Maprotiline Prompt the Anti-tumor Effect by Inhibiting the PD-L1 in Mice Burdened Melanoma

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Abstract

Programmed cell death 1 ligand 1 (PD-L1) binds with programmed cell death protein 1 (PD-1) to inhibit the responses of T cells. PD-L1 is significantly upregulated on tumor cells and blocking the PD-L1/PD-1 signal has become an important target of immunotherapy in clinic. At present, some old drugs of non-antitumor have been found that could play the effect of anti-tumor. Maprotiline, as a tetracyclic antidepressant, has been widely used for treating mental depression. Here, we study the anti-tumor effect of maprotiline by strengthening the immune response of mice. In vitro, treatment with maprotiline inhibits the proliferation and migration of B16 cells, increases the cell apoptosis. Importantly, treatment with maprotiline reduces the expression of PD-L1 in tumor tissue, prompts the ratios of CD4⁺ T cells, CD8⁺ T cells and NK cells in spleens, increases the infiltration of CD4⁺ and CD8⁺ T cells in tumor-tissues. In brief, we determine that maprotiline could prompt the anti-tumor immune response by inhibiting the PD-L1 in mice. This study may find a new inhibitor of PD-L1, which provides a new drug treated tumor in clinical.

Introduction

Melanoma is one of the most common fatal malignancies in the world. In the past, the outcomes of patients have been improved dramatically, but the overall survival rates with 5-year is only 30% to 40% [1]. Most patients don't achieve the therapeutic effect as expected. It is still necessary to study new drug for the treatment of melanoma.

It is considered an effective and safe route to find novel indications of old drug. As known, antidepressants have been widely used for the treatment of a variety of diseases including depressive states and mood in clinical [2]. Though antidepressants are found that may promote tumor growth and it is correlating with dose dependent in some studies [3,4], other studies showed the conflict results that antidepressants could play the anti-tumor effect [5,6] and did not revealed the disadvantageous risk in tumor patients [7,8]. Thus, the new functions of antidepressant to treat tumors still need to discuss stirringly in the future.

Maprotiline, as a tetracyclic antidepressant, has been widely used for treating mental depression in clinical [9,10]. With the development of studies, the most antidepressants have been demonstrated that could play an anti-tumor role [11,12]. Of course, maprotiline also is been proved that have potent anti-proliferative effect on Burkitt lymphoma [13]. In addition, a study found that maprotiline has a dual effect on autophagy of Neuro-2a cells. It not only induces apoptosis through the caspase-3 pathways, but also causes an anti-apoptotic response through the both pathways of Ca²⁺- and ERK-dependent [2]. Thus, it still needs to be further studied on the anti-tumor mechanism of maprotiline.

With the development of immunology, tumor immunotherapy is getting more and more attention. Checkpoint immunotherapy, as a strategy of tumor immunotherapies, has been demonstrated that could induce clinical benefit on treatment tumors by targeting the inhibitory co-receptors such as PD-1 [14,15]. PD-1 combination with PD-L1, which is expressed on the surface of tumor cells, inhibits the anti-tumor

function of T cells. It has been proved that PD-L1 overexpresses on the melanoma [16]. Inhibition of the pathway of PD-1/PD-L1 could attenuate melanoma growth by strengthening the anti-tumor effect of T cells [17]. However, it is not clear whether maprotiline influences the expression of PD-L1 on the melanoma and the immune response against tumor.

Material And Methods

Cell lines and animals

The B16 cells were presented by Professor Wang Liying (Department of molecular biology, Jilin University). C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were fed with food and water in sterile condition.

Wound healing assay

B16 cells were seeded into the 6-well plate with the density of 3.5×10^5 cells/well and incubated in an incubator (37°C, 5% CO₂) for 14-16h. Then the cells were scratched a hole in the well center with the tip of the 100 µl pipette and cultured for 24 hours or 48 hours with different concentrations of maprotiline (0, 0.625, 1.25, 2.5, 5 and 10 µg/ml). The scoring of cells in each well was recorded using the microscope.

Western blotting

The protein was extracted with lysis buffer and the concentration was determined using a bicinchoninic acid protein assay (Beyotime Institute of Biotechnology, China). The protein samples were then separated by 12% sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then incubated with the following primary antibodies of Tubulin (1:1000), Lc3b (1:1000), cleaved Caspase 3 (1:1000), MMP2 (1:1000), PD-L1 (1:1000), and p-Stat3 (1:1000) overnight at 4°C. All antibodies were purchased from Cell Signaling Technology, Inc. Specific immune complexes were visualized using enhanced chemiluminescence (Beyotime Institute of Biotechnology) and semi-quantified with the software of multifunctional chemiluminescence imaging system (Viliber, France).

Cell Counting Kit-8 analysis

B16 cells were prepared into 96-well plates with a density of 1.5×10^4 cells/well and incubated for 14-16h at 37°C with the condition of 5% CO₂. Then maprotiline with the different concentrations of 0, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20 and 40 µg/ml were added, respectively. After co-cultured for another 24 hours or 48 hours, CCK-8 was added to each well and incubated for 2 hours according to the instruction. Finally, the OD value at the wavelength of 450nm was detected by an enzyme-labeling instrument (Molecular Devices, USA). The cells in each well were photographed under the microscope.

Annexin V/PI analysis

B16 cells were seeded into the 6-well plate with the density of 3.5×10^5 cells/well and cultured in an incubator (37 °C, 5% CO₂) for 14-16h. The maprotiline with the different concentrations of 0, 0.625, 1.25, 2.5, 5 and 10µg/ml was respectively added into the well. After 24 hours or 48 hours, the cells were collected and mixed with 100µl Annexin V binding buffer. Then the cells were labeled with FITC and PI antibodies. The ratio of apoptosis was analyzed by the flow cytometry (Cytotflex, Beckman).

Establishment of animal models and treatment

Female C57BL/6 was used for establishing animal model of tumor burdened. The B16 cells at the dose of 1×10^6 per mouse were injected subcutaneously in the right leg of mice to establish the melanoma bearing mice model. At 7 days after tumor inoculation, mice were randomly divided into PBS group, maprotiline 100µg group and maprotiline 200µg group. After the treatment, the survival rate of mice was recorded every day. At 14 days after the treatment, the tumor was separated and weighed. The spleens also were collected for detecting the ratios of immune cells using the flow cytometry.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nickend labeling (TUNEL)

The apoptosis of cells in tumor-tissues is detected by TUNEL assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according the instruction. Firstly, the TUNEL detection solution is dropwise added on the surface of tumor sections and incubated in dark at 37°C for 60min. Then the sections are washed using PBS for 10min and 3 times. Finally, the sections are dried and sealed with the solution of anti-fluorescence quenching, and observed the image using the fluorescence microscope.

Immunofluorescence staining

The tumor sections were incubated with monoclonal antibodies of CD3 (1:100) CD4 (1:150) CD8 (1:600), (Cell Signaling Technology, USA) overnight at 4 °C. The sections were rinsed by PBS for three times and then incubated with the second antibody for 30min at room temperature. Finally, the sections were sealed with anti-fluorescence quenching and observed under confocal microscope (AR1⁺, Nikon).

Flow cytometry

The spleens were grinded and filtered through a nylon cell strainer filter, and then collected in PBS buffer. The Red Blood Cells (RBCs) were removed with Red Blood Cell Lysis Buffer (Beyotime Biotechnology, China). The spleen cells were resuspended in PBS buffer and adjust the concentration to 1×10^7 cells/ml. Each tube was added into 1×10^6 cell and labeled with anti-CD3, anti-CD4, anti-CD8 and NK1.1 antibody (Biolegend, USA) for 30 minutes. The ratios of immune cells were measured with flow cytometer (Cytotflex, Beckman).

Statistical analysis

Data are designated as the means $\pm$ SDs. One-way ANOVA was performed to test if mean values between different groups were significantly different ($P < 0.05$). For survival analysis, the Kaplan-Meier method

with a log-rank test was used.

Results

Maprotiline inhibited the proliferation of melanoma cells

Firstly, we tested the viability of maprotiline with different concentrations on melanoma B16 cells by CCK8 assay. The results showed that maprotiline at the concentration of 20µg/ml or 40µg/ml could effectively inhibit the proliferation of B16 cells at 24h after treatment with maprotiline, while at 48h after treatment, the cell proliferation was effectively inhibited at the concentration of 40µg/ml, indicating that maprotiline could effectively inhibit the proliferation of B16 cells (Fig. 1).

Maprotiline inhibited the migration of melanoma cells

The effects of maprotiline with different concentrations on the migration of B16 cells were detected by wound healing assay. The results showed that maprotiline could effectively inhibit cell migration when the concentration of maprotiline was greater than 2.5µg/ml for 24h after the action on B16 cells, and significantly inhibited when the concentration of maprotiline was more than 5µg/ml for 48h, indicating that maprotiline has the ability to inhibit the migration of B16 cells (Fig. 2).

Effect of maprotiline on autophagy, apoptosis and migrate of B16 cells in vivo

Then, we used Western blotting to detect the effect maprotiline on the expression of tumor related proteins in B16 cells. We found that maprotiline increased the expression of autophagy related protein LC3b and apoptosis related protein cleaved caspase 3 in B16 cells when the concentration was more than 2.5µg/ml for 24 hours, while maprotiline with the concentration of 10µg/ml inhibited the expression of migration related protein MMP2. When the concentration of maprotiline was more than 5µg/ml, the expression of LC3b and cleaved caspase-3 were increased significantly, but there was no significant effect on the expression of MMP2 (Fig. 3a and b). These results indicated that maprotiline did not inhibit B16 migration through MMP2 pathway. Subsequently, flow cytometry was used to detect the effect of maprotiline at different concentration on B16 cell apoptosis. The results showed that after treated for 24h or 48h, the concentration of maprotiline greater than 5µg/ml significantly increased B16 cell apoptosis, especially when the concentration was 10µg /ml (Fig. 3c and d).

Effect of Maprotiline on the mice burdened the B16 cells

Next, we further examined the therapeutic effect of maprotiline on melanoma tumor-bearing mice in vivo. The results showed that maprotiline with the concentration of 100µg/mouse and 200µg/mouse could effectively inhibit tumor growth in melanoma bearing mice and significantly prolonged the survival rate, but there was no significant difference between the two treatment groups (Fig. 4a-d).

Meanwhile, in order to further clarify the anti-melanoma effect of Maprotiline, we further examined the effect of Maprotiline on the expression of related proteins in tumor tissues. According to the results,

maprotiline with the concentration of 100µg/mouse or/and 200µg/mouse significantly increased the expression of apoptosis related protein Cleaved-Caspase3 (Fig. 4e) which was coincident with the TUNEL results (Fig. 4f), but there is no significant effect on the expression of LC3b. This is not consistent with that of the results of cell experiments, which might be related to the dose of the drug, or may also be related to the infiltration of a large number of proliferating T lymphocytes in tumor tissues, affecting the expression of autophagy-related proteins. In both vitro and vivo, maprotiline has no obvious effect on the expression of MMP2. Importantly, we found that maprotiline inhibited the expression of PD-L1 in tumor tissues (Fig. 4e).

Effect of maprotiline on the ratio of CD4⁺, CD8⁺ T lymphocytes and NK cells in spleen

As the largest peripheral immune organ in the body, the spleen is the main site of immune response. We measured the ratio of CD4⁺ and CD8⁺ T lymphocytes in the spleen. The results showed that compared with the PBS group, the rate of infiltration of CD4⁺ and CD8⁺ T lymphocytes in the spleen were significantly increased in the two maprotiline treatment groups. However, compared with the low-dose group, the proportion of T lymphocytes in the spleen of the high-dose group was significantly reduced (Fig. 5a and b). This may be related to the infiltration of numerous T lymphocytes into the tissue. In addition, we found that both treatment groups significantly increased the number of NK cells in the spleen compared with the PBS group, and there was no significant difference between the two groups (Fig. 5c).

Effect of maprotiline on the infiltration of T lymphocytes in tumor tissues

To further determine whether maprotiline enhanced the anti-tumor immune response in melanoma bearing mice, we used immunofluorescence techniques to detect the infiltration of CD4⁺ and CD8⁺ T lymphocytes in tumor tissues. The results showed that, compared with the PBS group, maprotiline significantly increased the infiltration of CD4⁺ and CD8⁺ T lymphocytes in the tissues. However, the infiltration of T lymphocytes in tumor tissue was more pronounced in the high-dose group (200µg/mouse) than in the low-dose group (100µg/mouse) (Fig. 6). This may be related to the apoptosis of mass tumor cells, which leads to the release of more antigens, thus chemotactic more T cells to the tumor tissue.

Discussion

Searching the safe and effective drugs for tumor treatment is the focus of research, but it is expensive and time consuming to develop of novel drugs. At present, some old drugs of non-antitumor have been found that could play the effect of anti-tumor in some studies [18-21]. Because these drugs have been successfully used to treat of non-tumor diseases in clinical, it is helpful to develop therapeutic drugs for cancer patients. Maprotiline, an antidepressant drug, has been found that could decrease proliferation of prostate cancer cells [22], but the anti-tumor mechanism in melanoma are needed to evaluate. Here, we find that maprotiline has a potent anti-tumor effect through prompting the immune response.

As known, the immune response of anti-tumor is impaired in patients, and the pathway of PD-1 and PD-L1 is an important cause of immune escape for tumor cells [23]. At present, the checkpoint inhibitors of PD-1 and PD-L1 have been approved or investigated for treatment tumor by FDA [24]. In this study, we confirm that maprotiline inhibited the growth of melanoma in mice burdened B16 cells, prolong the survival of mice. In order to explore the anti-tumor mechanism, we detect the expression of related protein and found that maprotiline inhibits the expression of PD-L1. Blocking the link of PD-1 and PD-L1 could recover the T cell function to destroy tumor cells [25]. It is might a reason that maprotiline delays the growth of melanoma. In addition, blocking the pathway of PD-1/PD-L1 could lead to significantly increase the ratios of T cells in spleen [26]. Our results also confirm that maprotiline increases the percentage of CD4⁺ and CD8⁺ T cells in the spleen. However, we find that the ratios of T cell are higher in mice treated with maprotiline at the dose of 100μg per mouse compared with maprotiline at the dose of 200μg per mouse. It might relate with that more T cells infiltrate in the tumor tissues.

In this study, we find that maprotiline significantly increase the T cell infiltration in tumor, and the degree of T cell infiltration is most remarkable in mice treated maprotiline at the dose of 200μg per mouse. Though Liu et al indicates that expression of PD-1 only influences the density of CD8⁺ T cell infiltration in tumor tissue, not correlates with CD4⁺ T cells [27], but other study points out that inhibition the bind of PD-1 and PD-L1 not only increases the percentage of T cells in the spleen, but also increases the number of T cells infiltration in tumor tissue [26]. We analyze that maprotiline causes the apoptosis of copious tumor cells [2] which could produce of more tumor antigens recognized by antigen presentation cells (APC). Finally, the APCs could further lead to T cell activation [28] and infiltration in tumor-tissues. In fact, maprotiline increases the apoptosis of B16 cells and the expression of cleaved-Caspase3. In addition, accumulating evidence suggests that chemotherapy could induce immunogenic cell death (ICD) and prompt the anti-tumor efficacy [28]. It also might be a reason that causes the strengthened T cell response. Of course, we convince that effect of maprotiline on T cell infiltration might be complex and need to be further explored.

Besides influencing the ratios of T cells, we also found that maprotiline increases the ratio of natural killer (NK) cells in the spleen. As known, NK cells belong to the innate lymphocytes that could destroy the tumor cells without MHC restricted manner [29]. However, the function of NK cells are suppressed by PD-L1 expressed on the surface of tumor cells [30]. Treatment with PD-1 monoclonal antibody could strengthen the NK cell function inhibited by PD-L1-mediated. Therefore, we detect the effect of maprotiline on the ratio of NK cells in spleen, and the maprotiline significantly raises the percentage of NK cells. It indicates that maprotiline might prompt the function of NK to kill the tumor cells by inhibiting the expression of PD-L1.

In this study, we confirm that maprotiline could increase the apoptosis of B16 cells, inhibit the tumor growth and improve the survival of mice burdened B16 cells. Importantly, treatment with maprotiline significantly inhibits the expression of PD-L1 in tumor-tissues, prompts the anti-tumor immune response of mice through increasing the ratios of immune cells in spleens and the infiltration of T cells in tumor-

tissues. This study may find a new inhibitor of PD-L1, which provides the theoretical and experimental basis to search for new drugs treated tumor in clinical.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants. The animal studies were approved by the Ethics Committee of Xinxiang Medical University (Xinxiang, China).

Consent for publication

The authors agree to publication in the journal of *Investigational New Drugs*.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

Conception and design: Huijie Jia. Acquisition of data: Yang Li, Miaomiao Liu, Lin Zhou, Zunge Wu, Haoqi Chen, Jiaming Guo, Mengdan Gao. Development of methodology: Yang Li, Zhiang Liu. Analysis and interpretation of data: Tiesuo Zhao, Yang Li. Writing, review, and/or revision of the manuscript: Zhiwei Feng, Huijie Jia, Feng Ren. Study supervision: Jiateng Zhong, Mingyong Wang. Administrative, technical, or material support: Zhiwei Feng, Huijie Jia, Feng Ren.

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Disclosure of potential conflicts of interest

The authors declare that they have no conflicts of interest.

Research involving Human Participants and/or Animals

This article does not describe any studies that include human participants performed by any of the authors. C57BL/6 mice were used for this study and the animal studies were approved by the Ethics Committee of Xinxiang Medical University (Xinxiang, China).

Informed consent

Formal consent is not required for this type of study.

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Figures

Figure 1

Effect of maprotiline on the proliferation of B16 cells in vitro. B16 cells are seeded into 96-well plates with concentration of 1.5×10^4 cells/well. 24h or 48h after being treated with maprotiline at the dose of 0, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20 and 40μg/ml, CCK-8 is added to each well and the OD value is detected according to the instruction. **a** Images of the representative cells in each well at 24h or 48h after being treated with maprotiline. **b** OD values of cells in each well at 24h or 48h after being treated with maprotiline. Data were presents as mean ± SD (n = 3). * $P < 0.05$ versus the Control group; # $P < 0.05$ versus the 20μg/ml group.

Figure 2

Effect of maprotiline on the migration of B16 cells in vitro. B16 cells are seeded into 6-well plates with the density of 3.5×10^5 cells/well. The cell migration is detected by the method of wound healing assay. **a** Images of the representative cells of wound healing assay at 0h, 24h or 48h after being treated with maprotiline. **b** Quantification analysis of the nick. Data were presents as mean $\pm$ SD ($n = 3$). * $P < 0.05$ versus the Control group; # $P < 0.05$ versus the 5 $\mu\text{g/ml}$ group.

Figure 3

Effect of treatment with maprotiline on the expression of relative proteins and cell apoptosis in vitro. B16 cells are seeded into 6-well plates with the density of 3.5×10^5 cells/well. At 24h or 48h after being treated with the maprotiline at different dose of 0, 0.625, 1.25, 2.5, 5 and 10 $\mu\text{g/ml}$, the expression of relative proteins is detected by the method of western blotting and the cell apoptosis is detected by the method of Annexin V/PI analysis. **a** Images of the relative protein expression at 24h after being treated with

maprotiline. **b** Images of the relative protein expression at 48h after being treated with maprotiline. **c** Images of cell apoptosis detected by the flow cytometry Images of the relative protein expression at 24h after being treated with maprotiline. **d** Images of cell apoptosis detected by the flow cytometry Images of the relative protein expression at 48h after being treated with maprotiline. * $P < 0.05$ versus the Control group.

Figure 4

Effect of treatment with maprotiline on mice burdened B16 cells. The C57BL/6 mice are injected with 1×10^6 B16 cells and treat with PBS, 100 μ g maprotiline and 200 μ g maprotiline. The tumor weight and survival are recorded. At 7 days after the last treatment, the tumor tissues are separated for the detection of protein expression and cell apoptosis. **a** The procedure of therapeutic time. **b** Images of the representative tumor in each group. **c** Histogram of tumor weight in each group. **d** Survival rate of the mice burdened the B16 cells. **e** The expression of relative proteins detected by Western blotting. **f** Representative images of TUNEL staining in tumor-tissues. Number of mice per group are six or ten ($n=6$ or 10). * $P < 0.05$ versus the PBS group.

Figure 5

Effect of treatment with maprotiline on the percentage of immune cells in the spleen. The C57BL/6 mice are injected with 1×10^6 B16 cells and treat with PBS, 100 μ g maprotiline and 200 μ g maprotiline. At 7 days after the last treatment, the spleens are separated for evaluating the percentage of immune cells. **a**

Figures of the representative CD4⁺ T lymphocyte detected by flow cytometry. **b** Figures of the representative CD8⁺ T lymphocyte detected by flow cytometry. **c** Figures of the representative NK cells detected by flow cytometry. * $P < 0.05$ versus the PBS group; # $P < 0.05$ versus 100μg/mouse group.

Figure 6

Effect of treatment with maprotiline on the T cell infiltration in tumor tissues. The C57BL/6 mice are injected with 1×10^6 B16 cells and treat with PBS, 100μg maprotiline and 200μg maprotiline. At day 7th after the last treatment, the tumor tissues are separated for the detection T cell infiltration by immunofluorescence staining. **a** Images of CD4⁺ T lymphocyte infiltration in tumor tissues. **b** Images of CD8⁺ T lymphocyte infiltration in tumor tissues.