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Chondroitin sulphate enhances the antitumor activity of gemcitabine and mitomycin-C in bladder cancer cells with different mechanisms

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Abstract. Non-muscle invasive bladder cancer is the most common type of bladder cancer in Western countries. The glycosaminoglycan (GAG) layer at the bladder surface non-specifically blocks the adherence of bacteria, ions and molecules to the bladder epithelium and bladder cancer cells express CD44 that binds GAG. Currently, there are few options other than cystectomy for the management of non-muscle invasive bladder cancer with intravesical chemotherapy using several drugs such as gemcitabine (GEM) and mitomycin-C (MMC) with poor prophylactic activity. In this study, we investigated the effects of the GAG chondroitin sulphate (CS) on the growth inhibition of human bladder cancer cell lines HT-1376 and effects of the combination between GEM or MMC with CS. We have found that CS, MMC and GEM induced 50% growth inhibition at 72 h. Therefore, we have evaluated the growth inhibition induced by different concentration of CS in combination with MMC or GEM, respectively, at 72 h. We have observed, at Calcusyn analysis, a synergism when HT-1376 cells were treated with CS in combination with MMC or GEM, respectively, if used at an equimolar ratio. We have also found that CS/GEM combination induced a strong potentiation of apoptosis with the consequent activation of caspases 9 and 3. On the other hand, HT-1376 cells were necrotic if exposed to the CS/MMC combination and no signs of caspase activation was observed. In conclusion, in the human bladder cancer cell line HT-1376 pharmacological combination of CS with GEM or MMC resulted in a strong synergism on cell growth inhibition.

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Abbreviations: BCG, Bacille Calmette-Guèrin; CIS, carcinoma *in situ*; CS, chondroitin sulphate; GEM, gemcitabine; MMC, mitomycin-C; TUR, transurethral resection

Key words: bladder cancer, chondroitin sulphate, gemcitabine, mitomycin-C, HT-1376

Introduction

Non-muscle invasive bladder cancer is the most common type of bladder cancer in Western countries. There are multiple risk factors for bladder cancer, which include exposure to tobacco and industrial chemicals, ingestion of arsenic-laced water, radiation therapy to organs adjacent to the bladder, therapeutic use of alkylating agents in chemotherapy regimens, and infection with the trematode *Schistosoma haematobium*. The glycosaminoglycan layer at the bladder surface non-specifically blocks the adherence of bacteria, ions and molecules to the epithelium. Therefore, it may be an important element in the first line of defence against infection, calculi and even carcinogens for the transitional cells of the bladder. Qualitative or quantitative defects in the glycosaminoglycan(s) expression may influence an individual's susceptibility to the development of bladder tumors (1-3).

At initial presentation 70% of bladder cancers are superficial and include carcinoma *in situ* (CIS), Ta, and T1 disease. Transurethral resection (TUR) is the primary mode of clinical management for both diagnosis and treatment of superficial bladder cancer, but 60 to 70% of these cancers recur and 20% progress to a higher stage (4). TUR is commonly followed by intravesical infusion of either chemotherapy or immune-modulating agents in order to reduce the incidence of recurrence and progression. Bacille Calmette-Guèrin (BCG) is the most effective agent in the prevention of recurrence resulting in a decrease in the rate of progression; however, only one third of patients respond to BCG an effect that can induce a range

of adverse effects from mild dysuria to systemic tuberculosis (5). Several conventional cytotoxic agents have been used for prophylaxis of recurrences after resection (6). Adjuvant intravesical infusion in recurrent tumors with chemotherapy or immunotherapy is not yet clearly established. Patients in whom BCG fails are a challenge for both urologists and oncologists, with the need for careful individualization of therapy in experienced hands. Among the compounds used in intravesical therapy, mitomycin C (MMC) is one of the most common. This anti-tumor antibiotic, according to the manufacturer's labeling, is indicated in intravesical infusion at the dose of 40 mg (7). The European Urological Association guidelines recommend 20 to 40 mg as the standard dose of MMC (7). MMC has been shown to be active in treating superficial bladder cancer, and given in multiple infusions produces response rates ranging between 40 and 50% (8). MMC is, at the present, one of the standard chemotherapy agents in the treatment of superficial bladder cancer (9). In two recent studies, MMC was compared with BCG showing a slightly decreased activity in the treatment of the disease and prevention of recurrence (10,11). Multiple infusions of MMC, however, are associated with an increased incidence of side effects (12). In fact, chemical cystitis and allergic reactions are quite common and disappear after cessation of therapy. The new pyrimidine analogue gemcitabine (GEM) exhibits anti-tumor activity against a variety of solid tumors including advanced bladder cancer. In fact, it is active and well-tolerated when used in the treatment of metastatic bladder cancer (13). The proven efficacy of systemic therapy against advanced bladder cancer led urologists to consider GEM as a potential new agent for the treatment of superficial transitional cell carcinoma by intravesical administration (14). Recently, we have demonstrated that GEM has better efficacy and lower toxicity than MMC in a phase III randomized clinical trial in the prevention of the recurrence of superficial bladder cancer and correlates with significantly lower side effects.

In this study, we investigated the effects of chondroitin sulphate (CS) (3) on the growth inhibition of human bladder cancer cell line HT-1376 and effects of the combination between GEM and MMC with chondroitin sulphate on the antitumor activity of these drugs *in vitro*.

Materials and methods

Materials. DMEM, BSA and FBS were purchased from Flow Laboratories (Milan, Italy). Tissue culture plasticware was from Becton-Dickinson (Lincoln Park, NJ, USA). Chondroitin sulphate (Uracyst®) was a gift of Stellar Pharmaceuticals, Inc., Gemcitabine (Gemzar®) was a gift of Lilly, mitomycin-C was a gift of Kyowa Hakko Kirin. Caspase-3 Mab (31A1067) and caspase-9 pAb were purchased from Enzo Life Sciences. Anti caspase-6 human Mab was purchased from Alexis. Anti α-tubulin Mab (DM1A) and anti-rabbit peroxidase conjugate were purchased from Calbiochem. GTX anti-mouse IgG was purchased from Millipore.

Cell culture. The human bladder carcinoma HT-1376 cell line, obtained from the American Type Tissue Culture Collection (Rockville, MD) was grown in DMEM supplemented with heat-inactivated 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine and 1% sodium pyruvate. The cells were grown in a humidified atmosphere of 95% air/5% CO_2 at 37˚C.

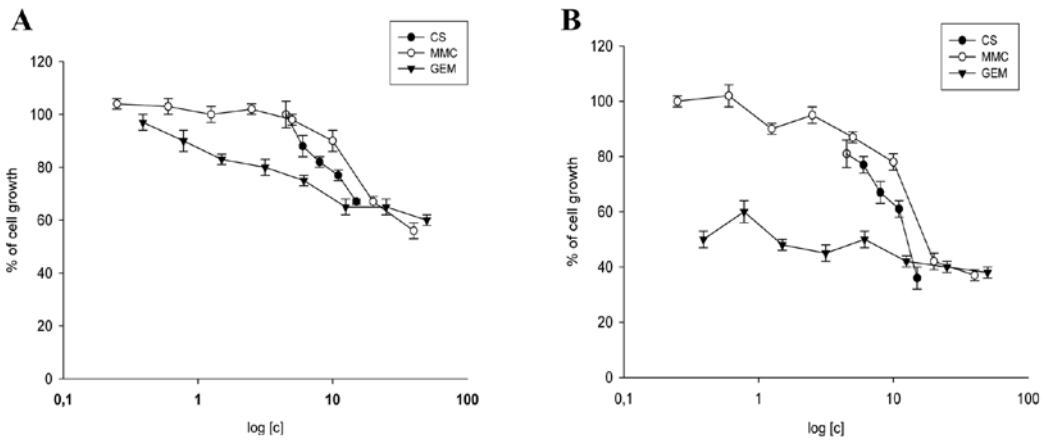
Drug combination studies. For the study of the synergism between CS and MMC or GEM on growth inhibition of HT-1376 cells, the cells were seeded in 96-multiwell plates at the density of $5x10^3$ cells/well. After 24 h incubation at 37˚C the cells were treated with different concentrations of CS or GEM or MMC and their combinations. Drug combination studies were based on concentration-effect curves generated as a plot of the fraction of unaffected (surviving) cells vs. drug concentration after 72 h of treatment. Assessment of synergy was performed quantitating drug interaction by the Calcusyn computer program (Biosoft, Ferguson, MO). Combination index (CI) values of <1, 1, and >1 indicate synergy, additivity, and antagonism, respectively (15). Furthermore, we analyzed the specific contribution of CS, GEM and MMC on the cytotoxic effect of the combination by calculating the potentiation factor (PF), defined as the ratio of the IC_{50} of either CS or GEM or MMC alone to the IC_{50} of CS/GEM or CS/MMC combinations, respectively, as described before; a higher PF indicates a greater cytotoxicity.

Flow cytometric analysis of apoptosis. Annexin V-FITC (fluorescein isothiocyanate) was used in conjunction with a vital dye, propidium iodide (PI), to distinguish apoptotic (Annexin V-FITC positive, PI negative) from necrotic (Annexin V-FITC positive, PI positive) cells. Briefly, cells were incubated with Annexin V-FITC (MedSystems Diagnostics) and propidium iodide (Sigma) in a binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$) for 10 min at room temperature, washed and resuspended in the same buffer. Analysis of apoptotic cells was performed by flow cytometry (FACScan, Becton-Dickinson). For each sample, $2x10^4$ events were acquired. Analysis was carried out by triplicate determination on at least three separate experiments.

Western blot analysis. HT-1376 cells were grown for 48 h with or without CS and/or GEM or CS and/or MMC at 37˚C. For cell extract preparation, the cells were washed twice with ice-cold PBS/BSA, scraped, and centrifuged for 30 min at 4˚C in 1 ml of lysis buffer (1% Triton, 0.5% sodium deoxycholate, 0.1 M NaCl, 1 mM EDTA, pH 7.5, 10 mM Na_2HPO_4, pH 7.4, 10 mM PMSF, 25 mM benzamidine, 1 mM leupeptin, 0.025 U/ml aprotinin). Equal amounts of cell proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose and reacted with the different antibodies. Blots were then developed using enhanced chemiluminescence detection reagents (SuperSignal West Pico, Pierce) and exposed to X-ray film. All films were scanned by using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Results

Effects of CS, GEM and MMC alone on the cell growth of bladder cancer cells. We evaluated the effects of CS, MMC and GEM on the growth inhibition of human HT-1376 cells at 48 and 72 h from the beginning of the treatment (Fig. 1). All



Drug	IC_{50}
CS	12 mg/ml
MMC	18 μg/ml
GEM	0.5 μg/ml

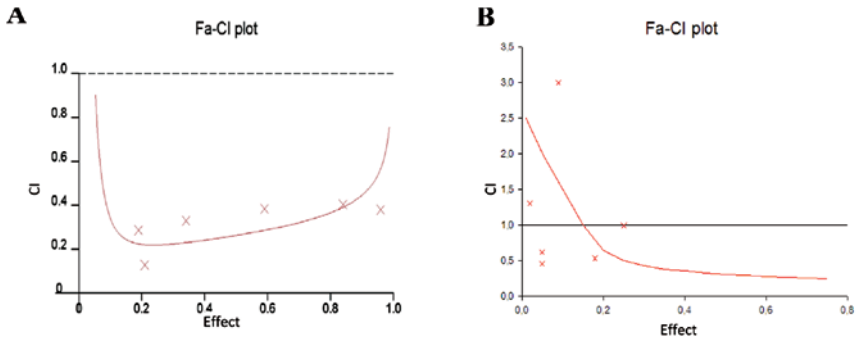
Figure 1. Effects of CS alone and in combination with GEM and MMC on cell growth of bladder cancer cells. HT-1376 cells were cultured for (A) 48 h and (B) 72 h and treated with chondroitin sulphate (CS) or gemcitabine (GEM) or mitomycin-C (MMC) alone at increasing concentrations. The effects on the cell growth were expressed as % of control after 48 and 72 h of treatment. Cell growth was evaluated by the MTT assay as described in Materials and methods. Bars, SEs.

the drugs induced a time- and dose-dependent growth inhibition and the 50% growth inhibition was reached at 72 h with CS at the concentration of 12 mg/ml (IC_{50}) and with MMC, and GEM at the concentrations of 18 and 0.5 μg/ml, respectively (Fig. 1B). The drugs did not induce cytotoxic effects at these concentrations and the growth was resumed upon withdrawal of the drugs from the media (data not shown).

Synergistic effects of CS in combination with GEM or MMC on cell growth of bladder cancer cells. On the basis of these results, we have evaluated if the agents could be synergistic in inducing cell growth inhibition of HT-1376 bladder carcinoma cells. Specifically, we evaluated the growth inhibition induced by different concentrations of CS in combination with MMC or GEM at 72 h on HT-1376 bladder carcinoma cell line.

We used Calcusyn (15), a dedicated software, to examine the synergism of our treatments. With this software, synergistic conditions occur when the combination index (CI) is <1.0. When CI is <0.5 the combination is highly synergistic. We found that the combination of CS and MMC was highly synergistic when the two drugs were used at ratios with higher concentrations of CS on HT-1376 (Fig. 2A). On the other hand, antagonism was recorded when ratios with higher concentrations of MMC were used (data not shown). In synergistic drug combination the CI_{50} (the combination index calculated for 50% cell survival by isobologram analysis) was 0.39 (Fig. 2C). On the other hand, the combination of CS and GEM was highly synergistic when the two drugs were used at ratios with higher concentrations of GEM on HT-1376 (Fig. 2B) and antagonism was again recorded when ratios with higher concentrations of CS were used (data not shown). In synergistic drug combination the CI_{50} was 0.73 (Fig. 2C). The dose reduction $index_{50}$ (DRI_{50}) represents the magnitude of dose reduction obtained for the 50% growth inhibitory effect in combination setting as compared to each drug alone. In our experimental conditions, the DRI_{50} of CS and MMC were equal to 459 and 3.82 and the DRI_{50} of CS and GEM were equal to 279 and 21.2 (Fig. 2C). Moreover, the potentiation factor (PF) values reported in Fig. 2C demonstrate that MMC had an important contribution to the cytotoxic effect of the combination CS/MMC while CS had an important role in the combination CS/GEM. Interestingly, the optimal results (lowest CI values with the best PF) were obtained when higher concentrations of CS were used with MMC and higher concentrations of GEM were used with CS (Fig. 2C). These results demonstrate that a strong or a very strong synergism can be recorded on cell proliferation when CS is used in combination with either drugs. Effective concentrations in the combination experiments are possible to be reached *in vivo*.

CS in combination with GEM, but not with MMC, induces apoptosis in bladder cancer cells. We selected two concentrations of CS and GEM that were highly synergistic as determined by Calcusyn elaboration and we evaluated the apoptotic effects of their combination with FACS analysis after double labeling with FITC-Annexin V and PI. We used this combination for all subsequent experiments on the perturbation of intracellular signaling. We found that treatment with 0.04 μg/ml CS and 0.15 μg/ml GEM alone for 48 h induced apoptosis in only 3-9% of the cell population vs. 4% of the untreated cells as demonstrated with FACS analysis (Fig. 3A-C). However, when the cells were treated with the two drugs in combination for 48 h 44% of apoptosis was detected (Fig. 3D). Addition to the cells of 50 μM VP-16 for 48 h was used as a positive control and it resulted in ~53% induction of apoptosis (data not shown).



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CS ED_{50} (µg/ml)	MMC ED_{50} (µg/ml)	Combination ratio	CI_{50}	DRI_{50}	P.F.	Interpretation
5,69	1,7	1:0,3	**0,39**	**MMC**: 3,82 **CS**: 459	**MMC**: 10,6 **CS**: 2,1	Strong synergism
CS ED_{50} (µg/ml)	**GEM** ED_{50} (µg/ml)	**Combination ratio**	**CI_{50}**	**DRI_{50}**	**P.F.**	**Interpretation**
0,04	0,15	1:3,75	**0,73**	**GEM**: 279 **CS**: 0,006	**GEM**: 3,3 **CS**: 300	Synergism

Figure 2. Evaluation of synergism of CS in combination with GEM and MMC by isobologram analysis. We evaluated the growth inhibition induced by different concentrations of CS in combination with either (A) MMC or (B) GEM at 72 h on HT-1376 cells. We performed these experiments with the MTT assay and the resulting data were elaborated with the dedicated software Calcusyn (developed by Chou and Talalay) as described in Materials and methods. Combination index (CI)/effect curves showed the CI vs. the fraction of cells affected/sacrificed by the two combinations on bladder cancer cells. Combinations were synergistic when CIs were <1. Each point is the mean of at least 4 different replicates. (C) DRI values (mean ± standard deviation from at least three separate experiments performed in quadruplicates) represent the order of magnitude (fold) of dose reduction obtained for IC_{50} (DRI_{50}) in combination setting compared with each drug alone. CI values (mean ± standard deviation from at least three separate experiments performed in quadruplicates) represent the assessment of synergy induced by drug interaction. Combination index (CI) values of <1, 1, and >1 indicate synergy, additivity, and antagonism, respectively. Potentiation factor (PF) values (mean ± standard deviation from at least 3 separate experiments performed in quadruplicates) defined the specific contribution of CS or MMC or GEM, respectively, evaluated as the ratio of the IC_{50} of CS or MMC or GEM, to the IC_{50} of CS/MMC or CS/GEM, respectively. The Friedman non-parametric rank test was used to analyze the impact of the different cytotoxic ratio on the whole cell line panel, P=0.018.

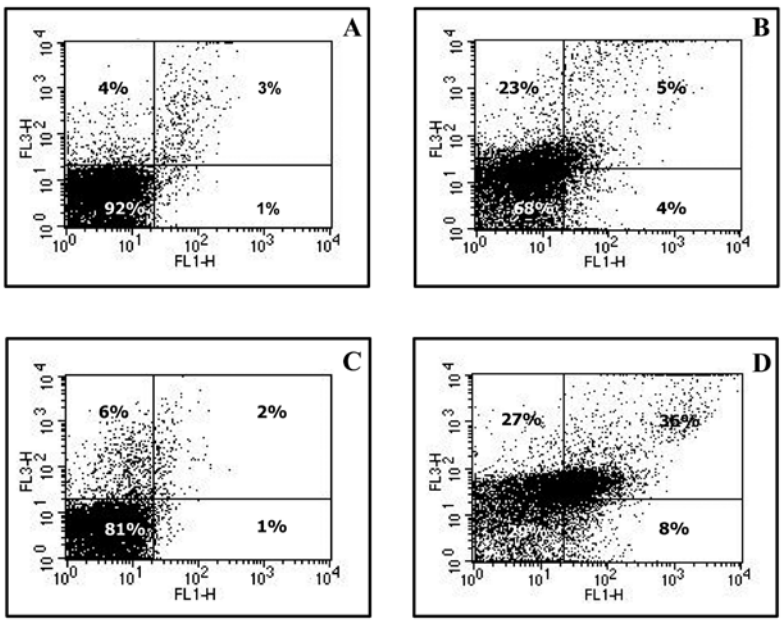

Figure 3. Effects of the CS/GEM combination on apoptosis. Apoptosis was evaluated by FACS analysis, after cell labeling with propidium iodide (PI) and FITC-Annexin V. HT-1376 cells were not treated (A) or treated with CS (B) and GEM (C) alone or in combination (D) for 48 h. The lower left quadrants of each panels show the viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper left quadrants contain the non-viable, necrotic cells, negative for FITC-Annexin V binding and positive for PI uptake. The lower right quadrants represent the cells in early apoptosis, that are FITC-Annexin V positive and PI negative. The upper right quadrants represent the cells in late apoptosis, positive for both FITC-Annexin V binding and for PI uptake. The experiments were performed at least three times with similar results.

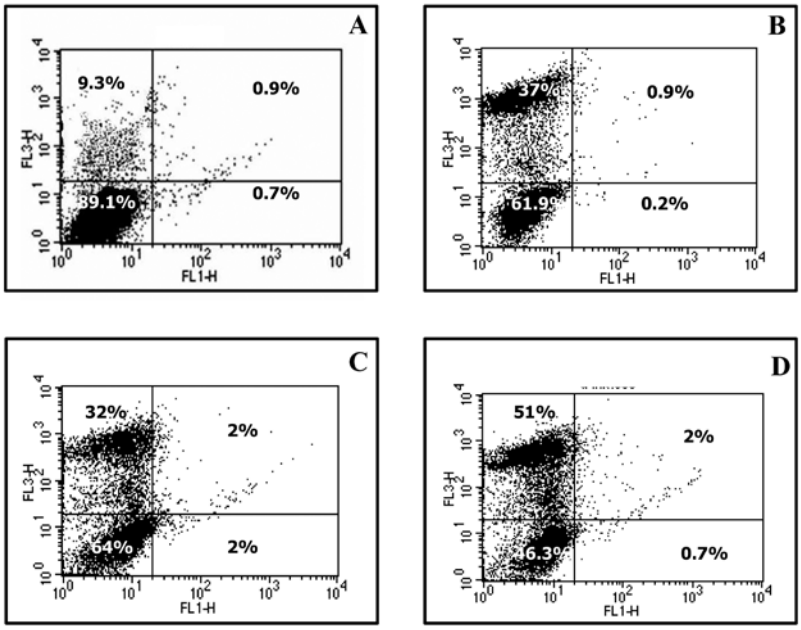

Figure 4. Effects of the CS/MMC combination on apoptosis. Apoptosis was evaluated by FACS analysis, after cell labeling with propidium iodide (PI) and FITC-Annexin V. HT-1376 cells were not treated (A) or treated with CS (B) and MMC (C) alone or in combination (D) for 48 h. The lower left quadrants of each panel show the viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper left quadrants contain the non-viable, necrotic cells, negative for FITC-Annexin V binding and positive for PI uptake. The lower right quadrants represent cells in early apoptosis that are FITC-Annexin V positive and PI negative. The upper right quadrants represent the cells in late apoptosis, positive for both FITC-Annexin V binding and for PI uptake. The experiments were performed at least three times and the results were always similar.

Different results were obtained with the combination between CS and MMC. Similarly to the other combination treatment case, we selected two concentrations of CS and MMC that were highly synergistic at Calcusyn elaboration and we evaluated the apoptotic effects of their combination with FACS analysis after double labeling with FITC-Annexin V and PI. We found that treatment with 5.69 μg/ml CS and 1.7 μg/ml MMC alone for 48 h induced apoptosis in only 1.1-4.0% of the cell population vs. 1.6% of untreated cells as demonstrated with FACS analysis (Fig. 4A-C). However, when the cells were treated with the two drugs in combination for 48 h only about 2.7% of apoptosis was found (Fig. 4D). On the other hand, 51% of cell population was necrotic when cells were exposed to the CS/MMC combination vs. 37 and 32% of the cell population in CS and MMC-treated cells, respectively (Fig. 4B-D).

Effects of either CS/GEM or CS/MMC combination on caspase activation. In order to evaluate the caspase cascade involved in the activation of apoptosis induced by the different combinations we assessed the expression of full length and fragmented caspases at 24 and 48 h after the beginning of the treatments. The different treatment had no effects on the expression of both full length and fragmented caspase 8 (data not shown) suggesting no effects on the extrinsic pathway of apoptosis. CS and GEM alone had little effects on the activation of caspase 9, 3 and 6 even if GEM alone induced a significant decrease of full length caspase 9 and fragmented caspase 3. On the other hand, a clear accumulation of the cleaved forms of caspase 9 and 3 paralleled by a decrease of their full length fragments was clearly detected after 48 h from the beginning of the treatment with the CS/GEM combination (Fig. 5A). The latter did not induce, in turn, any changes in the expression of full length caspase 6. These results correlated with the findings on apoptosis occurrence determined by CS/GEM combination at 72 h from the beginning of treatment suggesting the triggering of the mitochondrial-dependent apoptotic pathway mediated by caspase 9 (as initial caspase) and caspase 3 (as terminal caspase).

On the other hand, no variations of the expression of cleaved caspase 9 and full length caspase 3 and 6 were found in cells treated with CS and MMC either alone or in combination. The latter data additionally suggest that the CS/MMC combination did not induce apoptosis in human bladder cancer cells.

Discussion

Several studies indicate that the optimal intravesical schedule for patients with superficial bladder cancer has not yet been established, and cystectomy remains the only proven curative option. However, some patients are not candidates for radical surgery due to comorbidities and others refuse surgery. Many experimental modalities are now available for treating patients with superficial bladder cancer and optimized chemotherapy with MMC and GEM have shown some encouraging results (4). In a recent study by Addeo *et al* (16), the comparison of GEM and MMC shows that GEM has a better chemopreventive activity than MMC. The percentage of patients with recurrence on intravesical chemotherapy for GEM significantly differs from that observed in MMC-treated patients. Moreover, the incidence of toxic adverse effects was significantly lower with GEM than MMC. These results, according to previous studies, confirmed that chemical cystitis manifesting as irritative lower urinary tract symptoms represents the most adverse effect of MMC.

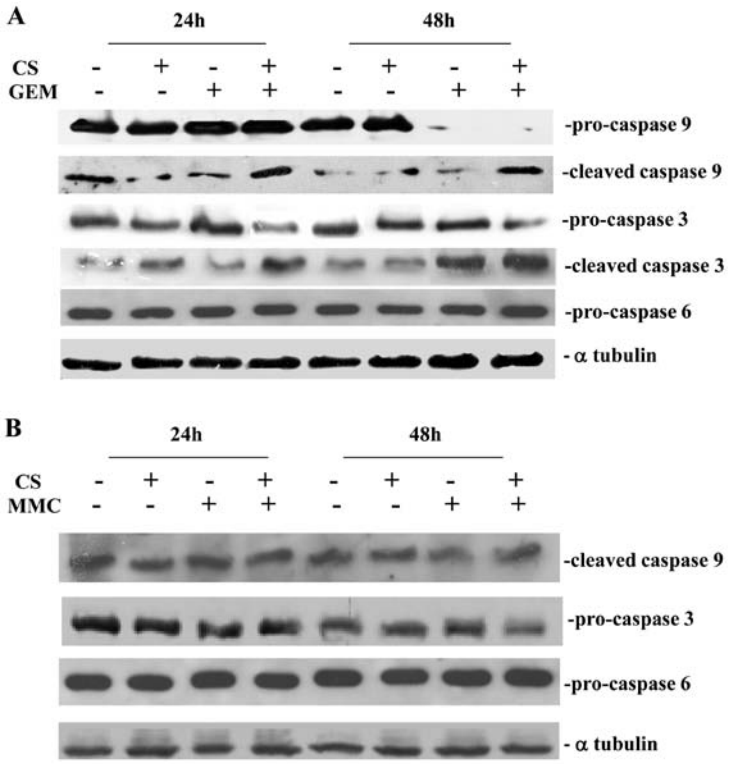

Figure 5. Effects of the CS/GEM and CS/MMC combination on the activation of caspases. (A) Effects of CS and GEM, alone or in combination, on caspases 3, 6 and 9 expression were evaluated by Western blotting with specific monoclonal antibodies, as described in Materials and methods after 24 and 48 h. (B) Effects of CS and MMC, alone or in combination on caspases 3, 6 and 9 expression were evaluated by Western blotting with specific monoclonal antibodies, after 48 and 72 h. Expression of the house-keeping protein α-tubulin was used as loading control. The experiments were performed at least three times and the results were always similar.

The glycosaminoglycan layer at the bladder surface non-specifically blocks the adherence of bacteria, ions and molecules to the epithelium reducing exposure to carcinogens of the transitional cells of the bladder. Nevertheless, there are few papers on glycosaminoglycan effects on bladder cancer. Volpi *et al* (17) have observed a decrease of proliferation and of differentiation in leukemia cells (U-937) in proportion to the increased amounts of CS with low sulphate. Therefore, in the present study, we have evaluated the growth inhibition induced by GEM, MMC and CS in the human bladder cancer cell line HT-1376, used as our experimental system. We have found that all the drugs induced a time- and dose-dependent growth inhibition and the 50% growth inhibition was reached at 72 h. In these experimental conditions, we have hypothesized that CS could be used in combination with GEM or MMC to increase the anti-tumor activity of drugs reducing their active concentrations and toxic adverse effects. For the evaluation of synergism, we have calculated the CI by the Calcusyn software and we have found that GEM or MMC and CS given in combination were strongly synergistic in the human bladder cancer cell line HT-1376, as a CI_{50} <0.5 was found after 72 h of treatment.

The mechanisms of action of GEM and MMC are quite different even if the two agents are both classical cytotoxic drugs affecting DNA integrity and inducing apoptosis (18,19). On the basis of our results we have evaluated the mechanisms of cell death that are induced by the synergistic combinations between CS and the two cytotoxic agents on HT-1376 cells. On this basis, we have selected two combinations of CS and GEM or MMC, respectively, that were highly synergistic with Calcusyn analysis and we have evaluated the effects of these combinations on apoptosis occurrence at FACS analysis. We have found that the CS/GEM combination increased apoptosis induced by GEM alone, suggesting that the synergism on growth inhibition was largely due to apoptosis. On the other hand, the cell population was necrotic when cells were exposed to the CS/MMC combination and poor apoptosis was observed in cells treated with MMC alone. Notably, the CS/GEM combination induced a significant activation of caspases 3, and 9, while no effect was found on caspase 6 degradation suggesting the triggering of the mitochondrial-dependent apoptotic pathway mediated by caspase 9 (as initial caspase) and caspase 3 (as terminal caspase). In contrast, no variations of the expression of cleaved caspase 9 and full length caspase 3 and 6 were found in cells treated with CS and MMC either alone or in combination. These findings suggest that CS/MMC combination did not induce apoptosis in human bladder cancer cells.

In this regard, it was previously reported that GEM in combination with cisplatin and 1,25 dihydroxyvitamin D3 induced a potent growth inhibition in human bladder cancer cells that appeared to be mediated by apoptosis (20). Moreover, it has been reported that MMC can induce apoptosis in T24 bladder cancer cells that can be antagonized by the binding of MMC to fibronectin and potentiated by the concomitant inhibition of insulin-like growth factor 1 receptor (19-21). In

our experimental model, MMC was not able to induce apoptosis either alone or in combination with CS. This could be, at least in part, explained by the difference in the cell line used (HT-1376 instead of T24).

It was demonstrated that another endocytic membrane lectin, the hyaluronan/chondroitin sulfate receptor also known as CD44, which has been purified and cloned, is responsible for the turnover in mammals of these glycosaminoglycans, which are important components of extracellular matrices (22,23). Moroever, CD44 is often expressed by cancer cells and is a marker of cancer stemness (24). On the basis of these considerations, several attempts have been developed in order to deliver anti-cancer drugs through their conjugation with hyaluronan and/or CS and several findings exist on the effective targeting of cancer cells with these bio-conjugates (25). In light of this finding, the positive interaction between GEM or MMC with CS is not surprising and could be, at least in part, explained with an increase of the internalization of the cytotoxic drugs by cancer cells. In addition, the intracellular route of anti-cancer drugs can influence their mechanism of action and, therefore, the different mechanisms of death induced by the two different combinations can be partially explained by the type of intracellular compartmentalization induced by CD44 endocytosis on the two drugs.

In conclusion, treatment with a pharmacological combination of CS with either GEM or MMC of HT-1376 human bladder cancer cells resulted in a strong synergism on cell growth inhibition. However, the mechanism of cell death induced by the two different combinations was different and strongly warrants additional investigation in order to determine the precise molecular mechanisms of the combinations. Moreover, these strategies may be useful in order to increase the therapeutic effectiveness of intravesical strategies for the prevention of bladder cancer recurrence. Therefore, these data encourage the additional investigations on the combined use of chondroitin sulphate with GEM and MMC in the treatment/prophylaxis of human superficial bladder cancer.

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