cm²; CCSW-1, GBC: 4.74×10⁴/cm²; SkChA-1: 5.53×10⁴/cm²; EGI-1, TFK-1: 6.32×10⁴/cm²; and MzChA-1: 7.11×10⁴/cm² of the culture receptacle in DMEM with 10% FBS. Cells were grown overnight in 35 mm cell culture dishes in sfDMEM and incubated in the presence or absence of 10 μM DZNep in DMEM for 14 days. Anchorage-independent growth was monitored with an inverted phase-contrast microscope (Motic AE31; Nikon Instruments, Melville, NY, USA) equipped with a CCD-1300B digital camera (Allied Vision Technologies/VDS Vosskühler, Stadtho, Germany), quantified using the CyQuant GR Dye (Cell Biolabs) on the Infinite M200 microplate reader and related to that of untreated (DMEM only) samples.

Drug cytotoxicity. Dose-, cell line- and time-dependent cytotoxic effects of DZNep were assayed on cells grown in 96-well microplates. For quantification of cell viability, the resazurin assay and an Infinite 200 Microplate Reader (Cell Biolabs, San Diego, CA, USA) were used as described previously (27, 29). For dose- and cell line-dependent cytotoxic effects, a dilution series of DZNep (0.04-20.00 μM) was added to the cells in sfDMEM for 72 h. Viability was related to that of untreated control samples (sfDMEM only). For time-course experiments, 10 μM DZNep was added to the cells in sfDMEM and viability was assayed at 0, 24, 48 and 72 h post-incubation with DZNep. Viability was related to that at the initial (0 h) time point.

Gene expression analysis. Cells were grown overnight in 35 mm cell culture dishes in sfDMEM and treated with 10 μM DZNep for 72 h. RNA was isolated with TRIzol Reagent (Ambion/Life Technologies, Vienna, Austria) using the Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). cDNA synthesis was performed with 1 μg of isolated total RNA using the ImProm-I Reverse Transcriptase system (Promega, Mannheim, Germany) according to the manufacturer’s protocol. For cDNA synthesis of EGI-1 spheres as well as aldehyde dehydrogenase-1 (ALDH)-positive and ALDH-negative cells, 0.1 μg total RNA was introduced for cDNA synthesis. Quantification of cDNA of cell cycle-related genes (CCNA2, CCNB1, Ki67), PRC2 core components (EED, EZH2, SUZ12) and stemness genes (ABCG2, CD133, CD24, CD44, C-MYC, EPCAM, HOTAIR, NANOG, OCT3/4 and SOX2) was performed by quantitative real-time reverse transcription polymerase chain reaction using the GoTaq qPCR Master Mix (Promega) and a ViiA7 real-time PCR system (Applied Biosystems/Life Technologies) using oligonucleotide primers (purchased from Sigma Aldrich; primer sequences are available from the corresponding author on request). All samples were measured in biological triplicates. Melting curve analysis was performed for all primer pairs to verify the specificity of PCR products. Samples were normalized to data for β-actin and data were analyzed according to the ΔΔCt method (30).

Expression of PRC2 core components in BTC cell lines. In a first step, we measured the expression of the three PRC2 core components EED, EZH2 and SUZ12 at the mRNA level in eight BTC cell lines using RT-qPCR. All tested cell lines expressed all three core components to a differing extent (Figure 1A).

DZNep reduces overall cell viability in a cell line- and dose-dependent manner. Next, we tested the effect of different concentrations of DZNep on the overall cell viability in all eight cell lines. DZNep reduced the viability in a cell line-and dose-dependent manner, ranging from 10% to 50% at the highest concentration (Figure 1B). We observed highly significant reduction of cell viability at low concentrations (0.16 μM) for five cell lines (BDC, EGI-1, GBC, SkChA-1 and TFK-1) and at higher concentrations (5 μM) for all eight cell lines.

Correlation analysis showed no correlation within the relative expression values of the PRC2 components (data not shown). Interestingly, the expression of EZH2 significantly positively correlated with viability after DZNep treatment at 2.5-20 μM; on the contrary, there was a negative trend for the association between EED and SUZ12 expression and the
viability after DZNep treatment (all concentrations, 0.039-20 μM; data not shown).

In one cell line (EGI-1), DZNep had a significant direct cytotoxic effect in a 72 h time course (Figure 1C), whereas in the other seven cell lines it slowed down-proliferation without causing direct cell death (data not shown). Therefore, we chose this cell line (EGI-1) for subsequent experiments.

DZNep reduces mRNA expression of PRC2 components, cyclins and stem cell markers. To address the effect of DZNep treatment on gene expression in EGI-1 cells, we measured mRNA levels after 72 h of DZNep treatment compared to untreated cells. All three PRC2 core components, EED, EZH2 and SUZ12, showed a significant decrease in mRNA levels to about 40-50% that of the controls. In addition, we observed significantly reduced expression levels of CCNA2, CCNB1 and KI67. Interestingly, mRNA levels for EPCAM \((p=0.009)\) and HOTAIR \((p=0.01)\) were also significantly reduced, the latter being a long non-coding RNA that has been recently linked to CSCs (31). The surface markers CD24 and CD133 showed a nonsignificant decrease in mRNA levels after DZNep treatment (Figure 2).

**DZNep affects stem cell properties in BTC cell line.** To test the hypothesis whether DZNep affects the CSC subpopulation as suggested by the mRNA expression results, we tested the effect of DZNep on established functional stem cell

Figure 1. Polycomb repressive complex 2 (PRC2) components expression and cytotoxic effects of 3-Deazaneplanocin A (DZNep) in biliary tract cancer (BTC) cell lines. A: mRNA expression of PRC2 core components embryonic ectoderm development (EED), enhancer of zeste homolog 2 (EZH2) and SUZ12 polycomb repressive complex 2 subunit (SUZ12) were measured in eight BTC cell lines using quantitative real-time reverse transcription polymerase chain reaction. Data were normalized to expression of β-actin. B: Dose-dependent effect of 72-h DZNep treatment on BTC cell lines assessed as overall viability related to untreated control. C: Time-dependent effect of treatment in EGI-1 cells with 10 μM DZNep after 0 h, 24 h, 48 h and 72 h, respectively. All data are related to the initial time point, i.e. start of incubation.
Figure 3. 3-Deazaneplanocin A (DZNep) impairs biliary tract cancer stem cell properties in vitro. A: Reduction of aldehyde dehydrogenase-1 positive (ALDH+) sub-population in EGI-1 cells after treatment with 20 μM for 72 h. B: Reduction of sphere formation of EGI-1 cells after two weeks growth in a semisolid agar in presence of 10 μM DZNep using the CyQuant GR Dye. C: Reduction of sphere formation of EGI-1 cells after 72 h pre-treatment with 20 μM DZNep using the CyQuant GR Dye. Panels on the left show representative flow cytometric (A) or microscopic data (B, C), respectively. rfu: Relative fluorescence units.
Figure 4. Characterization of putative cancer stem cells susceptible to 3-deazaneplanocin A (DZNep) in the EGI-1 biliary tract cancer cell line. Expression of cell cycle-related genes (A), polycomb repressive complex 2 (PRC2) core components (B) and stem cell-related genes (C) in EGI-1 aldehyde dehydrogenase-1 positive (ALDH+) cells and EGI-1 spheres by quantitative real-time polymerase chain reaction. All data were normalized to those for β-actin. Expression in ALDH+ cells were related to that of ALDH− cells and sphere-derived expression values were related to those of anchorage-dependent growing EGI-1 cells (parental cell line), respectively. Expression data are presented as raw data of the individual experiments (left) and box charts including mean (open square), median (horizontal line), standard error of mean (boxes) and maximum values (whisker). ABCG2: ATP-binding cassette sub-family G member 2; C-MYC: v-myc avian myelocytomatosis viral oncogene homolog; CCNA2: cyclin A2; CCNB1: cyclin B1; EED: embryonic ectoderm development; EPCAM: epithelial cell adhesion molecule; EZH2: enhancer of zeste homolog 2; HOTAIR: HOX transcript antisense RNA; NANOG: Nanog homeobox; OCT3/4: POU class 5 homeobox; SOX2: sex-determining region Y-box2; SUZ12: SUZ12 polycomb repressive complex 2 subunit.
properties. Increased ALDH activity was functionally linked to (cancer) stem cell properties in several studies, including BTC (32-34). Therefore, we analyzed EGI-1 cells for the proportion of an ALDH+ subpopulation and examined the effect of DZNep treatment on these putative CSCs. As shown in Figure 3A, we observed a clear trend of DZNep treatment for reducing the ALDH+ subpopulation by about 12%.

A second, independent assay for putative CSCs is anchorage-independent growth (35). To test possible effects of DZNep on this functional characteristic, EGI-1 cells were cultured in semisolid agar in the presence of DZNep for two weeks. Spheres became visible in wells without DZNep after one week and continued to grow within the two weeks’ time frame. As indicated in Figure 3B, quantitative analysis of sphere-formation capacity by fluorescence measurement revealed a significant reduction (by 80%) after 14 days of treatment with 10 μM DZNep. This effect was also seen by microscopy, where DZNep almost completely inhibited sphere formation (Figure 3B).

**DZNep irreversibly impairs CSC traits in BTC cells.** To assess whether DZNep impairs CSC properties (anchorage-independent growth) transiently or irreversibly, we treated EGI-1 cells for 72 h with 20 μM DZNep, removed the drug and reseeded the remaining viable cells in semi-solid agar without DZNep for an additional two weeks. This pre-treatment of cells with DZNep significantly reduced their ability to form spheres compared to untreated cells (Figure 3C), indicating that the effects of DZNep treatment on sphere formation were stable and irreversible throughout the experiment.

**Characterization of potential CSC subpopulations.** To verify the stem-cell characteristics of ALDH+, or sphere-forming, sub-populations, we compared the stemness gene signature between FACS-sorted ALDH+ cells, as well as cells isolated from anchorage-independent growth experiments (i.e. spheres) with ALDH+ cells or the parental cell line, respectively. As summarized in Table I (see Figure 4 for detailed results), putative ALDH+ CSCs exhibited enhanced expression of PRC2 components (significant for EED), proliferation markers and numerous stemness factors (significant for CD44) compared to ALDH+ cells. Cells derived from spheres exhibited (borderline) significantly elevated mRNAs of several stemness factors including ABCG2, C-MYC and OCT3/4 (Figure 4).

**Discussion**

BTC is a challenging malignancy with dismal prognosis at advanced stages due to the lack of efficient therapeutic strategies. In the current study, we show that DZNep reduces overall cell viability of BTC cell lines and, furthermore, impairs different CSC properties in vitro.

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**Table I. Summary of mRNA expression levels in putative cancer stem cell subpopulations (EGI-1 cells), based on Figure 4.**

<table>
<thead>
<tr>
<th>Sub-population†</th>
<th>ALDH+ cells</th>
<th>Sphere-derived</th>
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<tr>
<td>PRC2 components</td>
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<td>EED</td>
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<tr>
<td>EZH2</td>
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<td>HOTAIR</td>
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<tr>
<td>Proliferation markers</td>
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<tr>
<td>CCNA2</td>
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<tr>
<td>CCNB1</td>
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<td>KI67</td>
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<td>↓</td>
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<tr>
<td>Stemness factors and markers</td>
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<td>ABCG2</td>
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<tr>
<td>CD44</td>
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<td>C-MYC</td>
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<tr>
<td>EPCAM</td>
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<td>→</td>
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<td>NANOG</td>
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<td>↑*</td>
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<tr>
<td>OCT3/4</td>
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<td>↑*</td>
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<tr>
<td>SOX2</td>
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†Arrows (↑, →, ↓) stand for increased, unaltered or reduced expression compared to the parental cell line, respectively; *p<0.05, **p<0.01. n.q. Not quantifiable. ABCG2: ATP-binding cassette sub-family G member 2; C-MYC: v-myc avian myelocytomatosis viral oncogene homolog; CCNA2: cyclin A2; CCNB1: cyclin B1; CD24: cd24 molecule; CD44: cd44 molecule; EED: embryonic ectoderm development; EPCAM: epithelial cell adhesion molecule; EZH2: enhancer of zeste homolog 2; HOTAIR: HOX transcript antisense RNA; KI67: marker of proliferation Ki-67; NANOG: Nanog homeobox; OCT3/4: POU class 5 homeobox; SOX2: sex-determining region Y-box2; SUZ12: SUZ12 polycomb repressive complex 2 subunit.

We demonstrate here the expression of the three PRC2 core components EED, EZH2 and SUZ12 in a set of eight different BTC cell lines to a varying extent. DZNep significantly reduced cell viability in a cell line- and dose-dependent manner at doses ≥5 μM for all BTC cell lines. This is in line with a study carried out by Nakagawa et al., in which they showed an inhibitory effect of DZNep on BTC cell lines (19). However, in contrast to our results, they did not observe apoptosis induction following DZNep treatment. Interestingly, they observed enhanced effects of DZNep on proliferation inhibition and apoptosis when combined with standard chemotherapeutic gemcitabine (19).

In our study, DZNep reduced mRNA levels of all three PRC2 core components to about 40-50% that of untreated cells. Furthermore, we found that DZNep also reduced the two cyclins CCNA2 and CCNB1, indicating that DZNep causes cell cycle arrest at G2/M which was already demonstrated for other tumor types (19, 36). It is known that the cell-cycle inhibitor p16INK4a is a target of PRC2-mediated
gene silencing (7, 37, 38). Although several previous studies showed that DZNep treatment induced p16\textsuperscript{INK4a} expression (19, 39), we were not able to detect p16\textsuperscript{INK4a} mRNA levels after DZNep treatment in the tested EGI-1 cell line (data not shown). This might be due to a mutation or deletion of the \textit{INK4A} gene locus in this particular cell line. Another explanation could be that repression of the \textit{INK4A} locus is cooperatively carried out by the PRC2 and the PRC1 complex (37, 38) and that PRC2 inhibition alone may not be sufficient and has to be combined with inhibition of PRC1 in such tumor cells. Interestingly, Choudhury et al. showed that DZNep-induced reduction of H3K27me3 was partially reversed by vector-mediated maintenance of the PRC1 component BMI1 Polycomb Ring Finger Oncogene (BMI1) (17), which underlies the cooperation between these two epigenetic complexes – again suggesting that inhibition of both PRC1 and PRC2 might be an efficient strategy.

Additionally, our data show a negative effect of DZNep on the expression of \textit{CD24}, \textit{CD133} and \textit{EPCAM}, which are established stem cell markers in BTC and have been linked to higher aggressiveness of these tumors (13, 14, 40). Interestingly, we also recognized a significant down-regulation of the long non-coding RNA \textit{HOTAIR} after DZNep treatment. \textit{HOTAIR} acts as an interface between the PRC2 and the DNA and guides the protein complex to its target genes for subsequent H3K27me3-mediated silencing (9). Increased \textit{HOTAIR} expression was found in primary breast cancer samples associated with an increased metastatic potential (41) as well as in a colon CSC subpopulation (31). In light of these data, our findings suggest for the first time a possible effect of DZNep on putative CSC in BTC.

To further investigate this possibility, we analyzed the effect of DZNep on functional CSC characteristics in BTC cells. Increased ALDH activity was linked with stem cell properties and heightened expression of stem cell markers for e.g. breast (32) and nasopharyngeal (33) cancer. Here we show that the ALDH\textsuperscript{+} sub-population of EGI-1 cells not only has higher expression levels of the three PRC2 core components, but also of the stem cell markers \textit{CD24}, \textit{CD44}, \textit{C-MYC}, \textit{EPCAM}, \textit{NANOG} and \textit{OCT3/4} and that DZNep reduced this potential CSC sub-population. This is in line with a recent study by Shuang et al. which confirmed for another BTC cell line (TFK-1) that increased ALDH positivity is associated with a sub-population characterized by higher tumorigenic potential \textit{in vitro} and \textit{in vivo} (34). Additionally, this study investigated the correlation between ALDH expression and overall survival in patients with intrahepatic and extrahepatic cholangiocarcinoma. For both BTC types, the high ALDH-expressing group displayed lower overall survival. Together with our results, these data not only strengthen ALDH\textsuperscript{+} cells as one possible sub-population with CSC properties, but also underline their role in the aggressiveness of BTC. The effect of DZNep on anchoragem

independent growth, another stem cell characteristic, was more pronounced in our study: DZNep almost completely prevented sphere formation of EGI-1 cells during a 14-day incubation period. This result is underlined by published data demonstrating a similar effect of DZNep on sphere formation for various other cancer types (18, 36). Interestingly, pre-treatment with DZNep and subsequent sphere-forming assay yielded a similar and significant reduction of sphere formation. This indicates that treatment with DZNep causes stable and irreversible inhibition of this CSC property.

Characterization of cells isolated from EGI-1 spheres revealed an enhanced expression of \textit{CD24}, \textit{C-MYC}, \textit{HOTAIR}, \textit{NANOG}, \textit{OCT3/4} and \textit{SOX2}, a set of established stem cell markers or stemness factors (13, 31, 40, 42, 43) – thus firming-up the proposed stem cell status of these tumor cells. Additionally, spheres significantly over-expressed the drug efflux protein \textit{ABCG2}, a plasma membrane transporter which is implicated in cancer (multi-)drug resistance and thus related to CSCs (12, 44). Although the differences between putative CSC cells isolated by sphere formation and ALDH staining suggest the existence of probably functionally different CSC subpopulations, the effects of DZNep on both of these cell phenotypes underline its relevance as a potential CSC-targeting drug approach in BTC.

Since DZNep might not exclusively reduce H3K27me3, but may also lead to global decrease of histone methylation (45), several alternative and selective EZH2 inhibitors with a strong cytotoxic effect in lymphatic malignancy such as lymphoma (46, 47), as well as mesenchymal tumors such as rhabdoid tumor cells (48), have been developed. Subsequent studies need to clarify whether these small molecular inhibitors exert similar effects on BTC cell lines.

Taken together, we demonstrate that treatment with DZNep might be promising for targeting CSC in BTC. Further investigation of this agent, e.g. in combination with conventional tumor treatments both \textit{in vitro} and \textit{in vivo}, may provide novel and effective therapeutic approaches for future clinical trials.

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