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NDGA-P21, a novel derivative of nordihydroguaiaretic acid, inhibits glioma cell proliferation and stemness

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Nordihydroguaiaretic acid (NDGA) and its synthetic chiral analog dl-nordihydroguaiaretic acid (Nordy) show collective benefits in anti-tumor, and defending against viral and bacterial infections. Here, we synthetized a new derivative-NDGA-P21 based on NDGA structure. Regardless of the structural similarity, NDGA-P21 exhibited stronger capability in suppression of glioblastoma (GBM) cell growth as compared to Nordy. Mechanically, NDGA-P21 is able to arrest cell cycle of GBM cells in G0/G1 phase, and to block cell proliferation sequentially. It is important to note that NDGA-P21 is able to impair the stemness of glioma stem-like cells (GSLCs) via measurement of colony formation and sphere formation. Taken together, the novel NDGA-based compound NDGA-P21 exhibits potential therty -20 apeutic implications through inhibiting proliferation of glioma cells and self-renewal capability of GSLCs.
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Cancer has been a worldwide threat to public health, and the leading cause of death, particularly since 2010 in China.[1] Malignant brain tumors are leading cause for cancer death of children and adolescents, and glioma is one of the most malignant tumors up to now.[2] Recent decades, people have great progress in improving lifespan and life quality of the patients with such malignant burden, but to our regret, the beneficial effect is still quite limited on glioma.[2] The need for new drug or other methods to treat glioma is never too much.

Nordihydroguaiaretic acid (NDGA) is a naturel phenolic compound extracted from the leaves of *Larrea tridentata* (Creosote bush), a kind of evergreen desert shrub.[3] It is also known as masoprocol, an antineoplastic drug that has benefits as anti-viral remedy, and is used for the treatment of actinic keratosis. However, up to now, it remains elusive how NDGA exactly works.[4–6] In our previous studies, we synthesized Nordy (dl-NDGA), a chiral NDGA derivative which inhibits the growth of multiple tumors, including glioma.[7,8] Nordy could also induce differentiation and inhibit self-renewal of glioma stem-like cell (GSLC).[9]

In this paper, we reported to synthesize a novel small molecular compound—NDGA-P21, based on the structure of NDGA. According to several experiments, we found that NDGA-P21 obviously inhibited proliferation of glioma cells and self-renewal of GSLC.

MATERIALS AND METHODS

Synthesis and Chemical Structure of 1, 4—Bis (3, 4—Dimethoxy Phenyl)—2, 3—Dimethyl—2, 3—Epoxy Butane (NDGA-P21)

The substrate (E)-4, 4′-(2, 3-dimethylbut-2-ene-1, 4-diyl) bis (1, 2-dimethoxybenzene) (the detailed synthesis method could be found in Chinese patent: ZL02133700.4.; weighted as 0.87 g) was dissolved with 13 ml dichloromethane in a 25 ml round-bottom flask, cooled down to − 10 °C, and was then mixed with 0.90 g m-CPBA to stir and react for 2 h. The reaction was then quenched with 4.11 g Na_2SO_3 and stirred continuously for 3 h at room temperature. The product was extracted with ethyl acetate, washed with saturated NaCl, dried with Na_2SO_4 and concentrated to yield rude product (1.02 g). The rude product was subjected to flash silica gel column chromatography to obtain the pale yellow product (0.91 g) with the yield of 99.13%.

Identification of Compound

The HRESIMS of the product gave a peak at *m*/*z* 395.1835 $[M+Na]^+$, whose molecular weight was 16 Da more than the substrate, indicating an additional oxygen atom was present. Its ^1H NMR was almost identical to the substrate's one, except that the chemical shifts of the two methyl at C-2 and C-3 were moved to high field from δH 1.66 to δH 1.31. Furthermore,

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the two olefinic carbon resonances of δC 133.3 and 128.6 at C-2 and C-3 were absent, which were replaced by the two overlapped oxygen-carbon resonances of δC 65.1 in the ^{13}C NMR spectra of the product. All of these elucidated that the additional oxygen atom was cyclization between C-2 and C-3. The product was named as NDGA-P21.

Cell Culture

U87-MG cells (ATCC Number: HTB-14) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Primary GBM cells, named GBM1, were separated from a glioblastoma (GBM, WHO Grade IV) patient, and classified as proneural-like subtype owing to the positive staining of PDGFRA and P53, as described in more details previously.[10] Cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) complete medium, ie, DMEM (Gibco, Mount Waverley, VIC, Australia) containing 10% FBS (Gibco), 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco). Medium was changed every 1–2 days, and cells were passaged every 2–3 days. The 3rd–7th passaged cells were used for further experiments. U87-MG and primary GBM cells-derived GSLCs were maintained in serum-free neural stem cell culture medium, containing DMEM/F12 (Gibco), B27 supplements (Gibco), human recombinant basic FGF (20 ng/ml; Upstate, Lake Placid, NY, USA), and human recombinant EGF (20 ng/ml; Sigma-Aldrich, St Louis, MO, USA). Second or more passaged spheres were used in experiments. Sphere cells were dissociated with Accutase (Millipore, Bedford, MA, USA) during passage.

Cell Proliferation Assay

All compounds were dissolved in DMSO (Sigma-Aldrich) to make stock solution as 100 mmol/l. U87-MG or GBM1 cells were seeded in 24-well plates (Corning, Cambridge, MA, USA), at a concentration of 1×10^4 cells/well. Cells were starved for 12 h with serum-free DMEM medium, and then treated with 50 μmol/l NDGA-P21, 50 μmol/l Nordy, or 0.5‰ DMSO (drug vehicle). Cells stained with trypan blue were counted with a blood cell counting chamber each day until the indicated endpoint. IC_{50} of compounds was determined by counting cells with a 24-h-treatment of indicated compounds at concentration between 0 and 100 μmol/l. Each treatment was carried out in quadruplicate and the independent experiments were repeated for at least three times.

Apoptosis Assay

U87-MG cells were seeded in 6-well plates as 2×10^5 cells per well and starved 12 h before treatment. Then cells were treated with 50 μmol/l NDGA-P21, 50 μmol/l Nordy, or 0.05% DMSO. Cell apoptosis was tested with an Annexin V kit (eBioscience, San Diego, CA, USA), according to the protocol from manufacture. In brief, the treated U87-MG cells were digested with trypsin, and adjusted as 1×10^6 cells per test. Then the cells were washed with PBS once and the provided 1× binding buffer once, stained with Annexin V-APC for 30 min at 4 °C, and washed once with 1× binding buffer. The sample could stand on ice for 4 h before flow cytometry analysis. Propidium iodide (PI) was added to sample solution 5 min before testing with Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). All experimental procedures were carried out gently.

Cell Cycle Analysis

Administered U87-MG cells and GBM1 cells were starved 12 h before treatment. Then cells were treated with 25 μmol/l NDGA-P21, 25 μmol/l Nordy, or 0.025% DMSO. Adherent and non-adherent cells were collected, washed twice with cold PBS, and then fixed in ice-cold 70% ethanol for 6 h at 4 °C. Fixed cells were stained with freshly diluted PI staining buffer from a cell cycle and apoptosis kit (Beyotime Biotech, Nantong, China) for 30 min at 37 °C, and analyzed on a FACS Calibur flow cytometer within 2 h. The resulting histograms were analyzed for cell cycle distribution with ModFit software (Verity Software House, Topsham, ME, USA).

Colony Formation Assay

Soft agar colony formation assay was carried out by suspending 1000 GSLCs in DMEM complete medium containing 0.35% noble agar (Difco, BD) and 25 μmol/l NDGA-P21, 25 μmol/l Nordy, or 0.025% DMSO on top of a solid 0.6% noble agar DMEM complete medium layer using 6-well plates. Cells were incubated for 14 days and colonies were visualized by using an Olympus SZX-10 fluorescent microscope (Tokyo, Japan). Bright field images were captured with a QImaging digital camera (Surrey, BC, Canada) controlled by Image-Pro Plus 6.0 (Media Cybermetics, Rockville, MD, USA). Colonies with more than 50 cells were calculated under an invert microscope (Olympus CFX40, Tokyo, Japan), and each well was counted on 10 fields. Three independent assays were performed.

Sphere Formation Assay

Cells were sorted with a BD AriaII flow sorter, and seeded in a 96-well plate at a number of 10 cells per well. Cells cultured in serum-free stem cell culture medium with different treatment for 14 days. Then the formation of spheres was checked in each well, and was calculated for sphere formation rate. Ten wells were set up as a group in each treatment and three independent assays were performed.

Quantitative Real-Time PCR

Total RNA was extracted from cells treated with 25 μmol/l NDGA-P21, 25 μmol/l Nordy, or 0.025% DMSO by using RNAiso reagent (Takara, Shiga, Japan), according to TAKARA protocol book. Reverse transcription reaction was performed to transcript mRNA into cDNA with a PrimeScript RT reagent Kit (Takara) on C1000 Thermal Cycler. Real-time PCR was carried out to detect the expression of mRNAs in multiple groups by using SYBR Premix Ex Taq II (Takara) on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Primers were selected by NCBI primer

BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). The sequences for the primers of target gene are: GAPDH-Forward 5′-CAAGCTCATTTCCTGGTATGAC-3′; GAPDH-Reverse 5′-CAGTGAGGGTCTCTCTCTTCCT-3′; CCNE1-Forward 5′-GCCAGCCTTGGGACAATAATG-3′; CCNE1-Reverse 5′-CTTGCACGTTGAGTTTGGGT-3′; CDK2-Forward 5′-CCAGGAGTTACTTCTATGCCTGA-3′; CDK2-Reverse 5′-TTCATCCAGGGGAGGTACAAC-3′; CDK4-Forword 5′-ATGGCTACCTCTCGATATGAGC-3′; CDK4-Reverse 5′-CATTGGGGACTCTCACACTCT-3′; CDK6-Forward 5′-CCAGATGGCTCTAACCTCAGT-3′; CDK6-Reverse 5′-AACTTCCACGAAAAAGAGGCTT-3′; CCNA2-Forward 5′-AGCTATGAGTAAGACTGGCATCC-3′; CCNA2-Reverse 5′-TGGTTTTACTCTCATCTTGCCAC-3′; CCNB1-Forward 5′-TGCCACGAACAGGCCAATAA-3′; CCNB1-Reverse 5′-GAGAAGCAGAACACCGGAGG-3′; CDC25C-Forward 5′-TCAAGTCTTCGCCTGTGTCC-3′; CDC25C-Reverse 5′-CTGAGCCAGAGCTTCCTTCC-3′; CDK1-Forword 5′-TTTCTTTCGCGCTCTAGCCA-3′; CDK1-Reverse 5′-CAATCGGGTAGCCCGTAGAC-3′; CDKN1A-Forword 5′-AGTCAGTTCCTTGTGGAGCC-3′; CDKN1A-Reverse 5′-CATTAGCGCATCACAGTCGC-3′; GADD45A-Forword 5′-GAATTCTCGGCTGGAGAGCA-3′; GADD45A-Reverse 5′-CCACATCTCTGTCGTCGTCC-3′; Sox2-Forward 5′-CCGTTCATCGACGAGGCTAA-3′; Sox2-Reverse 5'-TAACTGTCCATGCGCTGGTT-3′; Oct4-Forward 5′-GCAGCGACTATGCACAACGA-3′; Oct4-Reverse 5′-CCAGAGTGGTGACGGAGACA-3′; Nanog-Forward 5′-GAGATGCCTCACACGGAGAC-3′; and Nanog-Reverse 5′-GGGTTGTTTGCCTTTGGGAC-3′.

Immunofluorescence

In a 24-well plate, U87-MG sphere cells were adhered to a 1 cm × 1 cm cover-slip coated with poly-D-lysin. GBM1 sphere cells were seeded in poly-D-lysin pre-treated glass bottom cell culture dish (Nest, Wuxi, China). After treatment with 25 μmol/l NDGA-P21 or 25 μmol/l Nordy for 48 h, cells were washed three times with PBS, and then fixed in 4% paraformaldehyde for 20 min at room temperature. For permeabilization, cells were incubated with PBS containing 0.2% Triton X-100 for 10 min at room temperature. After air-dry, cells were washed three times with PBS, blocked with 5% goat serum for 30 minutes at 4 °C, then stained with mouse-anti-human GFAP antibody (Boster, Wuhan, China) overnight at 4 °C, rinsed three times with PBS, followed by incubation with second antibody Goat anti-mouse Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C, washed twice with PBS and once with distilled water. Finally, the cover-slip was mounted to U87-MG cells with a drop of prolong gold anti-fade reagent with DAPI (Molecular Probes). GBM1 cells in dish were stained with DAPI (Molecular Probes) for 10 min at room temperature in dark. Fluorescent images were captured by a fluorescent microscope (Leica DM3000, Germany) or a laser confocal microscope (Zeiss LSM780, Germany).

Statistical Analysis

All experiments were repeated at least three times and each treatment was set up in triplicate, unless specially indicated otherwise. Data were presented as mean ± s.e.m. The statistical significance of difference among groups was obtained by the Student's *t* test or two-way ANOVA multiple comparisons with GraphPad Prism 6 (GraphPad, San Diego, California). All comparisons with a $P<0.05$ were considered statistically significant.

RESULTS

NDGA-P21 Inhibits GBM Cell Proliferation

NDGA is a natural product, which has been reported to inhibit growth of multiple tumors, such as breast cancer, cervical cancer, gastric cancer, lung cancer, prostate cancer, and neuroblastoma.[11–18] In previous studies, we found Nordy, as a synthetic chiral derivative of NDGA, repressed the growth of glioma cells both *in vitro* and *in vivo*.[7,19–21]

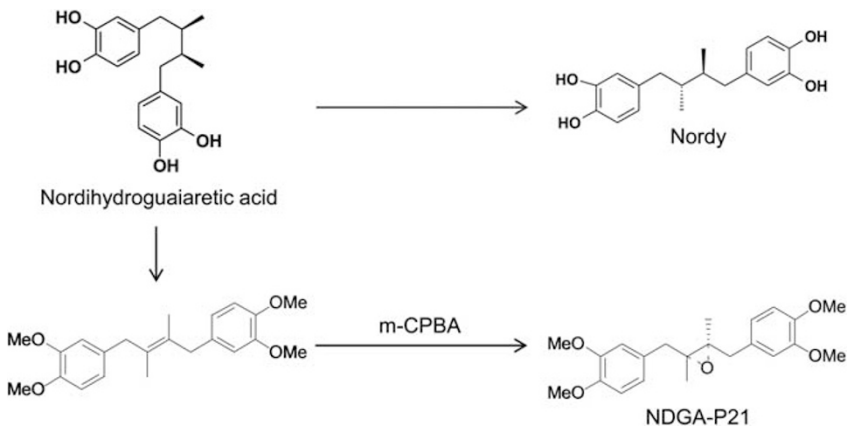

Figure 1 NDGA-P21 is synthesized based on the structure of nordihydroguaiaretic acid (NDGA), from the substrate (E)-4, 4′-(2, 3-dimethylbut-2-ene-1, 4-diyl) bis (1, 2-dimethoxybenzene). Both Nordy and NDGA-P21 are synthetized based on the structure of NDGA. NDGA-P21 came from the substrate (E)-4,4′-(2,3-dimethylbut-2-ene-1,4-diyl) bis (1, 2-dimethoxybenzene), and react with m-CPBA.

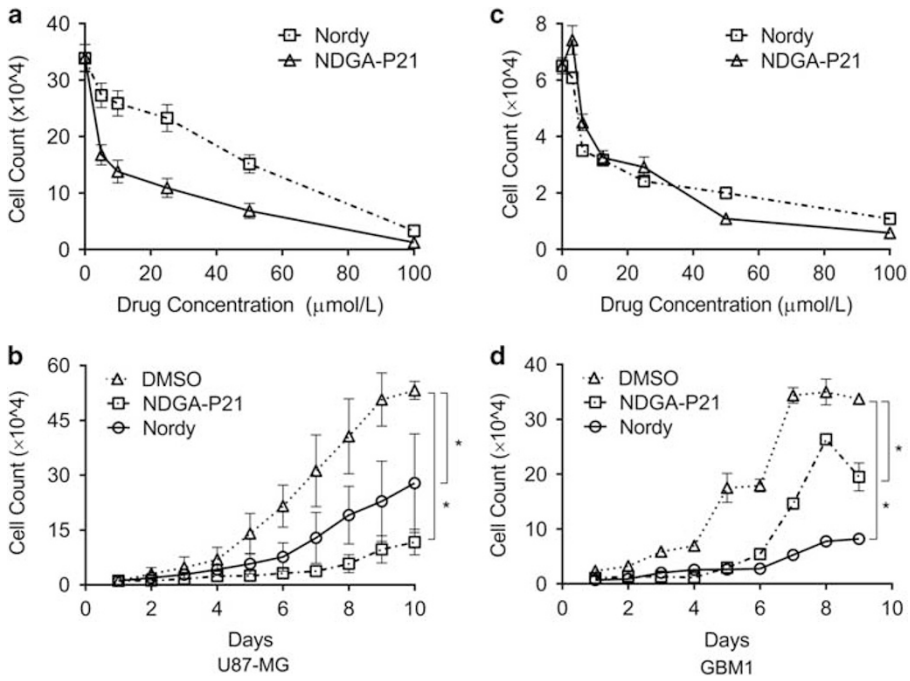

Figure 2 Nordihydroguaiaretic acid (NDGA)-P21 and Nordy inhibit glioma cell proliferation. (**a**) U87-MG cells were starved for 12 h, treated at different concentration of NDGA-P21, and Nordy for 3 days. IC_{50} of NDGA-P21 and Nordy were: 7.43 and 34.52 μmol/l. (**b**) U87-MG cells were treated with 50 μmol/l NDGA-P21, 50 μmol/l Nordy and 0.5‰ DMSO, respectively, until day 11. (**c**) GBM1 cells were treated as panel (**a**), IC_{50} of NDGA-P21 and Nordy were 47.56 and 41.91 μmol/l. (**d**) GBM1 cells were treated as panel (**b**). Here, P21 refers to NDGA-P21. DMSO-treated cells was used as control group. Data are presented as the mean ± s.e.m. of three independent experiments carried out in quadruplicate. $^*P < 0.05$.

Based on NDGA structure, NDGA-P21, as a newly synthetic compound from the substrate (E)-4, 4′-(2, 3-dimethylbut-2-ene-1, 4-diyl) bis (1, 2-dimethoxybenzene; Figure 1), showed stronger inhibitory effects on the growth of GBM cells than Nordy. We treated U87-MG cells and primary GBM cells with NDGA-P21 and Nordy for 3 days at the concentration of 0, 10, 25, 50, 100 μmol/l, calculated their IC_{50} values, and found that for U87-MG cell line, IC_{50} values of NDGA-P21, and Nordy were 7.43 and 34.52 μmol/l respectively (Figure 2a), and for primary GBM cells, IC_{50} values were 47.56 and 41.91 μmol/l respectively (Figure 2b). During administration, NDGA-P21-treated U87-MG cells began to show significant difference from DMSO-treated control group since day 7 until day 10 when cell proliferation reached a plateau. While Nordy-treated group began to show growth difference since day 8, the inhibition by Nordy on U87-MG proliferation was not as strong as NDGA-P21 (Figure 2c). In the proneural-like primary GBM cells, Nordy exhibited stronger inhibition than NDGA-P21 (Figure 2d). Meanwhile, we examined the effects of NDGA-P21 and Nordy on the proliferation of a normal glial cell line HEB at the concentration of 50 μmol/l, and found no significant difference among NDGA-P21, Nordy and DMSO group (Supplementary Data 1). As compared to Nordy, NDGA-P21 had stronger inhibitory effects on the proliferation of U87-MG cells, and similar effects in the primary GBM cells of proneural-like subtype.

NDGA-P21 Arrests Cell Cycle of Glioma Cells, but with Little Effect On Apoptosis

Previous studies of NDGA and Nordy suggest that cell cycle arrest, especially G1/S phase arrest, might be one of the underlying mechanisms for NDGA and its analogs to prevent cell proliferation in multiple tumors.[8,11,12,14,22] To determine whether cell cycle arrest was involved in the growth inhibition by NDGA-P21, we applied flow cytometry analysis on the NDGA-P21 treated U87-MG cell to examine their cell cycle status by ModFit software. As compared to DMSO-treated group, cells in G0/G1 phase increased by 19.46% after NDGA-P21 treatment, whereas cells in S phase decreased by 17.82% (Figure 3a). Cell cycle of primary GBM cells after treatment showed similar effects. With NDGA-P21 treatment, primary GBM cells in G0/G1 phase increased by 12.16% whereas cells in S phase decreased by 13.77% (Figure 3b). To further explorer the underlying mechanisms, we examined the mRNA expressions of several G1/S and G2/M phase cell cycle hallmarks and inhibitors. In consistent with flow cytometry analysis, qPCR data revealed a significant decrease in the mRNA expressions of CCNE1, cyclin-dependent kinase 2 (CDK2) and CDK6 after NDGA-P21 treatment for 24 h (Figure 3c). The mRNA expressions of several G2/M phase checkpoints such as CDK1, CDC25c and CCNB1 were also decreased whereas the mRNA expression of CCNB1 inhibitor GADD45A was increased after NDGA-P21 and Nordy

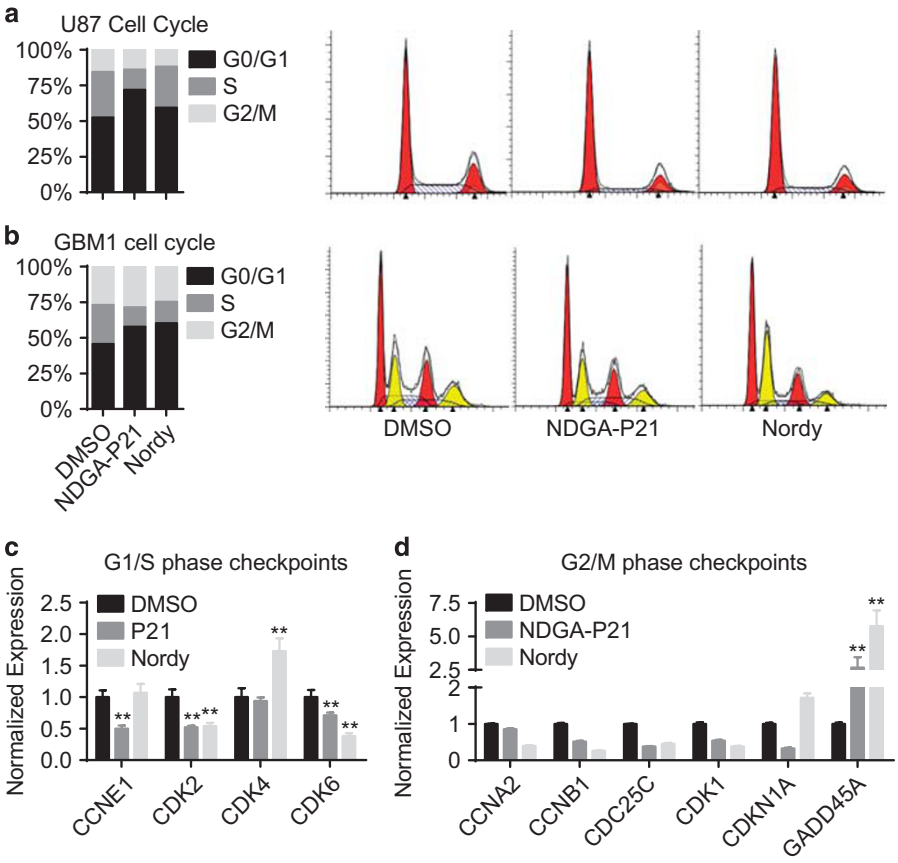

Figure 3 NDGA-P21 induces G1-to-S phase arrest in glioma cells. (**a**) Statistical analysis of cell cycle distribution tested with flow cytometer, U87-MG cells were pre-treated with 25 μmol/l NDGA-P21, Nordy or 0.25‰ DMSO. (**b**) Statistical analysis of cell cycle distribution tested with flow cytometer, GBM1 cells were pre-treated with 25 μmol/l NDGA-P21, Nordy or 0.25‰ DMSO. Three independent experiments were performed, and one representative data were exhibited. (**c** and **d**) Quantitative real-time PCR test for U87-MG cells were pre-treated with 25 μmol/l NDGA-P21, Nordy or 0.25‰ DMSO for 24 h. The column figure was the normalized mRNA expression as a fold change of the relevant GAPDH expression. Data are presented as the mean ± s.e.m. of three independent experiments carried out in triplicate. $*P<0.05$, $**P<0.01$.

treatment (Figure 3d). These results suggest that NDGA-P21 or Nordy treatment arrests the cell cycle of glioma cells in G1 phase.

Apoptosis is also suggested for NDGA inhibition of tumor cell proliferation.[18] Thus, we also examined U87-MG apoptosis after NDGA-P21 and Nordy treatment. We found that treatment by NDGA-P21 or Nordy had no significant influence on the apoptosis of U87-MG cells (Supplementary Data 2).

NDGA-P21 Inhibits GBM Cells Self-Renewal and Induces Differentiation

Cancer stem-like cells or tumor-initiating cells in tumor have been thought as a key reason for tumor survival and progression.[10,23] Self-renewal capability of GSLCs facilitates glioma cells to process self-copy, malignance, and differentiation into other cell types.[24,25] We performed both colony formation and sphere formation assays to determine whether NDGA-P21 exerts any effects on the self-renewal of GSLCs. As shown in Figure 4, ~9% of U87-MG cells in soft agar with DMSO formed colonies after culture for 14 days, while only 1% of the cells in the NDGA-P21-contained soft agar were able to into colonies, as compared to 3% in the presence of Nordy (Figure 4a). Colony formation of GBM1 was also decreased with NDGA-P21 and Nordy (Figure 4b). Akin to colony formation, sphere formation was significantly decreased in NDGA-P21 and Nordy-treated groups (Figure 4c and d). We also tested stemness markers of different drugs treated GSLCs, and observed an obviously decrease of Nanog mRNA expression in NDGA-P21 treated group (Figure 4e). GFAP is a protein usually expressed in glial cells and a differentiation marker of GSLC.[9,10] No matter for U87-MG or GBM1 cells, GFAP level of GSLCs after treatment of NDGA-P21 or Nordy was higher than control group, indicating that NDGA-P21 and Nordy promote differentiation of GSLCs (Figure 5a and b). Thus, NDGA-P21 could significantly suppress stemness and promote differentiation in GSLCs.

DISCUSSION

NDGA has been known as a potential medicine to overcome oxidization, angiogenesis, neurological disorders and virus associated infection.[3–6,26,27] Here, based on structure of

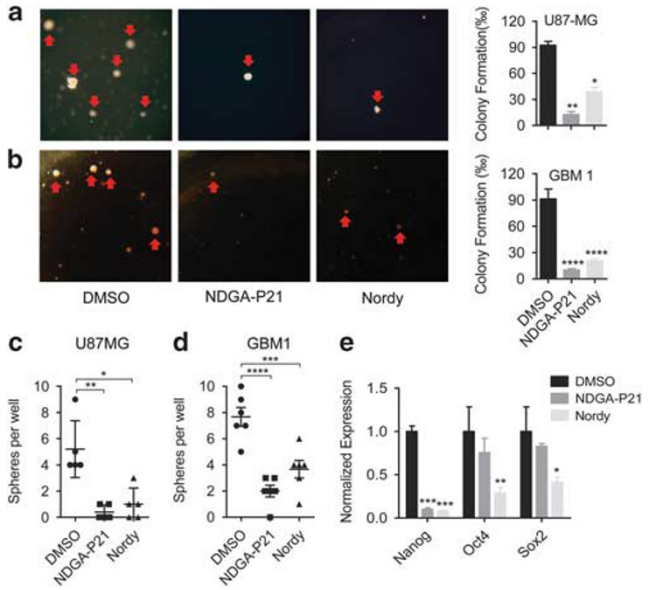

Figure 4 NDGA-P21 inhibits GBM cells self-renewal ability. (**a**) Bright field images of U87-MG colonies, under a stereo microscope. Cells were seeded in soft-agar containing NDGA-P21, Nordy or DMSO. Colonies with more than 50 cells were counted under a ×100 inverted microscope. Each well was counted at 10 fields. (**b**) Bright field images of GBM1 colonies, under a digital camera. Cells were seeded in soft-agar containing NDGA-P21, Nordy, or DMSO. Colonies with more than 50 cells were counted under a ×100 inverted microscope. Each well was counted at 10 fields. (**c** and **d**) U87-MG cells and GBM1 cells were maintained in neural stem cell culture medium for 14 days, with 25 μmol/l NDGA-P21, or 25 μmol/l Nordy. (**e**) Real-time qPCR test for stemness markers: Nanog, Oct4 and Sox2. U87-MG sphere cells were treated with 25 μmol/l NDGA-P21, 25 μmol/l Nordy, or 0.25‰ DMSO for 24 h before test. All statistical data are presented as the mean ± s.e.m. of three independent experiments carried out in triplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

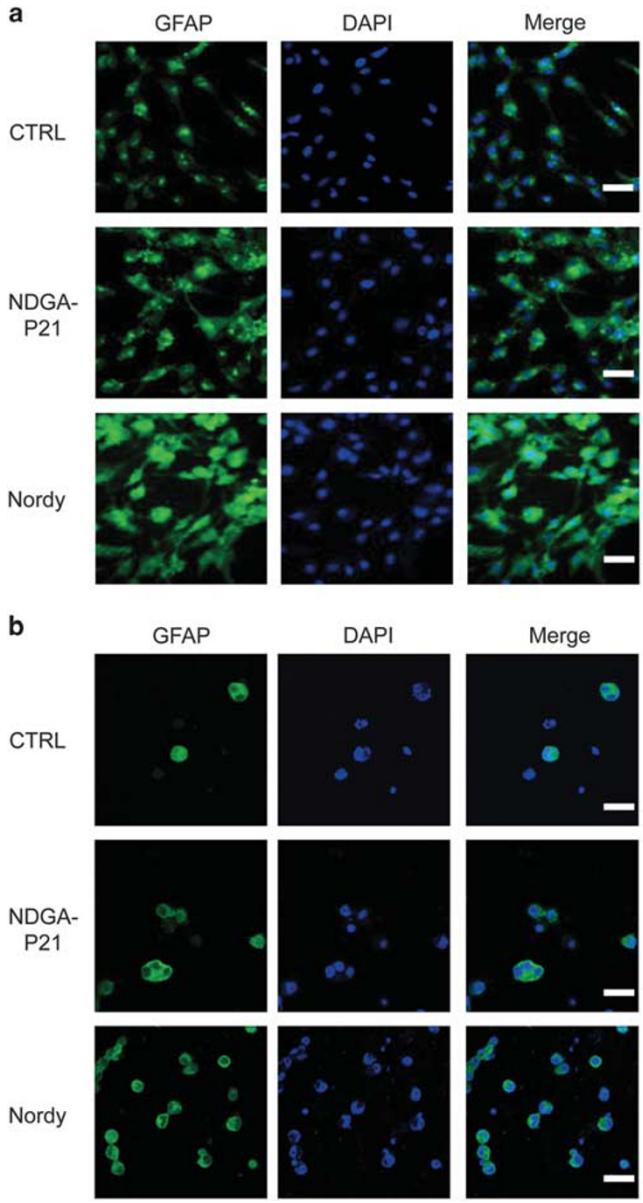

Figure 5 NDGA-P21 induces differentiation of GSLCs. (**a**) Immunofluorescence showing GFAP protein (green) in U87-MG GSLCs elicited by 25 μmol/l NDGA-P21 or Nordy treatment. (**b**) Immunofluorescence showing GFAP protein (green) in GBM1 GSLCs elicited by 25 μmol/l NDGA-P21 or Nordy treatment. Cell nucleuses were stained blue with DAPI. Scale bars of the figures showed as 50 μm in real distance. 0.25‰ DMSO-treated GSLCs were control group, labeled as CTRL.

NDGA, we synthesized NDGA-P21, which exerted extensive killing effects on glioma cells. NDGA-P21 can significantly inhibit the growth of glioma cells as similar as Nordy, while the inhibitory effects of NDGA-P21 on U87-MG cells appeared earlier and stronger than Nordy. Furthermore, NDGA-P21, similar to Nordy, can suppress stemness and promote differentiation of GSLC.

In general, phenolic groups are thought to be the key functional factors for modification of NDGA.[28] In our study, we changed the carbon chain of NDGA to yield NDGA-P21. And this novel compound showed strong inhibition effects of glioma cells. As there are many factors affecting cell growth, we focused on cell cycle and apoptosis as the potential mechanisms of NDGA-P21. By flow analysis of compounds-treated glioma cells, we found that there were more cells arrested in G1 phase but less in S phase after NDGA-P21 treatment. Then we analyzed mRNA expressions of several key cell cycle regulators. G1/S-specific cyclin-E1 (CCNE1), which accumulates at the G1-to-S phase boundary and is degraded as cells progress through S phase,[29,30] forms a complex with CDK2, and functions as a regulatory factor of CDK2 activity.[31,32] We found that both NDGA-P21 and Nordy were able to repress the mRNA expression of CCNE1 and CDK2. CDK6, as another important regulator during G1-to-S phase transition, was also downregulated with NDGA-P21 and Nordy. Importantly, CDK6 also has a specific oncogenic capability.[33,34] Suppression of CDK6 expression refers to another potential mechanism for NDGA-P21 to suppress tumor growth. Meanwhile, cyclin B1 (CCNB1) and CDK1 were suppressed by NDGA-P21, and

CCNB1 inhibitor GADD45A was strongly upregulated, indicating that G2-to-M phase arrest was also induced. Thus, NDGA-P21 arrested cell cycle of glioma cells. Apoptosis has been suggested as one of the mechanisms underlying the inhibition of NDGA and its derivatives in multiple tumors.[4,16,17,35,36] However, we found that similar to Nordy, NDGA-P21 had little effects on apoptosis. The results suggest different structure modification of NDGA leads an alternative mechanism for inhibition of tumor cells.

Glioma-stem-like cells are defined as glioma cells that retain extensive self-renewal potential through multiple generations, and are also known as a limited subpopulation of tumor-initiating cells.[37] GSLCs are thought to be the responsible cell population for glioma invasion, migration, progression, and recurrence.[38–41] Neurospheres and orospheres are used for propagation and enrichment of CSCs, including GSLCs.[42,43] Orospheres are non-adherent tumor cells that may be cultured in soft agar to colonies and retained stemness and self-renewal capability. In this study, we found NDGA-P21 and Nordy could suppress GSLC self-renewal and promote GSLC differentiation, which mainly owns to Nanog, a pluripotency sustaining transcription factor. Physiologically, Nanog only expresses by germline stem cells and during very early embryonic stages.[44] Nanog activates tumor suppressor P53 and facilitates proliferation of stem cells, but in the absence of P53, Nanog is overexpressed in a variety of human cancer types such as glioma.[45] Our study reveals that NDGA-P21 suppresses Nanog expression and inhibits self-renewal of GSLCs, suggesting that this compound could suppress tumorigenic capability of glioma.

NDGA-P21 has the potential therapeutic effects on glioma, not only because NDGA-P21 inhibits proliferation of glioma cells directly, but also because it inhibits the progression of most malignant groups of glioma. However, NDGA-P21 showed limit water solubility, and that might be an obstacle for further test for this molecule. So we are trying to modify this molecule for better solubility. Recently, NDGA and its analog terameprocol finished phase I/II clinical trials as anticancer agents, and showed limited severe adverse effects,[15,46,47] indicating that this family of small molecules has great potential on clinic treatment. Our novel NDGA-based small molecular compound strongly inhibited proliferation of glioma cells, suppressed self-renewal, and induced differentiation of glioma stem-like cells, at least *in vitro*, predicting the therapeutic application for glioma. Although our current study illustrated NDGA-P21 inhibition to GBM cell proliferation in several aspects *in vitro*, we still know little about the real effects after NDGA-P21 administration *in vivo*. Moreover, the underlying mechanisms involved in the suppression of multiple tumors by NDGA and its derivatives still need further exploration in the future.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org).

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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