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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Targeted selective degradation of Bruton's tyrosine kinase by PROTACs

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Abstract

Bruton's tyrosine kinase (BTK) is critical for B-cell receptor signaling and related to many types of human cancers. However, the drug resistance and off-target effect of present traditional BTK inhibitors occurred over time. As a new strategy for drug development, the proteolysis targeting chimera (PROTAC) has been proved to target varieties of proteins. Here SPB5208 (4-(2-(2-(2-(2-(3-(4-amino-3-(4-phenoxy phenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)ethoxy) ethoxy)ethoxy)ethoxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione) was synthesized as a new PROTAC degradation agent of BTK by linking Ibrutinib and Thalidomide. In vitro study indicated that SPB5208 reduced the BTK enzyme activity with high selectivity and inhibited effectively cancer cell proliferation. More than that, SPB5208 induced BTK protein degradation through a proteasome- and CRBN- dependent manner in JeKo-1 cells. In addition, SPB5208 was also confirmed to induce significantly BTK protein degradation in vivo. Thus, this study provides a new pharmacological tool for further study of new BTK PROTAC and their mechanism of action in physiological environment.

Keywords PROTAC · Bruton's tyrosine kinase · Targeted degradation

Abbreviations

PROTAC	Proteolysis targeting chimera
BTK	Bruton's tyrosine kinase

Introduction

Bruton's tyrosine kinase (BTK) is a nonreceptor kinase of the TEC kinase family which is a crucial integrant of B-cell receptor signaling and functions as a key regulator of cell proliferation and survival in B-cell malignancies (George et al. 2016; Khan 2001). B-cell malignancies include a series of hematologic malignancies, such as chronic lymphocytic leukemia (Sachdeva and Dhingra 2015), mantle cell lymphoma (Michael et al. 2013), follicular lymphoma (Radford et al. 2013). Most of cancers above-mentioned were mainly caused by the overexpression of BTK, therefore, the development of drug molecules targeting it has become an attractive therapeutic approach (Miao et al. 2013; Xin et al. 2019).

With the deepening of BTK research, many research groups have devoted great efforts to develop its inhibitors (Satterthwaite and Witte 2000). Ibrutinib, as the first-in-class BTK inhibitor, is an orally available and highly potent small-molecule inhibitor with subnanomolar activity (Kim 2019). Although Ibrutinib has remarkable achievement in clinical therapy, there were still many patients developed resistance to it after treated with Ibrutinib for 1–2 years. One reason is the critical mutation during tumorigenesis, and the other reason is its off-target activity (Jing et al. 2016). The limitation of Ibrutinib led to active developments of better selective second-generation BTK inhibitors, such as Acalabrutinib. However, the drug resistance and side effect still exist. Hence, it is very urgent to develop the new drug molecules targeted BTK or its mutant.

Currently, a new technology named proteolysis targeting chimera (PROTAC) is aimed at utilizing the ubiquitin-protease system to target a specific protein and induce its degradation (Gadd et al. 2017). Typically, PROTAC

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contains an E3 ligase targeting moiety, which is connected to a ligand for the target of interest via a linker. The chimera recruits E3 ubiquitin ligases to target proteins for their ubiquitination and proteasome-mediated degradation. Compared with the traditional enzyme inhibitors, PROTACs induce the degradation of the enzyme rather than inhibit it. Till now, the PROTACs can be used to target varieties of proteins, including transcription factors, skeleton proteins and regulatory proteins (Ottis and Crews 2017). Various PROTACs were reported to degrade specific kinases, such as CDK9 (Olson et al. 2017), BTK (Huang et al. 2017), epidermal growth factor receptors (EGFR), and TBK1 (Cheng et al. 2018).

Targeted degradation of BTK is explored as a new strategy for developing antineoplastic drugs. Consistently, PROTAC technology is aimed at developing a model system for the efficient targeted protein degradation (Sakamoto et al. 2001). PROTAC made use of the cell's own ubiquitin-proteasome mechanism to degrade targeted proteins. As a well BTK inhibitor, Ibrutinib is a good ligand for targeting BTK. More than that, it is reported that the type of linker is vital for the successful development of some PROTACs and small-molecule inhibitors (Ravichandiran et al. 2014). On the other, many studies shown that some small-molecule compounds have good solubility and cell activity with a piperidine group extent linker (Ravichandiran et al. 2014). So, we designed a series of PROTACs and chose the most potential compound by linking Ibrutinib and Thalidomide with a PEG linker and a piperidine group extent linker. The effects of SPB5208 on BTK protein degradation were investigated in vitro and in vivo.

Materials and methods

Chemicals and antibodies

The triethylene glycol and bis[2-(2-hydroxyethoxy)ethyl] ether was purchased from Energy Chemical (Shanghai, China). All organic solvents were purchased from Sigma Aldrich. The Ibrutinib was from Taiwo (Shandong, China). The antibodies included β-actin (4970 s) and BTK (3533 S) were purchased from Cell Signaling Technology.

Chemical analysis methods

The purities of the compounds were monitored by thin layer chromatography (Silica gel 60 F 254 aluminum from Merck Germany). The mobile phase was ethyl acetate, hexane, dichloromethane or methanol (95:5–80:20). The detection was made using UV light. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian-Mercury 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer using DMSO-d6 as solvent and TMS (Tetramethyl silane) as internal standard. All chemical shifts were reported in ppm. The mass spectra were carried out on Thermo Scientific Quantum Access max LC-MS spectrometer.

Cell culture

JeKo-1 cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum and 1% penicillin and streptomycin. Cells were cultured at 37 °C with 5% CO_2.

Cell proliferation assay

Effects of compounds on cell viability were determined by using cell counting kit-8 (CCK-8, Meilun MA0218). JeKo-1 cells were seeded in 96-well plates at a density of 10^4 cells per well in triplicate. After the treatment of SPB5208 for 72 h, 10 μL CCK-8 was added and incubated for 4 h. The absorbance was read at 450 nm using a micro plate reader (TECAN Infinite M200pro Switzerland). Each concentration was analyzed in triplicate.

Western blot assay

Briefly, equivalent amounts of protein for each sample was separated by 8% SDS-PAGE gels and transferred to the polyvinylidene difluoride membranes. Then, membrane was blocked in 5% BSA (Albumin from bovine serum) in the TBST for 1 h, incubated with a primary antibody overnight at 4 °C and treated with secondary antibody for 1 h at room temperature. The band signals were imaged with extreme hypersensitivity ECL chemiluminescence kit. The total gray of each band was quantified via the ImageJ software.

Kinases selectivity

All kinases, peptide FAM-P2, ATP, staurosporine and some labwares were bought from Carna and Sangon. The compound was transferred in tubes to one well on 96-well storage plate and serially diluted for a series of concentrations. Then, 90 μL of 1× kinase buffer was added to each well of the intermediate plate. Transfer 5 μL of each well from the 96-well intermediate plate to a 384-well plate in duplicates. At last, prepare enzyme solution, peptide solution and 5 μL of compound in 10% DMSO for the Kinase reaction. Incubate at room temperature for 10 min and stop the reaction by adding 25 μL of stop buffer. Detect them with a micro plate reader and fit the data in XL fit excel to obtain IC_{50} values.

In vivo degradation study

The ICR mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (IACUC Issue: JN. No20190530i0150610). SPF-grade ICR male mice were housed in constant temperature (20–26 °C) and humidity (40–60%). The blank control group was intraperitoneally injected with 0.1 mL/10 g of Vehicle every day. The SPB5208 group was intraperitoneally injected with 15 mg/kg or 50 mg/kg of SPB5208 once a day for 5 days. After the last administration, the mice were anesthetized with 1% pentobarbital sodium (0.1 mL/10 g) and the spleens were harvested and frozen at −80 °C for further analysis. The tissue samples were analyzed through WB detection.

Statistical analysis

Data were analyzed using GraphPad Prism. The data are presented as mean ± standard deviation of three independent experiments. Student's *t* tests were performed to compare individual treatment to the control, and comparisons between different treatments were analyzed by one-way analysis of variance with a significance level of $p < 0.05$.

Results and discussion

Synthesis of SPB5208

As shown in Fig. 1, SPB5208 was synthesized by a common synthetic route. The mono-TBS protected linker 2b was obtained from tert-butyldimethylsilyl chloride and bis[2-(2-hydroxyethoxy) ethyl] ether (2a). Then, the linker 2b reacted with the 3-hydroxy thalidomide by the Mitsunobu reaction to afford the intermediate 2c (Lai and Crews 2016). Followed by the TBS deprotection with diluted HCl, the intermediate 2c was transformed to the intermediate 2d and reacted with Ibrutinib intermediate (2e) by oxidation and reductive amination (Ravichandiran et al. 2015) to get the target compound SPB5208. The structure of compound SPB5208 was confirmed by ^{1}H NMR, ^{13}C NMR spectroscopy and liquid chromatograph mass spectrometer. As the most important component, the type and length of linkers are vital for the successful development of PROTAC (Gadd et al. 2017). The piperidinyl group is solvent exposed relatively by analyzing the X-ray crystal structure of BTK in complex with Ibrutinib (PDBID: 5P9J) (Bender et al. 2017). Based on these studies, SPB5208 was developed with a PEG linker and a piperidine group extent linker.

SPB5208 effectively inhibit the proliferation of Jeko-1 cells

First, the JeKo-1 cells were used to evaluate the effects of SPB5208 on cell proliferation. The result suggested that the inhibition of SPB5208 on the proliferation of JeKo-1 cells was concentration-dependent manner (Fig. 2a). Although its activity was lower than the positive control (Ibrutinib), SPB5208 still showed good potential on inhibiting cell proliferation. It is well known that BTK is overexpressed in many human cancer cells (Sehn and Gascoyne 2015). Moreover, BTK protein is critical for proliferation and survival of leukemic cells in many B-cell malignancies (Qian et al. 2012). These researches indicated that it is a

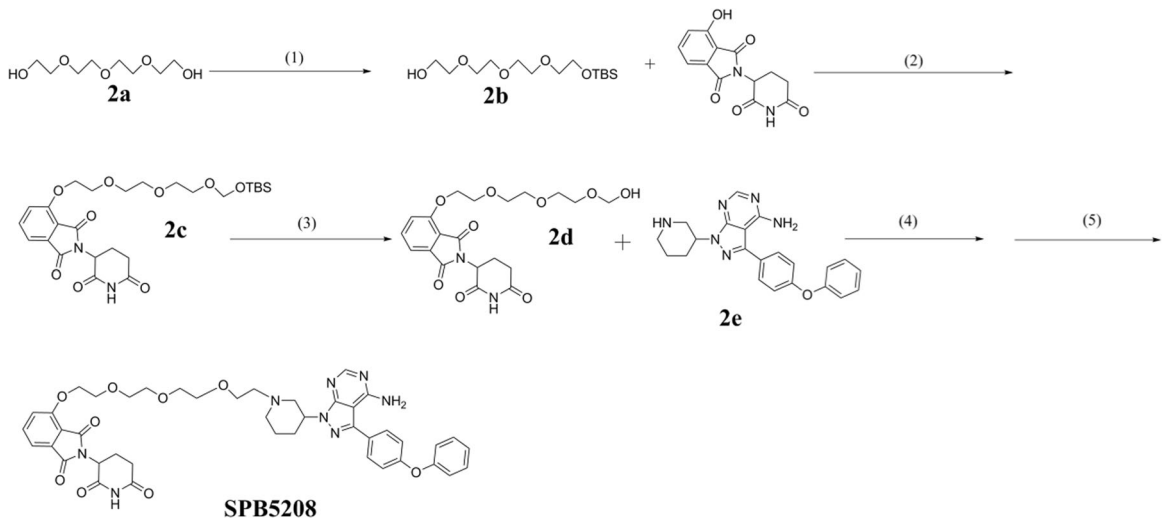

Fig. 1 Approach to develop SPB5208. Reagents and conditions: (1) TBS-Cl, Imidazle, DCM, 0 °C, overnight. (2) PPh_3, DIAD, THF, RT, 8 h. (3) HCl (aq), CH_3OH, RT, 2 h. (4) Dess-Martin periodinane, DCE, RT, 2 h. (5) HOAc, $NaHB(OAc)_3$, RT, overnight

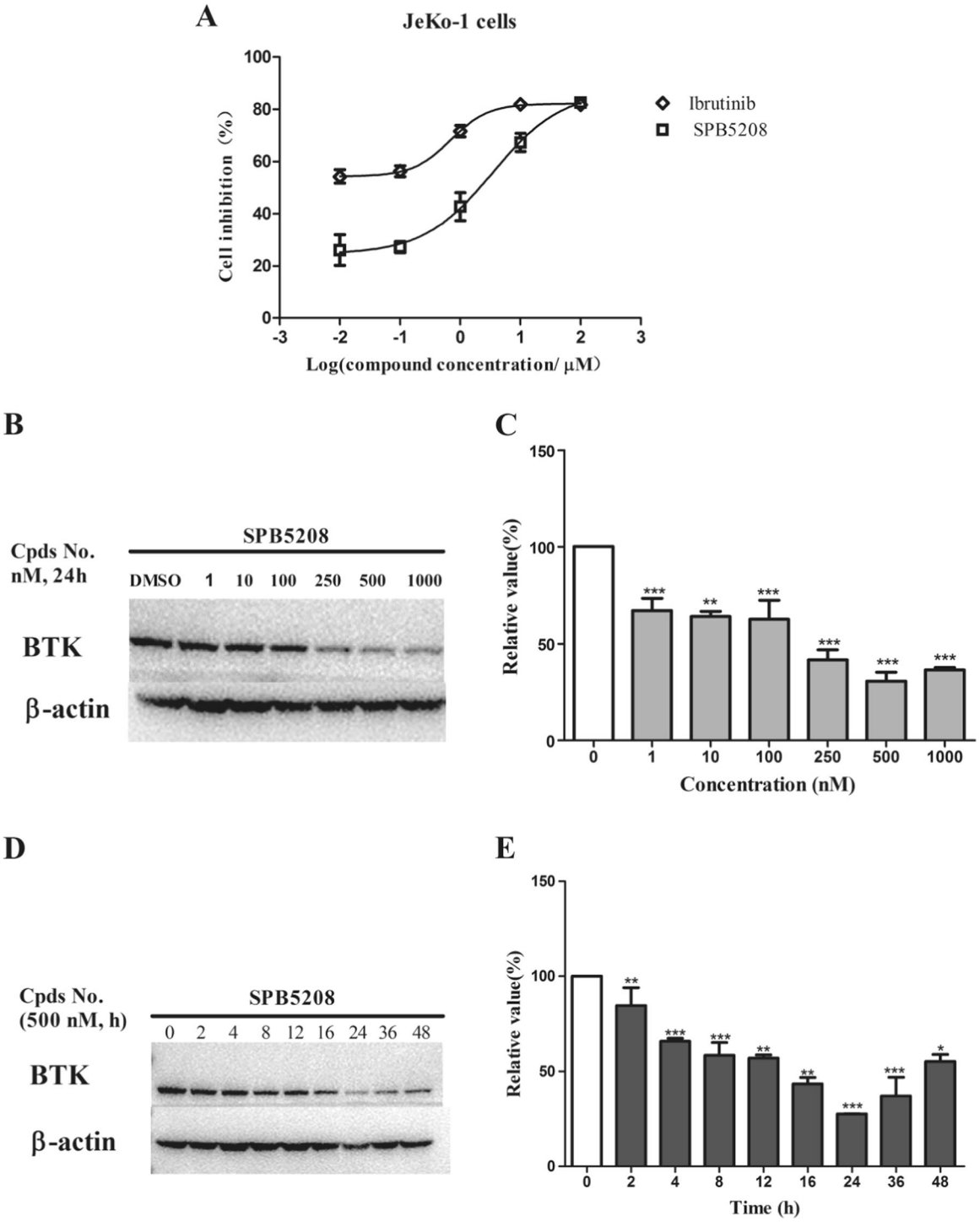

Fig. 2 The inhibition of SPB5208 and Ibrutinib on the proliferation of JeKo-1 cells (**a**). SPB5208 degraded the BTK protein in the JeKo-1 cells with a concentration-dependent manner. JeKo-1 cells were treated with DMSO or different concentrations of SPB5208 for 24 h (**b**). SPB5208 degraded the BTK protein in the JeKo-1 cells with a time-dependent manner. JeKo-1 cells were treated with SPB5208 for a series of times and Western blot analysis with specific antibodies toward BTK (**d**). Western blotting of JeKo-1 cells treated with SPB5208 with different time and concentrations gradients. The band intensities were quantified by densitometry, normalized to the level of β-actin, and calculated as the percentage of the basal response (**c**, **e**). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, compared with the control

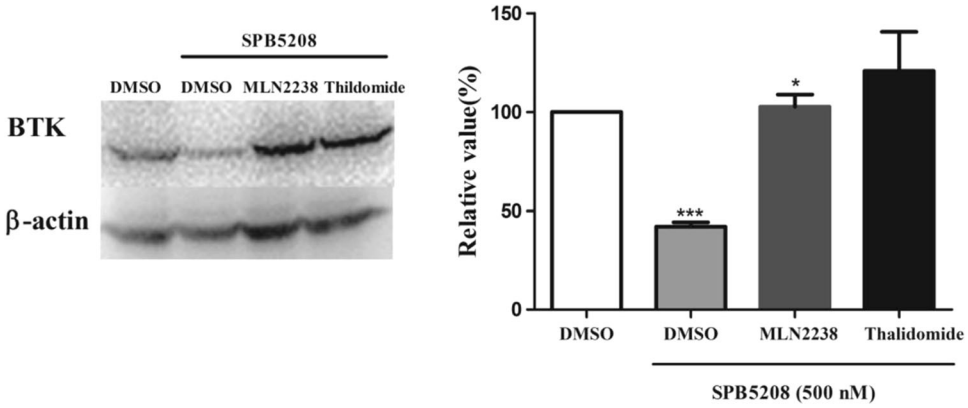

Fig. 3 The degradation of BTK protein induced by SPB5208 can be rescued. Cells were pretreated with DMSO, Thalidomide (50 μM), or MLN2238 (100 nM) for 2 h, and then were treated with the 100 nM SPB5208 for 24 h

valid strategy to inhibit the proliferation of Jeko-1 cells for some B-cell malignancies.

SPB5208 effectively induce the degradation of BTK in Jeko-1 cells

Next, the effect of SPB5208 on BTK protein was investigated. The result indicated that SPB5208 significantly reduced the BTK protein level in the JeKo-1 cell with a concentration-dependent manner (Fig. 2b). After treated with SPB5208 (500 nM) for 24 h, more than 70% of BTK protein was degraded (Fig. 2c). As expected, Ibrutinib did not change the BTK protein level under the investigated concentration (Fig. S2).

Based on the above results, the time course studies of SPB5208 induced BTK degradation in JeKo-1 cells were investigated. The result showed that the BTK protein began to degrade after treated with SPB5208 (500 nM) for 2 h. SPB5208 could degrade BTK protein with a time-dependent manner (Fig. 2d). The maximum degradation of BTK protein, over 70% degradation, was observed after 24 h treatment with SPB5208 (Fig. 2e).

Generally, traditional small chemical inhibitors were extensively synthesized to bind specific proteins and aimed to inhibit the activity of the protein. In recent years, many researches showed that the degradation of targeted protein is more efficient than inhibition, which is more likely to overcome drug resistance and some side effects (Matyskiela et al. 2016). Significantly, SPB5208 degraded BTK protein directly in vitro with a proteasome dependent manner.

SPB5208 recruits CRBN E3 ligase to degrade BTK protein

To explore the mechanism of BTK degradation by SPB5208, a rescued experiment was designed. First, the JeKo-1 cells were pretreated with an excess amount of the Thalidomide (the E3 ubiquitin ligase CRBN) and MLN2238 (a potent and selective reversible proteasome inhibitor) for 2 h. Then, it increased BTK protein levels significantly after treated with SPB5208 for 24 h. Both of two diminished the degradation effect of SPB5208 (Fig. 3). It indicated that SPB5208 degraded BTK proteins through a CRBN- and proteasome- dependent mechanism. We will try to obtain crystal structures of ternary complexes of BTK-SPB5208-CRBN and explore the concrete mechanism of the degradation on next work.

Table 1 The inhibition IC_{50} (nM) of compounds on 5 kinases

	JAK3	ITK	EGFR	BLK	BTK
SPB5208	>10,000	>10,000	4356	459	127
Ibrutinib	160	238	12	1.5	2.4
Staurosporine	0.35	5.3	221	2.4	152

SPB5208 exhibits high selectivity on BTK

Then, to investigate the kinase selectivity of SPB5208, in vitro inhibition activity of SPB5208 on five kinases was detected. Staurosporine and Ibrutinib were used as the positive control. As shown in Table 1, the SPB5208 showed no inhibitory activity against inducible T-cell kinase (ITK IL-2), EGFR, B-lymphoid tyrosine kinase (BLK) and Janus kinase 3 (JAK3). On the other, its inhibition IC_{50} was 127 nM, which showed the high selectivity on BTK (Table 1).

In recent researches, drug resistance, off-target and side effects occurred when a small-molecule inhibitor was continually used (Jing et al. 2016). For example, Rao's study showed that EGFR, ITK, and TEC family kinases can also be inhibited by low concentrations of Ibrutinib, resulting in serious side effects or off-target in patients (Yong et al. 2018). However, SPB5208 inhibited the BTK with high selectivity and almost showed no inhibitory activity against ITK or EGFR, which indicates that SPB5208 will unlikely lead to the side effects of Ibrutinib.

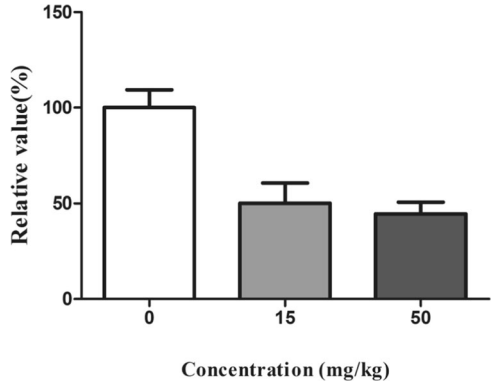

Fig. 4 SPB5208 reduced the BTK protein level in the spleen of mice with different concentration groups. The ICR mice were intraperitoneally injected with the SPB5208 or Vehicle once a day for 5 days. Western blot analysis was carried out on whole cell lysate from tissue samples of spleens in mice. The band intensities were quantified by densitometry, normalized to the level of β-actin, and calculated as the percentage of the basal response, compared with the control

SPB5208 effectively induce the degradation of BTK in vivo

At last, the in vivo degradation of SPB5208 on the BTK protein was investigated. The ICR mice were treated with SPB5208 by intraperitoneal injection once a day for 5 days. For the concentration of 15 mg/kg and 50 mg/kg, the result suggested that BTK protein could be degraded by SPB5208 in vivo (Fig. S3). Moreover, there was no obvious abnormality of viscera in mice and over 50% of spleen BTK protein was degraded by SPB5208 (Fig. 4).

Conclusion

Regulating protein function through targeted degradation as opposed to more conventional target inhibition has emerged as a new modality for the development of new therapeutics. In conclusion, SPB5208, a new PROTAC compound, was designed and synthesized in this study. SPB5208 could inhibit the growth of the JeKo-1 cells and induce BTK protein degradation in vitro and in vivo. Besides, SPB5208 suppressed BTK enzyme activity with high selectivity. Therefore, these results indicated that SPF5208 could be a potential pharmacological research tool in the development of antineoplastic drugs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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