The induction of potent anti-tumor efficacy in diffuse intrinsic pontine glioma by YF-PRJ8-1011 of a novel cyclin-dependent kinase 4/6 inhibitor

Pengcheng Zuo  
Capital Medical University

Yaopeng Li  
Tsinghua University

Tantan Wang  
Tsinghua University

Xingyu Lin  
Zhuhai Yufan Biotechnologies Co., Ltd

Zhen Wu  
Capital Medical University

Junting Zhang  
Capital Medical University

Xuebin Liao  
Tsinghua University

Liwei Zhang (✉ zhangliweittyy@163.com)  
Capital Medical University

Research Article

Keywords: Diffuse intrinsic pontine glioma, CDK4/6 inhibitor, Anti-tumor efficacy, Blood-brain barrier, Radiotherapy

Posted Date: March 13th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2668091/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: No competing interests reported.
Version of Record: A version of this preprint was published at Journal of Neuro-Oncology on May 3rd, 2023. See the published version at https://doi.org/10.1007/s11060-023-04323-5.
Abstract

Objective

Diffuse intrinsic pontine glioma (DIPG) is a lethal pediatric brainstem tumor. Despite numerous efforts to improve survival benefits, its prognosis remains poor. This study aimed to design and synthesize a novel CDK4/6 inhibitor YF-PRJ8-1011, which exhibited more potent antitumor activity against a panel of patient-derived DIPG tumor cells in vitro and in vivo compared with palbociclib.

Methods

Patient-derived DIPG cells were used to assess the antitumor efficacy of YF-PRJ8-1011 in vitro. The Liquid chromatography tandem-mass spectrometry (LC-MS/MS) method was used to measure the activity of YF-PRJ8-1011 passing through the blood-brain barrier. DIPG patient-derived xenograft models were established to detect the antitumor efficacy of YF-PRJ8-1011.

Results

The results showed that YF-PRJ8-1011 could inhibit the growth of DIPG cells both in vitro and in vivo. YF-PRJ8-1011 could well penetrate the blood-brain barrier. It also significantly inhibited the growth of DIPG tumors and prolonged the overall survival of mice compared with vehicle or palbociclib. Most notably, it exerted potent antitumor efficacy in DIPG in vitro and in vivo compared with palbociclib. In addition, we also found that YF-PRJ8-1011 combined with radiotherapy also showed more significant inhibition of DIPG xenograft tumor growth than radiotherapy alone.

Conclusion

Collectively, YF-PRJ8-1011 is a novel, safe, and selective CDK4/6 inhibitor for DIPG treatment.

Introduction

Diffuse intrinsic pontine glioma (DIPG) is a rare but fatal brainstem tumor in children with a mean overall survival of 8–14 months[1]. Surgery, chemotherapy, and radiotherapy have not shown an optimistic effect on patient outcomes[1]. To date, there has yet been no effective treatment available for DIPG[2]. Therefore, developing novel drugs with favorable clinical efficacy for patients is an unmet need in DIPG therapy. Cyclin-dependent kinases 4 and 6 (CDK4/6), which belong to the retinoblastoma signaling pathway (CDK4/6-cyclin D1-Rb-p16/ink4a), are indispensable kinases regulating the cell cycle[3, 4]. In a previous study, it was found through the analysis of more than 1000 cases of DIPG and pediatric high-grade glioma that DIPG is characterized by dysregulation of the G1/S cell cycle checkpoint[5]. So far, three CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) have been approved by the Food and
Drug Administration (FDA). Moreover, FDA-approved CDK4/6 inhibitors, such as palbociclib, are used for the treatment of DIPG in vitro. However, its inhibitory effects on tumor growth are only shown at relatively high concentrations (5–10µM)[6, 7]. Collectively, these research data motivated us to develop a more potent CDK4/6 inhibitor.

In this study, we synthesized a novel CDK4/6 inhibitor YF-PRJ8-1011, which showed selective inhibition of CDK4 and CDK6, but little activity against other CDK kinases. YF-PRJ8-1011 can effectively block the phosphorylation of Rb at Ser780, as well as G1/S cell cycle transition, inhibit DIPG cell proliferation at the concentration of 100nM. In our RNA-seq data, Gene ontology analysis showed that most of the affected genes were enriched in cell division–related functional pathways. It was also confirmed that the drug can penetrate the Blood-Brain Barrier (BBB) in vivo. Notably, YF-PRJ8-1011 can significantly inhibit tumor growth in patient-derived xenograft models and prolong the survival in tumor-bearing mice than palbociclib did. Collectively, YF-PRJ8-1011 is a potent orally active, BBB-permeable CDK4/6 inhibitor with robust anti-tumor efficacy in DIPG. We also found that YF-PRJ8-1011 combined with radiotherapy also showed more significant inhibition of DIPG xenograft tumor growth than radiotherapy alone. These findings supported additional studies in vivo, as well as clinical trials on safety and efficacy, in the future.

Materials And Methods

1. YF-PRJ8-1011 tested against CDK kinases

CDK4 and CDK6 kinase activity was measured by using GST-Rb rotein as a general substrate. Compounds were tested in 10-dose IC50 duplicate mode starting at 5 µM with a 3-fold serial dilution. The control compound, Staurosporine was tested in 10-dose IC50 mode starting at 20 µM with 4-fold serial dilution. Reactions were carried out at Km ATP according to the RBC Km binning structure: CDK4/cyclin D1 (100 µM ATP), CDK4/cyclin D3 (5 µM ATP), CDK6/cyclin D1 (100 µM ATP), CDK6/cyclin D3 (100 µM ATP). The ADP-Glo kinase assay kit (Promega) was adopted to measure IC50 values of YF-PRJ8-1011. When the enzyme activities at the highest concentration of compounds were less than 65%, curve fits were performed.

2. Cell Establishment And Culture Conditions

The patient-derived DIPG cells (including TT150630, TT150714, TT150728 and DIPG17) and primary pontine neural progenitor cells (PPCs) were kind gifts from Dr. Yu Sun. TT190326 was established as other DIPG cells. The cell culture method was performed as previously reported by Sun et al[8]. DIPG cells and PPCs were cultured in matrigel (356,234, BD, 1%, 4–12 h at 37°C) coated plates with serum-free medium containing DMEM (Invitrogen), B27 (Gibco, 1:50), N2 (Gibco, 1:100), Insulin-Transferrin-Selenium (ITS, Macgene, 20 µg/ml), bFGF (PeproTech, 20 ng/ml), EGF (PeproTech, 20 ng/ml) and penicillin streptomycin (Hyclone, 1:100). All the cells were cultured at 37°C in a 5% CO2 atmosphere.
3. Western Blot Analyses

The total protein was extracted with RIPA lysis buffer (Solarbio) according to the manufacturer's protocol. Whole cell lysates of DIPG cells and PPCs were generated by lysing 2×10^6 washed cells in 200 µL RIPA lysis buffer plus 1% phenylmethylsulfonyl fluoride (PMSF), followed by incubation on ice for 30 min. The lysed sample was centrifuged at 10000-14000g for 3–5 min, and western blots were then performed on the supernatant of centrifuged samples. After the determination of protein concentration with BCA Protein Assay Kit (Thermo), the samples were denatured by boiling them with 5×loading buffer (Scintol) at 100°C for 5 min. A 50 µg protein sample was separated by 10% SDS-polyacrylamide gels at 120V for 1.5 h and then transferred to PVDF Transfer Membranes (Millipore) at 300mA for 1 h. After they were blocked with 5% skimmed milk powder in PBST buffer (including 3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl and 0.05% Tween-20, pH 7.4) for at least 1 h, the membranes were incubated with primary antibodies, including anti-pRb (ab173289, 1:5000), anti-Rb (ab181616, 1:2000), anti-CDK4 (CST,12790, 1:1000), anti-CDK6 (CST, 13331, 1:1000), anti-CyclinD1 (CST, 2922, 1:1000), anti-P16 (CST, 18769, 1:1000) and anti-β-Actin (Easybio, 1:3000), at 4°C overnight. The membranes were washed 3 times with PBST buffer for 10 min, and then incubated with secondary antibodies Goat anti-mouse-IgG(H + L)-HRP-conjugated (Easybio, 1:3000), and Goat anti-rabbit IgG(H + L) (Easybio, 1:3000) for approximately 1 h at room temperature. The membranes were scanned by the Tanon 5200 Chemiluminescent Imaging System.

4. Cell Viability Assays

DIPG cells were first counted, and approximately 10^4 cells were seeded onto each well in 96-well plates. The cells were treated with YF-PRJ8-1011 at concentrations of 6.25 nM, 25 nM, 100 nM, 400 nM, 1600 nM, 6400 nM, and 25600 nM, respectively. Cell viability was determined by CellTiter-Glo assays (G7572, Promega) according to the manufacturer's protocol. In brief, CellTiter-Glo® Buffer and CellTiter-Glo® Substrate were thoroughly mixed to form CellTiter-Glo® Reagent. 100µl CellTiter-Glo® Reagent was added to each well in 96-well plates and the contents were mixed on an orbital shaker to induce cell lysis. The plates were incubated at room temperature for 10 min to stabilize the luminescent signal. The luminescence was recorded to measure cell viability. Cell viability was assessed 48 h after treatment (T48) and calculated as T48h. The value for control-treated cells was set to 100%. Triplicate wells were used for each concentration. IC50 values were calculated by Graphpad prism 8.

5. Cell Proliferation Analysis

Flow cytometric analysis of Ki-67 was adopted to evaluate cell proliferation. Foxp3 Transcription Factor Staining Buffer Set (eBioscience) was used for Ki67 staining, according to its protocol. In brief, 1 portion of Foxp3 Fixation/Permeabilization Concentrate was mixed with 3 portions of Foxp3 Fixation/Permeabilization Diluent to generate fresh Foxp3 Fixation/Permeabilization working solution. 1
portion of 10X Permeabilization Buffer was mixed with 9 portions of distilled water to generate 1X working solution of Permeabilization Buffer. The cells were washed three times, followed by the addition of 1 mL of Foxp3 Fixation/Permeabilization working solution to each tube, and incubated for 30–60 min at room temperature with foil. 2 mL 1X Permeabilization Buffer was added to each tube, followed by the centrifugation of samples at 500 g for 4 min. After the supernatant was discarded, the pellet was resuspended in residual volume of 1X Permeabilization Buffer, followed by the addition of Ki-67 (Biolegend) to incubate with foil at room temperature for 30 min. The cells were washed with 2 mL 1X Permeabilization, followed by the resuspension of stained cells in PBS. The samples and the data were analyzed by flow cytometry (BD FACS Calibur) and FlowJo7.6, respectively.

6. Rna-seq Library Preparation And Sequencing

RNA integrity was evaluated by the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Total RNA was used as input materials for RNA sample preparations. In brief, poly-T oligo-attached magnetic beads were adopted to purify mRNA from total RNA. Divalent cations were used to perform fragmentation under elevated temperature in First Strand Synthesis Reaction Buffer(5X). First-strand cDNA was synthesized by using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H). Subsequently, second-strand cDNA synthesis was performed by using DNA Polymerase I and RNase H. Remaining overhangs were converted to blunt ends through exonuclease/polymerase activities. After adenylation of 3’ ends of DNA fragments, the Adaptor with a hairpin loop structure was ligated to prepare hybridization. In order to preferentially select cDNA fragments with a length of 370 ~ 420 bp, the RNA-seq library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then, PCR was performed by using Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified with AMPure XP system, followed by the evaluation of library quality with the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System by TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer’s instructions. After cluster generation, library preparations were sequenced on an Illumina Novaseq platform, followed by the generation of 150 bp paired-end reads.

7. Rna-seq Analysis

Gene Ontology (GO) enrichment analysis of differentially expressed genes was performed by the cluster Profiler R package, in which gene length bias was adjusted. GO terms with adjusted P value (< 0.05) were considered to be significantly enriched by differential expressed genes. KEGG is a database resource for understanding advanced functions and utilities of the biological system, such as cells, organisms and the ecosystem, from the information at the molecular-level, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). Cluster Profiler R package was adopted to detect the statistical enrichment of differentially expressed genes in KEGG pathways. Gene Set Enrichment Analysis (GSEA) is
a computational method to determine whether a pre-defined gene set can show a significantly consistent difference between two biological states. The genes in the gene set were ranked according to the degree of differential expression in the two samples, and then the predefined gene set was detected to see whether it was enriched at the top or bottom of the list. In gene set enrichment analysis, expression changes in sets of genes rather than individual ones are detected and thus subtle expression changes in them can also be included. The local version of the GSEA analysis tool (http://www.broadinstitute.org/gsea/index.jsp) as well as GO and KEGG datasets were independently used for GSEA analysis.

8. Bbb Analysis Of Yf-prj8-1011

Plasma, cerebrospinal fluid (CSF) and brain sample concentrations were measured by a LC/MS/MS method. Briefly, 50 µL aliquots of plasma or brain samples and 3 µL aliquots of CSF ones were processed according to a protein precipitation procedure, with a mixture of methanol, acetonitrile, and Terfenadine used as internal standards. The 5-fold diluted supernatant was injected onto an ACE 5 C4 (50 mm * 2.1 mm) column, and the YF-PRJ8-1011 and internal standards were eluted and directed into Sciex API5500 mass spectrometer for detection with 5 mM NH4OAc (0.05% FA) – ACN (0.1% FA). The acquisition was performed for quantitation in positive ionization mode with multiple reaction monitoring (MRM), and the MRM channels for YF-PRJ8-1011 and the internal standard (Terfenadine) were 379.20/293.20 and 472.33/436.20, respectively. Both Data collection and linear regression with weighting (1/X2) were performed by Analyst Software version 1.6.3. Non-compartmental analysis was carried out to determine the PK parameters of YF-PRJ8-1011 by WinNonlin Version 8.0.

9. Maximum Tolerated Dose (Mtd)

In order to help determine the optimal concentration of YF-PRJ8-1011 for further in vivo experiments, the MTD values of the compound for female B6 mice were evaluated. After administration of 50mg/kg, 100 mg/kg, 250 mg/kg and 500 mg/kg, respectively, weight changes were measured every 3 or 4 days for 2 consecutive weeks. Heart, brain, kidney, spleen, lung and liver were all removed for HE staining to observe whether there was related organ damage for them 24h after administration of 500mg/kg.

10. Dipg Xenograft Mouse Models And Administration Of Yf-prj8-1011

6-week female NOD/ShiLtJGpt-Prkdcem26Cd52Il2rgem26Cd22/Gpt mice (GemPharmatech) were used to establish Orthotopic xenograft mouse models. The method for establishing the animal model was as described previously. Briefly, NCG mice were anesthetized with 2.5% avertin i.p. and positioned in a stereotaxic instrument (KDS Legato 130). The posterior skull skin of the mice was incised and lambdoid suture was located as a mark. A skull hole was made by an electric drill (RWD). Luciferase engineered DIPG cells were re-suspended in 5 µl PBS at a density of 10^5 cells/µl and implanted into the brainstem of NCG mice. The tumor burden was measured by bioluminescence imaging 2 weeks after the
implantation. D-luciferin (Goldbio) suspended in PBS (15 mg/mL) was injected (150 mg/kg, i.p.) 10 min before acquisitions. Bioluminescence images were taken by Xenogen IVIS Spectrum Imaging System (Caliper Life Sciences). The ROI tool was adopted to measure Bioluminescence imaging data by Living Image software Version 3.0 (Caliper Life Sciences). Once the luminescence signal was detected, the tumor-bearing mice were randomly assigned to the control, YF-PRJ8-1011 or palbociclib treated groups. Then, the two drugs or vehicle was orally administered at a dose of 100 mg/kg/day for 14 consecutive days.

11. Radiation And Yf-prj8-1011 Treatment For Dipg Xenograft Mouse Models

DIPG xenograft mouse models were randomized into groups (treatment and control groups) before treatment. Treatment groups included YF-PRJ8-1011 alone, 4Gy whole brain XRT alone and 4Gy whole brain XRT with YF-PRJ8-1011 groups. YF-PRJ8-1011 was administered orally at 100 mg/kg/day for 21 consecutive days.

12. Hematoxylin And Eosin (H&E) Staining And Immunohistochemistry (IHC)

Mouse brainstem tissue was fixed in 4% paraformaldehyde (PFA) for 48 h. PFA-fixed brainstem tissue was paraffin-embedded after dehydration in ethanol gradients. The sections were cut to 5µm-thickness by a Paraffin microtome (Leica RM2235). After the slides were placed in a 56–60°C oven for 45 min, the sections were deparaffinized with xylene three times, for 5 min each. The slides were transferred to fresh absolute ethanol and then successively to 95%, 70%, and 50% alcohols for 5 min each. The slides were stained with H&E and IHC. The methods for H&E and immunohistochemical (IHC) staining were described as previously reported[7].

13. Plasmids And Reagents

Luciferase-GFP were cloned into pLEX based lentivirus vector, which was used to establish DIPG cells with luciferase-GFP. The human antibodies for western blot test in this study included anti-pRb (ab173289, 1:5000), anti-Rb (ab181616, 1:2000) and anti-β-Actin (Easybio, 1:3000). Secondary antibodies included Goat anti-mouse-IgG(H + L)-HRP-conjugated (Easybio, 1:3000), and Goat anti-rabbit IgG(H + L) (Easybio, 1:3000).

14. Statistical Analysis

All the experiments were performed three times. Graphpad prism 8 was adopted for statistical analysis. Statistical significance was calculated by an unpaired two-tailed t-test between groups. P < 0.05 indicates
15. Ethics Approval

This study was approved by Animal Welfare Ethics Committee of Beijing Neurosurgical Institute.

Results

1. YF-PRJ8-1011 of a Potent and Selective CDK4/6 Inhibitor

The binding modes of YF-PRJ8-1011 and CDK4/6 kinases are shown in Fig. 1a and 1b. The 2D pattern for the binding pocket of YF-PRJ8-1011 with CDK4/6 kinase is shown in Fig. 1a. LYS35 forms a hydrogen bond with the carbonyl oxygen atom of YF-PRJ8-1011. VAL96 forms a hydrogen bond with the pyrimidine nitrogen atom of 1011 and also with the amino group connecting pyrimidine and pyridine. The 3D pattern for the binding pocket of YF-PRJ8-1011 with CDK4/6 kinase is shown in Fig. 1b. Amino hydrogen atom of LYS35 forms a hydrogen bond with the carbonyl oxygen atom of YF-PRJ8-1011. Amino hydrogen atom of VAL96 forms a hydrogen bond with the nitrogen atom of pyrimidine, and also with the amino group connecting pyrimidine and pyridine. The synthetic route of YF-PRJ8-1011 is shown in Fig. 1c. 1H nuclear magnetic resonance (1H-NMR) spectrum of YF-PRJ8-1011 was shown in Supplementary Fig. 1. A panel of enzymes in parallel with staurosporine and YF-PRJ8-1011 was adopted to detect the kinase activity of YF-PRJ8-1011, and YF-PRJ8-1011 was found to show selective inhibition of CDK4/cyclin D1 (IC50 = 3.7 nmol/L), CDK4/cyclin D3 (IC50 = 3.4 nmol/L), CDK6/cyclin D1 (IC50 = 1.7 nmol/L), and CDK6/cyclin D3 (IC50 = 8.3 nmol/L) (Fig. 1d). The IC50 of palbociclib against CDK4 or CDK6 was 11 nmol/L or 16 nmol/L, respectively[9]. These data showed that YF-PRJ8-1011 was a potent CDK4/6 inhibitor.

2. Inhibition of Patient-Derived DIPG Cell Proliferation in Vitro by YF-PRJ8-1011

Next, it was decided to confirm the anti-tumor efficacy of YF-PRJ8-1011 in patient-derived DIPG cells. The molecular and pathological information of these tumor cells is shown in Supplementary Table 1. First, considering that Rb protein was a well-known target for cyclin D-Cdk4,6, which inhibited cell cycle progression until its inactivation by phosphorylation (ref)[3], Rb expression was tested and found to highly elevate in DIPG cells (Fig. 2a), compared with PPCs. Also, 100nM YF-PRJ8-1011 was sufficient to block Rb phosphorylation at Ser780 and reduce total Rb protein levels in DIPG tumor cells (Fig. 2b). Then, the cell viability of YF-PRJ8-1011 in all DIPG cells was tested and it was found that the IC50 value ranging from 98.64nM to 300.9nM was much lower than that in palbociclib-treated DIPG cells (2056nM-15319nM) (Supplementary Table 2). After that, the effect of YF-PRJ8-1011 on cell proliferation was tested by flow cytometry analysis. It was found that 100 nM YF-PRJ8-1011 and 100 nM palbociclib both could significantly decrease Ki67 expression. Moreover, 100nM YF-PRJ8-1011 was more effective in inhibiting Ki67 expression compared with 100nM palbociclib (Fig. 2c). Moreover, G1 phase arrest was observed in cells treated with YF-PRJ8-1011 and palbociclib, respectively (Fig. 2d). Five DIPG cells were treated with 1µM palbociclib, 1µM ribociclib, 1µM abemaciclib, and 1µM YF-PRJ8-1011 for 48 h to verify
whether YF-PRJ8-1011 exhibited better in vitro anti-tumor efficacy compared with the other three FDA-approved CDK4/6 inhibitors. The cell viability was evaluated 48 h after treatment. The relative cell viability was evaluated by T48h/T0: 4.40–34.00% in the YF-PRJ8-1011-treated group, 54.04–86.03% in the palbociclib-treated group, 58.9–92.33% in the ribociclib-treated group, and 7.55–54.14% in the abemaciclib-treated group (Fig. 2e). These data showed that YF-PRJ8-1011 had better in vitro anti-tumor activity in DIPG than the other three FDA-approved CDK4/6 inhibitors. In conclusion, these findings demonstrated that YF-PRJ8-1011 is a potential anti-tumor drug in DIPG with more potent anti-tumor activity than approved CDK4/6 inhibitors.

3. Genome-wide Effects Of Yf-prj8-1011 On Dipg

High-throughput RNA sequencing (RNA-seq) was performed to explore the biological effect of YF-PRJ8-1011 treatment on DIPG cells at the transcriptome level, and 310 downregulated genes and 128 upregulated genes were identified in the YF-PRJ8-1011 treated group, compared with the normal group (p.adj < 0.05 & |log2FoldChange| > 1, Fig. 3a), suggesting that YF-PRJ8-1011 mainly represses the gene expression of DIPG cells. GO (Gene Ontology) enrichment analysis of differentially expressed genes revealed that, downregulated genes were mainly enriched in cell division–related pathways (Fig. 3b). The gene set enrichment analysis (GSEA) revealed that many genes involved in G1/S transition were enriched in the downregulated genes (Fig. 3c). Therefore, these results demonstrated that YF-PRJ8-1011 can inhibit the tumor cell cycle.

4. Bbb Permeability Of Yf-prj8-1011

Although some small-molecule drugs show in vitro anti-tumor activity, their efficacy is limited by the BBB[10–14]. At 0.5, 1, 2, 4, 8, and 24 h after oral administration of palbociclib or YF-PRJ8-1011 at a dose of 100 mg/kg to female BALB/c mice, the blood, brain tissue, and cerebrospinal fluid (CSF) were collected to explore the BBB permeability of YF-PRJ8-1011. The data on the pharmacokinetics of palbociclib was showed in Supplementary Table 3. The AUC$_{24h}$ of palbociclib in the brain, plasma, and CSF was 7134.4 h×ng/g, 55067.5 h×ng/mL, and 171.87 h×ng/mL, respectively. The YF-PRJ8-1011 concentrations in the plasma at the six time points were 511.50 ± 113.84 ng/mL, 735.50 ± 0.71 ng/mL, 517.50 ± 40.31 ng/mL, 321.00 ± 93.34 ng/mL, 247.00 ± 60.81 ng/mL, and 64.80 ± 21.64 ng/mL, respectively (Fig. 4a). The drug concentrations in the CSF were 5.12 ± 2.30 ng/mL, 7.94 ± 2.52 ng/mL, 7.06 ± 1.95 ng/mL, 6.70 ± 4.57 ng/mL, 5.07 ± 4.26 ng/mL, and 2.74 ± 0.10 ng/mL, respectively (Fig. 4b). The drug concentrations in the brain were 73.00 ± 7.07 ng/g, 164.50 ± 12.73 ng/g, 197.75 ± 41.37 ng/g, 233.25 ± 51.27 ng/g, 267.00 ± 19.80 ng/g, and 214.50 ± 10.61 ng/g, respectively (Fig. 4c). The brain–plasma ratio was 0.145 ± 0.02, 0.22 ± 0.02, 0.38 ± 0.05, 0.75 ± 0.07, 1.103 ± 0.19, and 3.48 ± 1.00, respectively. The CSF–plasma ratio was 0.0975 ± 0.0023, 0.0108 ± 0.0034, 0.0139 ± 0.0049, 0.0197 ± 0.0086, 0.0190 ± 0.0127, and 0.0451 ± 0.0166, respectively (Fig. 4d). The AUC$_{24h}$ in the brain, plasma, and CSF was 5539.75 h×ng/g, 5535.03
h×ng/mL, and 111.75 h×ng/mL, respectively. The results demonstrated that YF-PRJ8-1011 can effectively pass through the BBB and reach a stable concentration in brain tissue.

5. The Inhibition Of The Growth Of DIPG In Orthotopic Xenograft Mouse Models By Yf-prj8-1011

Firstly, in order to help determine the optimal concentration of YF-PRJ8-1011 for further in vivo experiment, the MTD values of the compound in female B6 mice were evaluated. After administration of one dose (50mg/kg, 100 mg/kg, 250 mg/kg and 500 mg/kg, respectively), weight changes were measured every 3 or 4 days for 2 consecutive weeks and weight loss was observed only in 500mg/kg group (Supplementary Fig. 2a). Heart, brain, kidney, spleen, lung and liver were removed for HE staining 24h after administration of one dose (500mg/kg) and the results showed that there was no acute damage to all organs (Supplementary 2b). Then, two luciferase-modified DIPG cells, TT150630 and TT190326 were used to establish orthotopic xenograft mouse models by injecting tumor cells into the brainstem of NCG mice so as to further examine the in vivo anti-tumor efficacy of YF-PRJ8-1011. Tumor cells modified with firefly luciferase enabled us to monitor the intracranial tumor growth by bioluminescence imaging. The bioluminescence images were acquired 2 weeks after the tumor cell implantation. The mice were randomly divided into vehicle, YF-PRJ8-1011-treated, and palbociclib-treated groups, and there was no statistical difference in tumor volume among the three groups. Furthermore, the oral administration of YF-PRJ8-1011, palbociclib and vehicle at a dose of 100 mg/kg was given daily in the three groups for 14 consecutive days, respectively. The bioluminescence images were acquired every week to measure the tumor volume. For both luciferase-modified DIPG xenografts, significant inhibition of tumor growth was observed in the YF-PRJ8-1011-treated group compared with the control group (p = 0.0064 and p = 0.0003 for TT150630 and TT190326, respectively) and palbociclib-treated group (p = 0.0070 and p = 0.0026 for TT150630 and TT190326, respectively) (Fig. 5a and 5b). The overall survival was significantly longer in the YF-PRJ8-1011 treated group than that in the control group (p = 0.0044 and p = 0.0067 for TT150630 and TT190326, respectively, with a median survival of 60 vs 38 days for TT150630 and 96 vs 50 days for TT190326) and palbociclib-treated group (p = 0.0333 and p = 0.0266 for TT150630 and TT190326, respectively, with a median survival 60 vs 39 days for TT150630 and 79.5 days for TT190326), respectively (Fig. 5c). After 14-day treatment, H&E staining of brainstem sections was performed to observe tumor cells in vivo (Supplementary Fig. 3a). The immunohistochemical (IHC) staining for p-Rb was performed to detect the proliferation of tumor cells in vivo. The result showed that the p-Rb staining in brainstem sections in the control group was significantly more than that in the YF-PRJ8-1011 treated group (Supplementary Fig. 3b). Tumor bioluminescence for TT150630-bearing NCG mice treated with YF-PRJ8-1011 alone, 4 Gy whole-brain irradiation and YF-PRJ8-1011 combined with 4 Gy whole-brain irradiation was measured. Tumor growth was significantly inhibited in the combination treatment group, compared with the control group (p = 0.0085), YF-PRJ8-1011-treated group (p = 0.0273), and irradiation group (p = 0.0078) (Fig. 5d and 5e). The overall survival in the combination therapy group was significantly longer than that in the control group (p = 0.0067), YF-PRJ8-1011-treated group (p = 0.0067) and irradiation group (p = 0.0169), respectively (Fig. 5f).
Discussion

Brainstem gliomas account for 15–20% of all pediatric central nervous system (CNS) tumors, most of which are DIPGs[15, 16]. DIPG is a rare and lethal brainstem tumor with a mean overall survival (OS) of 8–14 months, and surgical treatment for it does not exhibit potent efficacy due to extensive tumor infiltration in the brainstem¹. Although radiotherapy has been the standard of care for DIPG for more than 50 years, it can merely increase the OS by 3–6 months[17, 18]. Chemotherapy has also failed to show benefits beyond radiation so far[19]. Recently, various therapeutic targets for DIPG have been discovered, and the corresponding small-molecule compounds have shown anti-tumor effects of different degrees[8]. However, no clinical trial has confirmed that any small-molecule drug can improve the clinical prognosis in patients with DIPG[2]. Thus, finding druggable therapeutic targets and synthesizing more effective drugs are still of great significance for the treatment of DIPG.

CDK4/6 are fundamental drivers of the cell cycle, which play a pivotal role in the transition from G1 to S phase in many tumor cells including DIPGs through the phosphorylation of retinoblastoma-related protein 1 (Rb1)[5, 20]. Targeting CDK4/6 activity is one of the options for blocking G1/S cell cycle transition, which has showed the induction of cell cycle arrest in cancer therapy. In this study, a novel small-molecule compound YF-PRJ8-1011 with selectivity to CDK4/6 and potent anti-tumor activity in DIPG cells was investigated. The results showed that YF-PRJ8-1011 has stronger effects on the inhibition of DIPG cell growth both in vitro and in vivo than palbociclib. Palbociclib, an FDA-approved selective CDK4/6 inhibitor, shows anti-tumor activity in DIPG cells in previous reports[6, 7]. According to these reports, the effect of inhibiting tumor growth is only shown at relatively high concentrations (5–10µM). Sun et al. treated the DIPG mouse model with 150 mg/kg palbociclib in vivo for 21 consecutive days and obtained positive results. However, Franshaw et al. pointed out that the dose of palbociclib in mouse models was much higher than the equivalent MTD in humans[8, 21]. In this study, the administration of YF-PRJ8-1011 at a dose of 100 mg/kg for 14 consecutive days was sufficient to induce a more potent tumor-suppressive effect compared with palbociclib.

The BBB is a bottleneck for brain tumor treatment because 98% of small-molecule drugs cannot pass through it[22]. Most small-molecule drugs for DIPG exhibited in vitro anti-tumor activity, but the results of experiments with them on the mouse model were not ideal[2], which might be due to the presence of BBB. In this study, YF-PRJ8-1011 has been proved to be a CDK4/6 inhibitor with good BBB penetration. However, the YF-PRJ8-1011 concentration in the mouse brain is not as high as palbociclib and it still needs improvement. Therefore, we plan to optimize the design of the CDK4/6 inhibitor to develop a more powerful inhibitor to penetrate the BBB in the next phase of research.

In addition, we also found that the potential of YF-PRJ8-1011 combined with radiotherapy to achieve better efficacy. In previous clinical study[23], palbociclib monotherapy was not an effective treatment for gliomas. Combination therapy may be a strategy that can be tried in future relevant clinical trials.

Declarations
Funding

This study was funded by Multicenter clinical big data study and multi-path tumorigenesis mechanisms and precision treatment research on brainstem glioma (JINGYIYAN2018-7).

Conflicts of interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Pengcheng Zuo: writing—original draft and conceptualization,
Yaopeng Li: formal analysis and investigation,
Tantan Wang: Bioinformatics analysis,
Xingyu Lin: Drug design,
Zhen Wu, Junting Zhang: methodology and resources,
Xuebin Liao and Liwei Zhang: review and editing, and supervision,

All authors contributed to the article and approved the submitted version.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Availability of data and material

The datasets generated during the current study are available from the corresponding author on reasonable request.

Ethics approval

This study was approved by the human research ethics committee of Beijing Tiantan Hospital. The written informed consent was obtained from the subjects.

Consent to participate

Written informed consent was obtained from the parents.

Consent for publication

Written informed consent for publication was obtained from all participants.
References


Figures

Figure 1

(a) Figure 1a shows the 2D pattern for the binding pocket of YF-PRJ8-1011 with CDK4/6 kinase. (b) Figure 1b shows the 3D pattern for the binding pocket of YF-PRJ8-1011 with CDK4/6 kinase. (c) Synthetic routes of compounds. Reagents and conditions: (i) NaN₃, Pd/C, H₂, EtOH, 60°C; (ii) NAtomic, 100°C; (iii) NaN₃, Pd/C, H₂, EtOH, 60°C; (iv) NAtomic, 100°C; (v) NaN₃, Pd/C, H₂, EtOH, 60°C; (vi) NAtomic, 100°C; (vii) NaN₃, Pd/C, H₂, EtOH, 60°C; (viii) NAtomic, 100°C.

<table>
<thead>
<tr>
<th>Protein kinase</th>
<th>YF-PRJ8-1011</th>
<th>ICS0 (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK1/cyclin B</td>
<td>262.9</td>
<td></td>
</tr>
<tr>
<td>CDK2/cyclin A</td>
<td>181.9</td>
<td></td>
</tr>
<tr>
<td>CDK4/cyclin D1</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>CDK4/cyclin D3</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>CDK6/cyclin D1</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>CDK6/cyclin D3</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>CDK7/cyclin H</td>
<td>2318</td>
<td></td>
</tr>
<tr>
<td>CDK9/cyclin T1</td>
<td>90.5</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1

Chemical structure and CDK-inhibitory activity of YF-PRJ8-1011. (a) Figure 1a shows the 2D pattern for the binding pocket of YF-PRJ8-1011 with CDK4/6 kinase. (b) Figure 1b shows the 3D pattern for the binding pocket of YF-PRJ8-1011 with CDK4/6 kinase. (c) Synthetic routes of compounds. Reagents and conditions: (i) NaN₃, Pd/C, H₂, EtOH, 60°C; (ii) NAtomic, 100°C; (iii) NaN₃, Pd/C, H₂, EtOH, 60°C; (iv) NAtomic, 100°C; (v) NaN₃, Pd/C, H₂, EtOH, 60°C; (vi) NAtomic, 100°C; (vii) NaN₃, Pd/C, H₂, EtOH, 60°C; (viii) NAtomic, 100°C.
conditions included as follows: ( ) the yield of 2,4-dichloro-5-fluoropyrimidine, tributyl(1-ethoxyvinyl)stannane, Pd(PPh3)2Cl2, and DMF at 120°C for 2 h was 70.1%, ( ) the yield of Br2, AcOH at room temperature for 4 h was 62.6%, ( ) the yield of piperidine-2,4-dione, NH4OAc, and EtOH at room temperature for 1 h was 69.4%, ( ) the yield of NaH, CH3I, and DMF at room temperature for 18 h was 40.3%, ( ) the yield of 6-nitronicotinic acid, 1-ethylpiperazine, HATU, DIEA, and DCM at room temperature for 5 h was 82.7%, ( ) the yield of BH3•THF and THF after reflux at 12 h was 20.4%, ( ) the yield of H2, Pd/C, and MeOH at room temperature for 18 h was 90.8%, ( ) the yield of compound 5, Pd2(dba)3, X-Phos, K2CO3, and MePh at 100 °C for 18 h was 8.6%. (d) Half-maximal inhibitory concentrations (IC50) of the compound for CDK4 and CDK6.
YF-PRJ8-1011 of a potential CDK4/6 inhibotor of DIPG *in vitro.* (a) Western blot analysis of Rb expression in primary DIPG cells. (b) Western blot analysis of Rb phosphorylation at Ser780 in primary DIPG cells treated with 100nM YF-PRJ8-1011, 100nM palbociclib, or 100nM vehicle for 48 h, respectively (c) Quantification of Ki-67 FACS staining in DIPG cells treated with YF-PRJ8-1011, palbociclib, or vehicle at 100nM for 48 h, respectively. (d) The induction of cell cycle G1 arrest in DIPG cells by YF-PRJ8-1011.
treatment. These cells were treated with YF-PRJ8-1011, palbociclib, or control at 100nM for 48 h, respectively. (e) Five DIPG cells were treated with palbociclib, ribociclib, abemaciclib, and YF-PRJ8-1011 at 1μM for 48 h, respectively. The cell viability was assessed at 48 h with no treatment (T0) and 48 h after treatment (T48). The relative cell viability was evaluated by T48h/T0. Unpaired t-tests were performed. p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****).

Figure 3

Genome-wide effects of YF-PRJ8-1011 on DIPG cells. (a) The volcano plot of differentially expressed genes in RNA-seq analysis, which showed that 310 of the genes were down-regulated, while 128 genes were upregulated (p.adj< 0.05 & |log2FoldChange| > 1). (b) Gene set enrichment analysis (GSEA) of the genes in CDK treated group and normal group (q < 0.0001). The Heatmap showed the expression of the
genes involved in G1/S transition. Color bars represent scaled gene expression values. (c) Gene Ontology analysis of downregulated genes in the volcano plot, which demonstrated the enriched pathways.

Figure 4

The ability of YF-PRJ8-1011 to effectively penetrate the blood-brain barrier. (a) Blood, (b) cerebrospinal fluid and (c) brain tissue, and were collected 0.5, 1, 2, 4, 8, and 24 h after the oral administration of YF-
PRJ8-1011 at the dose of 100 mg/kg to female BALB/c mice to measure drug concentrations, and (d) all data are shown in the table.

Figure 5

The potent anti-tumor efficacy of YF-PRJ8-1011 in NCG mice bearing DIPG cells. (a, b) Normalized luminescence was analyzed after 14-day treatment by Living Image software Version 3.0 (Caliper Life
(c) Kaplan–Meier survival curve are plotted. The overall survival in the YF-PRJ8-1011-treated group was significantly longer than that in the control group (p = 0.0044 and p = 0.0067 for TT150630 and TT190326, respectively) and palbociclib-treated (p = 0.275 and p = 0.0266 for TT150630 and TT190326, respectively), respectively. (d, e) The measurement of tumor bioluminescence in TT150630-bearing NCG mice treated with YF-PRJ8-1011 alone, 4 Gy whole-brain irradiation and YF-PRJ8-1011 combined with 4 Gy whole-brain irradiation, respectively. (f) The overall survival in the combination therapy group was significantly longer than that in the control group (p = 0.0067), YF-PRJ8-1011-treated group (p = 0.0067) and irradiation group (p = 0.0169), respectively. Log-rank tests were performed. p < 0.05 (∗), p < 0.01 (∗∗), p < 0.001 (∗∗∗), and p < 0.0001 (∗∗∗∗).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTablesandfigures.docx](SupplementaryTablesandfigures.docx)