Intravesical dual PI3K/mTOR complex 1/2 inhibitor NVP-BEZ235 therapy in an orthotopic bladder cancer model

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Abstract. NVP-BEZ235 is an inhibitor of both phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin complex 1/2 (mTORC1/2), and its antitumor activity is expected to be higher than that of mTORC1 inhibitors because it inhibits the upregulation of pAkt through mTORC2. We examined the efficacy of intravesical NVP-BEZ235 therapy in the treatment of bladder cancer using an orthotopic bladder cancer model. The cytotoxic effects of various concentrations of NVP-BEZ235 in MBT-2 cells were examined using a WST assay. The expression of pAkt, pS6 and p4EBP1 was evaluated in MBT-2 cells treated with NVP-BEZ235 using western blotting. Orthotopic models were established by implanting MBT-2 cells into the bladders of female C3H/He mice. We assigned C3H/He mice to 2 groups: a control group treated with vehicle control (n=15), and a group intravesically administered 40 µM (18.78 mg/l) of NVP-BEZ235 (n=15). NVP-BEZ235 inhibited the viability of MBT-2 cells in a dose-dependent manner. Furthermore, the expression of pAkt, pS6, and p4EBP1 was inhibited in NVP-BEZ235-treated MBT-2 cells. Bladder weights were significantly lower in the NVP-BEZ235-treated group than in the control group (P<0.05). An analysis of the tumor tissues revealed that the NVP-BEZ235 treatment strongly reduced pAkt, pS6 and p4EBP1 levels. An immunohistochemical analysis showed that NVP-BEZ235 significantly inhibited the expression of pS6. Intravesically administered NVP-BEZ235 exerted significant antitumor effects in the orthotopic bladder cancer model by inhibiting the PI3K/Akt/mTOR pathway. The intravesical instillation of a dual PI3K/mTORC1/2 inhibitor may represent a novel therapy for the treatment of bladder cancer.

Introduction

Between 70 and 80% of bladder tumor cases are non-muscle-invasive tumors confined to the mucosa. Patients with this type of tumor undergo conservative treatment consisting of transurethral resection of the bladder tumor (TURBT) followed by intravesical adjuvant treatment (1,2). Intravesical immunotherapy with bacillus Calmette-Guerin (BCG) is the most effective adjuvant therapy for intermediate or high risk non-muscle-invasive bladder cancer (NMIBC) (3). However, the recurrence rate of NMIBCs is 60-70%, and 30% of recurrent tumors eventually progress to muscle invasive tumors (3). Moreover, the intravesical instillation of BCG can lead to non-specific inflammation in the bladder and sometimes result in side effects of sufficient severity to cause the treatment to be discontinued (4). Thus, new strategies and therapeutic agents with superior antitumor effects to those of conventional intravesical therapy with BCG are highly desirable for the treatment of NMIBC.

The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway has been linked to tumorigenesis in many tissues (5,6). The PI3K/Akt/mTOR pathway controls processes involved in tumor cell growth, proliferation and survival after DNA damage (7). The kinase mTOR consists of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which are two functionally distinct multiprotein complexes (8). mTORC1 inhibitors, such as RAD001, are known to have potent preclinical activities with respect to a wide variety of cancers, and RAD001 has been clinically approved as a treatment for renal cancer (9,10). In urothelial carcinoma (UC), preclinical studies have shown that mTORC1 inhibitors exhibit antitumor effects both in vitro and in vivo (11-14).

NVP-BEZ235 is a dual PI3K and mTORC1/2 inhibitor, the antitumor activity of which is expected to be higher than that of mTORC1 inhibitors in various human cancers because
it inhibits the upregulation of phospho-Akt (pAkt) through mTORC2 (15). Preclinical studies demonstrated the antitumor activity of NVP-BEZ235 in various models (16,17). However, the efficacy of dual PI3K/mTORC1/2 inhibitors has not yet been examined in bladder cancer in vivo.

The orthotopic bladder cancer model is effective for the evaluation of new intravesical agents against bladder cancer. In our model, a high incidence of bladder cancer was observed after the simple instillation of MBT-2 cell suspensions into the bladders of syngeneic hosts (18). IL-2, IL-12 and IL-15 intravesical gene therapies have previously been reported by our group using this model (19-21).

We here examined the cytotoxic effects of the dual PI3K and mTORC1/2 inhibitor NVP-BEZ235 in vitro and determined whether intravesically administered NVP-BEZ235 exhibited therapeutic effects in a MBT-2 murine orthotopic bladder cancer model through inhibition of the PI3K/Akt/mTOR pathway.

Materials and methods

Reagents. Rabbit monoclonal antibodies for pAkt (Ser473), phospho-S6 ribosomal protein (pS6) (Ser235/236), and phospho-4E-BP1 (p4E-BP1) (Thr37/46) were obtained from Cell Signaling Technology (Boston, MA, USA), and a mouse monoclonal antibody for β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA). NVP-BEZ235 was kindly provided by Novartis (Basel, Switzerland) via a material transfer agreement.

Cell line and animals. The MBT-2 murine bladder cancer cell line was provided by Dr T. Ratliff (University of Iowa, Iowa City, IA, USA). MBT-2 was established from N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide-induced urothelial carcinoma of the bladder removed from a female C3H/He mouse. Cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% heat-inactivated fetal bovine serum at 37˚C in a humidified 5% CO2 atmosphere. The orthotopic bladder cancer model is effective for the evaluation of new intravesical agents against bladder cancer. In our model, a high incidence of bladder cancer was observed after the simple instillation of MBT-2 cell suspensions into the bladders of syngeneic hosts (18). IL-2, IL-12 and IL-15 intravesical gene therapies have previously been reported by our group using this model (19-21).

We here examined the cytotoxic effects of the dual PI3K and mTORC1/2 inhibitor NVP-BEZ235 in vitro and determined whether intravesically administered NVP-BEZ235 exhibited therapeutic effects in a MBT-2 murine orthotopic bladder cancer model through inhibition of the PI3K/Akt/mTOR pathway.

Establishment of an orthotopic MBT-2 bladder cancer model. An orthotopic bladder cancer model was created by the intravesical implantation of MBT-2 cells, as previously described (18). Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (1.5 mg/200 µl) and mild pressure was applied to the abdomen to obtain urine samples evacuated from the bladder. MBT-2 cells (5x10^5 in a 50-µl suspension of serum-free RPMI-1640 medium) were injected into the bladder through a 24-gauge Teflon-coated catheter that had been introduced into the lumen of the bladder through the urethra. The catheter was then removed, and, to prevent the voiding of MBT-2 cells, the urethra was tied with 4-0 silk thread for 2 h. We previously investigated serial tumor growth in this orthotopic MBT-2 bladder tumor model and found that very small superficial tumors had been established 3 days after the implantation of MBT-2 cell suspensions and gradually grew into viable masses (18). All the procedures were tolerated well, and none of the mice died postoperatively.

Intravesical NVP-BEZ235 therapy in an orthotopic bladder cancer model. Regarding intravesical administration, 100 µl of PBS or 40 µM (18.78 mg/l) of NVP-BEZ235 in 100 µl of PBS was instilled transurethrally (n=15 mice per group). The NVP-BEZ235 and PBS instillation experiments were started 5 days after tumor implantation and were carried out 5 times at 3-day intervals (i.e., on days 5, 8, 11, 14 and 17). Transurethral injections were carried out under anesthesia induced by an intraperitoneal injection of pentobarbital sodium. All the surviving mice were sacrificed on day 21 and necropsied. To assess the growth of bladder tumors, the weights of the bladders were measured. Additionally, to assess the potential toxicity of intravesical NVP-BEZ235 therapy, we examined the histological appearances of the major organs, including the heart, lung, liver, and kidney. The serum concentrations of albumin, total bilirubin, creatinine, and alanine aminotransferase were also determined.
Analysis of MBT-2 tumor extracts from mice treated with NVP-BEZ235. A second set of in vivo experiments was performed to evaluate changes in the PI3K/Akt/mTOR pathway after the intravesical administration of NVP-BEZ235 in tumors. Mice treated with NVP-BEZ235 (40 µM, 18.78 mg/l) or vehicle control were sacrificed 12 h after the last dose (on day 18) (n=3 mice per group), whole tumor extracts in most parts of the bladder were obtained using RIPA buffer, and the protein expression of pAkt, pS6 and p4EBP1 was evaluated by western blotting, as described above. Furthermore, some of the tumors in the bladders of mice treated with NVP-BEZ235 (40 µM, 18.78 mg/l) or vehicle control were immunohistochemically evaluated for the expression of the pS6 protein. Formalin-fixed, paraffin-embedded sections (4 µm) were analyzed. Antigen retrieval with citric acid (pH 6.0) was carried out, and then endogenous peroxidase activity was blocked by a 20-min treatment with 0.3% hydrogen peroxide. Sections were incubated for 15 min in a blocking solution (Protein Block Serum-Free Ready-to-use, Dako) and were then incubated with an antibody specific for pS6 (dilution 1:100) (Ser235/236, D57.2.2E, Cell Signaling Technology) for 2 h. Slides were incubated for 30 min with secondary antibodies conjugated to a peroxidase-labeled dextran polymer. Visualization of the immunoreaction was achieved using diaminobenzidine followed by counterstaining with 10% hematoxylin.

Statistical analysis. All values are presented as the mean ± standard deviation (SD). The significance of differences between groups was assessed using ANOVA and/or the Student’s t-test, as appropriate. Significance was determined as P<0.05. These analyses were performed using IBM® SPSS® Statistics Version 21 (International Business Machines Corp., Armonk, NY, USA).

Results

Cytotoxic effects of NVP-BEZ235 on MBT-2 cells in vitro. We examined the cytotoxic effects of NVP-BEZ235 on MBT-2 cells in vitro (Fig. 1). After being exposed to NVP-BEZ235 at concentrations of 100, 250, 500 and 1000 nM for 12 h, the mean relative cell viabilities to vehicle were 60.4±9.1%, 50.0±5.3%, 40.4±7.5%, and 23.0±3.5%, respectively. The inhibition of cell growth was significantly greater in cells treated at any concentration of NVP-BEZ235 than in those treated with vehicle control after 12 h of exposure to NVP-BEZ235 in a dose-dependent manner. Equivalent or stronger cytotoxic effects were observed after 24 and 48 h of exposure to NVP-BEZ235 in a dose-dependent manner.

NVP-BEZ235 inhibited pAkt, pS6, and p4EBP1 in MBT-2 cells in vitro. To assess the inhibitory effect of NVP-BEZ235 on the PI3K/Akt/mTOR signaling pathway, we evaluated the protein expression of pAkt, pS6, and p4EBP1 in MBT-2 cells treated with or without NVP-BEZ235 by western blotting. The expression of pS6 in MBT-2 cells was significantly inhibited by NVP-BEZ235 at a dose of 100 nM or higher (Fig. 2A). The expression of pAkt and p4EBP1 was also significantly inhibited by NVP-BEZ235 in a dose-dependent manner (Fig. 2A). The expression of pS6, pAkt, and p4EBP1 was inhibited in cells exposed to NVP-BEZ235 for 12 h, and these inhibitory effects persisted for 24 h (Fig. 2B).
Therapeutic effects of intravesical NVP-BEZ235 in an orthotopic bladder cancer model. Five days after the intravesical implantation of MBT-2 cells, PBS (vehicle control) or NVP-BEZ235 (40 μM, 18.78 mg/l) was administered intravesically into mouse bladders on days 5, 8, 11, 14 and 17. All mice tolerated the 5 instillations of PBS or NVP-BEZ235 well. The mice were sacrificed on day 21, and the weights of their bladders were measured to assess the growth of bladder tumors. Bladder weights were significantly lower in the intravesical NVP-BEZ235 therapy in situ group than in the control group (P=0.0038).

Immunohistochemical analysis of tumor tissues. pS6 immunostaining on tumor tissue was evaluated in mice treated with NVP-BEZ235 or vehicle control. The expression of the pS6 protein was significantly lower in bladder tumor tissue treated with NVP-BEZ235 (40 μM) than in those treated with vehicle control (P=0.0038).

Staining with hematoxylin and eosin showed that there were no significant differences in the histological appearances of or damage to the major organs of the control or NVP-BEZ235–treated mice.

Analysis of orthotopic MBT-2 tumor tissues by western blotting. Mice treated with vehicle control or NVP-BEZ235 were sacrificed 12 h after the last dose. The tumors were then removed and analyzed using western blotting to determine the protein expression of pAkt, pS6, and p4EBP1. An analysis of the tumor tissues revealed that the NVP-BEZ235 treatment strongly reduced the expression of pAkt, pS6 and p4EBP1.

The serum concentrations of albumin, total bilirubin, creatinine, and alanine aminotransferase on day 21 were all within normal ranges in the 2 groups, which indicated that intravesical NVP-BEZ235 therapy did not result in any systemic side effects. We also analyzed the histological appearances of the major organs, including the liver, kidneys, heart and lungs.
with NVP-BEZ235 than in the tissue treated with vehicle control (Fig. 5).

Discussion

In the present study, the cytotoxic effects of NVP-BEZ235 were observed in MBT-2 tumor cells in vitro through the inhibition of pAkt protein expression, as well as the downstream targets of pS6 and p4EBP1. Furthermore, the intravesical NVP-BEZ235 therapy had significant inhibitory effects on tumor growth in the MBT-2 orthotopic bladder cancer model. An analysis of tumor tissues by western blotting revealed that the NVP-BEZ235 treatment strongly reduced the expression of pAkt, pS6, and p4EBP1. An immunohistochemical analysis revealed that the expression of pS6 was significantly inhibited by the intravesical instillation of NVP-BEZ235. These results indicated that targeting the PI3K/Akt/mTOR pathway with NVP-BEZ235 may represent a novel therapeutic modality for NMIBC.

The PI3K/Akt/mTOR pathway has attracted significant interest among UC researchers because the clinical importance of members of the PI3K/Akt/mTOR signaling pathway in UC has been demonstrated in previous studies (22-26). The expression of various PI3K/Akt/mTOR signaling pathway members including p-mTOR, pAkt and p4EBP1 was shown to be higher in UCs than in normal urothelia (24,25). Korkolopoulou et al (24) reported that the cytoplasmic expression levels of p-mTOR and pAkt in UCs were higher than those in normal urothelia. Several studies have also shown that the expression of various PI3K/Akt/mTOR signaling pathway members (p-mTOR, pS6 and pAkt) correlated positively with higher grades and/or stages (23,25,26). Sun et al (26) demonstrated that the expression of pS6 and pAkt correlated positively with higher grades in 887 cases of bladder cancer. Furthermore, recent studies reported that the expression levels of p-mTOR, pS6, and p4EBP1 were independent prognostic factors of bladder cancer-specific survival (CSS) (23-26). The strong expression of pS6 was previously shown to predict shorter recurrence-free survival, especially in patients with NMIBC (22,25). These findings indicated that upregulating the PI3K/Akt/mTOR pathway may be strongly associated with tumor aggressiveness, invasion and survival in patients with UC.
Previous preclinical studies reported that rapamycin and its derivative RAD001, reduced cancer cell viability and decreased the expression of PI3K/Akt/mTOR signaling pathway members such as p-mTOR, pS6, p4EBP1 and p-p70S6K in bladder cancer cell lines (11-14). The effects of inhibiting mTORC1 on bladder tumor growth have also been reported in a xenograft mouse model (11,13,14). Only one previous study on intravesical mTOR inhibitor therapy used the mTORC1 inhibitor rapamycin (27). They demonstrated that this therapy successfully suppressed bladder tumorigenesis, and subsequently concluded that the inhibition of mTOR signaling may have potential therapeutic benefits for patients at high risk of developing invasive bladder cancer. However, Vasconcelos-Nóbrega et al (28) recently reported that RAD001 only exerted modest effects in a chemically-induced UC animal model. Furthermore, clinical phase II trials on metastatic UC using temsirolimus, an inhibitor of mTORC1, failed to demonstrate any clinical activity (29). These controversial findings for mTORC1 inhibitors suggest the existence of mechanisms that result in rapamycin resistance. mTORC2 is considered to be regulated by rapamycin and activates the Akt signal pathway. This Akt-driven feedback loop has been identified as one of the mechanisms by which resistance to mTORC1 inhibitors develops (30,31). Thus, the inhibition of not only mTORC1, but also mTORC2 may be required to achieve a significant clinical effect (17).

mTORC2 is known to control processes that involve cytoskeletal remodeling, such as cell spreading and migration, in several cancers including UC (32). One study demonstrated that increased mTORC2 activity was associated with bladder cancer invasion that was primarily mediated through Rac1 as a major downstream target of mTORC2 (32). In the present study, we speculated that the dual inhibition of PI3K and mTORC1/2 may have strong therapeutic effects on bladder tumors in vivo and, thus, investigated the inhibitory effects of NVP-BEZ235, which is a novel dual PI3K and mTORC1/2 inhibitor, on orthotopic bladder tumors through an intravesical administration protocol. Several preclinical studies have reported promising activities for NVP-BEZ235 against various tumors (16,17), and various phase 1/2 clinical trials on NVP-BEZ235 as a breast cancer treatment are now being performed in different countries. We demonstrated that NVP-BEZ235 effectively blocked the PI3K/Akt/mTOR pathway and reduced tumor growth in the MBT-2 orthotopic bladder cancer model. To the best of our knowledge, intravesical dual PI3K and mTORC1/2 inhibitor therapy has not previously been assessed using an orthotopic bladder cancer model. Although no clinical trial has examined the effects of NVP-BEZ235 in UC patients, the experimental data generated in the present study strongly support the potential clinical application of this strategy in the future.

It is important to recognize that when tumor cells are removed from their natural milieu, they behave differently from those in the organ of origin (33,34). The orthotopic bladder cancer model used in the present study closely mimics the clinical situation, and has consequently helped to shed light on the mechanisms involved, which is a prerequisite for drawing meaningful conclusions (35). Various orthotopic bladder cancer models have been described; however, limitations have been associated with all these models, including low rates of tumor implantation and damage to the urothelium as a result of electrocautery or the application of chemical agents to facilitate tumor uptake, which induces the tumors to become invasive (36,37). The incidence of tumor establishment was high in our model following the simple instillation of MBT-2 cell suspensions into the bladders of syngeneic hosts (18). Our model was easily established by different investigators working in different institutions, is highly reproducible, and avoids the need for any open surgical procedures for implantation. The model can also be used to evaluate new intravesical agents against bladder tumors in situ. IL-2, IL-12 and IL-15 intravesical gene therapies have previously been examined by our group using this model (19-21).

Although the toxicity of NVP-BEZ235 remains unknown, the systemic absorption of agents rarely occurs through intravesical administration. The intravesical instillation of biological and chemotherapeutic agents facilitates the direct access of these agents to the bladder mucosa and tumor (38). Therefore, a distinct benefit of this localized approach is that agents can be used at high doses with a minimal risk of systemic adverse effects (39). The intravesical NVP-BEZ235 therapy did not cause any systemic side effects in our study.

In conclusion, we herein demonstrated that intravesically administered NVP-BEZ235 therapy exhibited therapeutic effects in the MBT-2 orthotopic bladder cancer model by inhibiting the PI3K/Akt/mTOR signaling pathway. This administration route is unique. The results obtained in the present study provide molecular-level evidence that supports the clinical use of a dual PI3K and mTORC1/2 inhibitor and may indicate that this is a useful approach for treating patients with bladder cancer.

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References


