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Pharmacology of novel small-molecule tubulin inhibitors in glioblastoma cells with enhanced EGFR signalling

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A B S T R A C T

We recently reported that CMPD1, originally developed as an inhibitor of MK2 activation, primarily inhibits tubulin polymerisation and induces apoptosis in glioblastoma cells. In the present study we provide detailed pharmacological investigation of CMPD1 analogues with improved molecular properties. We determined their anti-cancer efficacy in glioblastoma cells with enhanced EGFR signalling, as deregulated EGFR often leads to chemoresistance. Eight analogues of CMPD1 with varying lipophilicity and basicity were synthesised and tested for efficacy in the cell viability assay using established glioblastoma cell lines and patient-derived primary glioblastoma cells. The mechanism of action for the most potent analogue **15** was determined using MK2 activation and tubulin polymerisation assays, together with the immunofluorescence analysis of the mitotic spindle formation. Apoptosis was analysed by Annexin V staining, imunoblotting analysis of bcl-2 proteins and PARP cleavage. The apoptotic activity of CMPD1 and analogue **15** was comparable across glioblastoma cell lines regardless of the EGFR status. Primary glioblastoma cells of the classical subtype that are characterized by enhanced EGFR activity were most sensitive to the treatment with CMPD1 and **15**. In summary, we present mechanism of action for a novel small molecule tubulin inhibitor, compound **15** that inhibits tubulin polymerisation and mitotic spindle formation, induces degradation of anti-apoptotic bcl-2 proteins and leads to apoptosis of glioblastoma cells. We also demonstrate that the enhanced EGFR activity does not decrease the efficacy of tubulin inhibitors developed in this study.

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1. Introduction

Glioblastoma is a heterogeneous brain cancer, classified into four molecular subtypes – classical, proneural, neural and mesenchymal – each characterised by a set of molecular traits [1,2]. The most common (60%) are tumours of the classical subtype, which are characterised by amplification of the epidermal growth factor receptor (EGFR). Approximately half of the tumours with EGFR amplification also express a constitutively active EGFR variant III (EGFRvIII) [3]. Glioblastoma tumours of the proneural subtype are defined by the over-expression of platelet-derived growth factor receptor (PDGFR) and these tumours represent 13%

Abbreviations: Bak, Bcl-2 antagonist/killer-1; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-X_L, B-cell lymphoma-extra large; cMet, hepatocyte growth factor receptor; cmpd, compound; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; Mcl-1, myeloid cell leukemia 1; MK2, MAPK-activated protein kinase 2; MTA, microtubule targeting agents; PARP, poly-(ADP-ribose)-polymerase; PDGFR, platelet-derived growth factor receptor; RTK, receptor tyrosine kinase.

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of all glioblastomas [4]. Amplification of the hepatocyte growth factor receptor (cMet) has been assigned to the mesenchymal subtype (1.3%), whereas the neural subtype is characterised by over-expression of neuronal markers. The over-expression and mutation of receptor tyrosine kinases (RTKs) underlies enhanced proliferation, impaired apoptosis, angiogenesis and self-renewal capacity of glioblastoma cells. Thus, much of the glioblastoma research has focused on targeting RTKs, however results of trials using RTK inhibitors have been disappointing [5].

Microtubules are hollow tubes composed of α- and β-tubulin that polymerise parallel to a cylindrical axis. Microtubules act as essential elements of the cytoskeleton and are crucial for cell division, intracellular transport and migration [6,7]. Microtubules are attractive pharmacological targets for cancer therapy, and with the long history of clinical efficacy microtubule targeting agents (MTA) remain to date the most classical yet reliable chemotherapeutics. Microtubule targeting *Vinca* alkaloids (vinblastine, vincristine) and taxanes (paclitaxel, cabazitaxel) are frontline treatments for breast, ovarian and hormone-refractory prostate cancers. However, the acquired resistance developed over time has plagued the success of these drugs. In addition, in the case of invasive brain cancers, these agents are ineffective because their large molecular weight (>800 g/mol) renders them unable to cross the blood–brain barrier. Hence, there has been increasing research interest towards the development of effective MTA delivery methods [8–10] or identification of small-molecule tubulin inhibitors able to cross the blood–brain barrier [11,12].

In our recent work, we have discovered that a small-molecule CMPD1, initially developed as an allosteric inhibitor binding to p38 mitogen-activated protein kinase (p38 MAPK) and blocking activation of the MAPK-activated protein kinase 2 (MK2) [13], primarily inhibits tubulin polymerisation [14]. CMPD1 possesses anti-mitotic and apoptotic activity in a panel of human cancer cells, but had only cytostatic effect on non-malignant cells. This cytotoxic selectivity towards cancer cells and the small molecular weight (349 g/mol) make CMPD1 an attractive lead for the development of potential chemotherapeutic agents for glioblastoma.

In this study we present CMPD1 analogues with improved molecular properties and their detailed pharmacological investigation in glioblastoma cells with amplified and/or mutated EGFR. We have focused on EGFR-dependent glioblastoma models, because (a) EGFR is the prime oncogenic driver of glioblastomas [3,5,15] and (b) EGFR amplification/mutation is linked to the resistance to chemotherapy [16–20]. We demonstrate that novel agents presented in this study are inhibitors of tubulin polymerisation and induce comparable degree of apoptosis in U87 (low EGFR levels) and U87-EGFRvIII (over-expression of EGFRvIII) cell lines. Furthermore, we also demonstrate efficacy of these agents in patient-derived primary glioblastoma cells of classical, mesenchymal, proneural and neural subtypes. Intriguingly, glioblastoma cells of the classical subtype with enhanced EGFR expression were most sensitive to this novel class of tubulin inhibitors.

2. Methods

2.1. Reagents and materials

The physicochemical and pharmacokinetic properties of analogues were calculated using ACD/Labs v12 (Advanced Chemistry Development Inc., Toronto, ON, Canada) and QikProp v3.5 (Schrödinger Inc., New York, NY, USA), respectively. All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA), except for boronic acids that were purchased from Frontiers Scientific (Logan, UT, USA). Microwave irradiation was carried out using the CEM-Discover microwave reactor (Kamp-Lintfort, Germany). ^{1}H NMR spectra were obtained on a BRUKER "Avance 300" 300 MHz NMR spectrometer (Bruker Corp., Billerica, MA, USA). d_6-DMSO or $CDCl_3$ were obtained from Cambridge Isotope Laboratories. High performance liquid chromatography was conducted on an Agilent series 1200 LC system with an Agilent 1260 Infinity binary pump and integrated vacuum degasser, autosampler and diode array detector (Agilent Technologies, Santa Clara, CA, USA). The samples were analysed on an Agilent C18 column (particle size: 5 μM, 150 $\times$ 4.6 mm internal diameter) using an acetonitrile:water gradient of 0–100% over 40 min and at a flow rate of 0.2 mL/min. A sample of elution was analysed using the Agilent 6120 quadrupole mass spectrometer. Agilent OpenLAB Chromatography Data System (CDS) ChemStation Edition was used for data acquisition and processing.

Primary antibodies against pMK2 (T222; #3316), MK2 (#3042), pBcl-2 (S70; #2827), Bcl-2 (#2870), Bcl-X_L (#2764), Mcl-1 (#5453), PARP (#95425), secondary anti-rabbit (#7074) and anti-mouse (#7076) HRP-linked antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). EGFR (#sc03) and GAPDH (#sc737179) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). Antibody against β-tubulin (#ab11308) was purchased from Abcam (Cambridge, UK). Alexa488-conjugated anti-mouse secondary antibody and DAPI (4′,6′-diamino-2-phenylindole) were from Life Technologies (Carlsbad, CA, USA). CMPD1 (#sc-203138) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Paclitaxel and vinblastine were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Chemical synthesis

2.2.1. 4-(4-Bromophenyl)-4-oxobutanoic acid *3*

Bromobenzene **1** (48 g, 300 mmol) and succinic anhydride **2** (5.0 g, 50 mmol) were cooled to 0 °C. Aluminium chloride (13.3 g, 100 mmol) was added and the mixture was stirred for 4 h at 0 °C under nitrogen atmosphere. The reaction was allowed to warm to room temperature and stirred for 96 h under nitrogen atmosphere. The reaction was cooled to 0 °C, concentrated HCl (125 mL) was added and reaction stirred under nitrogen for a further 1 h. The reaction was filtered and washed with water (1 L) to obtain a pale yellow solid which was re-crystallised from toluene to yield 4-(4-bromophenyl)-4-oxobutanoic acid **3** (12 g, 46.68 mmol, 93.4%). LRMS (ESI-QUADRUPOLE) m/z: calc. for $C_{10}H_9BrO_3$ 255.97, 257.97; found 211.0 (17%), 213.0 (18%), 255.0 ($[M-H]^-$, 100%), 256.0 (12%), 257.0 ($[M-H]^-$, 98%), 258.0 (11%) (negative ions). HPLC: t_r = 24.4 min. ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm) 12.19 (1H, br s, OH), 7.96 (2H, d, J = 8.8 Hz, ArH), 7.88 (2H, d, J = 8.8 Hz, ArH), 3.21 (2H, t, J = 6.5 Hz, —CH_2—), 2.59 (2H, t, J = 6.5 Hz, —CH_2—).

2.2.2. 4-(4-Bromophenyl)butanoic acid *4*

Zinc (13.0 g, 200 mmol) and mercury chloride (1.00 g, 480 mmol) were stirred with water (10 mL) and concentrated HCl (0.6 mL) for 5 min. The liquid was decanted off and toluene (20 mL), concentrated HCl (20 mL) and water (8 mL) were added consecutively. 4-(4-Bromophenyl)-4-oxobutanoic acid **3** (2.55 g, 10.5 mmol) was added and heated under reflux at 100 °C for 24 h adding 1 mL of HCl every 6 h. The reaction was allowed to cool to room temperature, filtered and the solvent removed from the organic layer to give a clear liquid which gave white crystals upon cooling. These were purified with silica gel chromatography (ethyl acetate:hexane 1:3) to yield 4-(4-bromophenyl)butanoic acid **4** (2.33 g, 9.63 mmol, 91.4%). LRMS (ESI-QUADRUPOLE) m/z: calc. for $C_{10}H_{11}BrO_2$ 241.99, 243.99, found 241.0 ($[M-H]^-$, 100%), 242.0 (11%), 243.0 ($[M-H]^-$, 98%), 244.0 (10%) (negative ions). HPLC: t_r = 27.7 min. ^{1}H NMR (300 MHz, $CDCl_3$) (δ/ppm) 10.93 (1H, br s, OH), 7.39 (2H, d, J = 8.4 Hz, ArH), 7.04 (2H, d, J = 8.4 Hz, ArH), 2.62

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