Cancer Letters 381 (2016) 391–403

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

CAT3, a novel agent for medulloblastoma and glioblastoma treatment, inhibits tumor growth by disrupting the Hedgehog signaling pathway

Ju Chen [a,1], Haining Lv [a,1], Jinping Hu [a], Ming Ji [a], Nina Xue [a], Chao Li [a], Shuanggang Ma [a], Qin Zhou [a], Bin Lin [b], Yan Li [a], Shishan Yu [a,*], Xiaoguang Chen [a,**]

[a] State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China
[b] Shenyang Pharmaceutical University, Shenyang 110015, China

ARTICLE INFO

Article history:
Received 5 March 2016
Received in revised form 31 May 2016
Accepted 21 July 2016

Keywords:
Medulloblastoma
Glioblastoma
Hedgehog
Smo
Gli1

ABSTRACT

Medulloblastoma (MB) and glioblastoma (GBM) are the most prevalent malignant brain tumors. The identification of novel therapeutic strategies is urgent for MB and GBM patients. Herein, we discovered 13a-(*S*)-3-Hydroxyl-6,7-dimethoxyphenanthro[9,10-*b*]-indolizidine (PF403) strongly exhibited inhibitory activity against Hedgehog (Hh) pathway-hyperactivated MB and GBM cells with a 50% inhibitory concentration (IC_{50}) of 0.01 nM. CAT3 was designed and synthesized as the prodrug of PF403 and displayed significant in vivo efficacy against MB and GBM. Mechanistic study revealed that CAT3 inhibited MB and GBM primarily by interrupting the Hh signaling pathway. At the molecular level, PF403 inhibited the cell surface accumulation of the Smoothened (Smo) receptor by directly binding or enhancing the interaction of Smo with the repressor Ptch1. Furthermore, PF403 significantly repressed Gli1 nuclear accumulation and transcription by promoting Sufu-Gli1 and PKA-Gli1 interactions. Collectively, our studies support the hypothesis that CAT3 is a promising therapeutic agent for the treatment of Hh-driven MB and GBM.

Introduction

Malignant brain tumors, such as medulloblastoma (MB), neuroblastoma (NB) and glioblastoma (GBM), are characterized as highly invasive, motile tumors, and are associated with poor survival rates and prognoses. MB and NB are fatal extracranial tumors that commonly develop in children, and are associated with a median survival of less than 6 months to one year. The 2-year survival rates are approximately 9% and <40%, respectively, even with intensive therapy [1,2]. GBM more commonly occurs in adults and is associated with a median survival of 15–17 months even with the best available treatment [3]. Temozolomide (TMZ), an alkylating agent, is the principal first-line chemotherapeutic agent used for the treatment of recurrent, high-grade glioma and is also used as a conventional therapy for other malignant brain tumors [4,5]. TMZ has been proven to effectively inhibit GBM proliferation and survival. However, intrinsic and acquired TMZ resistance in GBM cells ultimately limits its efficacy [6–8]. Consequently, the identification of new alternative therapeutic approaches for brain tumors is imperative.

Recently, activation of the Hedgehog (Hh) signaling pathway was reported to be closely linked to tumor initiation and MB, NB and GBM maintenance [9–12]. The Hh signaling pathway is initiated by the binding of the key Sonic hedgehog (Shh) ligand to the Patched1 (Ptch1) receptor. This binding event relieves the inhibitory action of Ptch1 on the seven-transmembrane protein Smoothened (Smo), which ultimately leads to the transcription of Gli target genes, such as CMYC and BMI-1, along with GLI1 itself [13–17]. Thompson et al. reported that up to one third of all human MB patients exhibit hyperactive Hh signaling, often due to mutations in the Smo, Ptch1 or Sufu genes [18]. In GBM, up-regulated Hh signaling is important for tumor cell proliferation, survival and migration, and is associated with the poor treatment outcome in GBM [9,19]. Importantly, several antagonists targeting Smo, such as GDC-0449, NVP-LDE225, BMS-833923 and PF-04449913, are in advanced-stage clinical trials. Among these Smo antagonists, GDC-0449 is the first small molecule inhibitor of Smo to be approved by the United States Food and Drug Administration (FDA) for the treatment of advanced basal cell carcinoma (BCC), a type of skin cancer also driven by mutations in Hh pathway components [20,21]. Patients with BCC are predisposed to other types of cancer, particularly MB. Furthermore, GDC-0449 rapidly inhibited tumor growth and reduced symptoms in adult patients with recurrent GBM (NCT00930484) and in a young patient with metastatic MB that had not previously

Xiaoguang Chen and Shishan Yu are willing to handle correspondence at all stages of refereeing and publication, also post-publication.
* Corresponding author. Fax: 86-10-6316-5326.
E-mail address: yushishan@imm.ac.cn (S. Yu).
** Corresponding author. Fax: 86-10-6316-5207.
E-mail address: chxg@imm.ac.cn (X. Chen).
[1] The first two authors contributed equally.

http://dx.doi.org/10.1016/j.canlet.2016.07.030
0304-3835/

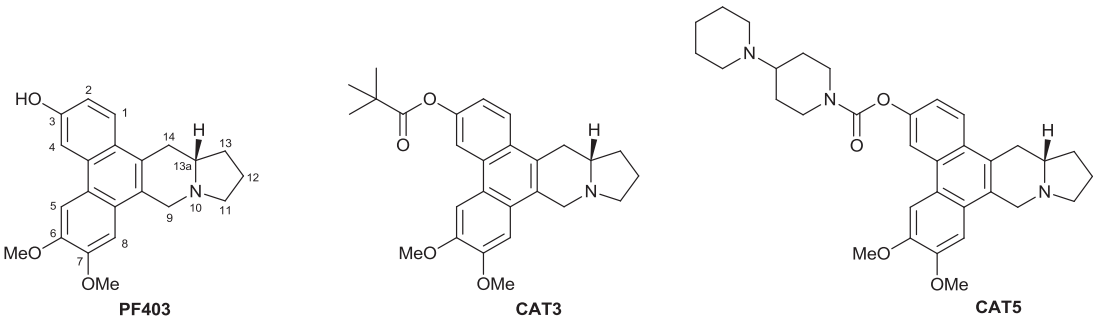

Fig. 1. The structures of PF403 and the selected prodrugs CAT3 and CAT5.

responded to conventional therapies (NCT00822458) [10]. Therefore, targeting the Hh signaling pathway is a reasonable strategy for treating Hh pathway-driven malignant brain tumors.

Natural products and their derivatives have served as major sources of therapeutic agents and lead molecules in drug discovery. Currently, over 70% of antitumor compounds are either natural products or their derivatives [22,23]. 13a-(*S*)-3-Hydroxyl-6, 7-dimethoxyphenanthro[9, 10-*b*]-indolizidine (PF403) (Fig. 1), a metabolite of the bioactive natural product, 13a-(*S*)-deoxytylophorinine [24,25], demonstrated potent anticancer activity with a 50% inhibitory concentration (IC_{50}) greater than 0.01 nM in multiple cancer cell lines in vitro [26]. Our previous study had demonstrated that PF403 had a potent antitumor activity against NB [27]. Moreover, PF403 was capable of penetrating the blood–brain barrier (BBB) and readily localized to brain tissue after administration [27], indicating that it has the potential to treat other brain tumors. However, in vivo studies with PF403 demonstrated negative results (Supplementary Table S1). We speculated that the lack of PF403 efficacy was due to the compound's poor pharmacokinetic properties. The presence of a polar functional group in PF403 might promote its rapid metabolism and elimination before it reaches its therapeutic target. To address this issue, the prodrug strategy was employed in subsequent studies. 11 prodrug candidates have been designed and synthesized (the structures and the synthesis methods are provided in Supplementary 1 A, B respectively). Using in vitro and in vivo screening assays, two prodrug candidates, 13a-(*S*)-3-pivaloyloxyl-6,7-dimethoxyphenanthro[9, 10-*b*]-indolizidine (CAT3, Fig. 1) and 13a-(*S*)-3-[4'-(piperidyl-1'')-piperidyl-1']acyloxyl-6,7-dimethoxy-phenanthro[9,10-*b*]-indolizidine (CAT5, Fig. 1) were selected for further analysis (Supplementary Fig. S1A and Supplementary Tables S2, S4). These two compounds effectively inhibit the growth of brain tumor in xenograft tumor models in a dose-dependent manner (Supplementary Fig. S1B, C and Supplementary Table S5). Pharmacokinetic assays revealed that PF403, but not the prodrugs evaluated, was detected in the plasma of mice following CAT3 and CAT5 oral administration, which is consistent with our hypothesis. Importantly, in brain tissue, high concentrations of PF403 were detected only after the administration of CAT3, but not CAT5. Thus, CAT3 was selected for additional in vivo studies utilizing orthotopic brain tumor models.

In this study, we investigated its antitumor effects against MB and GBM. The in vivo results demonstrated that CAT3 significantly suppressed Daoy and U87MG orthotopic xenograft tumor growth with inhibition rates of 78.8% and 65.4% at a dose of 12 mg/kg, compared with an inhibition rate of 71.9% with TMZ at a dose of 50 mg/kg. No significant toxicity was observed in the CAT3-treated mice bearing intracranial tumors. Interestingly, PF403, the metabolite of CAT3, exerted stronger inhibitory effects against Daoy and U87MG cells in which the Hh pathway is hyperactivated, compared with the Gli1-negative A172 cells, indicating that the inhibitory effect of PF403 might be mediated by a disruption of the Hh signaling pathway. Mechanistic study revealed that PF403 appeared to preferentially bind Smo in a similar manner as GDC-0449 to enhance the association of Smo with the Ptch1 repressor thereby inhibiting Smo activity. Furthermore, PF403 reduced Gli1 translocation to the nucleus by promoting the interactions of Sufu and PKA with Gli1. These results strongly suggested that CAT3 might be a promising novel agent for the treatment of Hh pathway-driven MB and GBM.

Materials and methods

Reagents and general methods

Antibodies against Shh, Smo, Sufu, Gli1, Flag and Sodium Potassium ATPase were purchased from Abcam. Antibody against Ptch1, PKA and Histone-3 were obtained from Cell Signaling Technology. β-actin antibody, normal mouse or rabbit IgG and Protein A/G PLUS-Agarose were purchased from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL) western blot detection reagents were purchased from Biotanon. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma.

The synthesis and structure verification of the PF403 prodrug

Reagents and solvents for chemical synthesis were purchased from commercial sources including Aldrich, Alfa Aesar and Acros and used directly without any purification. ^{1}H NMR and ^{13}C NMR spectra were acquired on an INOVA-500 spectrometer. The abbreviations used in the NMR analysis are as follows: s = singlet, d = doublet, dd = double-doublet, m = multiplet. HRESIMS data were obtained on an Agilent 6520 Accurate-Mass-Q-TOF LC/MS spectrometer. Optical rotations were measured on a JASCO P-2000 automatic polarimeter. TLC was conducted with glass precoated with silica gel GF254 (Qingdao Marine Chemical Inc., China) to monitor the progression of the reactions. Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., China) was used for column chromatography (CC).

The PF403 compound was synthesized according to the synthetic route we previously reported [26]. The PF403 prodrug candidates were synthesized by esterification with acyl chloride or acid. The specific method, the final structure and spectral data are available in Supplementary 1.

Pharmacokinetics assay

Male ICR mice weighing 22–25 g were supplied by the Animal Center of the Chinese Academy of Medical Science. The tested compounds were dissolved in water containing 20% PEG400. CAT3 and CAT5 were orally administrated to mice at a dose of 10 mg/kg. Blood sample and brain tissues were collected at 5, 15, 30 min, 1, 2, 4, 6, 8, 12 and 24 h after treatment. All experimental procedures were performed in accordance with the guidelines of the Beijing Municipal Ethics Committee for the care and use of laboratory animals. Plasma was prepared by the centrifugation of blood at 5000 rpm for 10 min and brain tissues were homogenized with saline (1:3 g/v). Then, 100 μL of plasma or brain homogenate samples were precipitated by adding two equivalent volumes of acetonitrile and were centrifuged at 14,000 rpm for 5 min. A 5 μL aliquot of plasma or brain extract was analyzed using a liquid chromatography tandem mass spectrometry (LC-MS/MS) system consisting of an Agilent 1260 Series high-performance LC apparatus, fitted with a Zorbax SB-C18 analytical column (3.5 mm, 100 mm × 2.1 mm) (Agilent, Santa Clara, USA) and an API 4000 triple quadruple mass spectrometer (AB SCIEX, USA).

Cell culture

The human medulloblastoma cell line (Daoy) was purchased from the American Type Culture Collection (ATCC, Manassas, VA), and the human glioblastoma cell lines (U87MG, U251 and A172) were obtained from the Cell Culture Center at the