



Synthetic Triterpenoid CDDO-Me Inhibits Proliferation, Migration, and Invasion in GBM8401 and GBM8901

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Objectives: We determined the anticancer potency of CDDO-Me in glioblastoma cell lines and the underlying mechanisms *in vitro*.

Summary: CDDO-Me is a synthetic triterpenoid with more potent anticancer and cancer preventive actions compared with the original triterpenoid CDDO.

Methods: Two glioblastoma cell lines, GBM8401 and GBM8901, were utilized to test the effect of CDDO-Me on cell viability, cell migration, and cell invasion using the MTT, wound healing, and transwell migration assays, respectively. Additionally, Western blotting was used to determine the protein expression levels of N-cadherin, cyclin D1, and vascular endothelial growth factor.

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Results: At nanomolar concentrations, CDDO-Me inhibited proliferation, migration, and invasion in both cell lines. In addition, CDDO-Me exhibited a dose-dependent downregulation in the protein levels of N-cadherin, cyclin D1, and vascular endothelial growth factor in GBM8401 and GBM8901 cells.

Conclusions: CDDO-Me exhibited anticancer effects at low nanomolar concentrations and should be considered as a potential chemotherapeutic agent for glioblastoma.

Key words: Triterpenoids – Glioblastoma – GBM8401 – GBM8901 – CDDO-Me – Proliferation – Migration – Invasion

Glioma is a common type of brain tumor in human. Grade IV astrocytoma, also called glioblastoma, is the most aggressive human malignant primary brain tumor and is characterized by histopathologic features, such as vascular thrombosis, microvascular proliferation, or necrosis. Despite advances in surgical treatment, radiotherapy, and chemotherapy, the overall survival of glioblastoma patients remains poor. Specifically, less than 20% of patients will survive longer than 1 year and less than 3% will survive beyond 3 years.^{1,2}

Triterpenoids are a large family of compounds synthesized in some plants, such as the chrysanthemum flower through the cyclization of squalene, that have been used in traditional Asian medicine for disease management.^{3,4} Bardoxolone methyl, CDDO-me, is more potent than CDDO in anticancer and cancer-preventive activities⁵⁻⁸ and in the activation of Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1/nuclear factor erythroid 2-related factor 2/antioxidant response element (Keap1/Nrf2/ARE) pathway,^{9,10} which is involved in cytoprotection in the presence of excessive electrophiles or oxidative stress. As a noncytotoxic and multifunctional drug, CDDO-me has applications for the prevention and treatment of not only cancer, but also of many other diseases with inflammation.^{11,12} The therapeutic effects of CDDO-me have been tested in phase III clinical trials for chronic kidney disease (CKD)¹³ and phase I/II clinical trials for malignant diseases.^{14,15} In this review, we will update our knowledge of the pharmacokinetics, therapeutic effects, and mechanisms of action of CDDO-me when used for the treatment of CKD, cancer, and other diseases. CDDO-me is a multi-targeting molecule exerting the potent anticancer effect in the treatment of various types of cancer in preclinical and clinical studies.^{16,17} CDDO-me inhibited the proliferation and induces apoptosis in many cancer cell types *in vitro*¹⁸⁻²¹ and inhibit the growth of tumor size and prevent development of cancers *in vivo*.²²⁻²⁴ In this

study, GBM cell lines GBM8401 and GBM8901 were treated with titration doses of CDDO-me and the effect on cell viability and migration was determined.

Materials and Methods

Cell culture

GBM8401 and GBM8901 were GBM cell lines from BCRC. GBM8401 and GBM8901 were cultured with RPMI medium plus 10% FBS. All cells incubated at 37°C with 5% CO₂.

Cell viability

The GBM cells were obtained by dissolving the cells in the RPMI culture medium containing 10% serum and will be placed in a 6-well plate with about 1 × 10⁶ cells of 2 mL volume in each well. These cells will be incubated under the condition of 5% CO₂, saturated humidity, and 37°C for 24 hours. Cell viability was assessed by an MTT assay (M5655; Sigma Aldrich Corp, St. Louis, Missouri). Titrating doses of 50, 100, 200, 400, and 500 NM CDDO-me were tested using an incubation period of the GBM cell lines of 24 hours.

Western blot

All samples lysis in 200 µL lysis buffer; 50 µg protein of every sample load in the wells of SDS-PAGE with 50V for 4 hours. Transfer the protein from the gel to the PVDF membrane. After 1 hour blocking buffer, the membranes were incubated with primary antibody for 90 minutes. ECL solution (Western Lightning; 205-14621, PerkinElmer, Waltham, Massachusetts) detect specific band with MINICHEMI (Thermo Fisher Scientific, Waltham, Massachusetts).

Invasion assay *in vitro*

Cell invasion assays was performed using a Transwell (COR3452, CORNING, Corning, New York) in

vitro. Cells seeded 5×10^5 per insert and the lower chamber of the Transwell was filled with 0.2 mL medium with 50 nM CDDO-me. After 24 hours of incubation, cells remaining on the upper surface of the Transwell membrane removed by a cotton swab. Cells that had invaded through the Transwell to the bottom of the insert were fixed, stained, photographed and quantified by counting them in 6 random high-powered fields.

Migration assay in vitro

Cell invasion assay used through wound healing assay (ibidi; 80209). Wound healing assay for 6 well plates will be coated with and culture at 37°C for 12 hours. Cells seeded 1×10^5 and add 50 nM CDDO-me after 24 hours. After 1 day, washing twice with PBS and take a picture.

Data analysis

SPSS 19.0 (Chicago, Illinois) used for statistical analysis. The result of Western blot analysis through Lane 1D. A value of $P < 0.05$ will be considered statistically significant.

Results

CDDO-me attenuated GBM cell lines proliferation

To evaluate CDDO-me treatment on proliferation, we used an MTT assay to detect GBM8401 cells and GBM8901 cells proliferation and compared the results of the CDDO-me. After 24 hours incubation with CDDO-me, cell viability was assayed via MTT assay. In GBM8401 cells, the results indicated reduced cell viability in the 50 nM CDDO-me group relative to the control on 24 hours, and the reduced cell viability in the 100, 200, 400, and 500 nM CDDO-me relative to the control (Fig. 1A). In GBM8901 cells, the results indicated reduced cell viability in the 50 nM CDDO-me group relative to the control on 24 hours, and the reduced cell viability in the 100, 200, 400, and 500 nM CDDO-me relative to the control (Fig. 1B). To avoid the cell death to interference the result of migration and invasion, we used 50 nM CDDO-me to treat GBM8401 and GBM8901 to detect them.

CDDO-me inhibited GBM cell lines migration

To evaluate cell migration, we used a wound healing assay and compared between control group and CDDO-me group. In GBM8401 cells, CDDO-me

markedly inhibited the migratory capability at 50 nM on 6 hours (Figs. 2A and 2C). In GBM8901 cells, CDDO-me markedly inhibited the migratory capabilities at 50 nM on 12 hours (Figs. 2B and 2D). These data suggested that CDDO-me is able to inhibit migration in astrocytoma.

CDDO-me inhibited GBM cell lines invasion

To evaluate cell invasion, we used a Matrigel invasion assay and compared between control group and CDDO-me group. In GBM8401 cells, 50 nM CDDO-me markedly inhibited the invasive capability (Figs. 3A and 3C). In GBM8901 cells, 50 nM CDDO-me markedly also inhibited the invasive capability (Fig. 3B and 3D). These data suggested that CDDO-me inhibit invasion in astrocytoma.

CDDO-me downregulated the protein expression of N-cadherin, cyclin D1, Ki-67, and VEGF in GBM cell lines

To evaluate the expression of proteins related to cell proliferation and migration, a Western blot analysis of VEGF, N-cadherin, and cyclin D1 was conducted. Cyclin D1 is a markers of astrocytoma proliferation, N-cadherin is a biomarker of metastasis, and VEGF is a marker of angiogenesis. In GBM8401 (Fig. 4A), western blotting revealed that CDDO-me led to the downregulation of N-cadherin (Fig. 4C), cyclin D1 (Fig. 4D), and VEGF (Fig. 4E) expression. In GBM8901 (Fig. 4B), Western blotting revealed that CDDO-me led to the downregulation of N-cadherin (Fig. 4F), cyclin D1 (Fig. 4G), and VEGF (Fig. 4H) expression. In addition, CDDO-me was a dose-dependent on regulation of N-cadherin, cyclin D1, and VEGF in GBM8401 and GBM8901.

Discussion

Previously studies showed that the anti-carcinogenic mechanisms of CDDO-me involves inhibiting a number of prosurvival signaling pathways, such as MAPK (Erk1/2), NF- κ B, and Akt/mTOR signaling.²⁵⁻²⁷ In our result, CDDO-me was able to inhibit the tumor progression of glioblastoma cells *in vitro* including proliferation, migration, and invasion.

In previously studies, cyclin D1 was usually found overexpression in many cancers and an important role to regulate cell cycle.²⁸⁻³² Gain of N-cadherin was usually association with metastasis and be a biomarker as migration in many cancers.^{32,33} In our result, CDDO-me was a dose-dependent on regulation of N-cadherin, cyclin D1

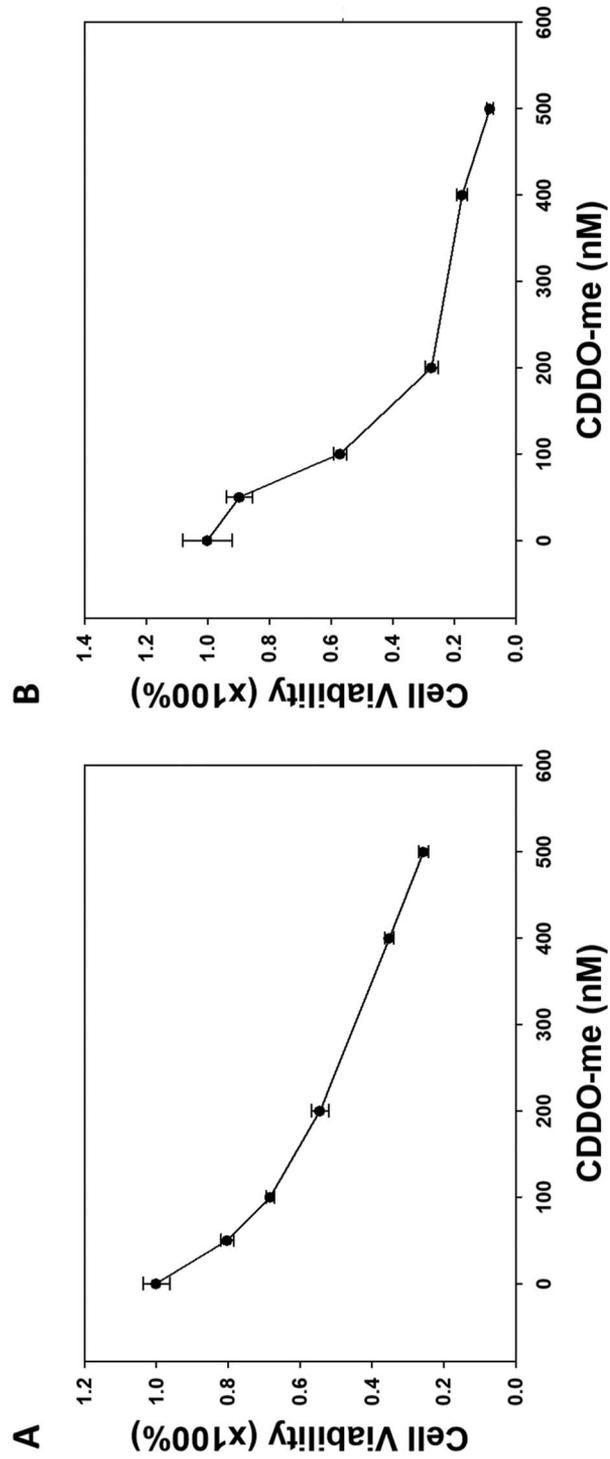


Fig. 1 The cell viability of GBM8401 (A) and GBM8901 (B) cells cultured in 24-well plates at 0, 50, 100, 200, 400, and 500 nM CDDO-me with MTT assay after 24 hours.

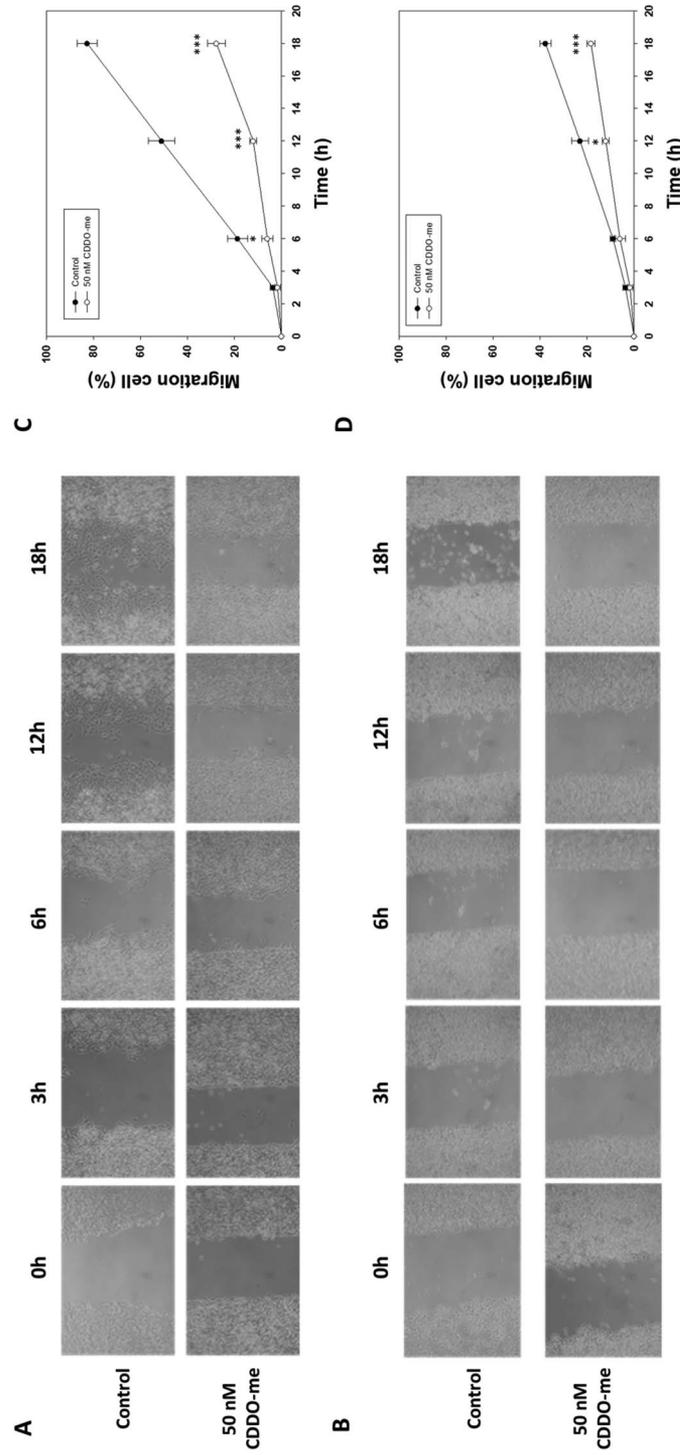


Fig. 2 Wound healing assay on GBM8401 (A, C) and GBM8901 (B, D) cells at 0, 3, 6, 12, and 18 hours treated with 50 nM CDDO-me. (A–B) Wounding healing assay (C–D) The percentages of migration cells (* $P < 0.05$; *** $P < 0.001$).

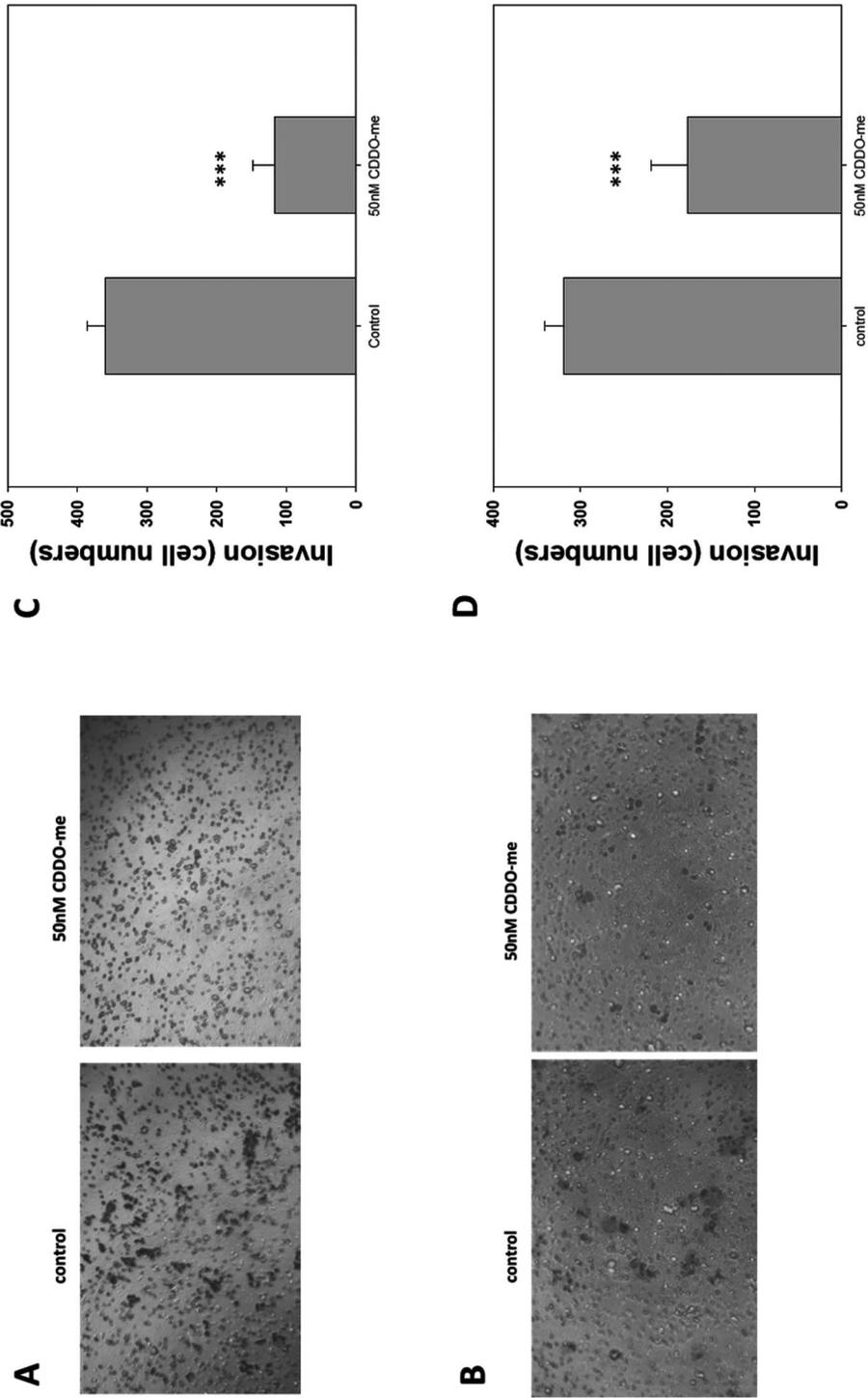


Fig. 3 Transwell invasion assay on GBM8401 (A, C) and GBM8901 (B, D) cell during 1 day treated with 50 nM CDDO-me. (A, B) Invasion assay. (C, D) The number of invaded cells (* $P < 0.001$).

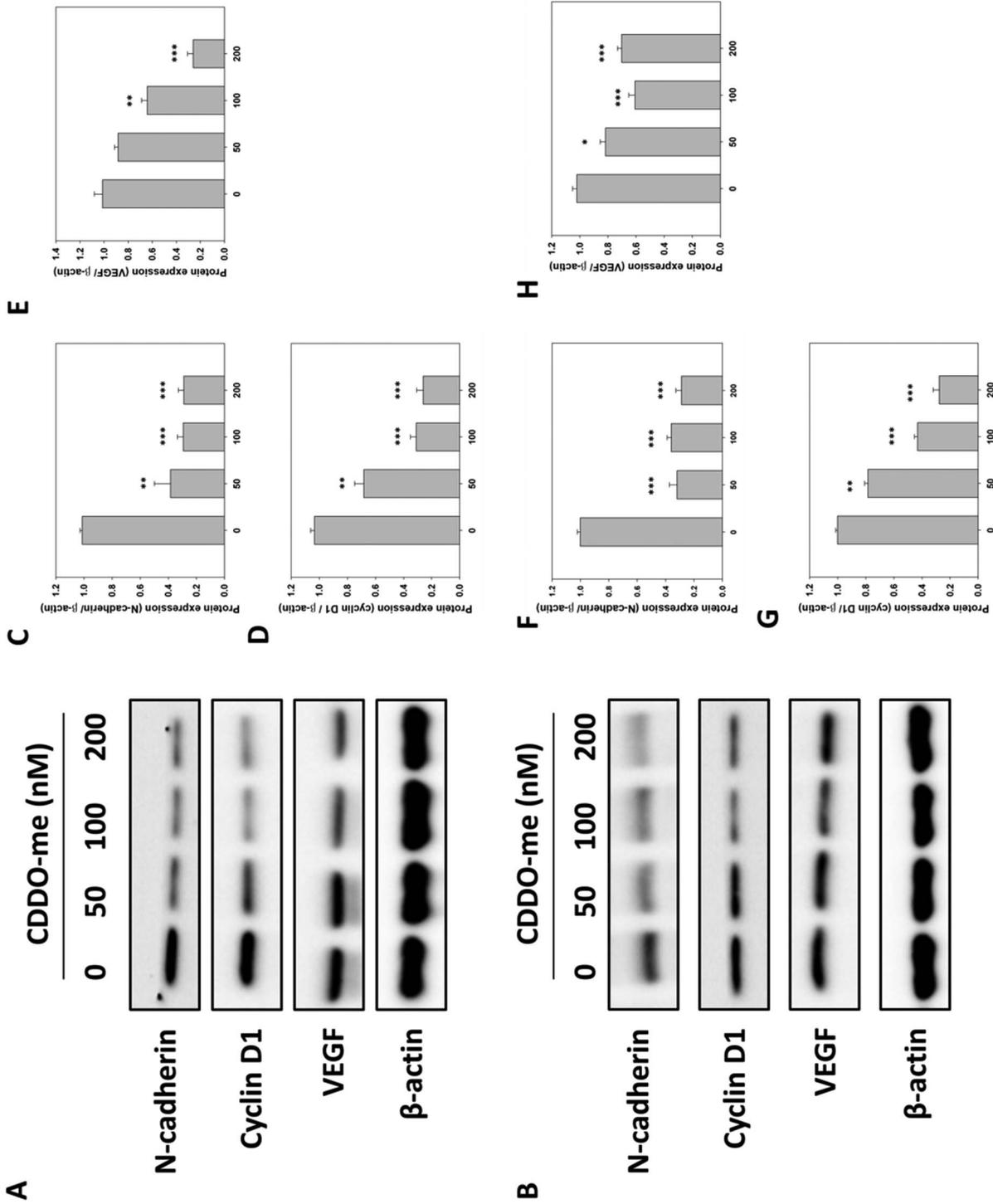


Fig. 4 The protein expressions of VEGF, N-cadherin, and cyclin D1 with different concentration of CDDO-me in GBM8401 (A, C-E) and GBM8901 (B, F-H) cells. (A, B) Western blot for N-cadherin, cyclin D1, and VEGF. (C-H) The relative protein expressions of N-cadherin, cyclin D1, and VEGF (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

and VEGF in GBM8401 and GBM8901. Eukaryotic cell cycle was controlled by the MEK1-ERK1/2 signaling.³⁴ ERK was reported to active the regulation of N-cadherin in prostate cancer stem cell.³⁵ In addition, VEGF induced angiogenesis also through ERK1/2 pathway in cancers.³⁶ These reports supported that CDDO-me regulated the proliferation, migration, invasion and angiogenesis following ERK1/2 pathway.

The limitation of this study, normal cells was not yet treated with CDDO-me and compared the concentration in GBM cells. Besides, we did not understand the ability of CDDO-me for passing through blood-brain barrier (BBB). In the future, we will detect the pathway, like p-ERK, p-JNK, and p-p38, and pharmacologic activity of CDDO-me including toxicity for normal cells and ability for passing through BBB.

In conclusion, CDDO-me was found the ability of inhibiting proliferation, migration, and invasion. This study establishes that even low nanomolar doses of CDDO-me have an effect in vitro on glioblastoma cell cycling, viability, potential for migration and angiogenesis. Further study is warranted.

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References

1. El-Sayed M, Taha MM. Immunohistochemical expression of cyclooxygenase-2 in astrocytoma: correlation with angiogenesis, tumor progression and survival. *Turk Neurosurg* 2011;**21**(1): 27–35
2. Santosh V, Arivazhagan A, Sreekanthreddy P, Srinivasan H, Thota B, Srividya MR *et al.* Grade-specific expression of insulin-like growth factor-binding proteins-2, -3, and -5 in astrocytomas: IGFBP-3 emerges as a strong predictor of survival in patients with newly diagnosed glioblastoma. *Cancer Epidemiol Biomarkers Prev* 2010;**19**(6):1399–1408
3. Huang MT, Ho CT, Wang ZY, Ferraro T, Lou YR, Stauber K *et al.* Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. *Cancer Res* 1994;**54**(3): 701–708
4. Nishino H, Nishino A, Takayasu J, Hasegawa T, Iwashima A, Hirabayashi K *et al.* Inhibition of the tumor-promoting action of 12-O-tetradecanoylphorbol-13-acetate by some oleanane-type triterpenoid compounds. *Cancer Res* 1988;**48**(18):5210–5215
5. Chintharlapalli S, Papineni S, Konopleva M, Andreef M, Samudio I, Safe S. 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid and related compounds inhibit growth of colon cancer cells through peroxisome proliferator-activated receptor gamma-dependent and -independent pathways *Mol Pharmacol* 2005;**68**(1):119–128
6. Konopleva M, Tsao T, Ruvolo P, Stiof I, Estrov Z, Leysath CE *et al.* Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia. *Blood* 2002;**99**(1):326–335
7. Ikeda T, Sporn M, Honda T, Gribble GW, Kufe D. The novel triterpenoid CDDO and its derivatives induce apoptosis by disruption of intracellular redox balance. *Cancer Res* 2003;**63**(17):5551–5558
8. Hyer ML, Shi R, Krajewska M, Meyer C, Lebedeva IV, Fisher PB *et al.* Apoptotic activity and mechanism of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic-acid and related synthetic triterpenoids in prostate cancer. *Cancer Res* 2008;**68**(8):2927–2933
9. Yates MS, Tauchi M, Katsuoka F, Flanders KC, Liby KT, Honda T *et al.* Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes. *Mol Cancer Ther* 2007;**6**:154–162
10. Dinkova-Kostova AT, Liby KT, Stephenson KK, Holtzclaw WD, Gao X, Suh N, Williams C *et al.* Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. *Proc Natl Acad Sci U S A* 2005;**102**(12):4584–4589
11. Liby KT, Yore MM, Sporn MB. Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. *Nat Rev Cancer* 2007;**7**(5):357–369
12. Liby KT, Sporn MB. Synthetic oleanane triterpenoids: multifunctional drugs with a broad range of applications for prevention and treatment of chronic disease. *Pharmacol Rev* 2012;**64**(4):972–1003
13. de Zeeuw D, Akizawa T, Audhya P, Bakris GL, Chin M, Christ-Schmidt H *et al.* Bardoxolone methyl in type 2 diabetes and stage 4 chronic kidney disease. *N Engl J Med* 2013;**369**(369):2492–2503
14. Nagaraj S, Youn JI, Weber H, Iclozan C, Lu L, Cotter MJ *et al.* Anti-inflammatory triterpenoid blocks immune suppressive function of MDSCs and improves immune response in cancer. *Clin Cancer Res* 2010;**16**(6):1812–1823
15. Hong DS, Kurzrock R, Supko JG, He X, Naing A, Wheler J *et al.* A phase I first-in-human trial of bardoxolone methyl in patients with advanced solid tumors and lymphomas. *Clin Cancer Res* 2012;**18**(12):3396–3406
16. Wang YY, Yang YX, Zhao R, Pan ST, Zhe H, He ZX *et al.* Bardoxolone methyl induces apoptosis and autophagy and inhibits epithelial-to-mesenchymal transition and stemness in esophageal squamous cancer cells. *Drug Des Dev Ther* 2015;**9**: 993–1026

17. Shanmugam MK, Dai X, Kumar AP, Tan BK, Sethi G, Bishayee A. Oleanolic acid and its synthetic derivatives for the prevention and therapy of cancer: preclinical and clinical evidence. *Cancer Lett* 2014;(2);**346**:206–216
18. Ito Y, Pandey P, Sporn MB, Datta R, Kharbanda S, Kufe D. The novel triterpenoid CDDO induces apoptosis and differentiation of human osteosarcoma cells by a caspase-8 dependent mechanism. *Mol Pharmacol* 2001;**59**(5):1094–1099
19. Konopleva M, Tsao T, Estrov Z, Lee R-m, Wang R-Y, Jackson CE *et al.* The synthetic triterpenoid 2-cyano-3,12-dioxoleana-1,9-dien-28-oic acid induces caspase-dependent and -independent apoptosis in acute myelogenous leukemia. *Cancer Res* 2004;**64**:7927–7935
20. Gao X, Deeb D, Jiang H, Liu Y, Dulchavsky SA, Gautam SC. Synthetic triterpenoids inhibit growth and induce apoptosis in human glioblastoma and neuroblastoma cells through inhibition of prosurvival Akt, NF-kappaB and Notch1 signaling. *J Neurooncol* 2007;**84**(2):147–157
21. Deeb D, Gao X, Arbab AS, Barton K, Dulchavsky SA, Gautam SC. CDDO-Me: A Novel Synthetic Triterpenoid for the Treatment of Pancreatic Cancer. *Cancers (Basel)* 2010; **2**(14):779–1793
22. Ling X, Konopleva M, Zeng Z, Ruvolo V, Stephens LC, Schober W *et al.* The novel triterpenoid C-28 methyl ester of 2-cyano-3, 12-dioxoolen-1, 9-dien-28-oic acid inhibits metastatic murine breast tumor growth through inactivation of STAT3 signaling. *Cancer Res* 2007;**67**(9):4210–4218
23. Liby KT, Royce DB, Risingsong R, Williams CR, Maitra A, Hruban RH *et al.* Synthetic triterpenoids prolong survival in a transgenic mouse model of pancreatic cancer. *Cancer Prev Res (Phila)* 2010;**3**(11):1427–1434
24. Deeb D, Gao X, Liu Y, Jiang D, Divine GW, Arbab AS *et al.* Synthetic triterpenoid CDDO prevents the progression and metastasis of prostate cancer in TRAMP mice by inhibiting survival signaling. *Carcinogenesis* 2011;(5)**32**:757–764
25. Konopleva M, Contractor R, Kurinna SM, Chen W, Andreeff M, Ruvolo PP. The novel triterpenoid CDDO-Me suppresses MAPK pathways and promotes p38 activation in acute myeloid leukemia cells. *Leukemia* 2005;**19**(8):1350–1354
26. Ahmad R, Raina D, Meyer C, Kharbanda S, Kufe D. Triterpenoid CDDO-Me blocks the NF-kappaB pathway by direct inhibition of IKKbeta on Cys-179. *J Biol Chem* 2006; **281**(47):35764–35769
27. Deeb D, Gao X, Jiang H, Dulchavsky SA, Gautam SC. Oleanane triterpenoid CDDO-Me inhibits growth and induces apoptosis in prostate cancer cells by independently targeting pro-survival Akt and mTOR. *Prostate* 2009;**69**(3):851–860
28. Monga SP. Role and regulation of beta-catenin signaling during physiological liver growth. *Gene Expr* 2014;**16**(2):51–62
29. Huang M, Tang SN, Upadhyay G, Marsh JL, Jackman CP, Srivastava RK *et al.* Rottlerin suppresses growth of human pancreatic tumors in nude mice, and pancreatic cancer cells isolated from Kras(G12D) mice. *Cancer letters* 2014;**353**(1):32–40
30. Butt S, Borgquist S, Anagnostaki L, Landberg G, Manjer J. Breastfeeding in relation to risk of different breast cancer characteristics. *BMC Res Notes* 2014;**7**:216
31. Atadja P, Wong H, Veillete C, Riabowol K. Overexpression of cyclin D1 blocks proliferation of normal diploid fibroblasts. *Exp Cell Res* 1995;**217**(2):205–216
32. Okumura N, Nakano S, Kay EP, Numata R, Ota A, Sowa Y *et al.* Involvement of cyclin D and p27 in cell proliferation mediated by ROCK inhibitors Y-27632 and Y-39983 during corneal endothelium wound healing. *Invest Ophthalmol Vis Sci* 2014; **55**(1):318–329
33. Lesser IM, Rubin RT, Rifkin A, Swinson RP, Ballenger JC, Burrows GD *et al.* Secondary depression in panic disorder and agoraphobia. II. Dimensions of depressive symptomatology and their response to treatment. *J Affect Disord* 1989;**16**:49–58
34. Schramek H. MAP kinases: from intracellular signals to physiology and disease. *News Physiol Sci* 2002;**17**(2):62–67
35. Ding Q, Miyazaki Y, Tsukasa K, Matsubara S, Yoshimitsu M, Takao S. CD133 facilitates epithelial-mesenchymal transition through interaction with the ERK pathway in pancreatic cancer metastasis. *Mol Cancer* 2014;**13**(1):15
36. Lichtenberger BM, Tan PK, Niederleithner H, Ferrara N, Petzelbauer P, Sibilina M. Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development. *Cell* 2010;**140**(1):268–279