

## Original Research Article

# Anti-glioma effect of 7-H-pyrrolo [2,3-di]pyrimidine derivative via induction of cell death

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### Abstract

**Purpose:** To investigate the effect of 7-H-pyrrolo[2,3-di]pyrimidine derivative (7-HPPD) on glioma cell growth.

**Methods:** Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, while apoptosis was assessed in Hoechst 33342 stained cells using flow cytometry. Changes in cell morphology were assessed by scanning electron microscopy (SEM).

**Results:** Glioma cell viability showed a concentration-dependent decrease on exposure to 7-HPPD for 72 h ( $p < 0.05$ ). The viabilities of C6, U251 and U87 cells were reduced by 24, 28 and 31 %, respectively, following treatment with 20  $\mu$ M 7-HPPD. Exposure to various concentrations of 7-HPPD resulted in a marked decrease in BrdU LI and cAMP levels in C6, U251 and U87 glioma cells ( $p < 0.05$ ). Moreover, 7-HPPD induced apoptosis in U251 and U87 cell cultures, as was evident in the condensation of chromatin material, presence of apoptotic bodies and intense blue fluorescence. Treatment of U251 cells with 7-HPPD for 72 h led to a significant increase in the proportion of cells in G0/G1 phase, and significant decrease in percentage of cells in G2/M and S phases. The population of rounded cells showed a significant rise with increase in 7-HPPD concentration from 10 to 20  $\mu$ M ( $p < 0.05$ ).

**Conclusion:** 7-HPPD inhibits growth and proliferation of glioma cells by inducing apoptosis. Therefore, it has a potential for application in glioma chemotherapy.

**Keywords:** Glioma, Fluorescence, Metastasis, Apoptosis, Infiltration

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## INTRODUCTION

Glioma is among the most commonly detected intracranial malignant tumors worldwide, and it accounts for more than half of the diagnosed intracranial tumors [1]. According to World Health Organization (WHO), gliomas are categorised into four grades: I, II, III and IV [2]. Grades I and

II are known as low-grade gliomas, whereas grades III and IV are classified as high-grade gliomas [2]. Glioma-associated mortality and disability are very high because of tumor metastasis and frequent tumor relapse.

The current treatment strategy for glioma involves surgery. However, because of deep infiltration, it is difficult to remove the tumor tissue

completely. Moreover, in most glioma patients, relapse has been observed after surgery [3]. The glioma treatment strategies have many challenges such as development of resistance to drugs and inability of the drugs to cross blood-brain barrier [4]. It has been observed that gliomas do not undergo metastasis but infiltrate into the local tissues, leading to the poor prognosis [5]. The currently-used chemotherapeutic agents and surgical methods for glioma treatment are not satisfactory. Therefore, there is need for development of novel therapeutic compounds.

Apoptosis, a highly regulated mechanism for elimination of undesired cells from body is controlled by several genes [6]. It is the vital process for regulation of cancer cell growth, and is explored usually for the development of various anti-cancer treatments [6]. There are several signalling pathways involved in the process of apoptosis, including activation of cysteine proteases and caspases [7]. Activation of the caspases during initial stage of apoptosis leads to death of cancer cells [8].

Natural products are great treasure of compounds for identification of therapeutic principles for glioma [9-11]. Indeed, several compounds have been reported as promising agents for inhibition of glioma growth, and are currently under clinical trials [9-11]. In the present study, the effect of 7-H-pyrrolo[2,3-d]pyrimidine derivative (7-HPPD) on glioma cell growth was investigated.

## EXPERIMENTAL

### Cell line and culture

The C6, U251 and U87 cell lines were obtained from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) containing 12% foetal calf serum and antibiotics i.e. penicillin (100 U/mL) and streptomycin (100 µg/mL). The cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere in an incubator.

### Cell viability assay

The viabilities of C6, U251 and U87 cells following 72 h of incubation with 2, 4, 6, 8, 10, 12 and 20 µM 7-HPPD were assessed with MTT assay. The cells were seeded in 96-well plates at a concentration of 1.5 x 10<sup>5</sup> cells/well, and cultured for 24 h. Thereafter, the cells were incubated in medium containing 2, 4, 6, 8, 10, 12 or 20 µM of 7-HPPD for 72 h, after which MTT

solution added to each well of the plate, followed by incubation of the cells for 4 h. Then, the medium was removed and DMSO was added to dissolve the solid formazan crystals formed. The absorbance of each plate was read at 495 nm in a microplate reader.

### Bromodeoxyuridine (BrdU) labelling analysis

The C6, U251 and U87 cells were plated in 24-well plates at a density of 2 x 10<sup>5</sup> cells per well. Following 24-h culture, the cells were exposed to 7-HPPD at concentrations of 2, 4, 6, 8, 10, 12 and 20 µM for 72 h. The cells were then washed, placed in cover slips and treated with 0.5% H<sub>2</sub>O<sub>2</sub> (alcoholic solution). Following PBS washing, the cells were incubated with BrdU antibody (dilution 1:100; catalog no. ab152095; Abcam, Cambridge, UK) at 4 °C. Then, the coverslips were washed with PBS, followed by incubation for 1 h with secondary antibody (1:5,000) at room temperature.

### Assessment of apoptosis

U251 and U87 cells seeded 96-well plates at a density of 1.5 x 10<sup>6</sup> cells/well were exposed to 7-HPPD at concentrations of 10, 12 and 20 µM for 72 h. Then, the cells were washed three times with PBS, and fixed with paraformaldehyde (4%) at room temperature for 15 min. The cells were then subjected to staining with Hoechst 33342 (10 µg/mL) at 37 °C for 20 min, after which the 7-HPPD-induced morphological changes were examined under a fluorescence microscope (Olympus Corporation).

### Cell cycle analysis

The U251 and U87 cells were put into 10-cm culture dishes at a density 1.5 x 10<sup>6</sup> cells/dish and exposed to 7-HPPD at concentrations of 10, 12 and 20 µM for 72 h. After 72 h of treatment, the cells were harvested, fixed in 70 % ethyl alcohol at -2°C and subsequently washed with PBS. Then, they were stained with 5 % PI solution in accordance with the manufacturer's instructions. Analysis of apoptosis was done using FACSCalibur flow cytometer connected to Cell Quest software Pro (5.1 version; BD Biosciences, Franklin Lakes, NJ, USA). Data analysis was performed using ModFit LT software package (version 2.0; Verity Software House, Inc., Topsham, ME, USA).

### Determination of cyclic 3',5'-monophosphate (cAMP) level

The C6, U251 and U87 cells were exposed to 8, 10, 12 and 20 µM 7-HPPD for 72 h at a density

of  $2 \times 10^5$  cells/well. Following 7-HPPD exposure, the cells were washed three times with PBS, and then treated with radioimmunoprecipitation lysis buffer. The cell lysate was centrifuged at 12000 g for 20 min, and the supernatant was subsequently mixed with 0.1 N HCl. The level of cAMP in the supernatant sample was determined using immunoassay. The cAMP level was determined by treating the supernatant overnight with mouse monoclonal antibody against cAMP (dilution 1:100; cat no. 250532; BI Biotech India Pvt., Ltd.) at 4 °C. The supernatant was then incubated with NorthernLights™ 557 conjugated anti-mouse immunoglobulin G secondary antibodies (cat. no. NL007, 1:5,000; BI Biotech India Pvt Ltd) at room temperature for 2 h.

### Determination of cell morphology

The U251 cells were exposed to 10, 12 and 20  $\mu$ M 7-HPPD for 72 h in 24-well culture plates at a density of  $2 \times 10^5$  cells per well. After incubation, the cells were fixed for 1 h, and then again for 70 h using glutaraldehyde (2.5%) and sodium cacodylate buffer at 4°C. The cells were again fixed for 1.5 h using osmium tetroxide at a temperature of 4°C, after which they were subjected to dehydration with gradient ethanol and amyl acetate. Then, the cells were fixed on gold-palladium alloy coated slides, and the morphological changes were determined using scanning electron microscopy (Jeol-JSM-5200).

### Statistical analysis

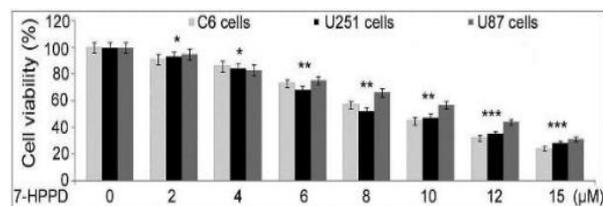
The data are expressed as mean  $\pm$  SD of triplicate experiments performed independently. Data analysis was carried out using one-way analysis of variance (ANOVA) and the Bonferroni post-test. Differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

### 7-HPPD inhibited C6, U251 and U87 glioma cell growth

Figure 1 shows the effect of 7-HPPD on growth of C6, U251 and U87 cell lines at 72 h, as measured using MTT assay. Concentration-based decreases in viabilities of all the three tested cell lines were observed on exposure to 2, 4, 6, 8, 10, 12 and 20  $\mu$ M 7-HPPD for 72 h. The viability of C6 cell was reduced to 91, 86, 73, 57, 45, 32 and 24 %, on treatment with 2, 4, 6, 8, 10, 12 and 20  $\mu$ M 7-HPPD, respectively. Treatment with 2, 4, 6, 8, 10, 12 and 20  $\mu$ M 7-HPPD reduced the viability of U251 cells to 93, 84, 68, 52, 47, 35 and 28 %, respectively. Similarly, in U87 cells, viability was reduced to 95, 83, 75, 66,

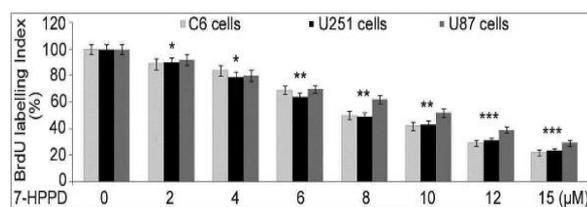
57, 44 and 31 %, on treatment with 2, 4, 6, 8, 10, 12 and 20  $\mu$ M 7-HPPD, respectively.



**Figure 1:** Effect of 7-HPPD on C6, U251 and U87 cell viability. The three cell lines were exposed for 72 h to 5, 10, 15, 20, 25, 30 and 50  $\mu$ M 7-HPPD, and cell viability was determined using MTT assay; \* $p < 0.05$ , \*\* $p < 0.002$ , \*\*\* $p < 0.001$ , versus untreated cells

### 7-HPPD decreased BrdU-labeling index in glioma cells

The C6, U251 and U87 cells were treated with 2, 4, 6, 8, 10, 12 and 20  $\mu$ M 7-HPPD (Figure 2). The treatment decreased BrdU-LI labelling in concentration-dependent fashion. The BrdU-LI labelling was reduced to 22, 23 and 29% in C6, U251 and U87 cells, respectively, on exposure to 20  $\mu$ M 7-HPPD.



**Figure 2:** Effect of 7-HPPD on BrdU-LI in glioma cells. The 7-HPPD exposure of C6, U251 and U87 cells was followed by analysis of BrdU uptake at 72 h. The labelling index was measured three times independently; \* $p < 0.05$ , \*\* $p < 0.002$ , \*\*\* $p < 0.001$ , relative to untreated cells

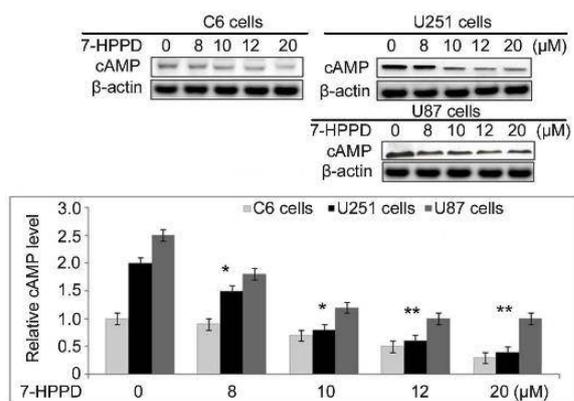
### 7-HPPD suppressed cAMP in C6, U251 and U87 glioma cells

The cAMP level in glioma cells was determined after 72 h of exposure to 8, 10, 12 and 20  $\mu$ M 7-HPPD (Figure 3). Exposure of C6, U251 and U87 cells to 7-HPPD significantly ( $p < 0.05$ ) decreased cAMP level in a concentration-dependent manner. Compared to the untreated cells, 7-HPPD treatment significantly decreased cAMP level in all the three cell lines from 8  $\mu$ M.

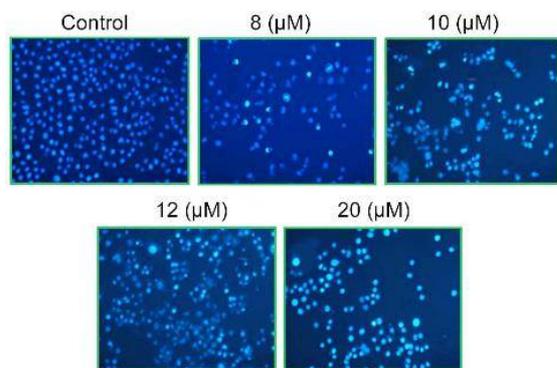
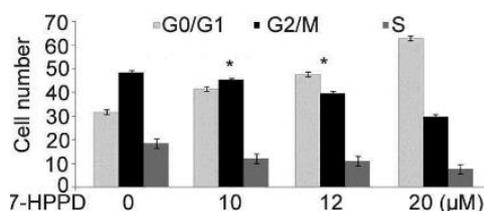
### 7-HPPD produced apoptosis in U251 and U87 cells

The U251 and U87 cells were exposed to 7-HPPD at concentrations of 8, 10, 12 and 20  $\mu$ M for 72 h, after which apoptosis was analysed using Hoechst 33342 staining (Figure 4).

Apoptosis induction in U251 and U87 cell cultures on exposure to 7-HPPD was evident from condensation of chromatin material, presence of apoptotic bodies and intense blue fluorescence. A significant increase in the population of apoptotic cells was observed in U251 and U87 cell cultures on exposure to 7-HPPD, when compared to the control cells.



**Figure 3:** Suppression of cAMP level by 7-HPPD. In C6, U251 and U87 cell cultures, cAMP level was assayed at 72 h of exposure to indicated concentrations of 7-HPPD. The experiments were performed three times independently; \* $p < 0.05$ , \*\* $p < 0.001$ , versus untreated cells

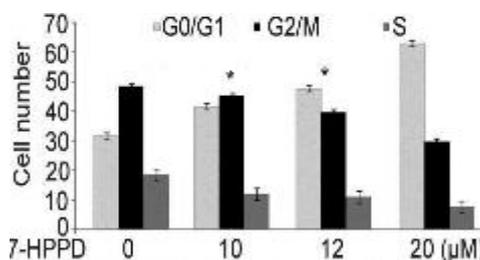


**Figure 4:** Effect of 7-HPPD on glioma cell apoptosis. The U251 and U87 cells were treated with 8, 10, 12 and 20 μM 7-HPPD for 72 h. Fluorescence microscopy was used for analysis of cell apoptosis. Magnification: x250

### 7-HPPD caused G0/G1 phase cell cycle arrest in U251 cells

Treatment of U251 cells with 10, 12 and 20 μM

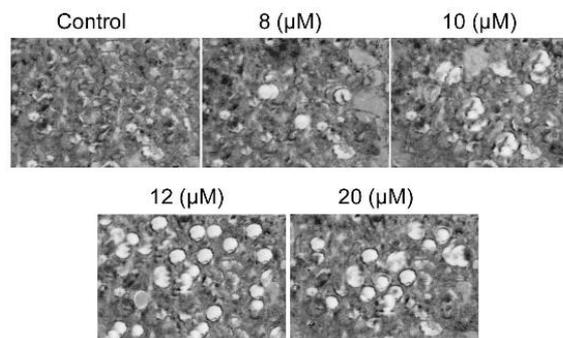
7-HPPD for 72 h led to significant increases in the proportion of cells in G0/G1 phase (Figure 5). The percentage of cells in G2/M and S phases decreased significantly on treatment with 7-HPPD, relative to the control cells. Exposure of U251 cells to 10, 12 and 20 μM 7-HPPD increased G0/G1 phase cell population to 41.54, 47.67 and 62.88 %, respectively. In G2/M phase, cell proportion was decreased to 45.08, 39.44 and 29.65%, respectively on exposure to 10, 12 and 20 μM of 7-HPPD, when compared to 48.23% in untreated cells. In S phase, cell percentage was decreased to 11.98, 10.89 and 7.53%, respectively on exposure to 10, 12 and 20 μM 7-HPPD.



**Figure 5:** 7-HPPD induced cell cycle arrest in U251 cells. The 7-HPPD-treated glioma cells were analysed for cell cycle distribution at 72 h with flow cytometry. The cells were exposed to 10, 12 and 20 μM 7-HPPD; \* $p < 0.05$ , \*\* $p < 0.002$ , \*\*\* $p < 0.001$  versus untreated cells

### 7-HPPD changes morphological of U251 cells

Treatment of U251 cells with 8, 10, 12 and 20 μM 7-HPPD caused marked changes in cell morphology (Figure 6). There was rounding of cells on treatment with 7-HPPD for 72 h. The population of rounded cells showed marked increases with increase in concentration of 7-HPPD from 10 to 20 μM. In contrast, the untreated cells were spindle-shaped and dividing at higher rates, as shown through scanning electron microscopy.



**Figure 6:** Effect of 7-HPPD on U251 cell morphology. The cells were exposed to 8, 10, 12 and 20 μM 7-HPPD for 72 h, and changes in morphology were determined using SEM. Images were taken at x750 magnification

## DISCUSSION

The development of treatment for glioblastoma multiforme is a serious challenge to clinicians throughout the world. Natural product screening has led to the identification of compounds which possess promising glioma-inhibitory potential [12-14]. In the present study, the effect of 7-*H*-pyrrolo[2,3-*di*]pyrimidine derivative (7-HPPD) on glioma cell growth was investigated. The study demonstrated that 7-HPPD inhibited growth and proliferation of glioma cells, arrested cell cycle, suppressed cAMP level and led to apoptosis induction. Higher proliferation of C6 glioma cells has been linked to the activation of platelet-derived growth factor receptors (PDGFRs) [15,16].

Studies have revealed that PDGFRs activation causes increase in the level of cAMP in glioma cells [15-18]. The interaction between activated PDGFRs and cellular  $\beta$ -adrenoreceptors enhances proliferation of the carcinoma cells through up-regulation of cAMP levels [15,16]. In the present study, 7-HPPD treatment markedly suppressed the viability of C6, U251 and U87 glioma cell lines. Concentration-based reductions in glioma cell viability was caused by 7-HPPD in C6, U251 and U87 cells. The level of cAMP was decreased significantly in U251 and U87 cells on treatment with 7-HPPD. Compared to the untreated cells, 7-HPPD treatment significantly decreased cAMP level in all the three cell lines.

Apoptosis, a highly regulated mechanism for the removal of unwanted cells from body, is controlled by several genes [6]. It is the vital process for regulation of cancer cell growth, and it is being explored for the development of various anti-cancer treatments [6]. In the present study, the effect of 7-HPPD on apoptosis induction in U251 and U87 cells was analysed flow cytometrically. The results showed that 7-HPPD exposure led to significant increases in the proportions of apoptotic cells in U251 and U87 cells. The 7-HPPD exposure also caused marked enhancement of the percentage of cells in G0/G1 phase, with subsequent reductions in the G2/M and S phases of cell cycle. These findings suggest that 7-HPPD suppresses glioma cell viability through apoptosis and cell cycle arrest.

Generally, apoptosis is due to changes in mitochondrial membrane permeability resulting in activation of the caspases [19-21]. Caspase activation causes formation of membrane projections, rounding of cells, and degeneration of the cell membrane [21]. In the present study, the effect of 7-HPPD on morphology of the

glioma cells was determined using scanning electron microscopy. The results showed that 7-HPPD treatment led to the development of membrane projections and rounding of U251 in concentration dependent manner.

## CONCLUSION

Treatment with 7-HPPD inhibits the growth and proliferation of glioma cells by inducing apoptosis. Therefore, 7-HPPD is a potential chemotherapeutic agent for glioma.

## DECLARATIONS

### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xing Xia designed the study and wrote the research paper. Jie Wang, Xiang Peng, Chengan Cao and Yan Zhao performed the experimental work, carried out the literature study and compiled the data. Jie Wang and Xiang Peng performed literature survey, analyzed the data and compiled the data. The research article was thoroughly read by all the authors before commination for the consideration of publication.

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