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In vitro and *in vivo* studies of pirarubicin-loaded SWNT for the treatment of bladder cancer

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Abstract

Intravesical chemotherapy is an important part of the treatment for superficial bladder cancer. However, the response to it is limited and its side effects are extensive. Functional single-walled carbon nanotubes (SWNT) have shown promise for tumor-targeted accumulation and low toxicity. In the present study, we performed *in vivo* and *in vitro* investigations to determine whether SWNT-based drug delivery could induce high tumor depression in rat bladder cancer and could decrease the side effects of pirarubicin (tetrahydropyranyl-adriamycin, THP). We modified SWNT with phospholipid-branched polyethylene glycol and constructed an SWNT-THP conjugate via a cleavable ester bond. The cytotoxicity of SWNT-THP against the human bladder cancer cell line BIU-87 was evaluated *in vitro*. Rat bladder cancer *in situ* models constructed by N-methyl-N-nitrosourea intravesical installation (1 g/L, 2 mg/rat once every 2 weeks for 8 weeks) were used for *in vivo* evaluation of the cytotoxicity of SWNT and SWNT-THP. Specific side effects in the THP group including urinary frequency (N = 12), macroscopic hematuria (N = 1), and vomiting (N = 7) were identified; however, no side effects were observed with SWNT-THP treatment. Flow cytometry was used to assess the cytotoxicity *in vitro* and *in vivo*. Results showed that SWNT alone did not yield significant tumor depression compared to saline (1.74 ± 0.56 and 1.23 ± 0.42%) *in vitro*. SWNT-THP exhibited higher tumor depression than THP-saline *in vitro* (74.35 ± 2.56 and 51.24 ± 1.45%) and *in vivo* (52.46 ± 2.41 and 96.85 ± 0.85%). The present findings indicate that SWNT delivery of THP for the treatment of bladder cancer leads to minimal side effects without loss of therapeutic efficacy. Therefore, this nanotechnology may play a crucial role in the improvement of intravesical treatment of bladder cancer.

Key words: Single-walled carbon nanotubes; Bladder cancer; Drug vehicle; THP; Intravesical chemotherapy

Introduction

Bladder cancer is the most common genitourinary malignancy in China and the second most common genitourinary malignancy in the rest of the world. The treatment of superficial bladder cancer consists of transurethral tumor resection of visible tumors, followed by intravesical chemotherapy (1). However, the response to intravesical chemotherapy is limited; up to 45% of patients with superficial bladder cancer will develop recurrent tumors, 20~30% of which progress to a higher stage or grade. Furthermore, some patients fail to accept intravesical chemotherapy because of intolerance. A phase III trial showed that improving the delivery of mitomycin C nearly doubled the recurrence-free rate in superficial bladder cancer patients (2). Therefore, new drug carriers for intravesical chemotherapy need to be developed.

Nanoparticles have been reported to accumulate in tumor tissues through the enhanced permeability and retention (EPR) effect (3), which permits tumor-targeted drug delivery applications. Single-walled carbon nanotubes (SWNT) have been explored as a novel drug delivery carrier *in vitro* because of their ultrahigh surface area and the EPR effect (4,5). NTs have been reported to be capable of carrying various biomolecules, including drugs, peptide, proteins, and DNA, into cells (6-9) through endocytosis and free diffusion (10). Some studies reported that long-term exposure to SWNT seemed to be nontoxic (11). NTs are excreted through the biliary pathway and into feces (12).

Pirarubicin (tetrahydropyranyl-adriamycin, THP) is a common chemotherapeutic drug used in intravesical therapy. It is an anthracycline obtained by hemisynthesis of daunorubicin or doxorubicin. It is rapidly incorporated into tumor cells and exerts anti-tumor activity by inhibiting nucleic acid synthesis, followed by cell death due to cessation of the cell cycle at the G2 phase (13).

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Thus, we speculated that SWNT-based drug carriers could play an important role in intravesical chemotherapy. We functionalized SWNT with polyethylene glycol (PEG), performed SWNT-THP conjugation, constructed rat bladder cancer models, and evaluated the cytotoxicity of SWNT and SWNT-THP *in vitro* and *in vivo*.

Material and Methods

Preparation of functional SWNT with phospholipid-branched PEG

Raw SWNT were sonicated in a 0.2 mM DSPE-PEG5000-4-arm-(PEG-amine) solution for 30 min, then centrifuged at 24,000 *g* for 6 h. The supernatant containing noncovalent phospholipid-branched PEG was collected. Excess untreated PEG was removed by repeated filtration using a 100-kDa molecular mass filter (Millipore, USA) and extensive washing with sterile water.

Preparation of THP and the SWNT-THP conjugate

As described by Liu et al. (14), we added a carboxyl acid group to THP using succinic anhydride (Aldrich, USA). PEG-modified SWNT (1 mg/L) was reacted on a shaking table with 0.5 mM prepared THP in the presence of 8 mM N-hydroxysulfosuccinimide (Pierce, USA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Aldrich). PBS, pH 7.4, was added to the solution at a ratio of 1:1 (v/v). The SWNT-THP conjugate was purified after 6 h of reaction. Unconjugated THP was removed using 5-kDa filters and extensive washing. The concentration of THP loaded on SWNT was measured by absorbance at 395 nm. Preparations of the SWNT-THP conjugate were stored at -20°C.

In vitro assay of cell cytotoxicity

Human bladder cancer cell line BIU-87 and human myofibroblast cell line C2C-12 (both purchased from ShangHai cell bank, China) were cultured in RPMI 1640 medium (HyClone, USA) with 10% fetal calf serum (HyClone). Carboxyl fluorescein succinimidyl ester (CFSE) was added to the cell suspension to a final concentration of 2.5 µM and incubated for 10 min at room temperature in the dark. Labeling was terminated by adding the same volume of RPMI 1640 with 10% fetal calf serum for 5 min. Labeled cells were washed five times with sterile PBS and resuspended in RPMI 1640 medium. They were then treated with SWNT (5 µg/mL), THP (5 µg/mL, 10 µL), and the SWNT-THP (5 µg/mL, 10 µL) conjugate for 8 h. Cultured cells were harvested, washed twice with sterile PBS, and then analyzed with a FACScan flow cytometer using the CellQuest software.

Rat models and treatment

Female Sprague-Dawley rats (6-8 weeks of age, weighing 200-250 g) were purchased from the Laboratory Animal Center of Chongqing Medical University, Chongqing, China. Animal experiments followed institutional guidelines for the use and care of animals. Animals were housed in microisolator cages under specific pathogen-free conditions on a 12-h light-dark cycle. Rat bladder cancer models were constructed by intravesical instillation of N-methyl-N-nitrosourea (MNU) at 2 mg/rat once every 2 weeks for 8 weeks. At the end of the 9th week, 3 rats were sacrificed and bladders were harvested for histological study. Tumor-bearing Sprague-Dawley rats were randomly divided into four groups: rats injected with SWNT (N = 5), with THP-saline solution (N = 15), with SWNT-THP (N = 15), and with saline (N = 5) for treatment. The injected doses of THP in THP-saline solution and SWNT-THP were normalized to 5 mg THP/kg body weight. The injected volume was 0.2 mL/rat and the injected substance was retained in the rat bladder for about half an hour.

In vivo cytotoxicity assay

Two days after the first intravesical chemotherapy, three rats in each treatment group were sacrificed. Tumor tissues were harvested and ground and tumor cells were then collected. Tumor cells (1×10^4) were stained with propidium iodide and analyzed by flow cytometry. Each experiment was done in triplicate.

THP concentration in rat bladder

The standard THP curve was established using standard samples provided by the Wanle Medical Company, China. Urine was collected by bladder puncture with a syringe at 0, 1, 2, and 4 h after intravesical chemotherapy. THP concentration was measured with a 721 spectrophotometer (Precise Co., Ltd., China) using absorbance at 395 nm.

T-lymph cell proliferation assay

T-lymph cells were isolated from normal rats and rats bearing bladder cancer treated with SWNT-THP (5 mg THP/kg body weight). T-cell proliferation was measured with CFSE-based proliferation kits as described previously (14). Fluorescence intensity was determined using an inverted fluorescence microscope and flow cytometry.

Interferon-γ (IFN-γ) release assay

T-cells were centrifuged for 5 min and the supernatant was collected and stored in -20°C. All supernatants were analyzed for IFN-γ release using a sandwich ELISA kit (eBioscience, Inc., USA) as described previously (15). Absorbance at 450 nm was measured using a microplate reader (Spectramax M2; Molecular Devices, USA).

Observation of side effects

The side effects of THP intravesical therapy include pain with micturition, urinary frequency, macroscopic hematuria, and hematologic and renal toxicity. We defined urinary frequency as micturition once every 15 min, and the appearance of any abnormal action during micturition was considered as a sign of pain. Hematologic and renal toxicity was evaluated by blood biochemical examinations.

Prior to treatment, blood was collected from the rat tail vein for hepatic and renal function analysis. Further evaluations of hepatic and renal function were performed every month after the original treatment.

Statistical analysis

Data are reported as means ± SD. Statistical differences between two groups were analyzed by one-way ANOVA or the Student *t*-test. A P value <0.05 was considered to be statistically significant.

Results

Preparation of functional SWNT and SWNT-THP conjugation

Functional SWNT was prepared by sonication of SWNT in a phospholipid-PEG water solution, centrifugation, and filtration for the removal of unreacted SWNT and phospholipid-PEG. The mean length of SWNT was ~150 nm. PEG chains provide amine groups that allow the association of more drug molecules. THP was activated by the addition of a carboxyl acid group from succinic anhydride. SWNT-PEG was linked to the carboxyl acid group-coupled THP through a cleavable ester. Unreacted THP molecules were removed by filtration through a 5-kDa membrane and extensive washing.

In vitro cytotoxicity assay

We evaluated the cytotoxicity of SWNT, THP, and SWNT-THP conjugate against human bladder cancer cell line BIU-87 by flow cytometry (Figure 1). No significant cytotoxicity was observed after treatment with SWNT, whereas significant cytotoxicity was found in the THP group. More obvious cytotoxicity was detected in the SWNT-THP group. The percent of killed cells after 8 h more in the SWNT, THP, and SWNT-THP groups were 1.74 ± 0.56, 51.24 ± 1.45, and 74.35 ± 2.56%, respectively. The differences between the THP and SWNT-THP groups were statistically significant ($P < 0.05$). Furthermore, we investigated the cytotoxicity of SWNT-THP against the normal human myofibroblast cell line C2C-12. SWNT-THP hardly reduced the proliferation of the human myofibroblast cell line C2C-12 (Table 1). These findings suggest that SWNT-THP could efficiently and specifically reduce cancer cell proliferation.

In vivo cytotoxicity assay

At the end of the 9th week after original intravesical instillation of MNU, three rats were sacrificed and their bladders harvested for histological study. Examination indicated that MNU successfully induced carcinogenesis (Figure 2). The cell apoptosis rates for SWNT, THP, SWNT-THP, and saline were 7.42 ± 1.85, 52.46 ± 2.41, 96.85 ± 0.85, and 1.02 ± 0.12%, respectively. The results indicate that SWNT-THP

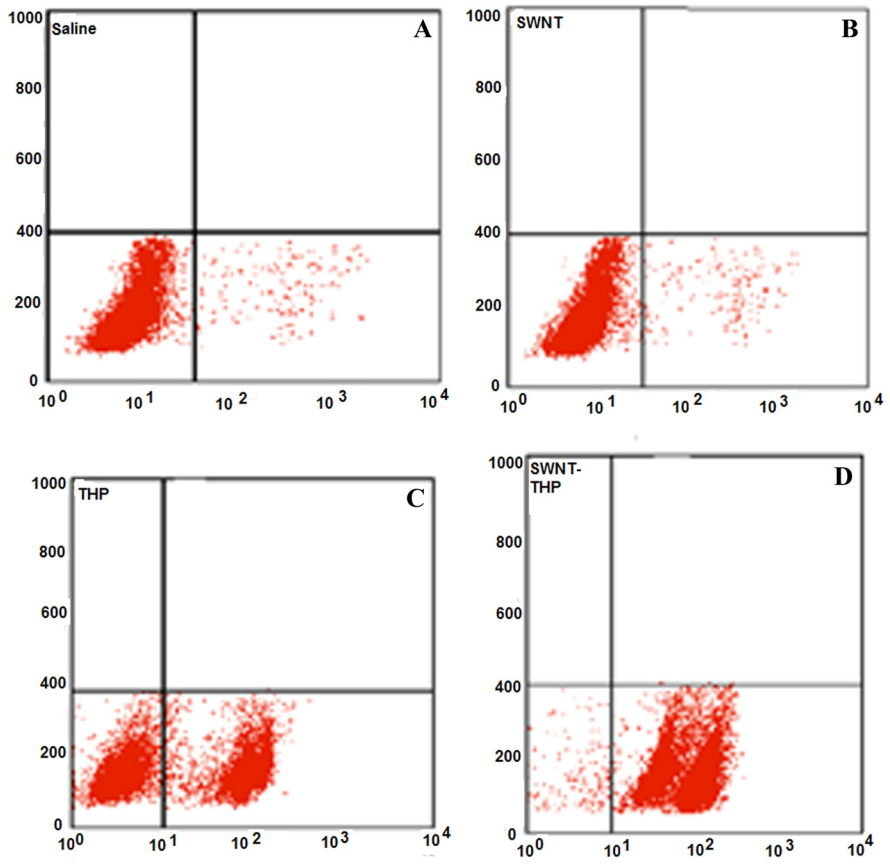

Figure 1. *In vitro* assay of saline (*A*), SWNT (*B*), THP (*C*), and SWNT-THP (*D*) toxicity against BIU-87 cells. SWNT and saline showed no toxicity against BIU-87 cells, whereas SWNT-THP showed more significant toxicity on BIU-87 cells than THP alone. BIU-87 cells were stained with CFSE (2.5 µM) for 10 min at 37°C and resuspended in RPMI 1640 medium at a concentration of 1 x 10^6 cells·mL^{-1}·well^{-1} on 24-well plates, following with stimulation of saline (10 µL), SWNT (5 µg/mL, 10 µL), THP (5 µg/mL, 10 µL), SWNT-THP (5 µg/mL, 10 µL) for 8 h. The cells were collected and cytotoxicity was analyzed by a FACScan flow cytometer. SWNT = single-walled carbon nanotubes; THP = pirarubicin; SWNT-THP = single-walled carbon nanotubes and pirarubicin conjugate.

could induce more tumor cell apoptosis than THP, whereas the saline group hardly induced tumor cell apoptosis.

SWNT enhance lymph cell proliferation and IFN-γ release

Eight hours after SWNT injection, blood was collected from the tail vein of the rats. Lymph cells were isolated for proliferation assay and IFN-γ release assay. The results show that SWNT could enhance the proliferation of lymph cells and the release of IFN-γ, as shown in Figure 3.

SWNT-THP induced long-term retention of THP in the bladder

We collected urine samples from rat bladder at 0, 1, 2, and 4 h after injection of THP or SWNT-THP conjugate. Urine was collected by bladder puncture to test the concentration of THP. THP concentration was higher in the THP group than that in the SWNT-THP group at 0 h, but was lower at all other times. THP concentration could hardly be tested 1 h after instillation in the THP group, but was still high in the SWNT-THP group. The concentration of THP retention indicated that SWNT could induce long-term retention of THP in rat bladder, as shown in Figure 4.

Table 1. *In vitro* cytotoxicity assay of SWNT, THP, and SWNT-THP against the BIU-87 and C2C-12 cell lines.

Cell line	SWNT	THP	SWNT-THP
BIU-87	1.74 ± 0.56*	51.24 ± 1.45	74.35 ± 2.56*+
C2C-12	1.89 ± 0.42	49.45 ± 0.89	23.47 ± 2.45

Cytotoxicity is reported as means ± SD of the percent of cells remaining after treatment for 8 h. BIU-87 and C2C-12 cells were stained with CFSE (2.5 µM) for 10 min at 37°C and resuspended in RPMI 1640 medium at a concentration of 1 x 10^6 cells·mL^{-1}·$well^{-1}$ on 24-well plates, followed by stimulation by SWNT (5 µg/mL, 10 µL), THP (5 µg/mL, 10 µL), SWNT-THP (5 µg/mL, 10 µL) for 8 h. The cells were collected and cytotoxicity was analyzed by a FACScan flow cytometer. FACS data analysis was performed using the CellQuest software. BIU-87 = human bladder cancer cell line; C2C-12 = human myofibroblast cell line; SWNT = single-walled carbon nanotubes; THP = pirarubicin; SWNT-THP = single-walled carbon nanotubes and pirarubicin conjugate. *P = 0.009 compared to THP; +P = 0.036 compared to C2C-12 (*t*-test).

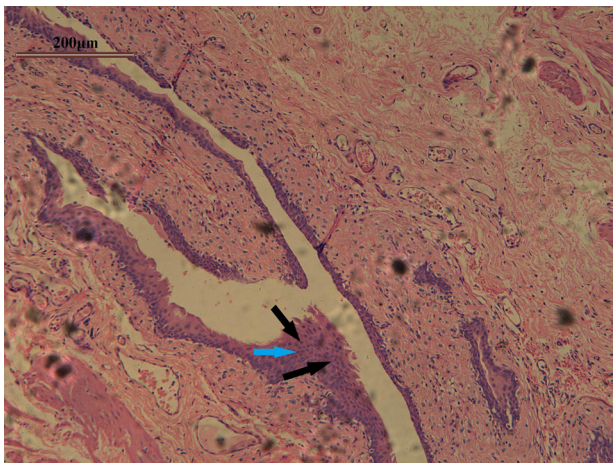

Figure 2. Histology of a rat bladder model (HE, 200X). At the end of 9 weeks after N-methyl-N-nitrosourea installation, rat bladder mucosa showed multicenter carcinogenesis (blue arrow). Disordered mucosa cells were evident (black arrows). Magnification bar = 200 µm.

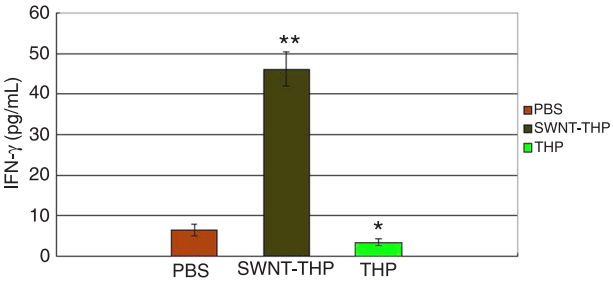

Figure 3. SWNT enhanced the proliferation of lymph cells and the release of IFN-γ. Eight hours after intravesical installation of saline (10 µL), SWNT (5 µg/mL, 10 µL), THP (5 µg/mL, 10 µL), and SWNT-THP (5 µg/mL, 10 µL) rat blood was harvested from the tail vein. T cells were isolated from PBMC and then cultured in RPMI 1640 medium with 10% fetal calf serum. The supernatants were collected and the release of IFN-γ were determined by ELISA. THP solution could hardly induce IFN-γ release; PBS group could induce IFN-γ release. However, the SWNT-THP induced the most significant IFN-γ release. PBS = phosphate-buffered saline; SWNT-THP = single-walled carbon nanotubes and pirarubicin conjugate; THP = pirarubicin; IFN-γ = interferon-γ. *P < 0.05, PBS group compared to SWNT-THP group (one-way ANOVA). **P < 0.05, THP group compared to SWNT-THP group (one-way ANOVA).

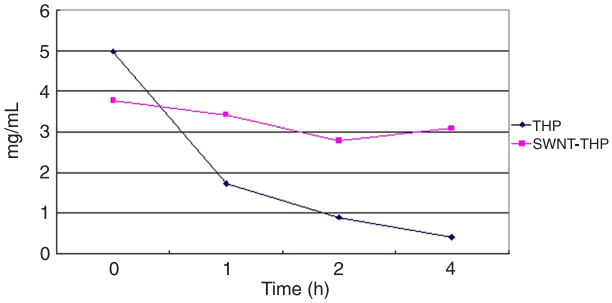

Figure 4. Retention of THP in the bladder after administration of THP or SWNT-THP. Urine samples were harvested from the bladder with a syringe from tumor-bearing rats at 0, 1, 2, and 4 h. Urine samples were then put into cuvettes at room temperature. THP concentration was measured with a 721 spectrophotometer using absorbance at 395 nm as soon as the samples were harvested. The figure shows that THP concentration was higher in the THP group at 0 h (purple curve). However, THP concentration remained high in the SWNT-THP group at 1, 2, and 4 h, whereas it rapidly decreased in the THP group. The findings indicate slow release of THP in the SWNT-THP group. THP = pirarubicin; SWNT = single-walled carbon nanotubes.

Observation of side effects

Throughout the month of intravesical instillation, 12 rats in the THP group experienced urinary frequency and 7 rats suffered from vomiting. In contrast, neither side effect was observed in the SWNT-THP group. Serum biochemical tests of hepatic and renal function revealed no significant changes after SWNT-THP treatment.

Discussion

Bladder cancer is the most common genitourinary malignancy in China. Although transurethral resection of bladder tumor is an effective therapy, up to 45% of patients experience a recurrence within 1 year after this procedure alone. Furthermore, there is a 3-15% risk of tumor progression to muscle invasion and/or metastatic cancer (16). Therefore, intravesical therapy, which includes intravesical chemotherapy and immunotherapy (1), has become an important part of bladder tumor treatment. The drugs commonly used in intravesical chemotherapy include mitomycin, doxorubicin, epirubicin, and THP (17-20), while the drugs used for intravesical immunotherapy are bacillus Calmette-Guérin vaccines. We selected THP for the present study because its prophylactic effect is greater than that of mitomycin and epirubicin (21,22).

However, the limited water solubility and the side effects of THP in intravesical chemotherapy limit its clinical application. Consequently, we activated THP by adding a carboxyl acid group from succinic anhydride. The carboxyl acid group conjugated to THP was linked to SWNT-PEG through a cleavable ester. The SWNT-THP conjugate exhibited high solubility and stability in saline. In addition, SWNT-THP significantly increased the inhibition of proliferation of the human bladder cancer cell BIU-87 *in vitro* compared to THP. We also found that the SWNT-THP conjugate improved the efficacy of *in vivo* treatment, as indicated by its ability to markedly induce apoptotic cells in rats in the SWNT-THP group. The side effects of intravesical THP instillation include pain with micturition, urinary frequency, macroscopic hematuria, and hematologic and renal toxicity (23). Interestingly, no side effects were observed in the SWNT-THP treatment.

The high anti-tumor effect of SWNT-THP conjugate could be attributed to the high tumor uptake of THP afforded by the functional SWNT-based drug carrier. High uptake of THP in an SWNT-based drug carrier is due to prolonged bladder retention time and EPR effects (24). In our investigation, we found that THP has poor solubility in saline and has a short bladder retention time. Little THP remained in the bladder after 1 h of intravesical chemotherapy at a dose of 5 mg/kg. THP carried by SWNT was present at high concentrations even 4 h after injection. SWNT-THP is highly water soluble, thus enabling full contact of THP with tumor tissue.

SWNT with a mean length of ~150 nm as used in drug delivery exceeded the threshold for renal clearance (25), a fact that makes it a typical drug delivery carrier in the blood circulation (26). However, as a drug vehicle used in intravesical therapy, the hydrophobicity of SWNT leads to increased nonspecific protein absorption on nanotube conjugations. This process greatly affects macrophages in the reticuloendothelial system (27). In addition, SWNT could penetrate tumor vessel walls (14). In the present study, we found that SWNT could cause aggregation of lymph cells, which is a promising property for the absorbance of the SWNT-THP conjugate.

High tumor toxicity suggests high uptake of THP by tumor tissue through the SWNT-based drug carrier. Furthermore, the presence of few side effects suggests low uptake of the SWNT-THP conjugate by normal tissue. The high uptake of the SWNT-THP conjugate by reticuloendothelial system organs such as the liver could harm these organs. In the present study, the SWNT-THP conjugate induced more apoptotic cells in bladder cancer tissue than in normal bladder tissue. We did not observe obvious liver toxicity. These results suggest that rapid THP release from the SWNT-THP conjugate through cleavage of the ester occurs *in vivo*.

SWNT exhibits promise for drug delivery. SWNT could increase the apparent solubility of poorly water-soluble drugs, thus ensuring that the chemotherapeutic drugs come into full contact with tumor tissue. In addition, SWNT could efficiently decrease side effects. Thus, SWNT has great potential as a tumor-targeted drug carrier cancer therapy. The application of SWNT-based drug carriers in intravesical therapy of rat bladder cancer may pave the way for further exploration of biomedical applications of SWNT in bladder cancer therapy in the future.

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