The Application Methods of Mitogen-activated Protein Kinase Signal Pathway Inhibitors SP600125 and SB203580 in Long-term *in Vivo* Experiments

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ABSTRACT

Objective: *To explore the application methods of mitogen-activated protein kinase signal pathway inhibitors SP600125 and SB203580 in long-term* in vivo *experiments.*

Methods: *A total of 55 healthy New Zealand rabbits were randomly divided into blank control group, model control group, SP low dose group, SP high dose group, SP blank group, SB low dose group, SB high dose group, SB blank group, dimethyl sulfoxide (DMSO) control group, DMSO blank group, and positive control group. Since the first day of the experiment, each group was administered the corresponding treatment for four weeks continuously. Then, the myocardial c-Jun N-terminal kinase (JNK) and the total protein of p38, protein phosphorylation and its gene expression levels were detected.*

Results: *After intravenous treatment with adriamycin, the myocardial phosphorylate-JNK (p-JNK) and phosphorylate-p38 (p-p38) levels in all groups were increased to varying degrees, of which the model control group increased the most significantly (*p < 0.05*). Compared with the model control group, the myocardial p-JNK and p-p38 increased more slowly in the SP low dose group, SP high dose group, SB low dose group, SB high dose group and positive control group (*p < 0.05*), of which the increase in the SP high dose group and the SB high dose group was the slowest (*p < 0.05*). After four weeks, the total protein and messenger ribonucleic acid of the myocardial JNK and p38 in all groups had no statistically significant difference (*p > 0.05*).*

Conclusion: *The continuous intravenous injection of SP600125 and SB203580 for four weeks significantly reduced the protein phosphorylation levels of JNK and p38, which provides a practical avenue for the long-term study* in vivo.

Keywords: C-Jun N-terminal kinase, *in vivo*, mitogen-activated protein kinases, p38, SB203580, SP600125

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Métodos de aplicación de los inhibidores SP600125 y SB203580 de la vía de señalización de la proteína quinasa activada por mitógeno en experimentos *in vivo* a largo plazo

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RESUMEN

Objetivo: *Explorar los métodos de aplicación de los inhibidores SP600125 y SB203580 de la vía de señalización de la proteína quinasa activada por mitógeno en experimentos* in vivo *a largo plazo.*

Métodos: *Un total de 55 conejos sanos de Nueva Zelandia fueron divididos aleatoriamente en los grupos siguientes: grupo de control en blanco, grupo de control modelo, grupo de dosis baja SP, grupo de dosis alta SP, grupo en blanco SP, grupo de dosis baja SB, grupo de dosis alta SB, grupo en blanco SB, grupo de control dimetilsulfóxido (DMSO), grupo en blanco DMSO, y grupo de control positivo. Desde el primer día del experimento, a cada grupo se le administró el tratamiento correspondiente por cuatro semanas continuas. Entonces, se detectaron la quinasa c-Jun N-terminal (JNK) miocárdica y la proteína p38 total, así como la fosforilación proteica y sus niveles de expresión génica.*

Resultados: *Después del tratamiento intravenoso con adriamicina, los niveles de fosfo-JNK (p-JNK) y fosfo-p38 (p-p38) del miocardio aumentaron en todos los grupos en diversos grados, siendo el aumento del grupo de control modelo el más significativo (*$p < 0.05$*). En comparación con el grupo de control modelo, p-JNK y p-p38 miocárdicos aumentaron más lentamente en el grupo de dosis baja SP, el grupo de dosis alta SP, el grupo de dosis baja SB, el grupo de dosis alta SB, y el grupo de control positivo (*$p < 0.05$*). De estos, el aumento en el grupo de dosis alta SP y el grupo de dosis alta SB fue el más lento (*$p < 0.05$*). Después de cuatro semanas, la proteína total y el ácido ribonucleico mensajero de JNK y p38 miocárdicos en todos los grupos, no tuvieron diferencias significativas (*$p > 0.05$*).*

Conclusión: *La inyección intravenosa continua de SP600125 y SB203580 durante cuatro semanas redujo significativamente los niveles de fosforilación proteica de JNK y p38, lo que proporciona una vía práctica para el estudio a largo plazo* in vivo.

Palabras clave: Quinasa c-Jun N-terminal, *in vivo*, proteínas quinasas activadas por mitógeno, p38, SB203580, SP600125

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are a prominent group of serine/threonine protein kinases that transduce from extracellular to intracellular and which can mediate a variety of signal transduction processes and pro-oncogene transcription. Thus, they are involved in physiological processes such as cell growth, cellular differentiation and apoptosis, mitogens. They are also associated with pathological processes, *eg* cell stress, inflammation, cell transformation and tumor metastasis. In the family of MAPKs, c-Jun N-terminal kinase (JNK) and p38 signalling cascades are two important intracellular signalling pathways, of which JNK can be specifically inhibited by SP600125, but SB203580 has more obvious inhibitory effects on p38 compared to SP600125. So far, inhibitors have been commonly used to block signalling pathways. Then the effector or indicator changes are detected. In addition, the application of SP600125 and SB203580 *in vitro* and *in vivo* for short-term experiments has been relatively mature. However, the utilization of SP600125 and SB203580 on interfering with the channel proteins such as JNK and p38 in long-term experiments is rarely reported. In this study, we explored the potential of the application of SP600125 and SB203580 in long-term *in vivo* experiments, providing theoretical evidence for related research.

SUBJECTS AND METHODS

Experimental materials

Animals

A total of 55 male New Zealand rabbits (weight: 1.84 ± 0.13 kg) were provided by the Laboratory Animal Center of Hunan University of Traditional Chinese Medicine, with approval from the State Committee of Science and Technology of the People's Republic of China [PRC] (animal licence number: SCXK (Xiang) 2009-0012). Rabbits were housed with free access to water and food in a room with controlled ambient temperature of 20 ± 3°C and humidity 50 ± 10% with a 12-hour light/dark cycle (illumination time: 6am to 6pm) and a background noise of 40 ± 10 db for a week to adapt to the environment.

Drugs and reagents

The following drugs and reagents were used:
- Adriamycin hydrochloride: Shenzhen Main Luck Pharmaceutical Inc, PRC;
- Enalapril tablets: Shandong Cisen Pharmaceutical Ltd, PRC;
- JNK antibody: Santa Cruz Biotechnology Inc, United States of America (USA);
- Phosphorylate-JNK (p-JNK) antibody: Santa Cruz Biotechnology Inc, USA;
- p38 antibody: Santa Cruz Biotechnology Inc, USA;
- Phosphorylate-p38 (p-p38) antibody: Santa Cruz Biotechnology Inc, USA;
- Inhibitor SP600125: Selleck Inc, USA; and
- Inhibitor SB203580: Selleck Inc, USA.

Methods

Grouping

The 55 New Zealand rabbits were randomly divided into 11 groups, with five rabbits in each group: the blank control group, model control group, SP low dose group, SP high dose group, SP blank group, SB low dose group, SB high dose group, SB blank group, dimethyl sulfoxide (DMSO) control group, DMSO blank group and positive control group.

Establishment of abnormal expression models of c-Jun N-terminal kinase and p38 signal pathway

Doxorubicin hydrochloride (adriamycin) was diluted with saline solution to 1.0 mg/ml and was injected into the marginal ear vein: 1.0 ml/kg each time and twice a week for four consecutive weeks (1, 2).

Drug administration

Each group was injected with adriamycin, except the blank control group, SP blank group, SB blank group and DMSO blank group. The inhibitor was diluted with DMSO solution to 1.0 mg/ml. Rabbits in the SP low dose group, SP high dose group, SP blank group, SB low dose group, SB high dose group and SB blank group were injected with inhibitor of 20 μg/kg/d, 40 μg/kg/d and 40 μg/kg/d, respectively. The DMSO control group and DMSO blank group were injected with 40 μL/kg/d of DMSO solution and 40 μL/kg/d of normal saline, respectively. Rabbits in the positive control group were fed with enalapril tablets of 5 mg/kg/d (3). The blank control group was fed with equivalent normal saline solution. All treatments were performed continuously in each group for four weeks.

Sample collection and detection

Extraction of total protein from myocardial tissues

To detect the protein changes in the progression of rabbit models induced by adriamycin, rabbits in each group were sacrificed, and left ventricular myocardial tissues were quickly taken out, placing in liquid nitrogen for cryopreservation. The frozen myocardial tissue in each group was weighed accurately. Using a 1:8 mass to volume ratio, the sample was added and mixed with lysis buffer, and the mixture was homogenized on ice by a mechanical homogenizer. After treatment by vortex blending, the mixture was kept on ice for one hour, and the homogenate was centrifuged at 18 000 g for 30 minutes at 4°C. Then, the supernatant was collected. The protein concentration was determined by Bradford method, and the samples were stored at -70°C for further experiments.

Western blots

A total of 50 μg of myocardial total proteins were added to 2 × sodium dodecyl sulfate (SDS) sample buffer (0.1 mol/L Tris pH 6.8, 0.2 mol/L DTT, 4% SDS, 20% glycerol and 0.02% bromophenol blue). The mixture was denatured at 100°C for eight minutes. Then, the samples were loaded. Using a constant current, electrophoresis was carried out at 6 mA/gel for the first 15 minutes. Then, electrophoresis was carried out to the end at 32 mA/gel. The current was set to 0.8 A/cm^2 for electro blotting in accordance with the membrane area, and the proteins were transferred to a nitrocellulose membrane for two hours. Then, the nitrocellulose membrane was blocked with tris-buffered saline and Polysorbate 20 (also known

as Tween 20) (TBST) containing 3% no-fat milk at room temperature for two hours. The membrane was removed and placed in a hybridization bag; anti-JNK, p-JNK, p38 and p-p38 antibody were added respectively, incubating at 4°C overnight. After rinsing three times with TBST (10 minutes each), the enzyme labelled second antibody was added, incubating at room temperature for two hours. Rinsing was performed three times with TBST (10 minutes each). The blots were developed with enhanced chemiluminescence (ECL) reagents and were scanned at 300 dpi and saved for analysis.

Detection of myocardial c-Jun N-terminal kinase messenger ribonucleic acid and p38 messenger ribonucleic acid expression
The left ventricular myocardial tissues were taken, referring to the manual of TRIquick Reagent. The total ribonucleic acids from myocardial tissues were extracted by using the Trizol one-step method. An ultraviolet spectrophotometer was used for purity measurement and quantification. The primers were designed according to rabbit JNK and p38 gene sequences in the Oryctolagus cuniculus. According to the instruction of the polymerase chain reaction reagent kit, JNK and p38 gene expression (messenger ribonucleic acid; mRNA) was analysed by fluorescence quantitative reverse transcription polymerase chain reaction. Primers were designed according to JNK, p38 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene sequences which are published in Oryctolagus cuniculus:

- JNK mRNA Oryctolagus cuniculus: XM_008250388
  Forward primer: 5'-aacgcatgctaagagggcctacaga-3'
  Reversed primer: 5'-tcgcatccatgagctccatgactat-3'
  Product size: 149
- p38 mRNA Oryctolagus cuniculus: XM_002714646
  Forward primer: 5'-cgcgacacactgatgatgaaatgac-3'
  Reversed primer: 5'-gcatccgaccgaccaaatatcaact-3'
  Product size: 119
- GAPDH Oryctolagus cuniculus: NM_001082253
  Forward primer: 5'-ttcaacagtgccacccactcctcta-3'
  Reversed primer: 5'-ccctgttgctgtagccaaattcgtt-3'
  Product size: 115

Statistical methods
All measurement data were expressed as $\overline{x} \pm s$. All data were tested for normality and processed by the SPSS 17.0 statistical analysis software package. The differences between the means of the multiple groups were analysed by one-way analysis of variance. The least

significant difference test was used when equal variances were assumed and Tamhane's T2 test when they were not. The significance level was $p < 0.05$.

RESULTS

Analysis of animal numbers
A rabbit from the DMSO control group died before the experiment ended. The possible reason may be acute poisoning caused by a high-speed injection of adriamycin. All other animals were normal in the experimental process.

Changes of the myocardial c-Jun N-terminal kinase expression level after various drug treatments
Table 1 and Figure 1 show the post-experiment myocardial JNK expression. After intervention with adriamycin, the myocardial p-JNK was increased after different administrations. Compared to the normal control group, the level of p-JNK was evaluated in the model control group ($p < 0.05$), as well as other drug treatment groups ($p < 0.05$). Compared to the model control group, the myocardial p-JNK level increased slightly in the SP low dose group, the SP high dose group and the positive control group ($p < 0.05$), of which the SP high dose group was suppressed the most significantly ($p < 0.05$). Compared to the model control group or blank control group, there was no statistically significant difference in the expression of myocardial p-JNK in the DMSO control group and the DMSO blank group ($p > 0.05$). Among all the experimental groups, there was no statistically significant difference in myocardial JNK and JNK mRNA levels ($p > 0.05$).

Table 1:   Myocardial JNK expression of each group after experiment ($\overline{x} \pm s$)

Group	n	JNK/Actin1	p-JNK/Actin2	JNK mRNA/ GADPH
Blank control group	5	0.29 ± 0.005	0.37 ± 0.011	0.27 ± 0.020
Model control group	5	0.28 ± 0.002[a]	0.77 ± 0.010[b]	0.28 ± 0.019[a]
SP low dose group	5	0.28 ± 0.002[a]	0.64 ± 0.012[b c]	0.28 ± 0.018[a]
SP high dose group	5	0.29 ± 0.003[a]	0.56 ± 0.012[b c e f]	0.27 ± 0.020[a]
SP blank group	5	0.29 ± 0.001[a]	0.15 ± 0.011[b c e]	0.28 ± 0.018[a]
DMSO control group	4	0.29 ± 0.003[a]	0.76 ± 0.010[b d e]	0.26 ± 0.020[a]
DMSO blank group	5	0.28 ± 0.002[a]	0.38 ± 0.012[a c e f]	0.27 ± 0.019[a]
Positive control group	5	0.29 ± 0.002[a]	0.70 ± 0.012[b c e]	0.27 ± 0.018[a]

[a] Compared with blank control group, $p > 0.05$.
[b] Compared with blank control group, $p < 0.05$.
[c] Compared with model control group, $p < 0.05$.
[d] Compared with model control group, $p > 0.05$.
[e] Compared with SP low dose group, $p < 0.05$.
[f] Compared with SP blank group, $p < 0.05$.
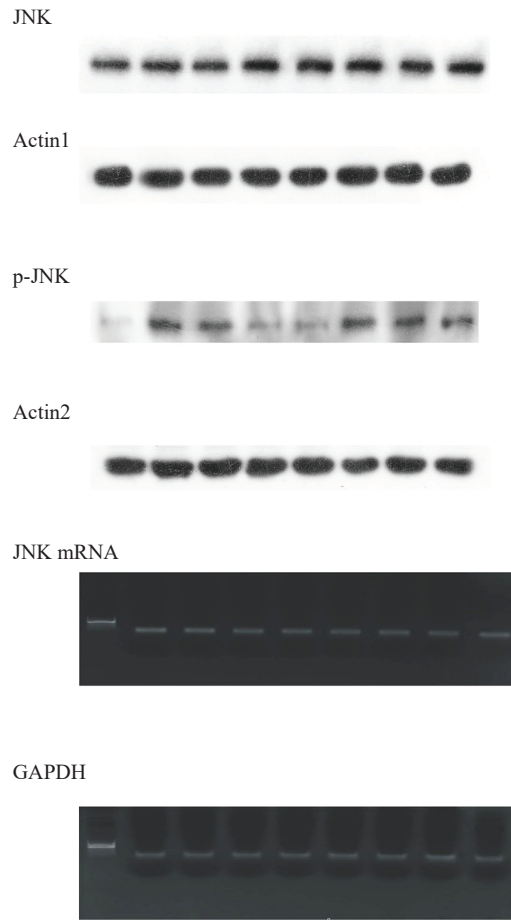
JNK

Actin1

p-JNK

Actin2

JNK mRNA

GAPDH

Fig. 1: Myocardial JNK expressions in rabbits in each group. From left to right: blank control group, model control group, SP low dose group, SP high dose group, SP blank group, DMSO control group, DMSO blank group and positive control group.

Changes of the myocardial p38 expression level after various drug treatments

Table 2 and Figure 2 present the myocardial p38 expression in the different groups. After intravenous administration of adriamycin, the myocardial p-p38 was increased (Table 2). Compared with the blank control group, the difference was statistically significant ($p < 0.05$), while the model control group increased the most significantly ($p < 0.05$). Compared with the model control group, myocardial p-p38 level was weakly enhanced in the SB low dose group, the SB high dose group and the positive control group ($p < 0.05$), while the increase in the SB high dose group was the most significant ($p < 0.05$). Compared with the model control group or blank control group, there was no statistically significant difference in the myocardial p-p38 changes in the DMSO control group and the DMSO blank group ($p > 0.05$). Among all the groups, there was no statistically significant difference in the total p38 levels in both mRNA and protein expression ($p > 0.05$).

Table 2: Myocardial p38 expression of each group after experiment ($\bar{x} \pm s$)

Group	n	p38/Actin3	p-p38/Actin4	p38 mRNA/GADPH
Blank control group	5	0.34 ± 0.03	0.36 ± 0.02	0.65 ± 0.05
Model control group	5	0.35 ± 0.03^a	0.67 ± 0.03^b	0.64 ± 0.06^a
SB low dose group	5	0.34 ± 0.04^a	0.51 ± 0.02^{bc}	0.64 ± 0.05^a
SB high dose group	5	0.35 ± 0.03^a	0.43 ± 0.03^{bcef}	0.65 ± 0.06^a
SB blank group	5	0.36 ± 0.04^a	0.12 ± 0.02^{bce}	0.63 ± 0.05^a
DMSO control group	4	0.35 ± 0.04^a	0.69 ± 0.03^{bde}	0.66 ± 0.06^a
DMSO blank group	5	0.35 ± 0.03^a	0.35 ± 0.03^{acef}	0.65 ± 0.05^a
Positive control group	5	0.37 ± 0.04^a	0.59 ± 0.02^{bce}	0.64 ± 0.04^a

[a] Compared with blank control group, $p > 0.05$.
[b] Compared with blank control group, $p < 0.05$.
[c] Compared with model control group, $p < 0.05$.
[d] Compared with model control group, $p > 0.05$.
[e] Compared with SB low dose group, $p < 0.05$.
[f] Compared with SB blank group, $p < 0.05$.

p38

Actin3

p-p38
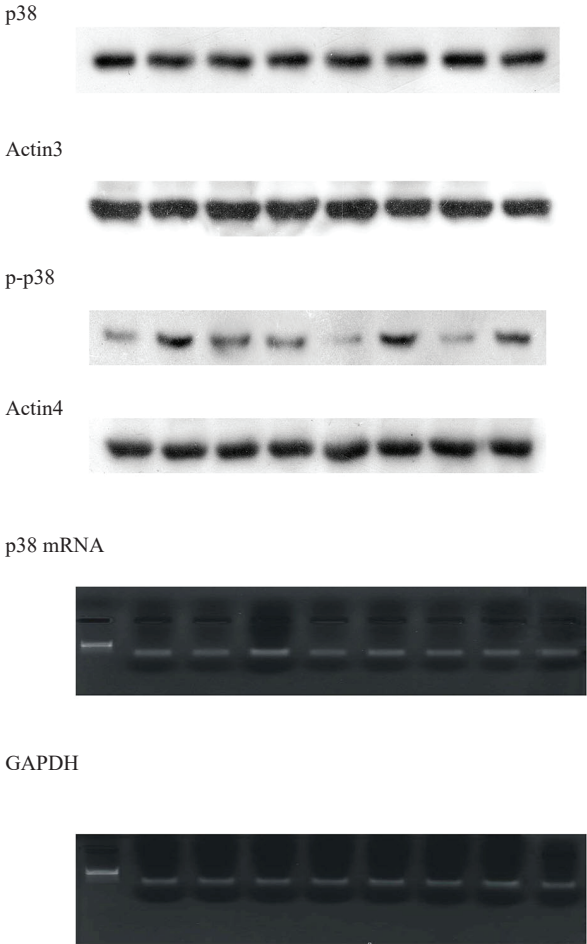
Actin4

p38 mRNA

GAPDH

Fig. 2: Myocardial p38 expressions in rabbits in each group. From left to right: blank control group, model control group, SB low dose group, SB high dose group, SB blank group, DMSO control group, DMSO blank group and positive control group.

DISCUSSION

Mitogen-activated protein kinases are widespread serine/threonine protein kinases in mammals and can be activated by a series of extracellular signals or stimuli. They participate in multiple physical processes and are also related to the development of a variety of diseases, such as neural inflammation, Alzhemier's disease, cancer and cardiovascular disease. C-Jun N-terminal kinase is one of the major members of the MAPK signal pathway, which is mainly involved in cell proliferation and differentiation, cell morphology, cell skeleton construction, and other biological reactions, such as apoptosis and cell malignant transformation (4). A recent study indicates that the relavant responses are mainly through regulation of the phosphorylation level of JNK (5). Patients who suffer from heart disease caused by hypertension show dramatically activated JNK and p38 changes (6), and the reactive oxygen species release also stimulates apoptosis signal kinase-l in the upstream of JNK and p38. Its excessive expression activates nuclear factor κB (NFκB) and subsequently causes myocardial hypertrophy, which further triggers the related apoptosis signal (7, 8). P38 is another central member of the MAPK family, which is located in the cytoplasm when not activated. Its serine/threonine residues can be phosphorylated and activated, which lead to a quick transfer from cytoplasm to the nucleus, which is applied to the corresponding targets (9). P38 is the main cell signal to respond to the body's stress. It can be activated by stimulating factors such as organized local neuroendocrine factors ET and AngII, which accelerate cell apoptosis (10). Metabolic stress can also be activated by p38, and p38 inhibition can eliminate the secondary 5-AMP activated protein kinase phosphorylation, improving the coupling in the process of glucose metabolism and restoring the heart function mediated by adenosine.

Pathway inhibitors play an important role in both basic research and clinical application. SP600125 is a competitive and selective JNK inhibitor of ATP. It can inhibit MAPK activation mediated by JNK. When the inhibitors act on JNK, its selectively inhibitory effect is 100 times higher than extracellular-signal-regulated kinase and p38. SB203580 is a common p38 inhibitor, which inhibits subsequent MAPK AP Kinase-2 and MAPK AP Kinase-3 activation of p38, thus effectively restraining part of the signal transduction induced by some inflammatory factors (eg IL-1, TNF-α). As far as we are aware, previous studies have only reported that SP600125 and SB203580 could be used in vitro. It has been confirmed that the pathway inhibitor can be applied to a variety of cells lines, causing obvious changes in indicators such as downstream proteins and inflammatory factors (11–14). There are also other in vivo reports focussing on SP600125 and SB203580, but most of these used executed experimental animals in 48–72 hours after high-dose intravenous or intraperitoneal injection, and then tested the related indicators (15). The value of the above reported literature is limited for observing long-term pathological development of chronic disease with animal models, parallelled with the intervention effect of SP600125 and SB203580. The present study used adriamycin to induce changes of JNK and p38, developing a more suitable method to use SP600125 and SB203580 in vivo for long-term processes, compared to the previous studies. Our results helped to clarify the theoretical basis for long-term MAPK examination, providing a promising strategy for relative basic research.

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