4SC-202 Exerts An Anti-tumor Effect in Cervical Cancer By Targeting PRLR Signaling Pathway

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Research Article

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Abstract

The aim of the present study is to investigate whether 4SC-202, a selective class I histone deacetylase inhibitor (HDACi), plays an anti-tumor role in cervical cancer (CC) by targeting prolactin receptor (PRLR). CCK-8 and colony formation assays were used to evaluate the effects of 4SC-202 on the proliferation of CC cells *in vitro*. Effects of 4SC-202 on the cell cycle distribution and apoptosis in SiHa cells were determined by flow cytometry and western blotting, respectively. Immunofluorescence and western blotting were performed to detect the activities of PRLR-related pathways and PRLR expression in CC cells. A xenograft tumor model in nude mice was established to examine effects of 4SC-202 on the tumor growth, apoptosis and PRLR-related pathways *in vivo*. The biochemical analyzer and H&E staining were used to detect the serum biochemical indexes and organ toxicity. 4SC-202 inhibited the proliferation of CC cells (SiHa, HeLa, and CaSki) *in vitro* in a time- and dose-dependent manner. SiHa cells were treated with 1 or 5 μ M 4SC-202 for 72 h and then subjected to various functional assays. The assays showed that 4SC-202 significantly induced G2/M phase arrest and apoptosis, while inhibiting the activities of PRLR-related pathways and PRLR expression. In addition, 4SC-202 reduced tumor growth and induced apoptosis *in vivo*. 4SC-202 down-regulated the expression of PRLR and activities of PRLR-related pathways in the mouse model, displayed no effects on serum biochemical indicators and caused no toxicity to mouse organs. This finding suggests that 4SC-202 may serve as a novel therapeutic agent for CC.

Introduction

In recent years, cervical cancer (CC) has become one of the leading malignancies that threaten the health and lives of women worldwide [1]. The vast majority of patients with CC are mainly caused by persistent infection with human papillomavirus (HPV) [2]. At present, while regular HPV screening, colposcopy directed biopsy, and HPV vaccination have been routinely used for the prevention of cervical cancer, it remains a high-risk disease for women [3]. Although HPV16 and HPV18 have been proven to be the main factors causing cervical cancer [4], some studies have shown that changes in hormone cofactors may be involved in pathogenesis of cervical tumors [5]. It has recently been demonstrated that prolactin receptor (PRLR) is highly expressed in cervical cancer cell lines [2, 6], suggesting that it may be implicated in the cancer development. However, the mechanism underlying role of PRLR in CC awaits further studies.

PRLR, a type I cytokine receptor family protein, promotes cell proliferation and differentiation through mediating the activity of prolactin (PRL) and triggering intramembrane signal cascades, such as signal transduction and the activators of transcription (STAT) family, extracellular regulated kinase (ERK1/2), and serine threonine kinase (AKT)[6–8]. Studies have shown that PRLR is highly expressed in CC tissues [8], and signal transduction and activation of STAT3 promotes anti-apoptotic effect of PRL [9]. Lopez-Pulido et al. [6] demonstrated that PRL/PRLR signal can be used as an important survival factor for CC. In a previous study, we showed that receptor dimerization is necessary for cytokines to activate signal transduction pathways in cells [10]. PRLR enhances lysine acetylation by recruiting CREB-binding protein (CBP) to neutralize the positive charge of the lysine side chain, and dimerizing the receptors, thereby

facilitating the acquisition of transcription factors to increase transcription [11, 12]. It has been reported that histone deacetylase inhibitors (HDACi) promote PRLR dimerization and subsequent signal transduction, whereas exogenous HDACs inhibit the processes [12]. While several HDACi have been shown to be involved in anti-tumoral immune responses [13], the underlying molecular mechanisms have not been fully understood. Domatinostat (4SC-202), a type I HDACi [14], shows potent anti-tumor activities in various cell lines and preclinical models. 4SC-202 has been found to markedly inhibit proliferation and survival of colorectal cancer (CRC) cells as well as the growth of colorectal tumors *in vivo* [15]. Moreover, 4SC-202 was capable of inhibiting the cell viability in urothelial carcinoma (UC) and inducing cell cycle disturbances and cell death [16]. Gruber et al found that 4SC-202 efficiently inhibits Hedgehog (HH)/GLI signaling in SMOi-sensitive and SMOi-resistant settings, while suppressing the growth of basal cell carcinoma (BCC) cells *in vivo*. [17]. Given that research on anti-tumor effects of 4SC-202 on cervical cancer and the underlying mechanism is still lacking, we intended to investigate whether 4SC-202 plays an anti-cervical cancer effect through targeting the PRLR signaling pathway.

Materials And Methods

Chemicals and reagents

4SC-202 was provided by Lan-jun Biotechnology Co.Ltd (Shanghai, China). Cell Counting Kit-8 (CCK-8) kit and DMSO were purchased from Sigma Aldrich (MO, USA). All cell culture reagents were purchased from Gibco Life Technologies (Grand Island, CA, USA).

Cell culture

Cervical cancer cell lines SiHa, Hela, and CaSki were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cell culture was conducted in a humidified atmosphere of 5% CO₂ at 37°C. Cells were grown to 80% confluence for further assays. All culture reagents were obtained from Gibco (Carlsbad, CA).

CCK-8 assay

SiHa, Hela, and CaSki cells were plated in 96-well plates and incubated with various concentrations of 4SC-202 (0, 0.01, 0.1, 1, 5, and 10 µM) for a certain period of time (24 h, 48 h, or 72 h). CCK-8 reagent (10 µL) was added and the mixture was incubated for 2 h in the dark. The optical density (OD) value for each well was measured at a wavelength of 450 nm. The half maximal inhibitory concentration (IC₅₀) was calculated by using SPSS software.

Colony formation assay

Colony formation assay was carried out as described previously [18]. SiHa cells (5×10³ cells/well) were plated in 6-well plates and incubated with various concentrations of 4SC-202 (0, 1, and 5 µM) for 72 h.

Culture media were refreshed every 2 days. The cells were stained with crystal violet, and the colonies were photographed and manually counted on day 10.

Cell cycle analysis and apoptosis detection

For cell cycle analysis, SiHa cells were incubated with various concentrations of 4SC-202 (0, 1, and 5 μM) for 72 h, digested with 0.25% trypsin and resuspended in phosphate-buffered saline (PBS) at a density of 1×10^6 cells/mL. Cell cycle distribution was detected by flow cytometry (BD Biosciences, CA, USA) and analyzed using Cell Quest software. For apoptosis detection, the cell suspension was gently mixed with 5 μL Annexin V-FITC and 10 μL PI, followed by an incubation at room temperature for 15 min in the dark. Apoptosis rate was determined by using the flow cytometry.

Western blotting

Total proteins were extracted from the cell lines using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Tissue lysates were centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatants were collected for further analysis. The protein concentration was determined using BCA protein Assay Kit (Beyotime). Fifty μg of total protein were mixed with loading buffer, denatured at 95°C for 5 min, resolved on a 10% SDS polyacrylamide gels and electro-transferred to PVDF membranes (Bio-RAD, CA). After being blocked with 5% milk and 1% bovine serum albumin solution, the membrane was incubated at 4°C overnight with the following primary antibodies: anti-Cleaved-caspase3, anti-BCL-2, anti-Bax, anti-p-STAT5a, anti-STAT5a, anti-p-AKT, anti-AKT, anti-p-ERK1/2, anti-ERK1/2, and anti-GAPDH (Acbam, diluted 1:1000). On the following day, the membrane was washed 3 times with TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies (Acbam, diluted 1:5,000) for 2 h at room temperature. The protein bands were developed with a chemiluminescence detection system (Immobilion, Millipore), and the relative band intensity was analyzed using ImageJ software.

Immunofluorescence

Cultured cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Fixed cells were blocked with 1% BSA at room temperature for 30 min. After the pretreatments, the cells were incubated with the primary antibodies (PRLR 1:20) at 4°C overnight, followed by an incubation with Alexa Flour-conjugated secondary antibody (Invitrogen) at room temperature for 1 h in the dark. Nuclear DNA was counterstained with 10 ng/ml DAPI at 4°C for 30 min in the dark. Stained cells were observed and photographed under a fluorescence microscope (200 $\times$).

Xenograft assay

Five-week-old female C57BL/6 nude mice were obtained from Bioscience (Beijing, China) and housed in a laboratory animal room under standard conditions (12-h light/dark cycle, 20-25°C, and 60-85% humidity), with ad libitum access to sterilized food and water. The mice were randomly divided into three groups ($n = 5$ per group): control, 50 mg/kg 4SC-202, and 100 mg/kg 4SC-202. SiHa cells were injected into the

upper right flank of 15 nude mice. 4SC-202 treatment was started on day 6 post-injection when the tumors were palpable. 4SC-202 was dissolved in DMSO and administered by gavage every two days. The tumor size was measured every 3 days, and tumor volumes were calculated as follows: $(V) = \text{width}^2 \times \text{length} \times 0.5$. In the meantime, body weights of the mice were recorded every week. All the animals were euthanized, and the xenografts were weighted after 30 days.

Subcutaneous tumors were dissected, removed and kept for immunohistochemical and western blot analyses. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by AU680 Chemistry System (Beckman Coulter, Brea, CA, USA). Organ tissues including lung, liver, kidney, and heart were removed, sectioned and subjected to H&E staining. Animal experiments were approved by the Ethics Committee of Medical Laboratory Animal Center of Chinese People's Liberation Army General Hospital and performed in accordance with National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals.

Immunohistochemical analysis

Paraffin-embedded sections were dewaxed with dimethyl benzene and rehydrated in dH₂O. The slides were immersed in citrate buffer (0.01 M, pH 6.0) with high-pressure heating for 2 min. After being blocked with 1% BSA (Sigma, USA) in TBST buffer for 5 min at room temperature, the slides were incubated with primary antibodies against Ki-67 (Proteintech, Wuhan, China) at a dilution of 1:16000 overnight at 4 °C, followed by an incubation with goat anti-rabbit IgG secondary antibody (Thermo Fisher, 1:5000 dilution) and DAB staining. Finally, the slides were counterstained with hematoxylin, dehydrated and sealed with neutral gum. Organ tissues were stained with H&E for histological analysis. The images were captured under a Nikon microscope.

Statistical analysis

Results were presented as the mean $\pm$ standard deviation (SD). At least three experimental replicates were evaluated for each sample. The significance of the experimental data was analyzed by using PRISM[®] GraphPad 8.0 software, one-way or two-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. The significance level was set at $P < 0.05$, and p-values were indicated accordingly.

Results

4SC-202 suppresses the proliferation of cervical cancer cells

To study the effect of 4SC-202 on the proliferation of cervical cancer cells, we performed CCK-8 assay to detect the cell proliferation in selected three human cervical cancer cell lines SiHa, Hela and CaSki. As depicted in Fig. 1A, 4SC-202 displayed a dose-dependent cytotoxicity against SiHa cells, while SiHa cells treated with 4SC-202 at a concentration of 1-10 μM showed significantly reduced cell viability. Moreover, we observed that while 4SC-202 exerted cytotoxic effect on SiHa cells in a time-dependent manner, the effect was the most obvious at 72 h. Strikingly, 4SC-202 exhibited the similar effect on both Hela (Fig. 1B)

and CaSki cells (Fig. 1C). Among the three cell lines, SiHa cells treated with 4SC-202 showed the most significant decrease in the cell viability (Fig. 1A). Meanwhile, the IC₅₀ values of 4SC-202 in all three cell lines decreased with increasing length of incubation. Collectively, these data indicated that 4SC-202 had anti-proliferative activity on cervical cancer cells. Besides, we performed colony formation assay and found that colony-forming ability of SiHa cells treated with 4SC-202 was significantly inhibited (Fig. 1D) ($P<0.01$), indicative of a suppressive effect of 4SC-202 on the growth of cervical cancer cells. We, therefore, chose SiHa cells for subsequent experiments.

4SC-202 induces cell cycle arrest and apoptosis

Effects of 4SC-202 on the cell cycle distribution of SiHa cells were analyzed by flow cytometry. As illustrated in Fig. 2A, treatment with 4SC-202 caused cell cycle arrest in SiHa cells. The quantitative analysis revealed a cell cycle arrest at G2/M phase in SiHa cells incubated with 4SC-202. The ratios of G2/M phase in SiHa cells treated with 0, 1 or 5 μ M 4SC-202 for 72 h were $21.49 \pm 0.81\%$, $30.01 \pm 2.18\%$ and $44.74 \pm 1.64\%$, respectively. These observations demonstrated that 4SC-202 caused cell cycle arrest at G2/M phase in SiHa cells in a dose-dependent manner.

We next performed western blotting to analyze the expression of several apoptosis-related proteins. As shown in Fig. 2B, 4SC-202 down-regulated the expression of BCL-2 (26 kDa), while up-regulating the expression of Cleaved-caspase3 (17 kDa) and Bax (21 kDa). In addition, we evaluated the effects of 4SC-202 on cell apoptosis by flow cytometry and found that 4SC-202 treatment led to a dose-dependent increase in the apoptosis rate of SiHa cells (Fig. 2C).

4SC-202 reduces the expression of PRLR in cervical cancer cells by inhibiting the PRLR pathway

We further examined the expression of PRLR pathway-related proteins by western blot analysis. As presented in Fig. 3A, compared with the control group, a significant decrease in phosphorylation levels of STAT5a and AKT and a marked increase in ERK1/2 phosphorylation level were evident in SiHa cells treated with 5 μ M 4SC-202 ($P<0.05$). Moreover, we performed an immunofluorescence assay and observed that 4SC-202 significantly down-regulated the expression of PRLR in SiHa cells compared with the control group (Fig. 3B) ($P<0.001$). Together, these results indicated that 4SC-202 reduced the expression of PRLR in CC cells through down-regulating PRLR pathway-related proteins.

4SC-202 inhibits tumor growth via the PRLR pathway.

To investigate effects of 4SC-202 on the growth of cervical cancer cells *in vivo*, the nude mice were injected subcutaneously with SiHa cells and then administered with 50 or 100 mg/kg 4SC-202 by gavage every two days for 30 days. As depicted in Figs. 4A-C, both tumor volume and tumor weight were significantly reduced in the mice treated with 4SC-202 as compared to the control group ($P<0.001$). Furthermore, we performed Ki67 immunostaining on tumor sections to assess tumor proliferation *in vivo*. The assay showed that compared with the control, 4SC-202 treatment led to a significant decrease in the percentage of Ki-67 positive cells in the tumor tissue (Fig. 4D).

We next evaluated the expression of apoptotic proteins in the nude mice treated with 4SC-202. Consistent with the *in vitro* data, we found that 4SC-202 remarkably down-regulates BCL-2 expression and up-regulated the expression of cleaved-caspase3 and Bax in the mice (Fig. 4E) ($P<0.001$). In addition, we observed a consistency in the expression of PRLR pathway related proteins between *in vivo* and *in vitro* studies (Fig. 4F) ($P<0.001$).

Finally, we found that 4SC-202 had no significant effects on the serum biochemical function in the nude mice (Fig. 4G) ($P>0.05$). H&E staining was performed to histologically analyze major organs such as lung, liver, spleen and heart. As shown in Fig. 4H, no significant histological changes were detected in the tissue sections of mice treated with 4SC-202.

Discussion

HDAC is essential for epigenetic control of cancer gene expression and functions, while being involved in the regulation of cell cycle progression, cell proliferation and differentiation, apoptosis, metastasis as well as angiogenesis [13, 19, 20]. HDACi can restore the homeostasis of cell acetylation and activate the expression of tumor suppressor genes [13], thus affecting various biological processes of cancer. And HDACi has been applied in some clinical trials [21–23]. DNA methylation and covalent histone modification have been identified as the most important epigenetic changes in cervical cancer [24]. To date, HDACi such as valproic acid (VPA) [25], suberoylanilide hydroxamic acid (SAHA) [26], and trichostatin A (TSA) [27] have been used as anti-tumor agents in cervical cancer. However, the research on anti-tumor effects of HDACi 4SC-202 on CC is still lacking.

In this study, we showed that 4SC-202, a novel oral class I specific HDACi of benzamide compounds [28], can effectively inhibit the growth, proliferation, and cell cycle progression of CC cells and promote the apoptosis. Moreover, we found that 4SC-202 treatment led to inhibition of the PRLR pathway as well as down-regulation of PRLR expression in CC cells. Notably, 4SC-202 displayed anti-tumor and anti-apoptotic effects in xenograft mouse model, dramatically decreased the percentage of Ki-67-positive cells and inhibited the PRLR pathway in tumor tissues. Meanwhile, we observed no significant alterations in serum biochemical function and histological morphology of organ tissues in the nude mice treated with 4SC-202, indicating that 4SC-202 was nontoxic *in vivo*. All these results suggested that 4SC-202 could serve as a potential therapeutic agent for CC.

HDACi can cause cell cycle arrest in G1/S or G2/M phase, highlighting HDACi as a promising target for cancer treatment [13]. In this study, we showed that 4SC-202 treatment led to cell cycle arrest of SiHa cells in G2/M phase (Fig. 2A). This observation is consistent with the previous study showing a G2/M phase arrest in colorectal cancer (CRC) cell lines and primary human CRC tumor cells following 4SC-202 treatment [29]. In addition, HDACi can regulate apoptosis in cancer cells through the mitochondrial pathway and the expression of some Bcl family proteins [30]. Zhang et al. demonstrated that 4SC-202 induced apoptosis in hepatocellular carcinoma [19]. Clearly, the present study confirmed that 4SC-202 promoted apoptosis in CC cells by up-regulating the expression of caspase3 and Bax and inhibiting Bcl-2

expression (Fig. 2B). In the meantime, flow cytometry analysis revealed that 4SC-202 significantly increased the apoptosis rate in CC cells (Fig. 2C).

Previous studies have shown that PRLR signaling pathways play an important role in initiating CC [2]. PRLR dimerization activates intracellular signaling cascades including downstream signaling pathways such as JAK/STAT5a, P13K/AKT, and ERK1/2, thereby promoting cell proliferation, differentiation and survival [7, 31]. It has been reported that PRL induced an increased expression of anti-apoptotic genes in CC cell lines by activating the STAT pathway, which was linked to cell survival [9]. In a previous study, we found that PRL had a protective effect on etoposide-induced apoptosis in CC cells [6]. A study on melanoma cells showed that HDACi mainly suppressed the expression of anti-apoptotic proteins by inhibiting transcription [11]. Cytotoxicity of 4SC-202 against CRC cells was enhanced by AKT inhibition or AKT1 knockdown [15]. Herein, we observed that the expression of PRLR and PRLR-related pathway components (STAT5a, AKT, and ERK1/2) was significantly inhibited in SiHa cells treated with 4SC-202 (Fig. 3). Furthermore, 4SC-202 markedly decreased the proliferation of CC cells (SiHa, HeLa, and CaSki) in a dose- and time-dependent manner (Fig. 1). Combined with the results of apoptosis, we speculated that 4SC-202 may promote apoptosis in CC cells by down-regulating the expression of PRLR and inhibiting the PRLR pathway, thereby reducing the cell proliferation.

The present study provided evidence that tumor growth was inhibited in the nude mice administered with 4SC-202, while no toxicity was detected in major organs of the mice. In addition, immunohistochemical assays revealed that the expression of Ki67, a nuclear and nucleolar protein related to cell proliferation and severity of cervical lesions [4, 32], decreased significantly with the increasing concentration of 4SC-202 in the tumor tissues. The expression of apoptotic protein and PRLR pathway components decreased in the tumor tissues, and 4SC-202 displayed no effect on the levels of ALT and AST in serum. All these findings indicated that 4SC-202 may affect the proliferation and activity of CC cells through inhibiting the expression of PRLR-related pathway components as well as induce cycle phase arrest *in vivo* and was non-toxic to organs.

Conclusion

In conclusion, the present study showed that 4SC-202 caused cell cycle arrest of CC cells at G2/M phase through inhibiting the expression of PRLR-related pathway components (STAT5a, AKT, and ERK1/2), induced apoptosis and inhibited CC cell proliferation. Moreover, 4SC-202 significantly suppressed the growth of CC *in vivo*, while exerting no toxicity to major organs in the nude mice. These findings suggest that 4SC-202 may potentially serve as a novel and efficacious therapeutic agent for CC.

Abbreviations

ALT: alanine aminotransferase; AST: aspartate aminotransferase; CC: cervical cancer; CCK-8: Cell Counting Kit-8; HDAC: histone deacetylase; HDACi: histone deacetylase inhibitor; IC50: half maximal inhibitory concentration; PRL: prolactin; PRLR: prolactin receptor.

Declarations

Ethics approval and consent to participate Animal experiments were approved by the Ethics Committee of Medical Laboratory Animal Center of Chinese People's Liberation Army General Hospital and performed in accordance with National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals.

Consent for publication Not applicable.

Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests Not applicable.

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Authors' contributions Huijuan Zhang and Yuanguang Meng conceived and designed the experiments. Mingxia Li, Wen Yang, Mingxia Ye and Hua Li performed the experiments and analyzed the data. Huijuan Zhang interpreted the results and wrote the manuscript. Yuanguang Meng contributed to the review of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest These authors declare that there is no conflict of interests.

Research involving human participants and/or animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Not applicable.

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Figures

Figure 1

4SC-202 inhibits the proliferation and growth of cervical cancer cells in a dose- and time-dependent manner. CCK-8 assay was performed to determine the cell viability of SiHa (A), HeLa (B), and CaSki (C) cells incubated with various concentrations of 4SC-202 (0, 0.1, 1, 5, and 10 μM) for a certain period of time (24 h, 48 h, or 72 h). (D) The colony formation assay of SiHa cells treated with 1 or 5 μM 4SC-202 for 72 h. **P<0.01 vs. the control group.

Figure 2

4SC-202 induces cell cycle arrest and apoptosis. (A) The cell cycle distribution of SiHa cells were evaluated by flow cytometry. (B) Western blot analysis was conducted to examine the expression of Cleaved-caspase3, BCL-2, and Bax in SiHa cells treated with 4SC-202. (C) Apoptosis of SiHa cells was determined by flow cytometry. * $P < 0.05$, *** $P < 0.001$ vs. the control group.

Figure 3

4SC-202 down-regulates the expression of related proteins by inhibiting the PRLR pathway. (A) The expression and phosphorylation levels of PRLR pathway-related proteins STAT5a, AKT and ERK1/2 were determined by western blot analysis. (B) Immunofluorescence was performed to detect the expression of PRL. The immunofluorescence intensity was quantified. Scale bar=30 μm. *P<0.05, **P<0.01, ***P<0.001 vs. the control group.

Figure 4

4SC-202 inhibits tumor growth in vivo. (A) The images of tumors in the nude mice administered with or without 4SC-202 (50 mg/kg and 100 mg/kg) for 30 days. (B) The tumor growth curve in the nude mice administered with or without 4SC-202. (C) The tumor weights in each group were measured. (D) The representative images of Ki-67 immunostaining of tumor sections. (E) The expression of apoptosis-related proteins was detected by western blot analysis. (F) The expression levels of PRLR pathway-related proteins were determined by western blot analysis. (G) Serum levels of AST and ALT in the mice were measured by using the biochemistry analyzer. (H) H&E staining-based histological examination of the mouse organs. Scale bar=100 μ m. **P<0.01, ***P<0.001 vs. the control group.