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Inhibitory and Apoptotic Effects of Mannan-Mitomycin C Conjugate Against Transitional Cell Carcinoma and Normal Mouse Fibroblasts

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Abstract

Background: Many studies have shown the anticancer effects of mannan and mitomycin C on tumor cells. In this regard, the aim of this study was to investigate the inhibitory and apoptotic effects of a mannan-mitomycin-C conjugate on transitional cell carcinoma (TCC) and normal mouse L929 fibroblast cells.
Methods: The conjugate was synthesized according to previous studies. Both cell lines were cultured and the cytotoxic and apoptotic effects of the compounds in different concentrations were assessed using MTT and flow cytometry, respectively. The mannan-mitomycin C conjugate inhibited proliferation of both cell lines in time and concentration -dependent manners.
Results: The conjugate inhibited TCC cell proliferation more than that of L929 cells. Mitomycin C alone inhibited proliferation of both cell lines in both time and concentration -dependent manners, and the effect was greater on L929 than on TCC cells. Mannan had a relatively low inhibitory effect on TCC and no significant effect on L929 cells. The percentage of apoptosis was greater in TCCs than in L929 cells at the highest concentration of conjugate. Mitomycin C induced apoptosis more extensively in L929 cells than in TCC cells at 25 and 400 μg/ml. The effect of mannan was similar on both cell lines.
Conclusions: The mannan-mitomycin C conjugate has greater inhibitory and apoptotic effects on TCC than on L929 cells and may inhibit TCC.

Keywords: Apoptosis, Inhibitory Effect, Mannan-Mitomycin C Conjugate, MTT, TCC.

Introduction

One of the most common genitourinary malignancies is bladder cancer (1). More than 90% of bladder malignancies are associated with transitional cell carcinoma (TCC) (2). Several factors can cause malignant cells in the bladder, including smoking, occupational exposure, chronic inflammation in the bladder due to schistosomiasis, or factors such as age, gender, race, and genetics (3, 4). Methods used to treat bladder cancer include transurethral resection of bladder (TURB), chemotherapy, Bacillus Calmette-Guérin (BCG) therapy, and cystectomy (5, 6). Chemotherapy with thiotepa, doxorubicin, and mitomycin is common. Although these methods lead to a relative improvement in cancer patients' conditions, the percentage of definitive treatment of the disease is low. Therefore, the use of complementary therapies, such as immunotherapy can help the treatment process (7). The most common bladder cancer immunotherapy treatment is intracellular BCG. One component of BCG is mannan (8). BCG induces immunological changes in bladder cells and stimulates the production of chemokines such as IL-8 and inflammatory cytokines (7).

Mannan is a polysaccharide found in the outer layers of yeast cell walls(9). The composition of mannan extracted from yeast *Saccharomyces (S.) cerevisiae* can be specifically linked to a receptor called toll-like receptor 4 (TLR4) from the pattern recognition

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receptors family (10, 11) which were originally found on immune cell membranes (10, 12) and also exist on bladder epithelial cells (12, 13). The binding of mannan with this receptor induces the production of inflammatory cytokines such as interleukins (ILs) 6, 8, and 12 and tumor necrosis factor-α (TNFα), and reduces or increases tumor growth (14, 15). Mannan also contributes to immune function by affecting monocytes and stimulating the production of interleukins and TNFα (11).

Mitomycin C, an antibiotic derived from *Streptomyces Caspitosus* (16), is used to treat many cancers, including bladder cancer. Unfortunately its side effects include anorexia, fatigue, hemolytic-uremic syndrome, mucositis, myelosuppression, thrombocytopenia, and renal failure (17).

In this study, we investigated the *in vitro* effects of mannan-mitomycin C conjugate, and mannan and mitomycin C alone on the proliferation and apoptosis of TCC and L929 cells, which may help us to develop new drugs that are more effective against cancer cells than normal cells and have fewer side effects than currently available drugs.

Materials and methods

Cell culture

The TCC and L929 cell lines were purchased from Ferdowsi University Cell Bank (Mashhad). The cells were cultured at 37°C in a humidified 5% CO2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM)-high glucose containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin.

Preparation and analysis of mannan-mitomycin C conjugate

The mannan-mitomycin C conjugate was synthesized according to the modified Matsumoto's method (18). In the first step, mannan from *S. cerevisiae* wild type strain was activated with cyanogen bromide. For this purpose, 0.1 g of mannan was dissolved in 10 mL of water. 55 mg of cyanogen bromide was added and the pH of the solution was adjusted to 10.7 with 1 M NaOH. Then, 0.1 g of 6-aminohexanoic acid was added and the pH was adjusted to 9 with 1 M HCl. To make the coupling reaction, the solution was stirred at room temperature for 24 hr. In the next step, the mannan-6-aminohexanoic acid product was dialyzed against sodium carbonate (Na2CO3) at pH 9 in dialysis tubes with 12000-MW cut-off for 24 hr. Then, 10 mg of mitomycin C was dissolved in mannan-6-amino-hexanoic acid solution and 0.2 g of 1-ethyl-3- (3-dimethyl-aminopropyl) carbodiimide hydrochloride was added. The pH was adjusted to 5.0-6.0 and the solution was stirred at room temperature for 24 hr. Finally, the solution was dialyzed for 24 hr. The amount of mitomycin C bound in the mannan-mitomycin C conjugate was determined by measuring the absorbance of mitomycin C at 364 nm on a spectrophotometer (Analytikjena, Germany).

MTT assay

The inhibitory effect of the mannan-mitomycin C conjugate and mannan and mitomycin C alone on TCC and L929 cell lines was measured by MTT assay. For this purpose, 20,000 cells per well were cultured in 96-well plates. Cells were incubated for 24 hr to allow them to attach to the plate. After this time, the TCC and L929 cells were incubated with 700, 350, 175, 87.5, 43.75, or 0 µg/mL mannan-mitomycin C conjugate, 400, 200, 100, 50, 25, or 0 µg/mL mitomycin C, and 10000, 5000, 2500, 1250, 625, or 0 µg/mL mannan for 24, 48, or 72 hr. At the end of the incubations the supernatants were removed and 20 µl of MTT solution was added. After 4 hr of incubation at 37 °C, 100 µl of DMSO and 10 µl of glycine buffer was added to each well. Absorbance of each well was measured on an ELISA reader (BioTek, USA) at a wavelength of 570 nm. All experiments were performed in triplicate. The results were reported as survival percentages at the various sample concentrations.

Cell Apoptosis Analysis

To determine cell apoptosis after treatment with mannan, mitomycin C, and the mannan-mitomycin C conjugate, flow cytometry with Propidium iodide (PI) and Annexin-V FITC was performed. For this purpose, 2×10^5 L929 and TCC cells per well were cultured in 6-well plates. After 24 hr of incubation to allow the cells to adhere to the wells, they were treated for 72 hr with 2 mL of the conjugate at 700 µg/ml, 2 mL of mitomycin C at 400 µg/ml, 2 mL of mitomycin C at 25 µg/ml, 2 mL of mannan at 1250 µg/ml, and a well for each cell line with no treatment as a negative control. The cells were then washed with PBS and trypsinized. The cell suspension was centrifuged and the cell pellet was washed 2x with PBS containing 1% FBS. The cells were then solubilized in 100 µl of ready-to-use binding buffer (cat # 422201). In the next step, 5 µl of Annexin-V FITC and 10 µl of PI solution were added to each tube. The solubilized cells were gently vortexed and incubated for 15 minutes at room temperature in darkness. Finally, 400 µl of binding buffer was added to each tube and apoptosis was analyzed on a BD FACSCalibur (USA).

Morphological studies

After treatment of the L929 and TCC cells with the mannan-mitomycin C conjugate, mitomycin C, or mannan at different concentrations for 24, 48, and 72 hr, the cell morphologies were examined by inverse optical microscopy.

Statistical analysis

The results were analyzed using GraphPad Prism 6 and one-way analysis of variance (ANOVA) and Tukey's post-HOC test. In all tests, P<0.05 was considered statistically significant.

Results

MTT results 24, 48, and 72 hr after adding mannan-mitomycin C conjugate to L929 and TCC cells

After 24 hr, no significant difference in cell viability was seen between the L929 and TCC cells treated either without or with the conjugate. The conjugate caused a slight decrease in viability of both the TCC and L929 cells, although this decrease was not significant (P>0.05) (Fig. 1). After 48 hr of incubation with the conjugate however, viability was significantly less in the TCC than in the L929 cells (P<0.0001). Cell viability decreased with increasing conjugate concentration. The mean (CI 95%) IC50 for the TCC cells was 457.9 (262.3 to 799.3) µg/ml, while the effect of conjugate was not sufficient to kill 50% of the L929 cells. Cell viability was significantly less in the TCC cells than in the L929 cells at all conjugate concentrations (P<0.0001). After 72 hr cell survival decreased for both cell lines with increasing conjugate concentrations and was significantly less in the TCC than in the L929 cells at conjugate concentrations of 175, 350, and 700 µg/mL (P<0.05, 0.001, and 0.0001, respectively). The mean (CI 95%) IC50 for the TCC cells was 187.0 (173.3 to 201.0) µg/ml, while in the L929 cell line, the mean (CI 95%) IC50 was 551.5 (427.2 to 712.0) µg/ml.

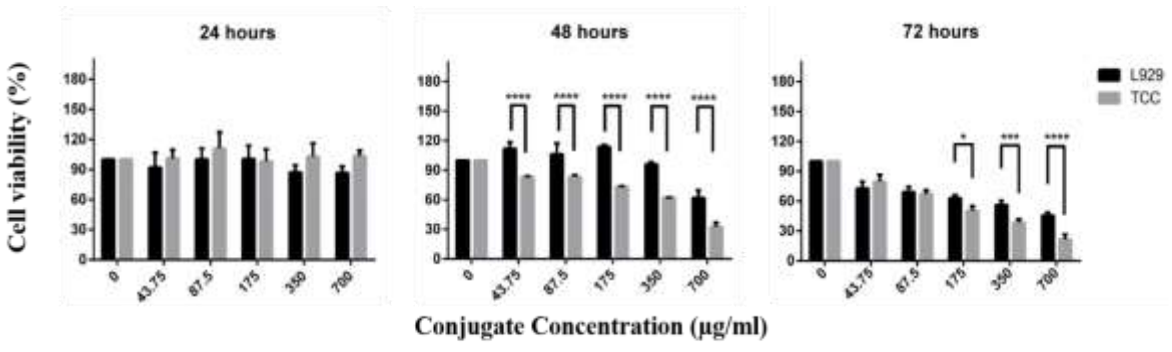

Fig. 1. Growth inhibitory effect of mannan-mitomycin C conjugate on L929 and TCC cells. Cells were grown in 96-well plates and incubated for 24, 48, or 72 hr with 0, 43.75, 87.5, 175, 350 or 700 µg/mL of the mannan-mitomycin conjugate. Cell viability was measured with MTT assays. Mean ± SD, ANOVA, n=3. (P<0.05*, P<0.01**, P<0.001***, P<0.0001****).

MTT results 24, 48, and 72 hr after adding mitomycin C to L929 and TCC cells

After 24 hr of mitomycin C treatment, viability decreased significantly in both cell lines (P<0.0001). The mean (CI 95%) IC50s were 74.8 (63.8 to 87.6) µg/mL and 57.3 (38.6 to 85.1) µg/mL for the TCC and L929 cells, respectively. After 48 the decreasing trend in survival rate was statistically significant (P<0.0001) (Fig. 2, 48 hr) and after 72 hr, the survival rate has decreased in both cell lines and its reduction trend is significant (P<0.0001) (Fig. 2, 72 hr).

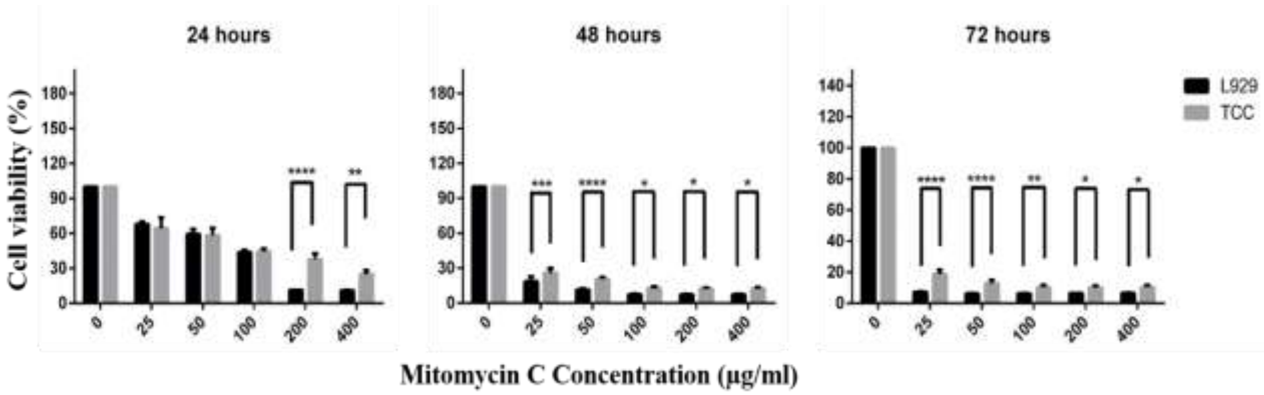

Fig. 1. Growth inhibitory effect of mitomycin C on L929 and TCC cells. Cells were grown in 96-well plates and incubated for 24, 48, or 72 hr with 0, 25, 50, 100, 200, or 400 µg/mL of mitomycin C. Cell viability was measured with MTT assays. Mean ± SD, ANOVA, n=3. (P<0.05*, P<0.01**, P<0.001***, P<0.0001****).

MTT results 24, 48, 72 hr after adding mannan to L929 and TCC cells

After 24 hr, viability was significantly less in the TCC than in the L929 cells at mannan concentrations of 623, 1250, 2500, and 5,000 µg/ml. (Fig. 3, 24 hr) The greatest decrease in TCC cell viability was at the lowest mannan concentration. After 48 hr, viability was significantly less in the TCC than in the L929 cells only at 5,000 µg/mL mannan. No significant differences were seen at any other mannan concentrations (Fig. 3, 48 hr). After 72 hr no viability differences were seen between the two cell lines at any mannan concentrations (Fig. 3, 72 hr).

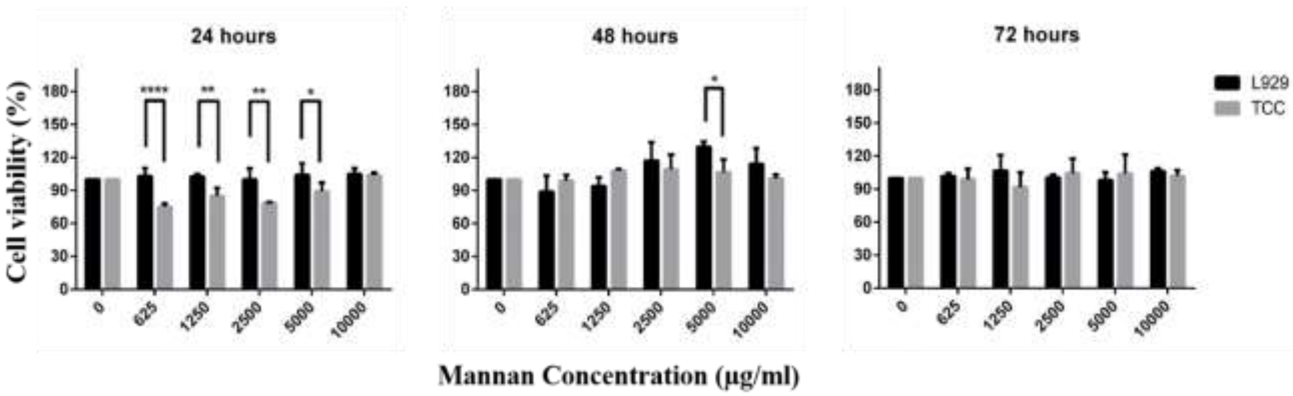

Fig. 2. Growth inhibitory effect of mannan on L929 and TCC cells. Cells were grown in 96-well plates and incubated for 24, 48, or 72 hr with 0, 625, 1250, 2500, 5000 or 10000 µg/mL of mannan. Cell viability was measured with MTT assays. Mean ± SD, ANOVA, n=3. (P<0.05*, P<0.01**, P<0.0001****).

Flow cytometry

Apoptosis was analyzed after 72 hr by flow cytometry. Table 2 shows the effects of mannan, mitomycin C, and conjugate samples at the concentrations listed. In this data, live cells that were negative for both Annexin-V and PI appear in Q4. Cells in Q3 were in early apoptosis and Annexin-V-positive and PI-negative. Cells in Q2 were in late apoptosis; their cell walls are slightly permeable, and they are positive for both Annexin V and PI. Cells in Q1 are necrotic or dead and stained only with PI. Apoptosis, shown in Q3+Q2, was induced by mannan in both cell lines at similar levels of 41.5 and 41% for L929 and TCC, respectively. Mitomycin C at 25 µg/mL induced apoptosis in 62.1% of L929 cells and 43.5% of TCC cells. Apoptosis was greater in cells treated with 400 µg/mL of mitomycin C than in those treated with 25 µg/ml. Apoptosis was less in the L929 conjugate-treated cells than in those treated with mannan or mitomycin C and similar in the TCC cells.

Table 1. The effects of the conjugate, mitomycin C, and mannan samples on L929 and TCC cells after 24,48, and 72 hr

Conjugate													
Cell Line		**L929**						**TCC**					
Concentration (µg/ml)		0	43.75	87.5	175	350	700	0	43.75	87.5	175	350	700
Time post incubation	24 hr	100±0	92.088±14.775	100.249± 10.992	100.661± 13.214	87.227±7.212	86.791±6.508	100±0	101.171±8.335	111.046±16.419	38.107±12.122	102.874±13.322	103.529±5.596
	48 hr	100±0	112.019±6.570	106.269± 11.057	113.778±1.980	96.011±2.485	62.127±7.408	100±0	83.183±1.001	83.227±1.950	73.049±0.791	52.198±0.299	32.813±4.033
	72 hr	100±0	72.831±6.916	69.074±5.373	62.997±2.941	56.132±4.625	45.550±3.284	100±0	79.614±6.738	67.171±3.804	50.189±4.830	38.367±3.564	21.404±5.123
Mitomycin C													
Cell Line		**L929**						**TCC**					
Concentration (µg/ml)		0	25	50	100	200	400	0.000	25	50	100	200	400
Time post incubation	24 hr	100±0	67.855± 2.468	59.483±4.249	43.784± 1.972	11.478±0.182	10.995±0.632	100±0	64.455±9.148	58.444±6.188	44.372±2.715	37.841±4.833	24.975±3.260
	48 hr	100±0	18.582±4.369	11.426±1.208	7.475±0.597	7.194±0.753	7.383±0.466	100±0	25.933±4.066	20.630±1.657	12.698±1.451	12.277±1.139	12.381±1.181
	72 hr	100±0	7.324±0.329	6.180±0.235	6.281±0.106	98.202±7.219	6.571±0.201	100±0	18.667±2.940	12.824±2.389	10.200±1.633	9.942±1.518	10.351±1.351
Mannan													
Cell Line		**L929**						**TCC**					
Concentration (µg/ml)		0	625	1250	2500	5000	10000	0	625	1250	2500	5000	10000
Time post incubation	24 hr	100±0	103.060±7.487	102.716±2.143	100.159±10.166	104.101±10.650	104.955±5.459	100±0	75.091±3.009	84.913±7.461	78.490±0.970	89.319±7.874	103.957±2.338
	48 hr	100±0	88.854±14.789	94.003±7.880	117.088±16.798	129.742±4.898	114.220±14.312	100±0	98.699±5.549	107.982±1.635	109.247±13.306	106.361±11.818	101.062±3.313
	72 hr	100±0	101.900±2.535	106.785±14.380	100.643±2.357	98.202±7.219	106.197±2.989	100±0	98.913±9.736	91.708±13.526	104.441±13.309	104.043±17.419	101.982±5.395

Table 2. Flow cytometry analysis of apoptosis induced by the mannan, mitomycin C, and conjugate samples on TCC and L929 and TCC cells after 72 hr

			Untreated (%)	Mannan 1250 µg/ml (%)	Mitomycin C 25 µg/ml (%)	Mitomycin C 400 µg/ml (%)	Conjugate 700 µg/ml (%)
72 hr' treatment	**L929 Cell Line**	Vital population (Q4)	72.5	53.5	34.5	16.5	68.5
		Early apoptosis (Q3)	14.4	16.8	50.2	79.3	13.0
		Late apoptosis (Q2)	11.4	24.7	11.9	4.18	13.6
		Necrosis (Q1)	1.75	4.94	3.45	0.04	4.84
	TCC Cell Line	Vital population (Q4)	71.1	51.1	54.1	42.9	57.4
		Early apoptosis (Q3)	13.6	16.5	21.7	16.7	27.0
		Late apoptosis (Q2)	13.4	24.5	21.8	39.6	15.6
		Necrosis (Q1)	1.88	7.91	2.39	0.87	0.04

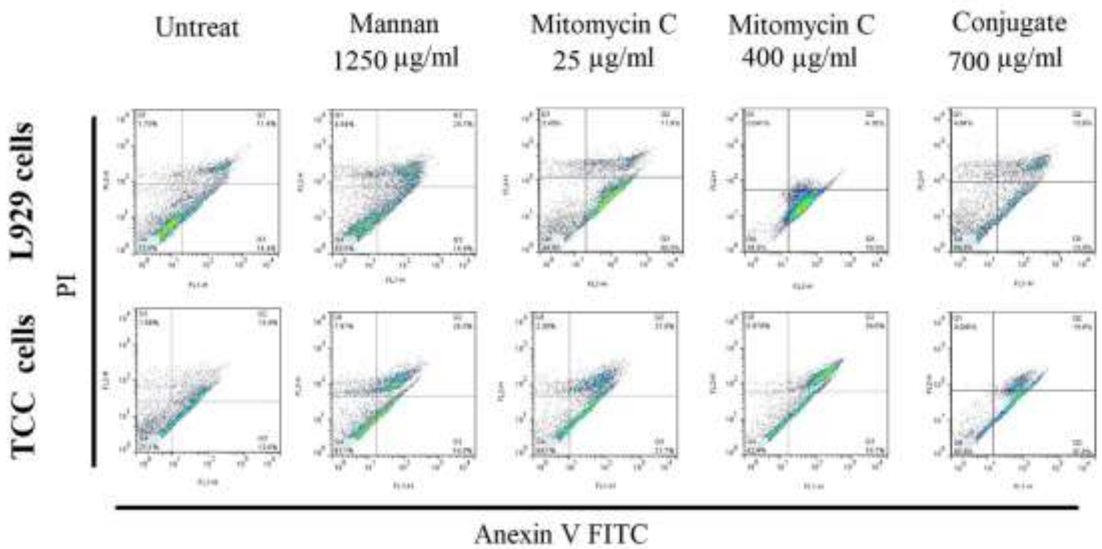

Fig. 3. Flow cytometry results of L929 and TCC cells following treatment with mannan, mitomycin C at 25 and 400 µg/ml, and mannan-mitomycin C conjugate. Untreated cells served as control.

Morphological alterations

After 72 hr the morphology of L929 and TCC cells treated with the highest concentration of each compound was observed with inverse optical microscopy L929 cells treated with the conjugate or mitomycin C had fewer cells than controls and cell shrinking, while those treated with mannan showed a population increase and no cell degradation (Fig. 5). After 72 hr conjugate-treated and mitomycin C-treated TCC cells showed decreased cell numbers, cytoplasmic granulation, and rounding, while those treated with mannan showed no substantial morphological changes (Fig. 6).

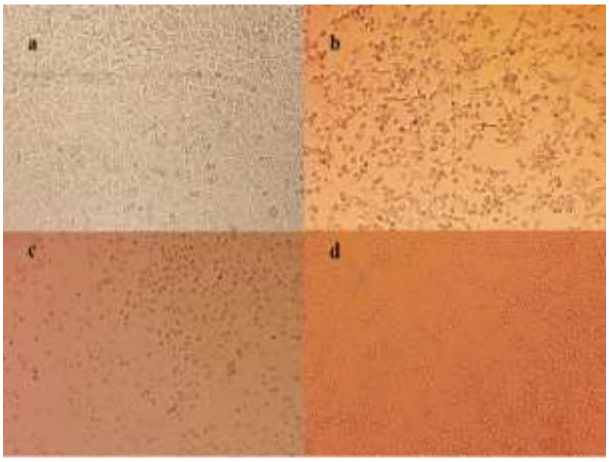

Fig. 5. Microscopy images of L929 cells treated with mannan-mitomycin C conjugate, mitomycin C, and mannan. L929 cells were incubated for 72 hr with 700 µg/mL mannan-mitomycin C conjugate, 400 µg/mL mitomycin C, and 10,000 ug/mL mannan. (a) untreated cells; (b) conjugate-treated cells; (c) mitomycin C-treated cells; (d) mannan-treated cells (10X).

Fig. 4. Microscopy images of TCC cells treated with mannan-mitomycin C conjugate, mitomycin C, and mannan. TCC cells were incubated for 72 hr with 700 µg/mL mannan-mitomycin C conjugate, 400 µg/mL mitomycin C, or 10,000 ug/mL mannan. (a) untreated cells; (b) conjugate-treated cells; (c) mitomycin C-treated cells; (d) mannan-treated cells (10X).

Discussion

To date, several methods have been used to treat various cancers, often with undesirable side effects and low response to treatment. Hence, researchers continue to search for new and more effective ways to control cancer with fewer side effects. Numerous studies have analyzed the effect of yeast extract on different types of cancers (19, 20). Research on fungal and yeast polysaccharides has shown their significant effects in terms of immunopotentiation and the ability to control carcinogenesis (18, 21). One polysaccharide with this feature is mannan in the cell wall of *S. cerevisiae*, a yeast known to be non-toxic in humans (22). This polysaccharide stimulates the immune system to produce cytokines that bind TLR-4 receptors on the surface of monocytes. Mitomycin C, an anti-cancer antibiotic, is activated and converted into an alkylating agent through an enzyme-mediated reduction, which causes DNA cross-linking (17). Mitomycin C is widely used to treat bladder cancer, which is why we chose it in this study. Tolley et al. reported that mitomycin C treatment reduced the number of subsequent returns and increased recurrence intervals in bladder cancer patients (23). Numerous studies on the effects of mitomycin C have been conducted including those by Bouffioux et al. (1995), Solsona (1999), Savino (2010), and James (2012), illustrating its importance in bladder cancer treatment (24-27). In this study the TCC cells were used because they express TLR-4 and thus can be specific targets for mannan (11, 12). Several cancer cell types express TLR-4, including those of the colon, stomach, prostate, breast, ovary, and brain (14, 15). The aim of this study was to investigate the inhibitory and apoptotic effects and selectivity of a mannan-mitomycin C conjugate, and these two compounds individually on L929 and TCC cells.

Inhibitory effect of mannan-mitomycin C conjugate, mitomycin C and mannan on L929 and TCC cell lines

The mannan-mitomycin C conjugate demonstrated its selective and inhibitory effects on L929 and TCC cells after 48 and 72 hr of treatment (Fig. 1). After 72 hr, the selective effect of the conjugate on the two cell lines at concentrations of 175, 350, and 700 µg/mL was statistically significant Mitomycin C alone had a time and dose -dependent inhibitory effect on both cell lines, and the normal cells were more sensitive to mitomycin C treatment than cancer cells.

Mannan alone had little inhibitory effect on either cell type and even increased to some extent the proliferation of normal cells after 48 and 72 hr of treatment. However, its growth-inhibitory effect on cancer cells was seen after 24 hr.

Apoptotic effect of mannan-mitomycin C conjugate, mitomycin C and mannan on L929 and TCC cell lines

Flow cytometry showed that the mannan-mitomycin C conjugate induced (early and late) apoptosis in both L929 and TCC cells. This effect was about two times greater on the TCC than on the L929 cells (Table 3). Mitomycin C alone induced apoptosis in both cell types at both 25 and 400 µg/mL in a dose-dependent manner. This effect was greater in the L929 than in the TCC cells (Table 2).

It is likely that the mannan-mitomycin C conjugate binds TCC cell TLR-4 receptors via the mannan moiety(14, 15). The mitomycin C moiety of the conjugate is likely to fragment the cell's DNA (28, 29), causing the level of cell cycle proteins such as P53 and P21 to increase (29, 30), and prevent the proliferation of cancer cells. If these proteins cannot induce repair of the damaged cell, apoptosis occurs. Mitomycin C, through the internal apoptosis pathway, reduces the level of anti- apoptotic proteins, such as Bcl-2, and activates Bax and Bad pro-apoptotic proteins, which translocate to the mitochondria. This changes the mitochondrial membrane potential, releasing cytochrome c from the mitochondria, from where it translocates to the cell cytoplasm. Cytochrome c, with ATP and dATP, causes oligomerization of Apaf-1, and procaspase 9 attaches to them to form the apoptosome. Finally, caspase 9 is activated and a caspase cascade begins, eventually leading to cell death (30, 31). Our study suggests that the mannan-mitomycin C conjugate may be more specific for bladder cancer cells than mitomycin C alone.

In this study the inhibitory and apoptotic effects of the mannan-mitomycin C conjugate was greater on cancer cells than on normal cells while mitomycin C was more cytotoxic to normal cells than cancer cells. These results may indicate conjugate selectivity for cancer cells due to the mannan moiety binding to TLR-4 on TCC cells. This was an in vitro study, but the results suggest that its effect on animals should be investigated.

Acknowledgment

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