Influence of Phosphatidylinositol-3-Kinase/Protein Kinase B-Mammalian Target of Rapamycin Signaling Pathway on the Neuropathic Pain Complicated by Nucleoside Reverse Transcriptase Inhibitors for the Treatment of HIV Infection

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Abstract

Background: Nucleoside reverse transcriptase inhibitors (NRTIs) are the earliest and most commonly used anti-human immunodeficiency virus drugs and play an important role in high active antiretroviral therapy. However, NRTI drug therapy can cause peripheral neuropathic pain. In this study, we aimed to investigate the mechanisms of rapamycin on the pain sensitization of model mice by *in vivo* experiments to explore the effect of mammalian target of rapamycin (mTOR) in the pathogenesis of neuropathic pain caused by NRTIs.

Methods: Male Kun Ming (KM) mice weighing 20–22 g were divided into control, 2 mg/kg rapamycin, 12 mg/kg stavudine, and CMC-Na groups. Drugs were orally administered to mice for 42 consecutive days. The von Frey filament detection and thermal pain tests were conducted on day 7, 14, 21, 28, 35, and 42 after drug administration. After the last behavioral tests, immunohistochemistry and western blotting assay were used for the measurement of mTOR and other biomarkers. Multivariate analysis of variance was used.

Results: The beneficial effects of rapamycin on neuropathic pain were attributed to a reduction in mammalian target of rapamycin sensitive complex 1 (mTORC1)-positive cells (70.80 ± 2.41 vs. 112.30 ± 5.66, $F = 34.36$, $P < 0.01$) and mTORC1 activity in the mouse spinal cord. Mechanistic studies revealed that Protein Kinase B (Akt)/mTOR signaling pathway blockade with rapamycin prevented the phosphorylation of mTORC1 in stavudine-intoxicated mice (0.72 ± 0.04 vs. 0.86 ± 0.03, $F = 4.24$, $P = 0.045$), as well as decreased the expression of phospho-p70S6K (0.47 ± 0.01 vs. 0.68 ± 0.03, $F = 6.01$, $P = 0.022$) and phospho-4EBP1 (0.90 ± 0.04 vs. 0.94 ± 0.06, $F = 0.28$, $P = 0.646$).

Conclusions: Taken together, these results suggest that stavudine elevates the expression and activity of mTORC1 in the spinal cord through activating the Akt/mTOR signaling pathway. The data also provide evidence that rapamycin might be useful for the treatment of peripheral neuropathic pain.

Key words: Human Immunodeficiency Virus Infection; Neuropathic Pain; Nucleoside Reverse Transcriptase Inhibitors; Phosphatidylinositol-3-Kinase/Protein Kinase B/Mammalian Target of Rapamycin Signaling Pathway; Rapamycin

INTRODUCTION

The use of nucleoside reverse transcriptase inhibitors (NRTIs) for high active antiretroviral therapy (HAART) has greatly prolonged the survival time and survival rate of human immunodeficiency virus (HIV)-infected patients.[1] However, NRTI drug therapy has been shown to lead to peripheral neuropathic pain, which is different from the slow progressive neuropathic pain caused by the HIV infection itself.[2,3] This neuropathic pain is mainly manifested as a sudden biting and burning pain after approximately 10 weeks of drug treatment and can extend to the upper limbs.[4]

This severe pain seriously affects the quality of life of the patient and even forces patients to stop treatment. However,

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treatment cessation leads to further exacerbation of the pain, potentially for up to several weeks or even months. In recent years, the rate of neuropathic pain due to NRTI treatment has been estimated at 20–50% in Europe and the United States, indicating that neuropathic pain has become a common and harmful complication, creating an even greater public health problem.[5] Beijing Ditan Hospital, affiliated with Capital Medical University, has prescribed NRTIs (stavudine, didanosine, and decitabine) to thousands of HIV-infected patients every year, and adverse events such as neurological pain associated with NRTIs have also been found in clinical cases. Therefore, the study of neuropathic pain in the treatment of HIV-infected patients with NRTIs is of great importance in improving the quality of life these patients.

The phosphatidylinositol-3-kinase/protein kinase B/ mammalian target of rapamycin (PI3K-Akt-mTOR) signaling pathway is one of the bridges that link intracellular and extracellular signal transduction and cell response. Recent study has shown that the Akt/mTOR signaling pathway is involved in the modulation of neuropathic pain from multiple levels, such as synaptic plasticity in the central nervous system, which may be related to the formation of pain sensitization after tissue injury.[6] At the same time, Akt/mTOR signaling pathway abnormalities are also closely related to tumour and neurodegenerative diseases and thus become the target of treatments for related diseases.[7,8] mTOR is an evolutionarily highly conserved serine/threonine protein kinase with a molecular weight of 289,000 Da, consisting of 2549 amino acids, and is widely found in various biological cells.[9] Recent study has shown that mTOR, as a downstream molecule of the Akt/mTOR signaling pathway, is extensively involved in the formation of neural synaptic plasticity and the conduction of pain signals in inflammatory pain, neuropathic pain, and cancer pain models.[10] The kinase catalytic domain (KIN) of the carboxyl terminal of the mTOR molecule is highly homologous to the P13K kinase lipase domain. KIN contains a negative regulatory domain (NRD) with a conserved phosphorylation site, and the phosphorylation sites Thr2448, Ser2448, and Ser2481 within NRD are associated with the activity level of mTOR.[11] The mTOR molecules and different proteins can form two different complexes, one in which mTOR and raptor (regulatory associated protein of mTOR) bind to form a rapamycin-sensitive complex called mTOR complex 1 (mTORC1) and the other in which mTOR and Rictor (rapamycin-insensitive companion of mTOR) combine to form a rapamycin-insensitive complex called mTORC2. mTORC1 is responsible for integrating growth factors and nutritional signals to control cell growth and proliferation. Once mTORCl is activated, two different downstream channels can be adjusted: ribosomal S6 protein kinase (S6K) and eukaryotic cell initiation factor 4E binding protein 1 (4EBPl). 4EBPl and S6K are the most widely studied mTOR substrates and are key regulators of protein translation. mTORC2 mainly regulates actin and phosphorylation of protein kinase C in the organism.[12]

Rapamycin is a specific inhibitor of mTOR. Rapamycin forms a complex with the intracellular receptor FKBP12 (12,000 Da immunophilin FK506-binding protein), which binds to the FRB (FKBPl2-rapamycin binding) domain of mTOR to specifically bind and inhibit mTOR activity; therefore, rapamycin is often used as a tool for studying the Akt/mTOR signaling pathway and is widely used in the study of physiological and pathological mechanisms.[13] Therefore, in order to further elucidate the etiology and pathogenesis of neuropathic pain associated with NRTIs, in this study, using stavudine-treated mouse models, we carried out a set of *in vivo* experiments to address the effects of rapamycin on neuropathic pain and the related mechanisms.

METHODS

Reagents and antibodies

Stavudine was supplied by the Department of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University (Beijing, China). Rapamycin was obtained from Selleck (Texas, USA). The drugs were suspended in 0.5% CMC-Na for oral administration. Bromophenol blue, acrylamide, methylenebisacrylamide, tetramethylene ethylenediamine, and Ammonium persulfate were products of Sigma-Aldrich (St Louis, MO, USA). TrisBase and SDS were products of the Original Pinghao Biotechnology Company (Beijing, China). Polyvinylidene fluoride film and nitrocellulose membranes were supplied by Millipore (Massachusetts, USA). Loading buffer, nondenaturing protease lysate, protein phosphatase inhibitor, protease inhibitor, and the hypersensitive luminescent solution were supplied by Beijing Applygen Technology Company (Beijing, China). The protein molecular weight marker was purchased from Beijing Transgen Biotechnology Company (Beijing, China). Rabbit anti-p70S6K (lot No: 2708), rabbit anti-phospho-p70S6K (lot No: 9234), rabbit anti-4EBP1 (lot No: 9644), and rabbit anti-phospho-4EBP1 (lot No: 2855) antibodies were purchased from Cell Signaling Technology (Massachusetts, USA). Rabbit anti-mTORC1 (lot No: 2587) and rabbit anti-phospho-mTORC1 (lot No: 5536) antibodies were purchased from Abcam (Cambridge, UK). The immunohistochemical kit was supplied by Beijing Sequoia Jinqiao Biotech Corporation (Beijing, China).

Animals and treatment

Male Kun Ming (KM) mice weighing 20–22 g were supplied by the Animal Center of Military Academy of Medical Sciences (No: 2012-0004). Mice were maintained in a 12-h light/dark cycle at 24°C in a room with a relative humidity of 60% and received food and water *ad libitum*. Animals were adapted for 1 week to the conditions before experimentation. All experimental procedures were performed in accordance with the guidelines of Beijing Municipal Ethic Committee for the Care and Use of

Laboratory Animals. All efforts were made to minimize animal suffering.

Drug administration

After 1 week of adaptive feeding, mice were randomly divided into control, 2 mg/kg rapamycin, 12 mg/kg stavudine, and CMC-Na groups. Each group was given the same dose of the drug or solvent control, and the CMC-Na group was given 0.5% CMC-Na. Rapamycin was given 10 min before the administration of stavudine in the 2 mg/kg rapamycin group. Drugs were orally administered to mice for 42 consecutive days. For the observation of mechanical and thermal hyperalgesia in mice, von Frey filament detection and thermal pain tests were conducted on day 7, 14, 21, 28, 35, and 42 after drug administration. After the last behavioral tests, 10 mice in each group were transcardially perfused with paraformaldehyde for mTOR immunohistochemistry. The remaining mouse spinal cords were rapidly removed for the measurement of mTOR and other biomarkers.

Behavioral testing

Mechanical hyperalgesia

The mouse was placed in a Plexiglas box with a metal mesh floor, and von Frey filaments were applied to the plantar surface of the bilateral hindpaws through the metal mesh floor. When the mouse was quiet after approximately 30 min of adaptation in the Plexiglas box, the mechanical pain threshold of the left and right hindpaw of each group, called the paw withdraw mechanical threshold (PWMT), was measured. The von Frey filament with a 2.00-g force was applied to the testing site of the hindpaw. For each filament, 5 trials with at least 5-min intervals were conducted with each mouse. The mean value after removal of the maximum and minimum values was expressed as the mechanical pain threshold.

Thermal hyperalgesia

For the examination of thermal hyperalgesia, the mouse was placed on a 2-mm-thick glass surface covered with a Plexiglass box. The sensitivity to heat stimuli was measured with a radiant heat stimulator. A radiant heat source was focused onto the plantar surface of the hindpaw. Measurements of the paw withdrawal thermal latency (PWTL) were obtained using a timer that was started by the activation of the heat source and stopped when withdrawal of the paw was detected with a photodetector. Five measurements of the PWTL were taken for each hindpaw and were averaged as the result of each test session. The ipsilateral hindpaw was tested with intervals of more than 5 min between consecutive tests.

Western blotting assay

Mouse spinal cords (from ten mice from each group) were lysed in nondenaturing lysis buffer. Samples containing 30 μg of protein per lane were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked in 5% skim milk-TBST (20 mmol/L Tris-HCL, pH 7.5, 500 mmol/L NaCl, 0.1% Tween 20) for 1 h. The appropriate primary antibodies were then added to the same milk (mTORC1: 1: 1000, phospho-mTORC1: 1: 500; 4EBP1: 1: 1000, phospho-4EBP1: 1: 500; p70S6K: 1: 1000, phospho-p70S6K: 1: 500), and the membranes were incubated overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody in TBST for 2 h at room temperature. The blotting was developed with the LAS3000 chemiluminescence system (Fujifilm, Tokyo, Japan), and the densities of the bands were determined using Gel-Pro Analyser 4.0 software (Media Cybernetics, Maryland, USA).

Immunohistochemical analysis

Ten mice from each group were anesthetized with pentobarbital and perfused transcardially with saline followed by cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. The spinal cords were then removed and post-fixed for 4 h in the same fixative. The fixed spinal cords were washed and cryoprotected in the same buffer containing 20% sucrose and finally sectioned into 40-μm-thick sections on a freezing microtome. Coronal sections through the intumescentia lumbalis were processed for mTOR immunohistochemistry. Briefly, after incubation for 1 h in 10% normal swine serum with 0.25% Triton X-100 in 0.02 mol/L potassium-phosphate-buffered saline containing 1% bovine serum albumin (KPBS-BSA), sections were incubated with the primary antibody (rabbit monoclonal antiserum to mTORC1, 1:500 in KPBS-BSA containing 2% normal swine serum and 0.25% Triton X-100) and then incubated first with the corresponding biotinylated secondary antibody followed by a 90-min incubation with Avidin-Peroxidase. Finally, the labeling was visualized with 0.04% hydrogen peroxidase and 0.05% 3,3′-diaminobenzidine (DAB) was visualized. The sections were observed with light microscopy (NIKON E600, Japan), and the intensity of the stained area in each group was analyzed by the Image-Pro plus system (Media Cybernetics, Silver Spring, MD). All evaluations were done by a researcher blind to the experimental design.

Statistical analysis

Data were expressed as the means ± standard deviation (SD). Multivariate analysis of variance (ANOVA) was used for repeated measurement of escape latency data, using a general linear model in SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Other data were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test. A $P < 0.05$ was considered to be statistically significant.

RESULTS

Rapamycin alleviated the pain caused by oral administration of stavudine

To observe the mechanical and thermal hyperalgesia of mice, we subjected the mice to von Frey filament detection and a thermal pain test on day 7, 14, 21, 28, 35, and 42 after drug administration. The results clearly showed that there were no significant differences in the PWMT or PWTL between

the four groups 1 day before drug administration, indicating that the selected mice were from the same population. As the drug administration period progressed, there were no differences in the PWMT or PWTL among the control group and the CMC-Na group of mice. The PWMT and PWTL in the 12 mg/kg stavudine and 2 mg/kg rapamycin groups were significantly decreased on day 7, 14, 21, 28, 35, and 42 after drug administration compared with those in the control group, and the PWMT and PWTL were the lowest on the 42nd day, indicating that oral administration of stavudine caused peripheral neuropathic pain.

As shown in Figure 1, treatment of mice with 2 mg/kg rapamycin increased the PWMT on day 7 (13.44 ± 0.25 vs. 12.35 ± 0.36, $F = 5.71$, $P = 0.026$), 21 (10.12 ± 0.29 vs. 9.16 ± 1.39, $F = 4.12$, $P = 0.046$), 28 (9.51 ± 0.20 vs. 8.54 ± 0.22, $F = 4.36$, $P = 0.042$), 35 (8.69 ± 0.20 vs. 7.64 ± 1.03, $F = 5.06$, $P = 0.031$), and 42 (8.41 ± 0.17 vs. 7.40 ± 0.13, $F = 4.89$, $P = 0.034$) after drug administration compared with treatment with 12 mg/kg stavudine alone, and the PWTL in the 2 mg/kg rapamycin group was significantly higher than that in the 12 mg/kg stavudine group on day 7 (8.56 ± 0.23 vs. 7.56 ± 0.35, $F = 4.57$, $P = 0.039$), 14 (8.29 ± 0.14 vs. 7.12 ± 0.32, $F = 5.92$, $P = 0.023$), 21 (7.88 ± 0.19 vs. 6.54 ± 0.20, $F = 6.23$, $P = 0.017$), 28 (7.27 ± 0.11 vs. 5.89 ± 0.16, $F = 6.68$, $P = 0.013$), 35 (6.90 ± 0.14 vs. 5.26 ± 0.23, $F = 15.09$, $P < 0.01$), and 42 (6.25 ± 0.16 vs. 4.43 ± 0.21, $F = 13.32$, $P < 0.01$) after drug administration. These results suggested that rapamycin had a beneficial effect on peripheral neuropathic pain induced by stavudine in mice.

Rapamycin inhibited the activation of mammalian target of rapamycin-sensitive complex 1, p70S6K, and 4E binding protein 1

The mTOR pathway is an evolutionarily conserved signaling module that regulates cell growth by directly phosphorylating the key translation regulators known as p70S6K and 4EBP1 and is also involved in the modulation of peripheral neuropathic pain from multiple levels.[14] In this study, the effects of rapamycin on the Akt/mTOR signaling pathway were assessed by Western blotting assay using antibodies against phosphorylation sites of different related proteins, including mTORC1, p70S6K, and 4EBP1. As shown in Figure 2, mice treated with 12 mg/kg stavudine exhibited a marked increase in the expression of phospho-mTORC1 (0.86 ± 0.03 vs. 0.58 ± 0.03, $F = 12.13$, $P < 0.01$), phospho-p70S6K (0.68 ± 0.03 vs. 0.35 ± 0.02, $F = 11.37$, $P < 0.01$), and phospho-4EBP1 (0.94 ± 0.06 vs. 0.46 ± 0.03, $F = 13.26$, $P < 0.01$) compared with the control mice. The addition of 2 mg/kg rapamycin significantly inhibited the phosphorylation of mTORC1 (0.72 ± 0.04 vs. 0.86 ± 0.03, $F = 4.24$, $P = 0.045$) and p70S6K (0.47 ± 0.01 vs. 0.68 ± 0.03, $F = 6.01$, $P = 0.022$) compared with stavudine treatment alone, and rapamycin also decreased phospho-4EBP1 expression, although not significantly (0.90 ± 0.04 vs. 0.94 ± 0.06, $F = 0.28$, $P = 0.646$). The results suggested that rapamycin alleviated peripheral neuropathic pain through activation of the Akt/mTOR signaling pathway.

Rapamycin reduced mammalian target of rapamycin-sensitive complex 1 expression in the spinal dorsal horn of mice treated with stavudine

In addition to the Western blotting assay, we further investigated the expression of mTORC1 in the spinal dorsal horn of mice using immunohistochemical analysis. The results showed that the mTORC1 protein was expressed in the spinal dorsal horn but primarily in the superficial laminae. In this study, we observed that compared with the control group, the stavudine-treated mice exhibited large areas of mTORC1 deposits in the superficial laminae of the spinal dorsal horn (112.30 ± 5.66 vs. 36.87 ± 2.24, $F = 36.12$, $P < 0.01$). Rapamycin treatment at 2 mg/kg significantly reduced mTORC1 deposits (70.80 ± 2.41 vs. 112.30 ± 5.66, $F = 34.36$, $P < 0.01$). These results suggested that rapamycin might have inhibited the production of mTORC1 that was induced by stavudine [Figure 3].

DISCUSSION

Currently, many HIV-infected patients use NRTI drugs for high active antiretroviral therapy, greatly extending their lifespan.[15] However, the patients taking NRTIs develop peripheral neuropathic pain, and the pathogenesis of this neuropathic pain has not yet been elucidated. Thus, there is no clear and effective treatment.[16,17] The Akt/mTOR signaling pathway is widely distributed in many kinds of cells and participates in cell metabolism, proliferation, differentiation, and apoptosis. In recent years, numerous studies have shown that the Akt/mTOR signaling pathway is involved in the regulation of neuropathic pain.[18,19]
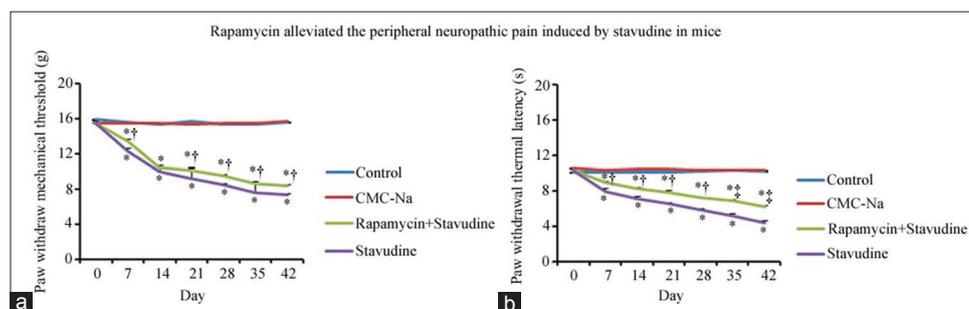
Figure 1: Rapamycin alleviated the peripheral neuropathic pain induced by stavudine in mice. (a) Paw withdraw mechanical threshold. (b) Paw withdrawal thermal latency. The results are expressed as the means ± standard deviation. $n = 20$ in each group. *$P < 0.01$ compared with control mice. †$P < 0.05$, ‡$P < 0.01$ compared with stavudine-treated mice.
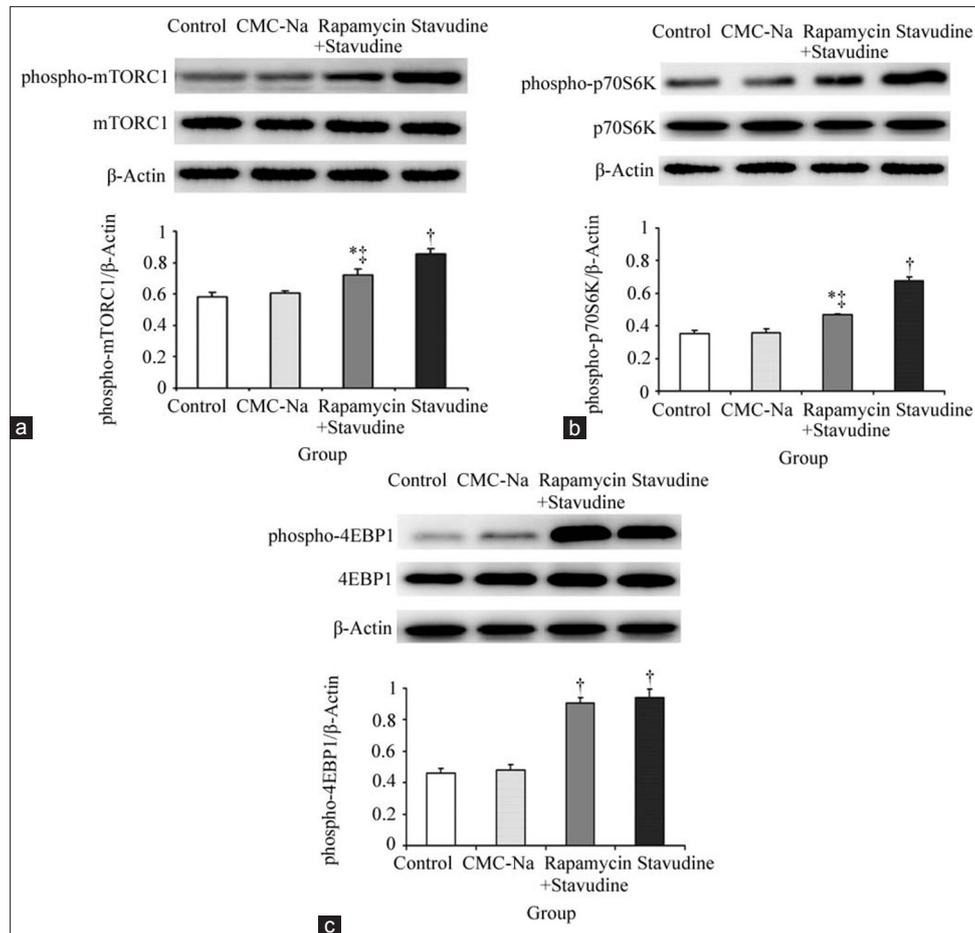
Figure 2: Rapamycin inhibited the expression of phospho-mTORC1, phospho-p70S6K, and phospho-4EBP1 in the spinal cord of mice treated with stavudine. (a) Western blotting assay of phospho-mTORC1 and mTORC1. (b) Western blotting assay of phospho-p70S6K and p70S6K. (c) Western blotting assay of phospho-4EBP1 and 4EBP1. Representative immunoblot of four experiments is shown. The results are expressed as the means ± standard deviation. *$P < 0.05$, †$P < 0.01$ compared with control mice. ‡$P < 0.05$ compared with stavudine-treated mice. mTOR: Mammalian target of rapamycin; S6K: Ribosomal S6 protein kinase; mTORC1: Mammalian target of rapamycin-sensitive complex 1; 4EBPl: Eukaryotic cell initiation factor 4E binding protein 1.

Rapamycin, which has mainly been used in the study of the pathogenesis and treatment of tumors and immune diseases, specifically inhibits the activity of mTOR.[20,21] Recent study has shown that rapamycin is a promising tool for the mechanistic study and treatment of pain.[22] In the present study, changes in behavior and protein expression related to the Akt/mTOR signaling pathway after the induction of neuropathic pain by oral administration of stavudine were investigated in mice. Using this stavudine-treated mouse model, we verified that rapamycin could alleviate peripheral neuropathic pain as well as mTOR production in the spinal cord. These data, together with the Akt/mTOR signaling pathway data, showed that the beneficial effects of rapamycin against NRTI-induced neuropathic pain were at least partially due to the suppression of the activation of the Akt/mTOR signaling pathway as indicated by reduced phosphorylation of the downstream proteins.

Neuropathic pain is caused by injury or dysfunction of the peripheral or central nervous system and includes the pain caused by central or somatic injury or disease. The clinical manifestations of neuropathic pain include pain in response to touch, hyperalgesia, and hyperthermia.[23,24] mTOR is a silk/threonine protein kinase expressed on mammalian neurons, and activation of the Akt/mTOR signaling pathway is involved in protein synthesis-dependent synaptic plasticity formation through the regulation of gene transcription as well as protein translation and transport.[25] Recent study has shown that the Akt/mTOR signaling pathway is involved in the production and maintenance of pain and should be a focus of pain studies. Recent study has shown that rapamycin, a tool for the study of the Akt/mTOR signaling pathway, can effectively reduce the mechanical sensitivity of rats with chronic inflammatory pain.[26] After pretreatment with rapamycin, the late maintenance of long-term potentiation and long-term memory formation in the hippocampus were blocked, and the hyperalgesia induced by formalin was reduced; moreover, the excitability of spinal dorsal horn neurons was also significantly decreased.[27,28] To better understand, the effect of rapamycin on neuropathic pain, we applied rapamycin to stavudine-treated mice to further explore the role of rapamycin in hyperthermia and mechanical pain
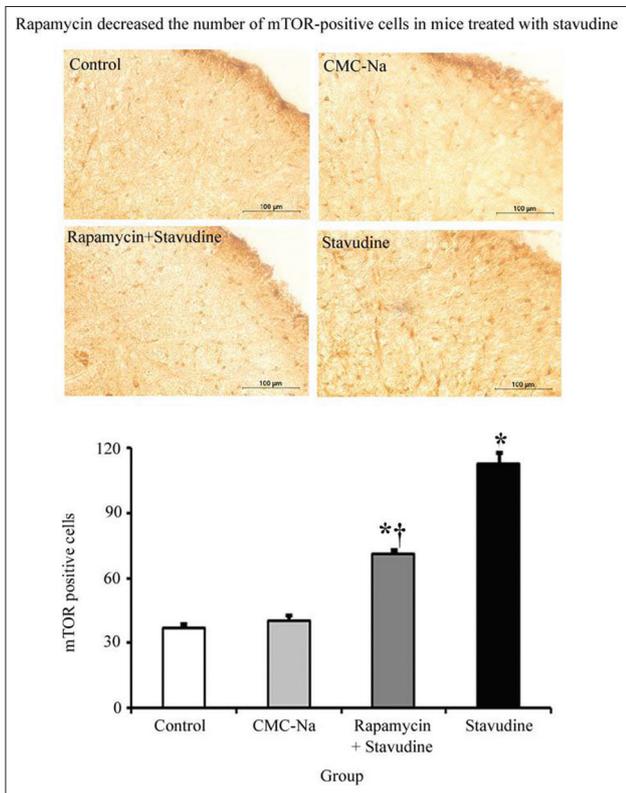
Figure 3: Rapamycin decreased the number of mTORC1-positive cells in mice treated with stavudine. Immunohistochemistry of mTORC1 in the spinal dorsal horn of mice. Representative sections of the spinal dorsal horn from 10 mice are shown. The results are expressed as the means ± standard deviation. $*P < 0.01$ compared with control mice. $†P < 0.01$ compared with stavudine-treated mice. mTOR: Mammalian target of rapamycin; mTORC1: Mammalian target of rapamycin-sensitive complex 1.

abnormalities. In the present study, rapamycin consistently elevated the mechanical pain and thermal pain thresholds compared to stavudine treatment alone, but the thresholds in the rapamycin-treated group were still lower than those in the control group, indicating that rapamycin could relieve the pain caused by stavudine but had no further analgesic effect.

To investigate the underlying mechanisms of rapamycin protective effects on peripheral neuropathic pain, the Akt/mTOR signaling pathway was studied. At present, research on the mechanism of neuropathic pain has mainly focused on the peripheral mechanism and the central mechanism, with the central mechanism expressed as changes in synaptic plasticity in the spinal dorsal horn, that is, central sensitization. The spinal dorsal horn is the aggregation site of peripheral sensory afferent nerve fibers and the downstream projection nerve fibers of the cerebral cortex and brain stem and is the primary center for nociceptive information transmission and integration. The superficial laminae of the spinal dorsal horn is an important site in the transmission of nociceptive information to the upper brain by afferent nerve fibers, and abnormalities in neuronal excitatory in this region are the pathophysiological and morphological bases of central sensitization in neuropathic pain.[29] In recent

years, mTOR has been found to regulate protein synthesis through mRNA translation and participate in the maintenance of chronic pain by mediating central sensitization and peripheral sensitization.[30] Changes in pain threshold and behavior are the expression of neuronal plasticity, which causes long-term pain. mTOR-mediated mRNA translation at the level of the spinal cord is a key factor in the formation of neuronal hyperhidrosis and behavioral allodynia.[31] mTOR is expressed in the sensory nerve fibers of the skin tissue where is regulates local protein synthesis and maintains the pain sensitivities of the nociceptors. A study from other laboratory has also demonstrated that local application of rapamycin could be used to relieve chronic pain caused by tissue damage.[32] In carrageenan and formalin inflammatory pain or spinal nerve ligation neuropathic pain animal models, mTOR has been shown to be activated and expressed in the superficial laminae of the spinal dorsal horn, and rapamycin treatment dose-dependently alleviates thermal hyperalgesia and mechanical hyperalgesia. In addition, researchers have used electrophysiological methods to show that mTOR is involved in the regulation of mRNA protein translation, which is crucial in neuronal synaptic plasticity and pain sensitivity, and direct application of rapamycin on the surface of the spinal cord inhibits the electrophysiological activity of neurons in rats with formalin-induced pain.[33] Other study had also shown that mTOR and its downstream translational elements are expressed in myelin, and local use of rapamycin reduces mechanical sensitivity in an spared nerve injury rat model, suggesting that the sensitivity of peripheral sensory nerve fibers is related to mTOR.[34] Collectively, these findings suggest that the mTOR signaling pathway regulates protein translation and synthesis, which is involved in neuropathic pain by mediating new protein synthesis in pain-related areas. Here, we focused on the central mechanism of the mTOR signaling pathway in neuropathic pain induced by stavudine; therefore, we observed the changes in the expression of mTOR-related proteins and their activated forms in the spinal cord. The results showed that oral administration of rapamycin decreased the expression of mTOR in the spinal cord of mice compared with stavudine treatment alone, and this change was negatively correlated with the increase in the mechanical and thermal pain threshold, indicating that the Akt/mTOR signaling pathway might be involved in the maintenance of neuropathic pain by modulating the expression of mTOR in the spinal cord.

Active Akt has many substrates, of which mTOR is an important one, with vital roles in cell survival. mTOR forms two complexes with distinct substrate specificities, mTORC1 and mTORC2, and mTORCl is sensitive to rapamycin.[35] mTORC1 largely controls translation and cell growth in response to nutrients and regulates key cellular functions. On the other hand, mTORC2 has been shown to control actin cytoskeleton dynamics. The effect of Akt has been demonstrated to be mediated through activation of mTOR. The mTORC1 complex controls protein translation by phosphorylating substrates such as p70S6K and 4EBP1. p70S6K regulates the biosynthesis of translational elements

by phosphorylating the S6 protein and upregulating the translation of mRNA for 5' terminal oligopyrimidine tract, which is a necessary kinase for protein synthesis. The phosphorylation of 4EBPl relieves the inhibition of eukaryotic initiation factor 4E and allows protein translation.[36] Our findings showed that the expression of phospho-mTORC1, phospho-4EBP1, and phospho-p70S6K were increased after stavudine administration, suggesting that stavudine might activate the Akt/mTOR signaling pathway by increasing the phosphorylation of mTORC1 and participate in the occurrence of neuropathic pain. Meanwhile, rapamycin significantly decreased the expression of phospho-mTORC1 and its substrates p70S6K and 4EBP1. The data indicated that the Akt/mTOR signaling pathway might be involved in rapamycin effect on neuropathic pain. However, some small differences in protein expression have been reported, and the response of 4EBPl to rapamycin has been observed to be non-specific.[37] Choo and Blenis reported that the different sensitivity of p70S6K and 4EBPl to rapamycin might be related to the difference in the affinity to mTORCl; the combination of 4EBPl and raptor is relatively tight, whereas p70S6K binds to raptor with a lower affinity.[38] Consistent with these reports, the study revealed that the inhibition of phospho-4EBPl by rapamycin was not sufficient to result in significant differences. Together with the results mentioned above, we inferred that mTOR, and its related Akt/mTOR signaling pathway might be the possible mechanism involved in the effect of rapamycin on neuropathic pain. However, this study only clarified that rapamycin might participate in the neuropathic pain caused by stavudine through the Akt/mTOR signaling pathway, but the interaction between proteins in the signaling pathway and whether there are other related signaling pathway involved in neuropathic pain has not been studied, thus, the mechanism by which the Akt/mTOR signaling pathway is regulated by rapamycin still needs further investigation.

Chronic pain, particularly neuropathic pain in humans, is difficult to manage with the current therapeutic approaches. The focus of the present work was to identify the mechanism of the neuropathic pain induced by stavudine. This study provides evidence that the application of rapamycin, which inhibits mTOR-regulated protein synthesis, attenuates persistent pain states in mice. We demonstrate that this effect is mediated by blocking mTOR activity in the spinal cord, specifically in a subset of dorsal horn projection neurons that convey nociceptive information to the brain. These findings suggest a new pharmacological route for therapeutic intervention in neuropathic pain.

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Conflicts of interest

There are no conflicts of interest.

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PI3K-Akt-mTOR信号通路有助于缓解核苷逆转录酶抑制剂治疗HIV感染所并发的神经性疼痛

摘要

背景： 核酸逆转录酶抑制剂（Nucleoside reverse transcriptase inhibitors, NRTIs）是临床应用最早且数量最多的抗人类免疫缺陷病毒（human immunodeficiency virus, HIV）药物，在高效抗病毒逆转录治疗（Highly active anti-retrov iral therapy, HAART）中至今仍发挥着重要的作用。但是，NRTIs类药物治疗可引起周围神经病理性疼痛。本研究将通过动物实验观察雷帕霉素干预后对造模小鼠痛敏的影响来探索哺乳动物雷帕霉素靶蛋白（mammalian target of rapamycin, mTOR）mTOR在NRTIs类药物所致神经病理性痛发生中所起的作用。

方法： 体重20–22g的雄性昆明（Kun Ming, KM）小鼠分为正常组，雷帕霉素（2mg/kg）组，司他夫定组（12mg/kg）和CMC-Na组，各组连续灌胃给药42天。在给药后7、14、21、28、35、42天利用热痛敏仪和von Frey纤维检测热痛敏和机械性痛敏。行为学实验结束24h后，利用免疫组织化学和免疫印迹实验检测mTOR和其他生物标志物。使用多元方差分析进行数据统计。

结果： 雷帕霉素对神经性疼痛的有益作用归因于减少小鼠脊髓中哺乳动物雷帕霉素靶蛋白敏感复合物（mammalian target of rapamycin-sensitive complex, mTORC1）阳性细胞的数目（70.80 ± 2.41 vs. 112.30 ± 5.66, $F = 34.36$, $P < 0.01$）和降低mTORC1的活性。机制研究表明雷帕霉素通过抑制mTORC1的磷酸化（0.72 ± 0.04 vs. 0.86 ± 0.03, $F = 4.24$, $P = 0.045$），同时减少磷酸化p70S6K（0.47 ± 0.01 vs. 0.68 ± 0.03, $F = 6.01$, $P = 0.022$）和磷酸化4EBP1（0.90 ± 0.04 vs. 0.94 ± 0.06, $F = 0.28$, $P = 0.646$）的表达进而抑制 Akt/mTOR信号通路的激活。

结论： 研究结果表明，司他夫定通过激活Akt/mTOR信号通路提高了脊髓中mTORC1的表达和活性，该数据还提供了雷帕霉素可能用于治疗神经性疼痛的证据。