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The proteasome inhibitor bortezomib induces testicular toxicity by upregulation of oxidative stress, AMP-activated protein kinase (AMPK) activation and deregulation of germ cell development in adult murine testis

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ABSTRACT

Understanding how chemotherapeutic agents mediate testicular toxicity is crucial in light of compelling evidence that male infertility, one of the severe late side effects of intensive cancer treatment, occurs more often than they are expected to. Previous study demonstrated that bortezomib (BTZ), a 26S proteasome inhibitor used to treat refractory multiple myeloma (MM), exerts deleterious impacts on spermatogenesis in pubertal mice *via* unknown mechanisms. Here, we showed that intermittent treatment with BTZ resulted in fertility impairment in adult mice, evidenced by testicular atrophy, desquamation of immature germ cells and reduced caudal sperm storage. These deleterious effects may originate from the elevated apoptosis in distinct germ cells during the acute phase and the subsequent disruption of Sertoli–germ cell anchoring junctions (AJs) during the late recovery. Mechanistically, balance between AMP-activated protein kinase (AMPK) activation and Akt/ERK pathway appeared to be indispensable for AJ integrity during the late testicular recovery. Of particular interest, the upregulated testicular apoptosis and the following disturbance of Sertoli–germ cell interaction may both stem from the excessive oxidative stress elicited by BTZ exposure. We also provided the *in vitro* evidence that AMPK-dependent mechanisms counteract follicle-stimulating hormone (FSH) proliferative effects in BTZ-exposed Sertoli cells. Collectively, BTZ appeared to efficiently prevent germ cells from normal development *via* multiple mechanisms in adult mice. Employment of antioxidants and/or AMPK inhibitor may represent an attractive strategy of fertility preservation in male MM patients exposed to conventional BTZ therapy and warrants further investigation.

Introduction

The 26S proteasome is the enzymatic core engine of the ubiquitin and proteasome dependent proteolytic system (UPS), the major eukaryotic pathway for regulated protein degradation (Frankland-Searby and Bhaumik, 2012; Durairaj and Kaiser, 2014). Growing bodies of evidence have documented the essential role of UPS in divergent cellular events including control of cell cycle (Kabani et al., 2014), regulation of gene expression (Baker et al., 2008), responses to cellular stress (Livnat-Levanon et al., 2014), signal transduction and cell differentiation and apoptosis (Vaeteewoottacharn et al., 2013). Bortezomib (Velcade®, formerly known as PS-341 and LDP-341) is a boronate-based, dipeptidyl inhibitor of 26S proteasome that has been approved for use by the United States Food and Drug Administration to treat relapsed/refractory multiple myeloma (MM) that primarily targets the

Abbreviations: BTZ, bortezomib; UPS, ubiquitin and proteasome dependent proteolytic system; MM, multiple myeloma; i.p., intraperitoneally; T, testosterone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; SCs, radioimmunoassay (RIA) Sertoli cells; GCs, germ cells; AMPK, AMP-activated protein kinase; Vit C, Vitamin C; Vit E, Vitamin E; oFSH, ovine follicle-stimulating hormone; SABC, Streptavidin–biotin complex; AJs, anchoring junctions; TJs, tight junctions; ROS, reactive oxygen species; PKB/Akt, phosphorylated protein kinase B; PAK-2, p21-activated kinase-2; LCs, Leydig cells; PSR, proteotoxic stress response; MEHP, mono-(2-ethylhexyl) phthalate; TER, transepithelial electrical resistance.

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chymotrypsin-like activity of the intracellular proteasome enzyme complex (Murray et al., 2014). In a recent phase I clinical study, bortezomib (BTZ) was proved to be efficacious in relapsed childhood acute lymphatic leukemia when applied together with traditional chemotherapeutic drugs (Messinger et al., 2010).

Defining parameters and potential underlying mechanisms important for side effects is a key objective in oncologic care, thus an important concern for patients may be whether/how proteasome inhibition will impact other physiological functions and clinical outcome. To this end, serious adverse effects including neutropenia (Berenson et al., 2014), thrombocytopenia (Lin et al., 2014) and heart failure (Subedi et al., 2014) have so far been reported in BTZ-treated patients. One recent study demonstrated that BTZ treatment causes long-term gonadal dysfunction, such as increased apoptosis, testicular atrophy, hypospermatogenesis and reduced caudal epididymal sperm storage, *via* unknown pathways in pubertal mice (Hou et al., 2014). Hence, new drugs or drug combinations that act as potential adjuncts are required to achieve successful eradication of BTZ-induced side effects. Understanding the mechanisms of side effects may therefore lead to improved treatment.

Due to the high metabolism rate in testis, rapid and efficient elimination of apoptotic cells and degraded protein *via* ubiquitin–proteasome pathway is essential for the normal spermatogenesis to occur (Sheng et al., 2014). Regulatory molecules involved in apoptosis have been identified as important substrates of the proteasome (Kwon, 2007; Shimizu et al., 2014). In this context, the core components of ubiquitin–proteasome pathway are believed to be indispensable for testicular function. For example, gain- and loss-of-function analyses both suggest that deregulation of UCHL1, a deubiquitinating enzyme responsible for regenerating monoubiquitin from the ubiquitin–protein complex, exerts deleterious effects on germ cell death and development (Kwon et al., 2005; Wang et al., 2006; Du et al., 2014). Mice lacking the UBC4-testis gene exhibit delays in postnatal testis development (Bedard et al., 2005). Additionally, it has been suggested that ubiquitin–proteasome signaling also functions as a regulator of oxidative stress during spermatogenesis (Yu et al., 2008).

Because MM usually happens in adulthood (Dimopoulos et al., 2015), and testis is one of the most toxicant/drug sensitive organs, we reasoned that understanding BTZ-induced pathologies and their underlying mechanisms during adult spermatogenesis will better explain the diverse role that this proteasome inhibitor plays in various physiological systems, thereby benefiting the clinical treatment in future. In the present communication, we evaluated the potential impacts of BTZ treatment on adult murine spermatogenesis. We also provided *in vitro* evidences that may be related to the mechanistic foundation. Our results should provide novel insight into BTZ action as an irreplaceable first-line drug for MM treatment.

Material and methods

Ethics statement. The Ethics Committee for Animal Experiments of the Fourth Military Medical University approved all animal work and the experimental protocols strictly complied with the institutional guidelines and the criteria outlined in the "*Guide for Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1996)". All surgery was performed under sodium pentobarbital anesthesia [0.04–0.05 mg/g body weight, intraperitoneally (i.p.)], and all efforts were made to minimize suffering. In all experiments, mice were euthanized under diethyl ether anesthesia, followed by cervical dislocation.

Animal treatment. Sexually mature C57/BL6 male mice (3 months of age) were obtained from the Animal Research Center of the Fourth Military Medical University, Xi'an (China), they were fed *ad libitum*, and maintained under a constant 12 h light:12 h darkness cycle (lights on at 08:00 a.m.) and controlled conditions of humidity (between 70 and 80%) and temperature (22 ± 1 °C). They were allowed to acclimatize for at least 7 days before the experiment. Mice were randomized into treatment and control groups (n = 6). Each mouse was injected i.p. with a relevant dose of BTZ (1.0 mg/kg of body weight, dissolved in 0.9% NaCl; LC Laboratories, Woburn, MA, USA) while controls received 0.9% of NaCl (Vehicle Ctrl) in intervals as illustrated in Fig. 1. Vehicle-treated mice were pair-fed with equal amounts of food as BTZ-treated mice in order to avoid any influence of nutritional status (Hou et al., 2014). At the 45th or 105th day after BTZ/Vehicle treatment, animals were killed and testes were immediately removed and decapsulated (free of surrounding epididymal fat). These two time points were chosen because the duration of mouse spermatogenesis is 35 days, which consists of 6-day mitosis, 14-day meiosis, 9-day spermiogenesis (differentiation of round spermatids into elongated spermatids), and 6-day appearance of testicular sperm (Zhu et al., 2013). To this end, effects of BTZ on testicular damage could be reflected within one cycle (acute effects) or three cycles (long-term impacts) of spermatogenesis. For histological studies, testes were fixed in Bouin's solution for 24 h and embedded in paraffin, followed by staining with hematoxylin–eosin (Sigma-Aldrich, Beijing, China) or other histological analyses. For biochemical analysis, testes were frozen in liquid nitrogen and stored at −80 °C until processing.

Determination of sperm concentrations. The number of living sperms in BTZ and vehicle-treated mice was determined as described previously (Ma et al., 2010). In brief, cauda epididymidis was excised and then rinsed with medium containing 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM HEPES, 10 mM glucose, 10 mM lactic acid, and 1 mM pyruvic acid (pH 7.4). After transfer to 1 ml of medium supplemented with 5 mg of bovine serum albumin per ml and 15 mM $NaHCO_3$, semen was allowed to exude (15 min at 37 °C, 5% CO_2) from three to five small incisions. Cells were collected twice by sedimentation (400 ×*g*; 5 min). Living sperms were stained with Trypan blue, followed by a calculation under a light microscope (Axio Imager M1 microscope; Zeiss).

Hormone assays. Mice were euthanized at defined time-points as described in Fig. 1. A mid-line sternotomy was performed and 1 ml of blood was drawn by cardiocenthesis. After 15 min of centrifugation at 3000 ×*g*, the serum was collected and stored at −20 °C until analysis. Levels of testosterone (T), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured by radioimmunoassay

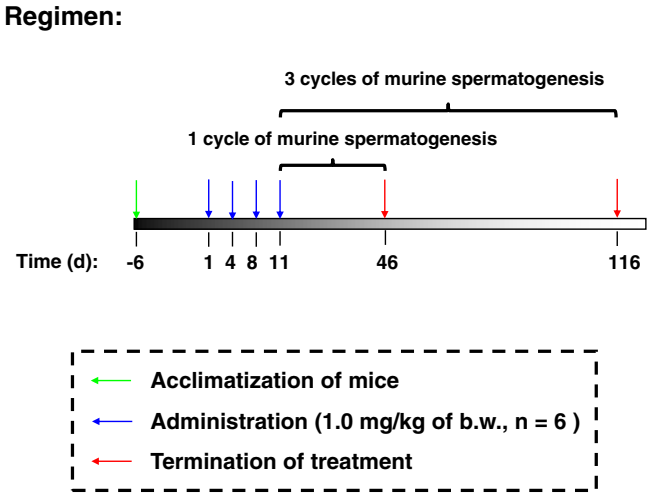

Fig. 1. Schematic representation of the experimental procedure used in the *in vivo* study. Mice were injected intraperitoneally with a relevant dose of bortezomib (BTZ, 1.0 mg/kg of body weight, dissolved in 0.9% NaCl) while controls received 0.9% of NaCl (Vehicle) in defined intervals as described in Fig. 1. At the 45th or 105th day after BTZ/vehicle treatment, animals were killed and effects of BTZ exposure on spermatogenesis were determined using different experimental assays.