Anti-androgen therapy with Hydroxyflutamide or androgen receptor degradation enhancer ASC-J9® enhances BCG efficacy to better suppress bladder cancer progression

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Running title: ASC-J9® with BCG therapy to better suppress bladder cancer

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Abstract

Recent studies suggested that the androgen receptor (AR) might play important roles to influence the bladder cancer (BCa) progression, yet its clinical application remains unclear. Here we developed a new combined therapy with Bacillus Calmette-Guérin (BCG) and the AR degradation enhancer ASC-J9® or anti-androgen hydroxyflutamide (HF) to better suppress the BCa progression. Mechanism dissection revealed that ASC-J9® treatment enhanced BCG efficacy to suppress BCa cell proliferation via increasing the recruitment of monocytes/macrophages that involved the promotion of BCG attachment/internalization to the BCa cells through increased integrin-α5β1 expression and IL-6 release. Such consequences might then enhance BCG-induced BCa cell death via increased TNF-α release. Interestingly, we also found ASC-J9® treatment could directly promote BCG-induced HMGB1 release to enhance the BCG cytotoxic effects for the suppression of BCa cell growth. In vivo approaches also concluded that ASC-J9® could enhance the BCG efficacy to better suppress the BCa progression in BBN-induced BCa mouse models. Together, these results suggest that the newly developed therapy combining BCG plusASC-J9® may become a novel therapy to better suppress BCa progress.
Introduction

Bladder cancer (BCa) has a high mortality with an estimated 74690 new cases and 15580 deaths in U.S. in 2014(1). Most BCa patients receive the complete transurethral resection of their tumor followed by intravesical instillation of anti-tumor agents, including Bacillus Calmette-Guerin (BCG) that represents a very successful adjuvant agent for treating non-invasive BCa (2,3). However, the BCG therapy still has its limitations: approximately 30% of patients treated with intravesical BCG failed to respond, and for those patients with initial positive response, many suffered the high rate of recurrence (4). Recently significant progress has been made toward understanding the mechanism of BCG anti-tumor response. With respect to the local response to BCG, growing data support a critical role for BCG/tumor interaction in initiating and directing the downstream antitumor response. By the combination of local effects and a systemic immune response, BCG therapy might have a remarkable anti-tumor effect.

Interestingly, a recent study suggested the potential linkage of androgen receptor (AR) with BCa initiation and progression (5), and another study also indicated that androgens might be able to modulate IL-6 to alter BCG interaction with BCa cells (6).

Here we found a new potential therapy with combination of BCG and anti-androgen hydroxyflutamide (HF) or AR degradation enhancer ASC-J9® better suppressed BCa progression (7-9).

Materials and Methods

Reagents. The compound ASC-J9® was a gift from AndroScience Corp. BCG, a lyophilized preparation containing 10.5±8.7×10^8 colony forming units (CFU), was re-suspended in PBS. HF was obtained from Sigma. Polyclonal antibodies against F4/80 and integrin-α5β1 were from Abcam, and BrdU was from BD. The liquid DAB+ substrate chromogen system-horseradish peroxidase used for immunocytochemistry was obtained from Dako Cytomation. Penicillin, streptomycin, MEM, and fetal bovine serum (FBS) were obtained from Invitrogen. Tris, glycine, NaCl, SDS, bovine serum albumin, and monoclonal antibody against β-actin were obtained from Sigma.
Cell lines and culture conditions. The T24 cell line was a gift from Dr. Timothy Ratliff, Greenville, WI, USA in 2009 and cultured in Hyclone McCoy’s 5A media supplemented with 10% FBS. The human BCa cell line 253J was generously provided by Dr. Colin P.N. Dinney, The University of Texas, Houston, TX, USA in 2010 and cultured in T media supplemented with 10% FBS. The MB49 cell line was a gift from Dr. Ron Wood, University of Rochester Medical Center, Rochester, NY, USA in 2009. The human THP-1 cell line was obtained from the American Type Culture Collection in Sep 10, 2009 and cultured in RPMI 1640 supplemented with 10% FBS, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin. All cell lines were authenticated and tested by PCR when we received them, and then were expanded and frozen down in several aliquots. Each aliquot was thawed and used for no more than six months.

Animals. FVB female mouse were obtained from the Jackson Labs. The animals were housed four per cage in a specific pathogen-free animal facility and fed with regular chow diet with water ad libitum. Animal protocols/usage was approved by the University of Rochester Committee on Animal Resources, and the mice were kept in a specific pathogen-free environment at the animal facilities of the University of Rochester Medical Center.

DNA extraction, RNA extraction, RT-PCR and Quantitative Real-Time PCR (Q-PCR)
For RT-PCR, 1μg of total RNAs were reverse-transcribed using the iScript synthesis kit (Bio-Rad Laboratories, c, s), according to the manufacturer’s protocol. The sequence of primers used in the PCR. RT-PCR and qPCR were as followed: human β-actin: 5'-ATCTGGCAC CAC ACC TTC TA-3' (sense) and 5'- CGT CAT ACT CCT GCTTGC TG -3' (antisense); human GAPDH: 5'-GCTCTCCAGAACATCATCC-3'(sense) and 5'-TGCTTCACCACCTTCTTG-3'(antisense); human integrin-α5 5'-CCT GGTC TGAT TAC TGC-3'(sense) and 5'-GTC GGG GGC TTC AAC TTA GAC-3'(antisense); human integrin-β1: 5'-TTA TTG GCC TTG GAT TAC TGC-3'(sense) and 5'-CCA CAG TTG TTA CGG CAC TCT-3'(antisense); BCG:5'-CCT GCG AGC GTA GGC GTC GG-3'(sense) and 5'-CTC
GTC CAG CGC CGC TTC GG-3' (antisense); human Interleukin 6: 5'-AAG CCA GAG CTG TGC AGA TGA GTA-3' (sense) and 5'-TGT CCT GCA GCC ACT GGT TC-3' (antisense).

**MTT assay.** The anti-proliferative effects of HF and ASC-J9® on BCG against BCa cell lines 253J and T24 were determined by MTT dye uptake method.

**BCG attachment and internalization assay**

We used PCR to detect BCG internalization and attachment to PCa cells. After incubation and washing out non-attached, excess BCG, the cells monolayer was washed twice with Hanks BSS (Gibco,). Cells were then harvested using Cell Disassociation Solution (Sigma). Genomic DNA was extracted according to procedure of the Dneasy Blood and Tissue kit (QiaGen). The BCa cell lines were each plated at a density of $10^5$ cells/well in 6-well plates (Costar, c, s) and pre-treated with 5μMASC-J9® or HF, then incubated at 37°C in complete culture media overnight. The following day $5 \times 10^6$ cfu BCG was added to each well, and placed at 37°C for 2 hours. At the time points indicated, the culture media with non-attached BCG was aspirated and the cell monolayer washed twice with Hanks BSS (Gibco). Cells were then harvested using Cell Disassociation Solution (Sigma). Parental 253J and T24 cells were incubated with a concentration of 50 cfu BCG per cell for 2 hours and compared to the negative threshold. Tests were performed in duplicate and repeated on a subsequent day.

**Migration assay.** 5$\times 10^4$ BCa cells were seeded into the bottom well (5μm pore size transwell, Corning Incorporated) treated with HF and ASC-J9® for 12 hours, treated with BCG for 2 hours. Then the excess BCG was removed and $4 \times 10^5$ THP-1 cells placed into the upper transwell, incubated for 2 hours, then we collected the THP-1 cells migrated into the bottom wells for counting.

**N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-Induced Mouse Bladder Cancer Model**

12 weeks old FVB female mice (Jackson Lab) were supplied ad libitum with tap water containing 0.05% BBN (TCI America, Portland, OR) in opaque bottles for 12 weeks and thereafter with tap water without BBN. The drinking water was prepared fresh twice a week,
and consumption was recorded to estimate BBN intake. Then mice were divided into 4 groups (10 mice/group) as soon as urine blood tested positive, and treated with 1) ETOH control, 2) BCG (2×10⁶ CFU/mouse, intravesical injection weekly) alone, 3) ASC-J9® (75 mg/kg body weight, every other day, IP) alone and 4) BCG (2×10⁶ CFU/mouse, intravesical injection weekly) + ASC-J9® (75 mg/kg body weight, every other day, IP). Mice were then sacrificed 48 hours after the 4th treatment and bladders were collected for further examination.

**Immunohistochemical analysis for F4/80, and BrdU in tumor tissues.** The expression of F4/80 and BrdU were evaluated using an immunohistochemical method described previously (10). The antibodies used were anti-F4/80 (Abcam) and anti-BrdU (BD). Results were expressed as percentage ± SE of positive cells per ×200 magnification field. A total of 5 × 200 fields was examined and counted from each group.

**Statistics.** Data are presented as mean ± SEM of at least 3 independent biological replicates. The values were compared using Student's t test (two-tailed). We considered a P-value inferior to 0.05 as significant.

**Results**

**ASC-J9® and HF enhance BCG attachment/internalization to better suppress the BCa cells.**

Early reports suggested that BCG was able to function through attachment/internalization into BCa urothelial cells to alter the immune responses to exert its chemotherapeutic effect (11-13). Other studies also indicated that AR might play important roles to influence the BCa progression (5). We therefore decided to use two BCa urothelial cell lines (T24 and 253J) to investigate the potential impacts of the anti-androgen HF and AR degradation enhancer ASC-J9® (Fig. 1A, structure) on the BCG therapeutic effect via studying their influence on BCG attachment/internalization to BCa cells. We first applied PCR to detect BCG internalization in BCa urothelial cells and found addition of either 5 μM HF or 5 μM ASC-J9® increased BCG internalization near 2 fold (Fig. 1B&C).

As early reports suggested that the fibronectin-integrin-α5β1 complex functioned as a
bridge complex to promote BCG attachment/internalization to BCa cells (14), we then estimated the integrin-α5β1 expression to examine its influence on BCG attachment/internalization to BCa urothelial cells. The results from Fig. 1D&E clearly demonstrated HF or ASC-J9® enhanced significantly the integrin-α5β1 mRNA expression in the two BCa urothelial cell lines.

We further applied the interruption approach with integrin-α5β1 antibody to see if neutralization of integrin-α5β1 could interrupt the HF or ASC-J9® enhanced BCG attachment/internalization to BCa urothelial cells, and results showed neutralization of integrin-α5β1 could reduce the ability of the HF or ASC-J9® enhanced BCG attachment/internalization to BCa urothelial cells (Fig. 1F&G).

Together, results from Fig. 1B-G demonstrated that HF or ASC-J9® could enhance BCG attachment/internalization to BCa urothelial cells via induction of integrin-α5β1 expression.

ASC-J9® and HF treatment increases IL6 expression to enhance the recruitment of monocytes/macrophages to the BCa cells.

To study the consequences after enhanced BCG attachment/internalization to BCa urothelial cells following treatment with HF or ASC-J9®, we then applied the co-culture system to examine if ASC-J9® or HF could affect BCG-induced immune responses in BCa cells as early reports suggested that BCG suppressed BCa progression was linked to the recruitment of immune cells including monocytes/macrophages (15). We seeded BCaT24 (or 253J) cells in the bottom chambers and monocytes/macrophage THP-1 cells on the top chambers (Fig. 2A), and co-cultured cells were treated with BCG, or BCG with HF or ASC-J9® for two hours. As shown in Fig. 2B&C, addition of BCG increased THP-1 cells migration to BCa cells, and addition of HF or ASC-J9® further increased significantly the THP-1 cells migration to BCa cells.

To dissect the mechanisms at the molecular level by which HF or ASC-J9® could enhance BCG efficacy to promote monocytes/macrophages migration toward BCa cells, we then examined the altered immune cytokines expression and found the expression of IL-6 increased after co-culture of THP-1 cells with T24 or 253J cells, and addition HF or ASC-J9® further enhanced IL-6 expression in both T24 and 253J cells (Fig. 2C-G). These results were in
agreement with an early report showing IL-6 promotes monocytes/macrophages migration to BCa cells (16)

Together, results from Fig.2A-G proved that HF or ASC-J9® could enhance IL6 expression in the BCa cells that recruits more monocytes/macrophages to the BCa cells.

More recruited monocytes/macrophages to BCa cells leads to more TNF-α secretion to kill more BCa cells

We then asked what the impacts of recruiting more monocytes/macrophages to BCa cells. Early studies suggested that BCG might function through recruitment of macrophages to suppress BCa that involved the release of soluble cytotoxic factors, including TNF-α, IFN-γ and nitrous oxide (17). We first demonstrated that addition of BCG to the THP-1 cells released more TNF-α compared to THP-1 only and importantly, adding ASC-J9® or HF released even more TNF-α compared to BCG only (Fig.3A). Importantly, interruption approach with addition of anti-TNF-α antibody also interrupted monocytes/macrophages/BCG/HF- or monocytes/macrophages/BCG/ASC-J9® induced BCa cell viability (Fig. 3B&C).

Together, results from Fig. 3A-C suggest that HF or ASC-J9® enhanced BCG efficacy to suppress more BCa cells might function through recruiting more monocytes/macrophages that involve the increase of TNF-α release.

ASC-J9® or HF enhances BCG efficacy to suppress BCa progression via High Mobility Group Box 1 (HMGB1) release

In addition to enhancing BCG efficacy via recruitment of more monocytes/macrophages to suppress BCa cells, we also examined the potential BCG direct intra-cellular cytotoxic effect after attachment/internalization into BCa urothelial cells. We first demonstrated that without co-culture with THP-1 cells, BCG alone (for 2 hours in every 48 hours) could suppress BCa cell proliferation in 4 different BCa cell lines (3 human cell lines, TCCSUP, T24, and 253J and 1 mouse cell line, MB49) and that BCG plus 5μM HF or 5μM ASC-J9® treatment further suppressed cell proliferation significantly (Fig. 4A-D).

To dissect the potential mechanism, we examined HMGB1 expression as an early report
suggested HMGB1 release from BCa could function as a paracrine factor to potentiate the direct cellular effects of BCG (18). Our results suggested that addition of BCG increased HMGB1 release from BCa cells and addition of HF or ASC-J9® further enhanced BCG-induced HMGB1 release (Fig. 4E).

Together, results from Fig. 4A-E demonstrated that HF or ASC-J9® could also enhance the BCG intra-cellular efficacy to suppress BCa cell proliferation that might involve the alteration of the HMGB1 release.

**ASC-J9® enhances the BCG efficacy to suppress BCa development in BBN-induced mouse BCa model.**

We then applied BCa mouse model to prove our above in vitro cell lines findings. 12-weeks-old FVB female mice were divided into 4 groups (10 mice/group), treated with BBN (0.05% in drinking water) for 12 weeks, each group of mice were then injected with 1) vehicle control, 2) BCG alone (2 × 10^6 cfu/mouse, intravesical injection weekly for 28 days), 3) ASC-J9® alone (75 mg/kg body weight, every other day for 28 days, IP) and 4) BCG (2 × 10^6 CFU/mouse, intravesical injection weekly for 28 days) + ASC-J9® (75 mg/kg body weight, every other day for 28 days, IP). Mice were then sacrificed 24 hours after 4th injection and bladders collected for further examination.

Using HE staining (Fig. 5A), we found mice receiving vehicle injection developed bladder papilloma and carcinoma in situ, and BCG treated mice developed less hyperplasia with little carcinoma. Importantly, mice injected with both BCG plus ASC-J9® developed little hyperplasia, suggesting ASC-J9® indeed enhanced BCG efficacy to prevent BCa development in the BBN-induced BCa mouse model.

We also applied BrdU staining to assay the proliferation in these BBN-induced BCa mouse model and found mice treated BCG alone or ASC-J9® alone had less BrdU staining than those mice receiving vehicle control, and mice treated with both BCG and ASC-J9® had least BrdU staining (Fig. 5B&D), which again confirmed the above findings showing ASC-J9® could enhance BCG efficacy to suppress BCa cell proliferation in the BBN-induced BCa mouse model.
Importantly, we examined the recruitment of monocytes/macrophages in this BBN-induced BCa mouse model with anti-macrophage F4/80 antibody and found BCG alone helps to recruit more monocytes/macrophages to BCa, and BCG plus ASC-J9® recruited many more monocytes/macrophages to BCa (Fig. 5C&E), which is in agreement with above in vitro co-culture system (Fig. 2) showing ASC-J9® could enhance BCG to recruit more monocytes/macrophages to BCa cells. Together, results from 2"nd BCa mouse model (Fig. 5C-E) clearly demonstrated that ASC-J9® could enhance BCG efficacy to suppress BCa development.

Discussion

BCG therapy has been used successfully to treat non-muscle invasive BCa since 1976(2, 19, 20). However, even BCG is quite effective, approximately 30% of BCa patients treated with intravesical BCG fail to respond [6], and many patients have higher rate of recurrence and/or progression within five years(21,22).How to improve the BCG efficacy to reduce the recurrence of BCa is therefore clinically important and essential to extend the BCa patients survival.

Understanding the mechanism of BCG is critical for improving the efficacy of therapy. Presently, it is generally assumed that the BCG-induced anti-tumor activity is critically dominated by a local nonspecific immunological reaction reflecting the activity of immunocompetent cells. After instillation, the BCG binds to fibronectin that expressed on the urothelium, which could then be internalized by the urothelial cells including cancer cells (23).Subsequently, BCG antigens can be presented at the cell surfaces of urothelial cells in the context of major histocompatibility complex (MHC) class II, which may then stimulate the CD4+ T cells and induce a primary T helper type 1 immune response (24). This complex and robust immune reaction evoked by BCG is evidenced by a massive transient secretion of cytokines in voided urine, including interleukin (IL)-1, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, IL-15, and IL-18, as well as interference of inducible protein (IP)-10, TNF-α, granulocyte-monocyte colony stimulating factor (GMCSF), and interferon (IFN)-γ (25). The final step in the eradication of BCa cells is the activation of cytotoxic effector cells, such as the Natural Killer cells, monocyte/macrophage cells and other innate immune cells(26,27).
The linkage of androgen/AR signals to alter the BCG efficacy, however, remains unclear, and few studies suggest that targeting AR could be an alternative therapy to suppress BCa progression. Using an ARKO mouse model, Miyamoto et al. found androgen/AR signals might play important roles to promote BCa development/progression, and targeting AR with ASC-J9® suppressed BCa development/progression (5). See et al also reported that one of the cytokines elicited in response to BCG, specifically IL-6, up-regulates the cellular expression of integrin-α5β1, the receptor complex on which BCG adherence depends (4). They further confirmed that androgen could down-regulate NF-κB mediated IL-6 expression by human TCC lines (6). These studies suggest that alteration of the autocrine IL-6 response to BCG via pharmacological manipulation of the androgen milieu may have therapeutic value for the BCG antitumor treatment efficacy for transitional carcinoma.

In this paper we used two methods to block androgen/AR signaling. One is the anti-androgen compound, hydroxyflutamide, which is an active metabolite of flutamide approved by the FDA and inhibited androgen/AR signals by blocking androgen binding to AR. On the other hand, we used ASC-J9® to inhibit androgen/AR signaling by degrading AR in T24 and 253J cells (Supplemental Fig1. A&B).Based on clinical BCG intravesical therapy guidelines and a previously published paper, we used BCG only or combined with ASC-J9® to treat BCa cell or intravesical therapy in BBN-induced BCa mouse model both for two hours (28), and the results showed that the combination of BCG and HF or ASC-J9® better suppressed BCa progression.

Importantly, we proved here that ASC-J9® and HF could also enhance the BCG therapeutic efficacy to suppress BCa cell proliferation in the absence of infiltrating monocytes/macrophages (Fig. 4). Mechanism dissection suggested that BCG combined with HF or ASC-J9® induced more HMGB1 release from BCa cells compared with BCG only. These results are in agreement with recent studies showing HMGB1 release from BCa after BCG treatment could be one of the direct cellular effects of BCG.

Interestingly, in addition to the direct effect, other indirect mechanisms also indicate that BCG could enter into BCa via adherence to the luminal surface of the bladder to promote immunological responses that involve the recruitment of the infiltrating
monocytes/macrophages to suppress BCa (29). Importantly, using qPCR to assay the M1- vs M2-type of macrophage (30) during BCG treatment, we further found BCG could enhance the recruitment of M1-type of macrophage that led to suppress BCa cell proliferation (Supplemental Fig2.). This result is in agreement with early studies showing infiltrated M1-type macrophages may suppress tumor cells (31, 32).

ASC-J9® could enhance BCG immunotherapeutic effects via alteration of those key factors including IL-6, integrin-α5β1 and TNF-α, which is also in agreement with early studies showing ASC-J9® had high efficacy to suppress other AR-related tumors including prostate, kidney and liver (33-38). We also found similar effects when we replaced ASC-J9® with HF. This result not only further supports the above findings showing targeting androgen/AR signals can enhance BCG efficacy to suppress BCa, but may also provide a much easier and quicker therapeutic approach to develop a new combined therapy of BCG with HF. In contrast, more time and effort will be needed in the future to see if ASC-J9® can also pass human clinical trials to translate its in vitro and in vivo mouse effects into humans.

In summary, our findings showing the combined therapy of BCG with HF or ASC-J9® can lead to better therapy than BCG alone to suppress BCa progression may help patients to better battle BCa in the near future.

Acknowledgments
Conflicts of interest: ASC-J9® was patented by the University of Rochester, University of North Carolina, and AndroScience, and then licensed to AndroScience. Both the University of Rochester and C.C. own royalties and equity in AndroScience.

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Figure Legends

**Figure 1.** HF/ASC-J9® promotes BCG attachment/internalization through regulating the integrin-α5β1 pathway in BCa cells. (A) Structure of ASC-J9®. (B&C) We seeded 4x10⁵ T24 and 253J cells into plates. Cells were treated with 5μM HF and 5μM ASC-J9® for 12 h, then incubated with BCG (2x10⁷ CFU) for 2 h. Excess BCG was washed away and genomic DNA was extracted to perform PCR using BCG primers. (D&E) The T24 and 253J cells were treated as it in Fig.1 B&C, RNA was extracted, and integrin-α5β1 expression was determined using Q-PCR. (F&G) We seeded 4x10⁵ T24 and 253J cells into the plates. Cells were treat with 5μM HF and ASC-J9® for 12 h, then incubated with BCG (2x10⁷ CFU) and integrin-α5β1 neutralizing antibody for 2h. After incubation, excess BCG was washed out by 1xPBS 3 times and genomic DNA collected to perform PCR using BCG primers. Each experiment was performed in triplicate. *P<0.05(Student’s t test)

**Figure 2.** HF/ASC-J9® promotes monocyte migration toward BCG treated bladder cancer cells. (A) 5x10⁴BCa cells were seeded into the bottom chambers of transwells and treated with HF or ASC-J9® for 12 h, then BCG was added and incubated for additional 2 h. After washing out excess BCG, 4x10⁵ THP-1 cells were added into the upper chambers and then co-incubated with BCa cells for 2 h. The media of bottom chambers were collected to count the migrated THP1 cells. (B&C) THP-1 cell migration assay were detected in T24 and 253J cell. Each experiment was performed in triplicate. (D-G) HF and ASC-J9® increased BCG induced IL-6 release from BCa cells. We seeded 4x10⁵ T24 and 253J cell into the plates. Cells were treated with 5μM HF or 5μM ASC-J9® in the presence of 10nM DHT for 12 h, and then incubated with BCG(2 x 10⁷ CFU) for 2 h. After washing away excess BCG, mRNA was extracted from BCa cells to determine IL-6 expression using Q-PCR. Each experiment was performed in triplicate. *P<0.05(Student’s t test)

**Figure 3.** Monocytes/macrophages recruitment to BCa cells under BCG plus ASC-J9®/HF treatment lead to decreased proliferation of BCa cells through TNF-α production. (A) 10⁶THP-1 cells were cultured in 6-well plates, treated with BCG only, BCG+ASC-J9®, or BCG+HF for 48 h, Those 3 different conditioned media (CM) were collected to detect TNF-α production by Elisa.
(B&C) The above condition mediums were used to treat T24 and 253J cells, cell viability were detected by MTT. Each experiment was performed in triplicate. *P<0.05 (Student's t test)

**Figure 4** HF and ASC-J9® potentiates the antitumor effects of BCG *in vitro*. (A-D) Four different BCa cells were seeded in the 24-well plates, treated with BCG only, HF, ASC-J9®, or BCG plus HF or ASC-J9®, then collected the cells, then MTT assay were performed. (E) Above CM were collected and used to detect HMGB1 release by Elisa. *P<0.05 (Student's t test)

**Figure 5.** ASC-J9® potentiates the antitumor effects of BCG in BBN-induced mouse BCa model. 12 weeks old FVB female mice were divided into 4 groups (10 mice/group) and treated with 0.05% BBN in drinking water for 12 weeks. Mice were then injected with 1) vehicle control, 2) BCG (2×10⁶ CFU/mouse, intravesical injection weekly) alone, 3) ASC-J9® (75 mg/kg body weight, every other day, IP) alone and 4) BCG (2×10⁶ CFU/mouse, intravesical injection weekly) + ASC-J9® (75 mg/kg body weight, every other day, IP). Mice were then sacrificed 48 h after the 4th injection and bladders collected for further examination including (A) H&E section and (B) BrdU stain for the BCa cell proliferation. (C) We used monocytes/macrophages marker F4/80 antibody to assay monocyte/macrophage cells infiltrate. (D&E) The positive expression cells of BrdU and F4/80 were quantified in three random fields. Bule arrows mean positive cells. *P<0.05 (Student's t test)
Figure 1

A

B

C

D

E

F

G
Figure 3

A

THP-1 Cell

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<th>HF</th>
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B

THP-1/T24

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C

THP-1/2B3J

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Figure 4

A

TCCSUP

- Mock
- BCG
- BCG+HF
- BCG+ASC-J9

B

T24

- Mock
- BCG
- BCG+HF
- BCG+ASC-J9

C

253J

- Mock
- BCG
- BCG+HF
- BCG+ASC-J9

D

MB49

- Mock
- BCG
- BCG+HF
- BCG+ASC-J9

E

HMGBl level (ng/ml)

Mock
BCG
HF
ASC-J9

* * *
Figure 5

A

HE(200X)

B

BrdU(400X)

C

F4/80(200X)

D

E

Mock  BCG  ASC-J9  ASC-J9+BCG

Mock  BCG  ASC-J9

Mock  BCG  ASC-J9

Mock  BCG  ASC-J9

BrdU+ No/Field

F4/80+ No/Field

*
Molecular Cancer Therapeutics

Anti-androgen therapy with Hydroxyflutamide or androgen receptor degradation enhancer ASC-J9® enhances BCG efficacy to better suppress bladder cancer progression

zhiqun shang, yanjun li, minghao zhang, et al.

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