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Impact of Methadone on Cisplatin Treatment of Bladder Cancer Cells

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Abstract. *Background: Cisplatin-based chemotherapy is the treatment of choice for advanced bladder cancer. Since many tumor cells show inherent or acquired cisplatin resistance, research is needed to improve the therapeutic efficacy. Since the analgesic methadone is discussed as being a sensitizer for chemotherapy, we tested its effects on the cisplatin treatment of bladder cancer cells. Materials and Methods: T24 and HT-1376 bladder cancer cells were incubated with cisplatin in combination with methadone. Cytotoxicity was examined using the WST-1 viability assay and induction of apoptosis was analyzed via phase-contrast microscopy, flow cytometry, and western blot analysis. Results: Methadone was shown to enhance the cytotoxic effects of cisplatin on T24 cells based on the induction of apoptosis. In contrast, HT-1376 cells were identified as non-responders to methadone. Conclusion: Methadone could act as a chemosensitizer in the future treatment of advanced bladder cancer. Further research is needed to identify the underlying molecular mechanisms.*

Bladder cancer is the ninth-most common cancer and the 13th most common cause for cancer-related deaths worldwide, particularly in most developed countries (1). For patients with advanced bladder cancer, cisplatin-based combination therapy is the treatment of choice. Cisplatin is a platinum-based chemotherapeutic agent, which forms inter- and intra-strand complexes with DNA (2). As a consequence, cell division is impaired, DNA repair mechanisms are activated and apoptosis is induced when DNA repair fails (3). The main problem in the treatment of bladder cancer is an inherent or acquired resistance of bladder cancer cells to cisplatin, which counteracts its efficacy (4). Therefore, further research is required to explore the mechanisms of molecular resistance and to improve the therapeutic outcome (5, 6).

Methadone is a synthetic opioid which is usually used as a racemic mixture (D,L-methadone) for pain management. It can be applied as a first- or second-line analgesic in patients with cancer pain, especially when effects of other opioids decreased during first-line treatment or when severe side-effects have appeared (7-9). Although the analgesic effects of methadone can vary widely from patient to patient due to intra-individual metabolism and potential drug interactions, the drug combines many advantages, such as oral administration, high bioavailability, rapid analgesic impact, and lack of active metabolites (10).

In preclinical studies with small cell lung carcinoma, leukemia, and glioblastoma cells, methadone was shown to enhance the action of chemotherapeutic agents and is, therefore, discussed as being a sensitizer for chemotherapy (11-15). In the present study, we examined the effects of methadone on cisplatin treatment of the urinary bladder carcinoma cell lines T24 and HT-1376 in view of cytotoxicity and induction of apoptosis.

Materials and Methods

Cell lines and reagents. Human urinary bladder carcinoma cell lines T24 (transitional cell carcinoma) and HT-1376 (grade 3, carcinoma) were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle medium (Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and penicillin/streptomycin (100 U/ml, 100 mg/l) at 37˚C and 5% CO_2. Verification of the cell lines was performed by short tandem repeat analysis (CLS GmbH, Eppelheim, Germany). D,L-Methadone hydrochloride and cisplatin (Hoelzel Biotech, Cologne, Germany) were freshly prepared for each experiment as 1 mg/ml solutions in ultra-pure water. The inducer of necrotic cell death, Triton X-100, was purchased from Sigma-Aldrich (Taufkirchen, Germany). The

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apoptosis inducer camptothecin (Bio-Techne GmbH, Wiesbaden, Germany) was solved in dimethylsulfoxide at a concentration of 10 mM and stored at −20˚C. The pan-caspase inhibitor (3S)-5-(2,6-difluorophenoxy)-3-[[(2S)-3-methyl-2-(quinoline-2-carbonylamino)butanoyl]amino]-4-oxopentanoic acid (QVD-OPh hydrate, Sigma-Aldrich) was stored as a 25 mM solution in dimethylsulfoxide at 4˚C.

WST-1 viability assay. A total of 5×10^3 cells were seeded in a 96-well plate and treated with methadone alone, cisplatin alone or a combination of both substances at different concentrations for 72 h. QVD-OPh hydrate was added at a final concentration of 25 μM. Cytotoxicity was determined *via* WST-1 viability assay (Roche Diagnostics, Indianapolis, IN, USA) as described previously (16). The statistical significance of the combination therapy was analyzed by unpaired *t*-test with Welch's correlation (GraphPad Prism 6; GraphPad Software Inc., San Diego, CA, USA).

Phase-contrast microscopy. Morphology of the bladder cancer cells was monitored before and after methadone and cisplatin treatment *via* phase-contrast microscopy. For this, 5×10^3 cells were grown in 96-well plates overnight and incubated with 0.63 μg/ml cisplatin alone, 10 μg/ml methadone alone or with a combination of both agents, supplemented with or without 25 μM QVD-OPh hydrate, for 72 h. Phase-contrast images were recorded with help of a Zeiss AxioObserver Z.1 inverted microscope (Carl Zeiss Microscopy GmbH, Munich, Germany).

Flow cytometry. For the detection of apoptosis, 1.5×10^5 T24 cells were cultured in 6-well plates and incubated with 0.63 μg/ml cisplatin alone, 10 μg/ml methadone alone or in combination of both substances for 72 h. Camptothecin (75 μM) as a positive control for apoptosis or 15% Triton X as a positive control for necrosis were added to the cells for 24 h or for 1 min, respectively. Afterwards, the supernatant and the trypsinized cells were pooled, washed with phosphate-buffered saline (PBS), and centrifuged at 470 × *g* for 3 min. Cell pellets were then resuspended in 100 μl PBS and transferred into 96-well plates, washed twice with PBS, centrifuged at 680 × *g* for 3 min, and incubated with Annexin V (eBioscience Annexin V-FITC Apoptosis Detection Kit; Thermo Fisher Scientific, Langenselbold, Germany) for 15 min in the dark. Finally, propidium iodide (PI) solution (2 μg/ml) was added and the samples were measured using a FACSCalibur flow cytometer with CellQuest Pro software (BD Biosciences, Heidelberg, Germany).

Western blotting. Cells were incubated with 0.63 μg/ml cisplatin, 10 μg/ml methadone alone or in combination for 72 h and treated with lysis buffer [150 mM NaCl, 30 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton-X, 200 μM phenylmethylsulfonylfluoride, 1 mM 1,4-dithiothreitol, EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics)]. Activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP) as markers for apoptosis were detected by western blotting using the detection antibodies mouse anti-human caspase-3 monoclonal IgG1 (ECM Biosciences, Cologne, Germany) and horseradish peroxidase- (HRP) conjugated goat anti-mouse IgG (Santa Cruz Biotech., Heidelberg, Germany) as well as rabbit anti-PARP (Cell Signaling Tech., Danvers, MA, USA) and HRP goat anti-rabbit IgG (Santa Cruz), respectively. β-Actin was detected as control (rabbit anti-human β-actin-HRP; Cell Signaling Tech). Protein bands were visualized with ECL chemiluminescence and Agfa Curix 60 device (Düsseldorf, Germany).

Results

Cytotoxicity of methadone and cisplatin towards bladder cancer cells. Human urinary bladder cancer cells T24 and HT-1376 were treated with increasing doses of methadone and cisplatin for 72 h to determine the individual cytotoxic effects of both substances. Methadone alone did not affect the viability of T24 and HT-1376 cells up to a concentration of 10 μg/ml (Figure 1A). In contrast, cisplatin treatment reduced the number of viable cells in both cell lines at concentrations up to 1.25 μg/ml (Figure 1B).

Cytotoxicity of methadone plus cisplatin towards bladder cancer cells. To examine whether methadone sensitizes bladder cancer cells to cisplatin treatment, the combination of both agents was tested by WST-1 viability assay. As shown in Figure 1C, addition of 1 or 10 μg/ml methadone enhanced the cytotoxicity of cisplatin towards T24 cancer cells after 72 h (Figure 1C). For example, when T24 cells were treated with 0.63 μg/ml cisplatin plus 10 μg/ml methadone, the percentage of viable cells significantly decreased from 74.8% (cisplatin alone) to 23.9% compared to the control ($p<0.0001$). In contrast, HT-1376 cells treated with equal doses did not respond to the combination and showed cytotoxic effects comparable to those observed with methadone and cisplatin alone (Figure 1C).

Induction of apoptosis of bladder cancer cells by methadone plus cisplatin. To investigate whether the significant reduction of cell viability after combination treatment was based on an induction of apoptosis, T24 cells were incubated with 0.63 μg/ml cisplatin and 10 μg/ml methadone. QVD-OPh hydrate was used as an inhibitor of apoptosis. As shown in Figure 2A, cytotoxicity of the combination therapy was significantly attenuated by QVD-OPh hydrate, whereas the effects of methadone and cisplatin alone were not influenced by the pan-caspase inhibitor. This indicates that the single substances did not induce programmed cell death in this setting. In contrast, the reduction of cell viability, elicited by combination therapy, was based on the induction of apoptosis. Phase-contrast microscopy confirmed these observations. Whereas no changes in cell morphology were detected after single-dose application of cisplatin and methadone, the combination of both substances led to cell shrinkage, rounding, fragmentation and formation of apoptotic bodies, the typical hallmarks of apoptosis. Intact morphology was restored in cells treated with methadone plus cisplatin by addition of QVD-OPh hydrate (Figure 2B).

To support the evidence that methadone in combination with cisplatin activates caspase-dependent apoptosis, annexin V/PI-based flow cytometry was performed. Cell viability of T24 cells incubated with the single substances was not

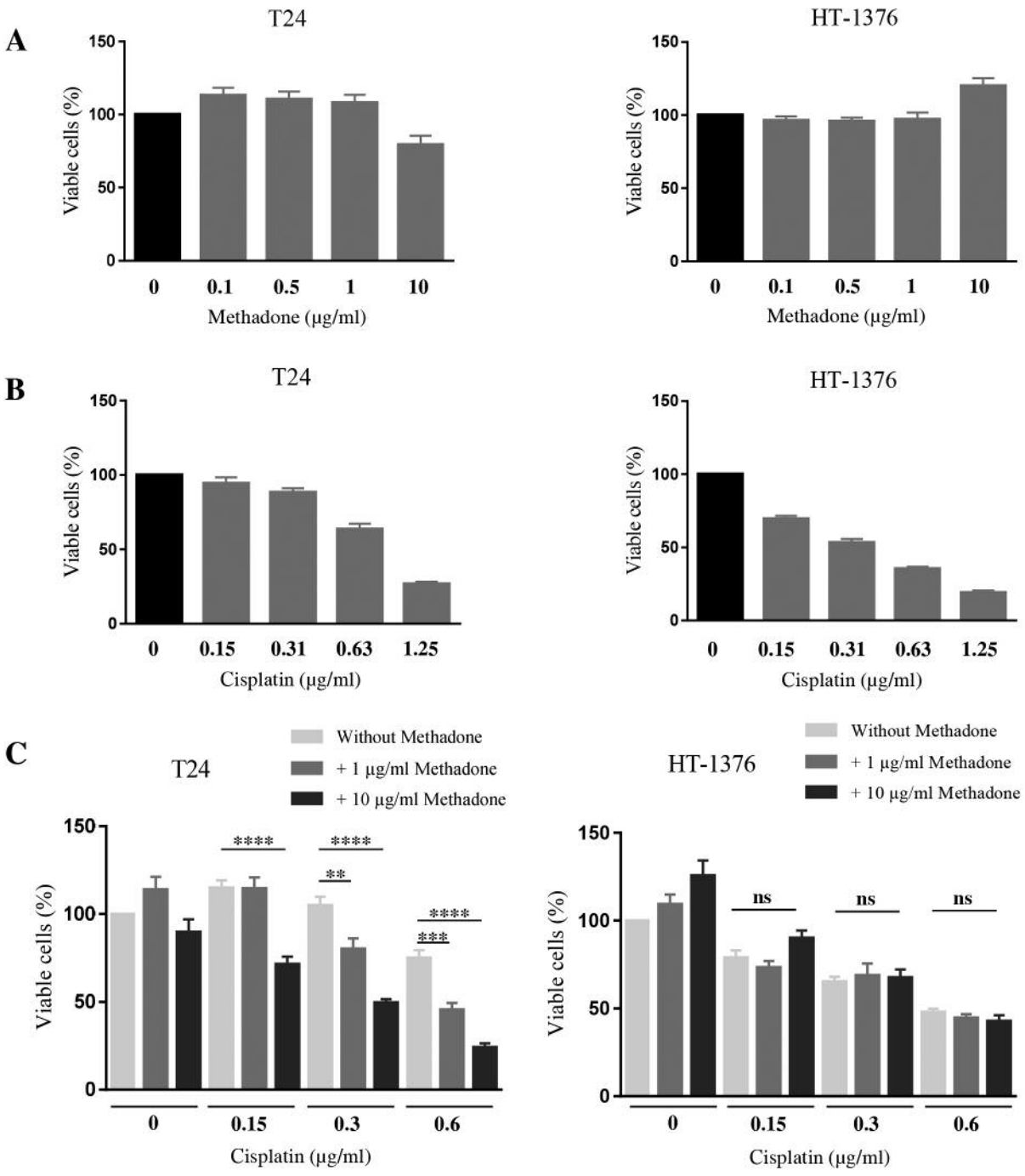


Figure 1. *Cytotoxicity of methadone (A), cisplatin (B) and combination of both agents (C) against urinary bladder cancer cells as measured by WST-1 viability assay after 72 h. Mean values±SD of 3-4 independent experiments in triplicates are shown. Significantly different at *p≤0.05, **p≤0.01, ***p≤0.001, and ****p≤0.0001; ns, not significant.*

impaired and the percentage of dead cells did not differ from that of untreated controls. However, treatment with methadone plus cisplatin resulted in 21.62% apoptotic (annexin V-positive) and 30.45% dead (PI-positive) cells (Figure 3A). Western blot experiments confirmed that methadone plus cisplatin treatment resulted in a significant induction of apoptosis characterized by PARP cleavage and caspase-3 activation (Figure 3B).

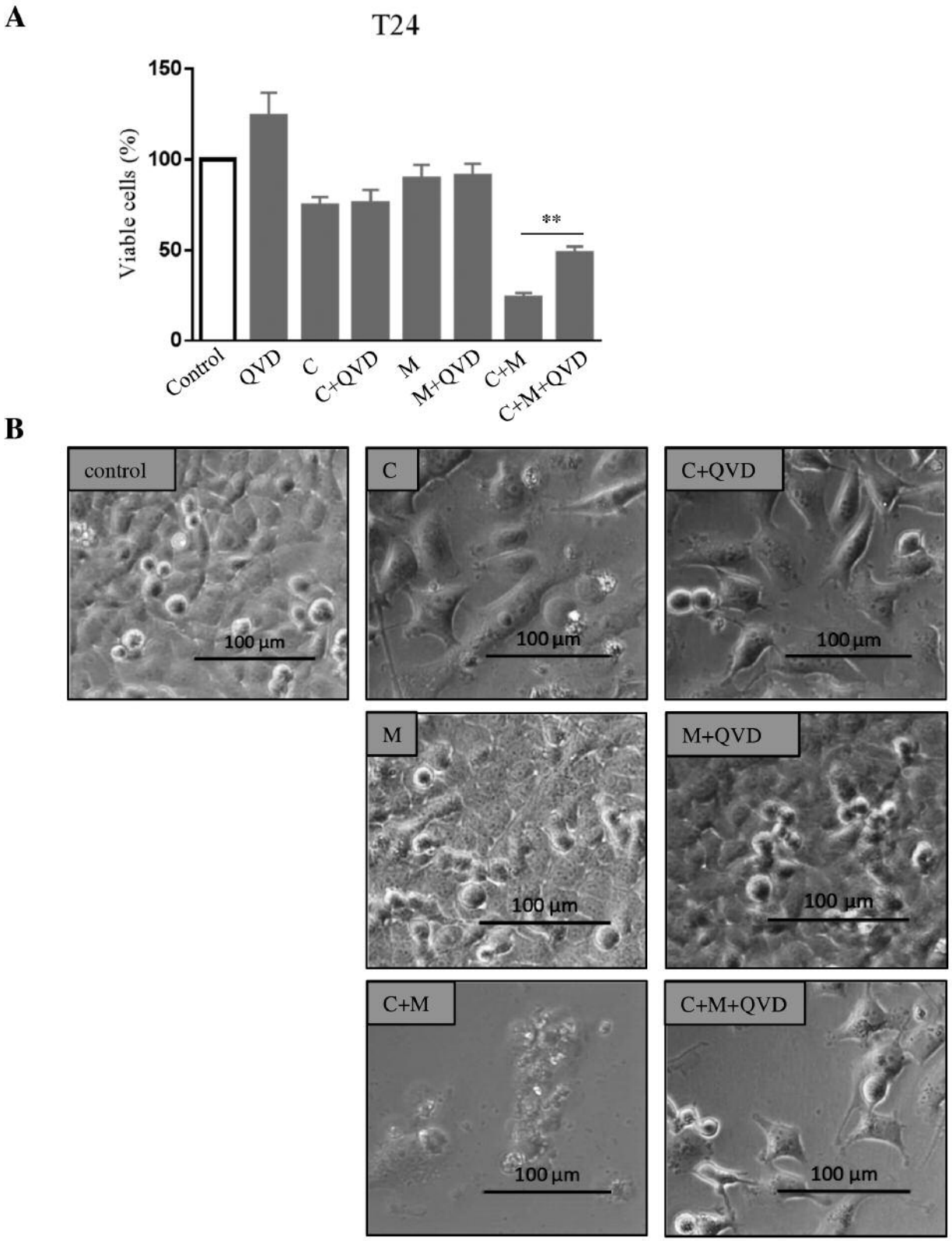
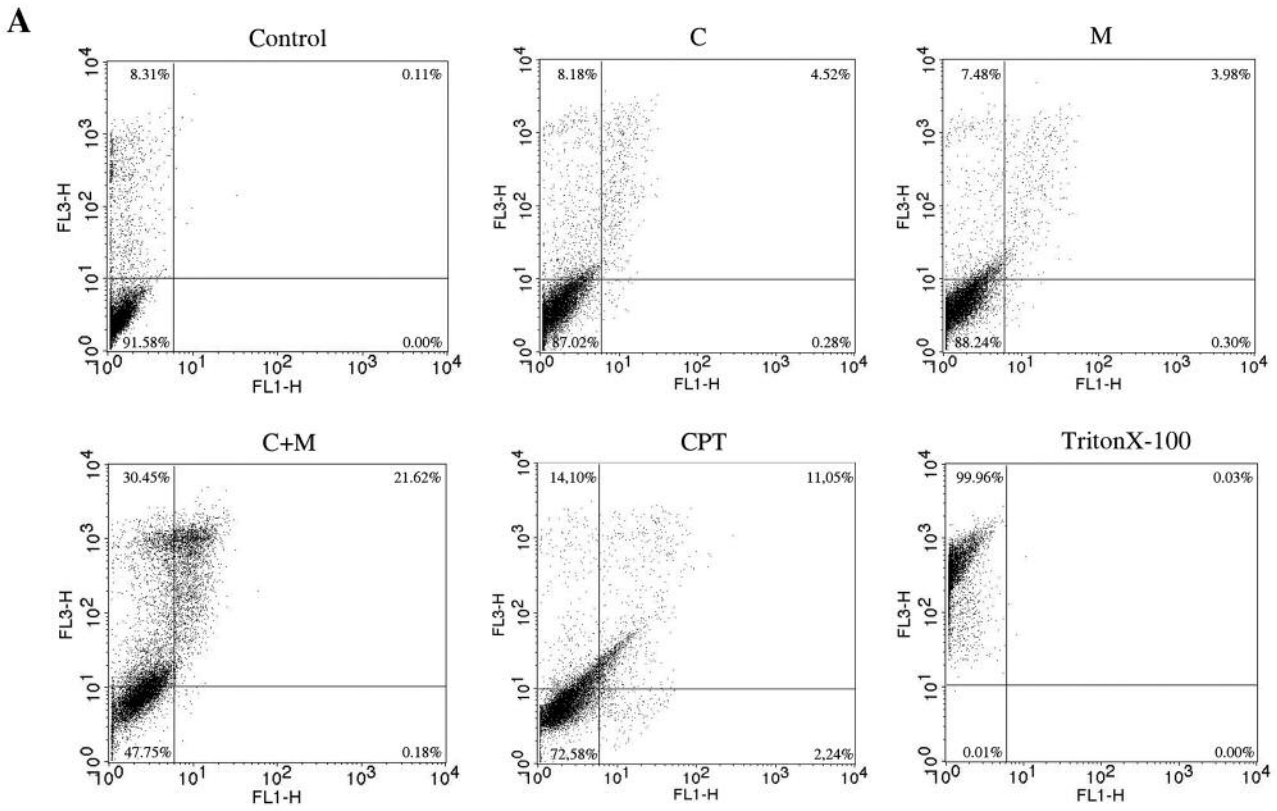


Figure 2. *A: Cytotoxicity of cisplatin (C), methadone (M), and the combination of both agents (C+M) on T24 cells as measured by WST-1 viability assay after 72 h. Pan-caspase inhibitor QVD-OPh hydrate (QVD) was used as an inhibitor of apoptosis. B: Phase-contrast images of T-24 cells treated with cisplatin (C), methadone (M) or in combination of both (C+M) with/without QVD after 72 h.*



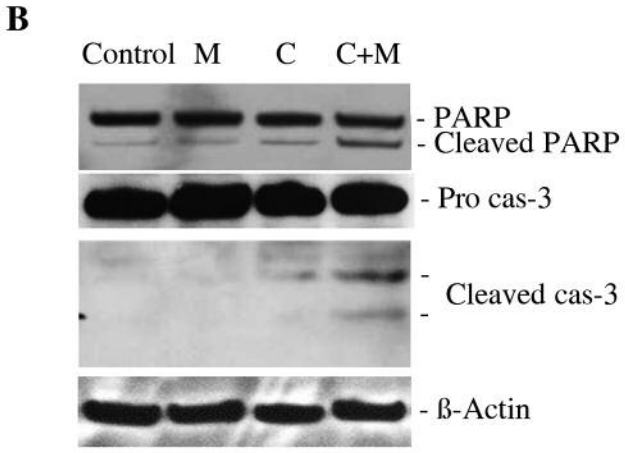


Figure 3. *A: Analysis of apoptosis by flow cytometry with annexin V (FL1-H) and propidium iodide (FL3-H). The percentage of positive cells in each quadrant is shown. T24 cells were incubated with cisplatin (C), methadone (M) or with both agents in combination (C+M) for 72 h. The apoptosis inducer camptothecin (CPT) and the necrosis inducer Triton X-100 were used as positive controls. B: Western blot analysis of cleaved poly (ADP-ribose) polymerase (PARP) and caspase-3 (cas-3) in lysed cells treated with cisplatin (C), methadone (M) or with a combination of both (C+M) after 72 h. β-Actin was detected as a loading control.*

Discussion

Based on preclinical studies, the analgesic methadone is discussed as being a possible sensitizer to the chemotherapeutic treatment of cancer cells (11-15).

In the present study, we demonstrated that methadone was able to enhance the cytotoxic effects of cisplatin on T24 bladder cancer cells. We used a setting with singles doses of cisplatin and methadone with an observation period of 72 h to avoid nonspecific effects from consumption of cell culture medium during longer incubation times. Methadone alone did not reduce the number of viable T24 cells significantly even at the highest concentration of 10 μg/ml. Cisplatin alone led to a concentration-dependent decrease of viable T24 cells, which was not based on apoptosis because the addition of apoptosis inhibitor QVD-OPh hydrate did not

influence this effect. Instead, the reduced number of viable cells can be traced back to cisplatin-induced cell-cycle arrest, which appears in T24 cells in this concentration range (17).

Interestingly, the addition of 10 μg/ml methadone significantly enhanced the cytotoxicity of cisplatin, based on an induction of apoptosis. In other cancer cell lines, the mechanism of chemosensitization by methadone was marked by a down-regulation of anti-apoptotic proteins such as B-cell lymphoma 2 (BCL-2) (13), Induced myeloid leukemia cell differentiation protein (MCL-1) (15), B-cell lymphoma-extra large (BCL-xl) or X-linked inhibitor of apoptosis protein (XIAP) (11, 12). Since cisplatin is known to down-regulate BCL-xL (18) and since BCL-2 overexpression is responsible for cisplatin resistance (19, 20), combination with methadone could have led to comprehensive down-regulation of anti-apoptotic proteins in T24 cells, lowering the hurdle to the induction of apoptosis. Further experiments are necessary to explore the cell-cycle regulatory mechanisms and the intrinsic apoptotic pathways in bladder cancer cells after combinatorial treatment.

We also identified the bladder cancer cell line HT-1376 as a non-responder to methadone. It can be speculated that this cell line could have a higher resistance to apoptosis due to a higher expression of anti-apoptotic proteins or due to other alterations in apoptotic signaling pathways. Longer incubation times or a repeated administration of methadone could possibly elicit apoptotic effects in combination with cisplatin. Moreover, it is conceivable that HT-1376 cells do not respond to methadone because of a lack of methadone receptors on the cell surface. In the central nervous system, the mu opioid receptors are mainly used to achieve the analgesic effects of methadone (21). The expression of opioid receptors was also described on the surface of cancer cells of different origin (*e.g.* breast, lung, stomach, esophagus, prostate) and was associated with survival, staging, grading or metastatic spread (22-26). There is evidence that methadone achieves its antitumor activity by binding to opioid receptors, because opioid receptor antagonists were shown to abolish the effects of methadone (11, 12, 14). However, a direct identification of opioid receptors as methadone-sensitive molecules on cancer cells is lacking. Further experiments will examine the molecular background of chemosensitization and will clarify the reasons why different bladder cancer cell lines show different responses to methadone.

Taken together, we showed that methadone is able to sensitize bladder cancer cells to cisplatin treatment. This could open a new therapeutic window for methadone in the future treatment of patients with bladder cancer to support cisplatin-based chemotherapy with simultaneous pain relief.

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