HSP90 inhibitor gedunin impairs glioblastoma proliferation and invasiveness

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43
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45 **Graphical Abstract**

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50 **Highlights**
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• Gedunin reduces GL261 cell proliferation, migration and invasion *in vitro*.

• Gedunin induces GL261 cell apoptosis and ERK activation *in vitro*.

• Gedunin reduces the presence of tumoral vascular structures *in vivo*.

Abstract

Glioblastoma is the most invasive and malignant brain tumor, for which no effective

treatment is currently available. Heat shock protein 90 (HSP90) is a potential target for

the treatment of different types of cancer. Herein, we investigated the effect of gedunin,

an HSP90 inhibitor, in murine GL261 glioblastoma *in vitro* and *in vivo* models.

Gedunin treatment decreased GL261 cell proliferation and triggered apoptosis in a

concentration-dependent manner. Gedunin also reduced GL261 cell migration and

metalloproteinase-2 activity, suggesting that it impairs glioblastoma cell invasion.

Despite the reduction of total protein content in gedunin-treated cells, the
phosphorylation of STAT3 and ERK1/2 pathways was enhanced within 24 h. *In situ*
treatment with gedunin did not significantly reduce tumor volume, but reduced the
presence of vascular structures in xenograft glioblastoma in C57BL/6 mice, a clinical
feature associated with morbidity and mortality. Our results demonstrate that gedunin is
a promising compound for the treatment of glioblastoma.

Keywords: Brain tumor, GL261, limonoids, natural products.

1. Introduction

Glioblastoma is a type of brain tumor that arises from phenotypically altered cells with
neural stem cell-like properties [1]. This highly invasive and malignant tumor is defined
by the World Health Organization as grade IV, based on histological,
immunohistochemical, and molecular features [2]. Patients bearing glioblastoma have
poor prognosis, with estimated median survival rates of eight months and a maximum
five-year survival rate of only 5% [3,4].

The current standard protocol for glioblastoma treatment (Stupp protocol) relies on total
tumor excision followed by high-dose radiotherapy with concurrent and adjuvant
chemotherapy with temozolomide, a DNA alkylating agent [5]. The most common
adverse events related to temozolomide treatment are myelosuppression (with cases of
neutropenia, thrombocytopenia, lymphopenia, and anemia) and hepatotoxicity (affecting
AST and ALT liver enzyme levels) [6–8]. Another limiting factor of glioblastoma
treatment is that most orally and intravenously administered alkylating agents have poor
blood-brain barrier permeability [9]. An alternative strategy is the usage of intracranial
wafer implants containing the alkylating agent carmustine after tumor removal surgery,
which, in combination with radiochemotherapy, has been shown to significantly
improve patient survival [10,11]. However, carmustine wafers induce severe side
effects, such as cerebral edema and hematological toxicity [6,12,13]. Another challenge
in glioblastoma treatment with alkylating agents is the induction of resistance by the
enhanced activity of the DNA-repair enzyme O6-methylguanine-DNA
methyltransferase (MGMT) that repairs alkylating agents-induced DNA damage [14].

Glioblastoma is still considered incurable [15]; however, treatments targeting multiple
signaling pathways are a promising strategy [16].

Modulation of the chaperone heat shock protein 90 (HSP90) is considered a potential
antitumor therapy [17]. HSP90 activity is implicated on several hallmarks of cancer,
such as proliferation, invasion, metastasis, angiogenesis, and resistance to apoptosis
[18]. Increased expression of HSP90 correlates with tumor malignancy and poor
prognosis in different types of cancer, including breast, colon, and melanoma [19–22].
Indeed, HSP90 chaperones several proteins involved in oncogenic processes, including
the human epidermal growth factor receptor 2 (HER2/neu; ErbB2), tyrosine-protein
kinase Src (Src), protein kinase B (AKT/PKB), breakpoint cluster region/Abelson
murine leukemia virus (BCR/ABL) and tumor protein 53 (p53) [23–25]. In line with
this, encouraging pre-clinical data have been obtained with HSP90 inhibitors in
different types of tumor cells and animal models, including lymphoma, melanoma,
breast, and lung cancer [26–30]. Currently, in the NIH´s Clinical Trials database
ClinicalTrials.gov, there are more than a hundred registered trials (11 currently active)
evaluating HSP90 inhibitors (alone or in combination with other drugs) for the
treatment of different types of cancer [31]. Worthy of note, off-target toxicity, including
ocular toxicity and hepatotoxicity, is a limiting factor for the use of these substances
[32,33].

Over the past two decades, our group and others have been studying the antitumoral and
anti-inflammatory effects of gedunin, a limonoid produced by vegetal species from the
Meliaceae family [34] or by semi-synthetic routes [35]. Gedunin negatively modulates
HSP90 activity by binding to the HSP90 co-chaperones p23 and cdc37 and, thus,
impairing the formation of the active HSP90 complex [36,37]. This limonoid has potent
antitumoral effects demonstrated against an array of tumor types *in vivo* and *in vitro*
[36,38–41]. However, gedunin anti-tumoral effects have not been evaluated in
glioblastoma.

Considering the urgent need for an effective therapy against glioblastoma and the high
antitumor activity of the HSP90 inhibitor gedunin, here we investigate the antitumoral
effect of this compound using the murine GL261 model both *in vitro* and *in vivo*.

2. Materials and Methods

2.1 Reagents and antibodies

Gedunin (Gaia Chemicals, USA) and the specific HSP90 inhibitor 17-AAG (17-N-allylamino-17-demethoxygeldanamycin, Tanespimycin, Sigma-Aldrich, USA) were dissolved in DMSO (dimethyl sulfoxide, Sigma-Aldrich) and frozen at -86 °C until use. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Dulbecco´s modified Eagle´s medium (DMEM), phosphate buffer saline (PBS) tablet and trypsin-EDTA (ethylenediamine tetraacetic acid) solution were purchased from Sigma Aldrich. Fetal bovine serum (FBS) was purchased from Cultilab (Brazil). Propidium iodide (PI) solution was obtained from Biolegend (USA). Vybrant™ CFDA-SE (carboxyfluorescein diacetate succinimidyl ester) Cell Tracer Kit and gentamicin were obtained from Thermo Fischer Scientific (USA). Annexin V-FITC (fluorescein isothiocyanate) conjugated was purchased from BD Pharmingen (USA). IgG anti-mouse STAT3 (signal transducer and activator of transcription 3, rabbit, C-20), anti-mouse-IgG-HRP (horseradish peroxidase), and anti-rabbit-IgG-HRP secondary antibodies were purchased from Santa Cruz Biotechnologies (USA). Antibodies against mouse p44/42 MAPK (mitogen-activated protein kinase, Thr202/Tyr204, D13.14.4E), ERK1/2 (extracellular signal-regulated kinase 1/2, rabbit), AKT (protein kinase B, PKB, rabbit, pan, 40D4), phospho-AKT [rabbit, Ser473, (D9E)XP] and GAPDH (glyceraldehyde-3-phosphate dehydrogenasemouse, D4C6R) were purchased from Cell Signaling (USA). Antibodies against human PCNA (proliferating cell nuclear antigen, rabbit) were purchased from Sino Biological (China). DNase I recombinant RNase free was purchased from Roche Life Sciences (Germany).

2.2 GL261 cell culture and treatment

Murine glioblastoma cell line (GL261, Cell Bank from Federal University of Rio de Janeiro - UFRJ, Brazil) was cultured in DMEM (Sigma Aldrich) supplemented with 10 % FBS (Cultilab) and 50 μg/mL gentamicin (Thermo Fisher Scientific) in humidified atmosphere (5 % CO_2 at 37 °C) until confluence. Cells were digested with 0.25 % trypsin at the 5th passage and washed with 1x PBS. Cell viability was determined by trypan blue (Sigma Aldrich) staining, under a light microscope, before further assays.

GL261 cells were incubated with 6.25-100 μM of gedunin for 24-72 h at 37 °C, 5 %
CO_2.

2.3 Animals

Adult male C57BL/6 mice (20-25 g) provided by Oswaldo Cruz Foundation (Fiocruz)
breeding unit (Institute for Biomodels Science and Technology, Rio de Janeiro, Brazil)
were kept in standard autoclavable polypropylene boxes, with free access to food and
fresh water in environmentally controlled room temperature (ranging from 22 to 24 °C)
on a light/dark cycle at Farmanguinhos (Fiocruz, Rio de Janeiro, Brazil) and at the
Laboratory of Neurophysiology (State University of Rio de Janeiro, Rio de Janeiro,
Brazil) experimental animal facilities. Animal procedures were performed in accordance
with the ethical guidelines of the Brazilian National Council for the Control of Animal
Experimentation (CONCEA) and reported following the ARRIVE guidelines [42].
Experimental procedures were approved by the Ethics Commission on Animal Use of
Oswaldo Cruz Foundation (license #LW-35/16) and the Ethics Commission on Animal
Use of the State University of Rio de Janeiro (license #025/2017).

2.4 Primary mixed glial cell culture

Under sterile conditions, mouse brain cortex tissue was rapidly dissected from
euthanized (150 mg/kg sodium thiopental, i.p.) young adult (4-week-old) C57BL/6 male
mice and transferred to ice-cold DMEM/F12 media (Sigma Aldrich) in 6 well plates.
After removal of the meninges, brain tissue was cut into small pieces, and washed three
times with 1x sterile PBS (Sigma Aldrich). Trypsin-DNAse solution (0.25 %-250
μg/mL) was added to the wells and the plate was incubated for 1 h in an orbital shaker
(Excella E24, New Brunswick Scientific, USA) at 37 °C and 150 rpm. Tissue samples
were vigorously triturated and dissociated with a 1 mL pipette tip. Cells were washed
with FBS-DNAse solution (10 %-250 μg/mL), added to a 20 % percoll gradient media,
and centrifuged at $800 \times g$ for 30 min at 25 °C. Recovered cells were washed ($400 \times g$,
10 min at 4 °C), distributed in T-25 bottle flasks, and incubated at 37 °C, 5 % CO_2.
DMEM/F12 media was changed every 3-4 days until use. Data were collected from two
independent experiments (n=5 mice per group; license #LW-35/16).

2.5 Proliferation assay

GL261 cells (4×10^7/mL) were incubated with 5 μM CFSE solution (Invitrogen

Molecular Probes, USA) diluted in PBS for 15 min at 37 °C, 5 % CO_2. Cells were

washed with 10 % PBS/FBS solution, seeded in 24 well plates (2×10^5 cells per well),

incubated overnight, and treated with gedunin (6.25-100 μM) for 72 h. Cell proliferation

was evaluated by analyzing CFSE fluorescence intensity (FL1) upon cell divisions, by

flow cytometry (FACScalibur, Becton Dickinson, USA). Results were analyzed using

FlowJo X Software (Tree Star, Becton Dickinson).

2.6 MTT assay

GL261 or primary mixed glial cells were seeded in a 96 flat bottom well plate (2×10^4

cells/well) and cultured in DMEM for 24 h (CO_2 5 % at 37 °C) for adhesion. Adherent

cells were cultured with different concentrations of gedunin (6.25-100 μM), 17-AAG (1

μM), or temozolomide (100 μM) for 20 or 68 h, when MTT solution (5 mg/mL; 22.5

μL/well) was added to the culture and incubated for 4 h. The analyzes were performed

at 24 and 72 h time points. Plates were centrifuged for 3 min ($720 \times g$ at 4 °C), the

supernatant was discharged and DMSO (150 μL/well) was added for formazan crystals

solubilization. The absorbance was read in a microplate reader (SpectraMax M5,

Molecular Devices, USA) at 540 nm.

2.7 Determination of apoptosis

Apoptosis was evaluated using annexin V-FITC and propidium iodide (PI) staining.

GL261 cells were seeded in 24 well cell culture plates (2×10^5 cells/well) and left to

adhere overnight. GL261 cells were then incubated with gedunin (6.25 - 100 μM) for 24

or 72 h, followed by trypsinization, resuspension in annexin V Binding Buffer (HEPES

10 mM pH 7.4, NaCl 140 mM, $CaCl_2$ 2.5 mM) and labeling with annexin V-FITC and

PI for 15 min at room temperature. Cells incubated with medium were used as the

negative control, whereas 17-AAG-incubated cells were used as the positive control.

Staining was analyzed by cytometry using FACScalibur (Becton Dickinson) and

FlowJo Software (Tree Star, Becton Dickinson). At least 10^4 cells were acquired per

sample. Data are presented in dot plots in a log scale of increasing FL-1 (annexin V-

FITC) and FL-3 (PI) intensity.

2.8 Scratch assay

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GL261 cell monolayers, seeded into 6 well plates, were scratched at the center of the
well, and then the migration path was assessed at the leading edge of the scratch by
phase-contrast optical microscopy (100 ×, Olympus IX70, Japan) at indicated time
points. Analyses were performed by Image J software (National Institute of Health,
USA). The rate of migration was determined by quantifying the total distance that cells
moved from the edge of the scratch toward the center of the scratch, as previously
described [43].

2.9 Chemotaxis assay

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GL261 cell migration was assayed in a 48-well micro-chemotaxis Boyden chamber
(Neuro Probe Inc., USA). The bottom wells of the chamber were filled with DMEM 10
% FBS (29 μL) or serum-free DMEM media as the negative control. Polycarbonate
membrane filters with 8 μm pore size (Neuro Probe) were placed between the lower and
upper chambers. GL261 cells were pre-treated with different concentrations of gedunin
(6.25-25 μM) for 1 h in serum-free DMEM and then loaded into the upper wells of
Boyden chamber (2×10^4 cells/50 μL/well). The chamber was incubated in a humidified
atmosphere with 5 % CO_2 at 37 °C for 24 h. The membranes were removed and stained
using a Diff-quick (Laborclin, Brazil). The bottom side of membranes was visualized by
light microscopy for counting GL261 migrating cells (1000 ×, Olympus IX70). GL261
chemotaxis was calculated and expressed as the mean number of migrated cells in ten
random high-power fields (HPFs) per well (in quadruplicate).

2.10 Zymography

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GL261 cells were plated (1×10^6/well) in a 6-well plate in DMEM. After 24 h,
supernatant was removed, and cells were washed 3 times with pure DMEM to eliminate
any FBS trace. Then, cells received gedunin (6.25-50 μM) or 17-AAG (1 μM) treatment
in serum-free DMEM. After 48 h, the supernatant was collected and stored at -70 °C
until lyophilization. Samples were dehydrated in a Gamma 2-16 LSCplus (Christ,
Germany) ice condenser and, resuspended with 200 μL of zymography extraction buffer
(Tris-HCl 50 mM pH 7.5, NaCl 0.2 M, Triton X-100 0.1 %, $CaCl_2$ 10 mM, protease

inhibitor cocktail). Lowry method was used for protein quantitation. Enzymatic activity

of matrix metalloproteinase (MMP)-2 was determined on a 10 % SDS-PAGE

containing 0.1 % gelatin from porcine skin (Sigma Aldrich).

2.11 Enzyme-linked immunosorbent assay

258 Levels of vascular endothelial growth factor (VEGF) were evaluated by sandwich

259 enzyme-linked immunosorbent assay (ELISA) in GL261 cell supernatants using

260 matched antibody pairs from R&D Systems (USA), according to the manufacturer's

261 instructions. Absorbance was determined by Spectramax M5 microplate reader

262 (Molecular Devices) at 490 nm.

2.12 Western blot

GL261 cells (1×10^5 cells) treated with gedunin (6.25-100 µM) or 17-AAG (1 µM) for

24 h were lysed in a buffer containing protease inhibitors. Protein extract (20 µg) was

resolved by SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF)

membranes (GE Healthcare, USA), and then probed with primary antibodies.

Antibodies were diluted in Tris-buffered saline containing 0.5 % Tween 20 and 5 %

non-fat milk or bovine serum albumin (BSA, Sigma -Aldrich, USA), and incubated

overnight at 4 °C. On the following day, the membranes were incubated with

horseradish peroxidase (HRP)-conjugated secondary IgG (Santa Cruz Biotechnology)

for 1 h at room temperature. The blots were developed in a Hyperfilm with the use of a

chemiluminescent substrate (ECL Western blotting Detection Reagents; Amersham

Biosciences, USA). The band intensity was quantified using ImageJ (NIH, USA), and

expressed in arbitrary units (a.u.). The raw intensity values of total proteins were

normalized using the values of the control protein (PCNA) and of the control group

(untreated cells) to allow comparison between different experiments. The phospho-

protein values were normalized by the specific total-protein content.

2.13 Orthotopic glioblastoma model and osmotic mini-pump implantation

Glioblastoma was induced in adult male C57BL/6 by stereotaxic injections of GL261

cells at the Laboratory of Neurophysiology experimental animal facility (UERJ, Rio de

Janeiro, Brazil). Mice were intraperitoneally anesthetized with 100 mg/kg ketamine

(Syntec, Brazil) and 10 mg/kg xylazine (Sedalex™ Venco, Brazil). Under sterile

conditions, a midline incision was made in the scalp, the skull was exposed to identify

bregma and lambda, and a small burr hole was drilled over the right hemisphere.

GL261 cells ($5 \times 10^4/\mu L$; final volume of 2 μL) were stereotaxically injected into the

caudate putamen with a 10 μL Hamilton syringe at the rate of 0.5 μL/min. The needle

was slowly removed 4 min after the injection was finished. The following coordinates

(in mm) were used for stereotaxic injections (anterior-posterior; medial-lateral; dorsal-

ventral): 0.1 AP; 2.32 ML; 2.35 DV. Coordinates were based on the Cha et al., 2003

[44] descriptions and confirmed on Paxinos and Franklin mouse brain atlas [45].

Fourteen days after GL261 injections, animals were implanted with Alzet osmotic

minipumps (model 2004, 0.25 μL/h; Durect Corporation, USA) filled with gedunin

(250 μM, n=8) or DMSO (2.5 %, n=8). Cannulas (1-2 mm long) were placed into the

site of the tumor following similar surgical procedures described above. Treatment

lasted between 14 and 17 days. Mice were monitored every day for clinical signs of

pain and distress, such as ruffled fur, hunched back, trembling, convulsions, paralysis,

and permanent recumbency [46]. If signs of pain and distress were observed, the

animal was excluded from the study and euthanized. Data were collected from two

independent experiments (n=8 randomized mice per group). Mice were euthanized 28

days after GL261 injection with sodium thiopental (150 mg/kg, i.p.).

2.14 Mouse Brain Computed Tomography Images

After 14-day treatment, anesthetized mice (100 mg/kg ketamine/10 mg/kg xylazine,

i.p.) were intravenously (i.v.) injected with 300 mg/mL of Omnipaque iohexol contrast

(GE Healthcare). Brains were scanned 3-6 min after contrast administration in a GE

Highspeed CT/E image system (Pedro Ernesto University Hospital, UERJ, Rio de

Janeiro, Brazil), according to the following protocol: 120 kV tube voltage, 55 mA, 1

mm slice thickness. Images were reconstructed in a 256 x 256-pixel matrix using

InVesalius 3.1.1 software (developed by Centro de Tecnologia da Informação Renato

Archer, CNPq, and Ministry of Health, Brazil). Tumor volumetry was determined by

selecting contrast-enhanced regions of interest in coronal sections.

2.15 Histology of Brain Tissue

Euthanized mice (150 mg/kg sodium thiopental, i.p.) were perfused with 4%
formaldehyde. Mouse brains were collected and transferred into a 4 % formaldehyde
solution for 2 days for tissue fixation. Brains were then sectioned and embedded in
paraffin. Coronal sections of brain tissue (5 μm) were made using a Shandon Finesse
325 microtome (Thermo Fisher Scientific). Brain slices were stained with hematoxylin
(Merck, USA) and eosin (Sigma Aldrich, USA). Lesion size, liquefactive necrosis area,
vascular structures, intratumoral hemorrhage, and mitosis were blindly analyzed in the
central slice tumor area. Lesion size area was defined by the percentage of tumor area in
relation to the size of the brain area in the slice. The liquefactive necrosis area was
determined by the percentage of necrosis area in relation to the lesion size. Vascular
structures that had their full composition were counted in intratumoral and peritumoral
regions. Intratumoral hemorrhage was described in a semi-quantitative form regarding
the intensity at the slice, with the following definitions: discrete (+), moderate (++), and
intense (+++). Mitosis was characterized by estimating the average amount of cells per
analyzed field, and thus: >50% (+), 50-75% (++), and >75% (+++). Multipoint brain
slice images were composed in NIS-Elements AR (Nikon Corporation). Each brain slice
was analyzed by an observer blind to the experimental groups.
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2.16 Statistical Analysis
All analyzes were performed using GraphPad Prism 5 (GraphPad Software, USA).
Western blot analyzes were performed using the non-parametric Kruskal-Wallis test
followed by Dunn's multiple comparisons. Histologic analyzes were performed by one-
way analysis of variance (ANOVA) and Tukey's post-test for multiple comparisons. All
other comparisons were carried out using one-way ANOVA followed by Student-
Newman-Keuls test. Data are shown as the mean $\pm$ SEM (unless otherwise mentioned).
Significance was assumed either at the level of $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$, as
specified.
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3. Results

3.1 Gedunin impairs GL261 cell growth and viability in vitro

To verify the effect of gedunin on GL261 cell growth, a 72 h CFSE proliferation assay was performed, in which staining decreases as cells proliferate. Incubation of CFDA-SE-stained GL261 cells with gedunin decreased cell proliferation in a concentration-dependent manner (**Fig. 1A**). Gedunin (25-100 μM) significantly inhibited GL261 cell growth, similar to what was observed with the selective HSP90 inhibitor 17-AAG (reference control). **Figure 1B** shows representative histograms of CFSE fluorescence intensity (FL-1) of GL261 cells depicted in **Figure 1A**.

The effect of gedunin on cell viability was evaluated by the MTT assay. The incubation of GL261 cells with gedunin decreased mitochondrial function in a concentration-dependent manner within 24 and 72 h (**Fig. 1C**). MTT assay was also performed on primary mixed glial cells to evaluate gedunin toxicity to non-tumoral cells. Gedunin effect on primary mixed glial cells was less toxic, and higher concentrations were required to induce cell death when compared to GL261 cells (**Fig. 1C**). The half-maximal inhibitory concentration [IC50] of gedunin on GL261 cells was 2.3 times lower than the IC_{50} observed on primary glial cells (**Fig. 1D**). Temozolomide, the first-line treatment for glioblastoma, also reduced GL261 viability within 72 h in a concentration-dependent manner (**Suppl. Fig. 1**). Considering the antiproliferative and cytotoxic effects of gedunin on GL261 cells, we further examined whether this limonoid induced apoptosis.

Figure 1. *Gedunin impairs GL261 cell proliferation.* (A) Effect of gedunin (6.25-100 μM) and 17-AAG (1 μM) on GL261 cell proliferation evaluated by CFSE staining and analyzed by FACScalibur (BD Biosciences) 72 h after incubation. Results are expressed as the mean ± SEM of median fluorescence intensity (MFI) from triplicate wells from three independent experiments. (B) Representative histogram shows CFSE fluorescence intensity (FL-1) from one experiment. (C) Effect of gedunin (6.25-100 μM) and 17-AAG (1 μM) on GL261 cell viability evaluated by MTT reduction assay (SpectraMax M5, Molecular Devices) 24 and 72 h after incubation. (D) IC50 values of gedunin treatment on GL261 and glial mixed cells 72 h after incubation. SI: selectivity index.

3.2 Gedunin induces GL261 cell apoptosis in vitro

Apoptosis is regulated by a series of signaling events that include disruption of the mitochondrial membrane and phosphatidylserine externalization [47,48]. Membrane phosphatidylserine staining by annexin V-FITC revealed that gedunin induced apoptosis of GL261 cells in a concentration- and time-dependent manner (**Fig. 2A**). Apoptosis was detected 24 h after treatment, and surpassed 90 % within 72 h, at the highest

concentrations. Representative dot plots of PI$^+$ and annexin V$^+$ cells are shown in

Figure 2B. Apoptotic cells are characterized by a reduced ability to scatter light either

in forward or right angle directions [49]. Indeed, GL261 cells incubated with gedunin

diminished FSC-H and increased SSC-H intensities, when compared to non-treated cells

(**Fig. 2C**). Representative dot-plots of the effect of 17-AAG (1 μM) on GL261 apoptosis

(24 h and 72 h) is available on **Supplemental Figure 2**.

Figure 2. *Gedunin induces time- and concentration-dependent GL261 apoptosis.* (A) Effect of gedunin (6.25-100 µM) on GL261 cell (2×10^4/well) apoptosis evaluated by propidium iodide and annexin V-FITC staining MTT (FacsCalibur – BD Biosciences) 24 h and 72 h after incubation. Results are expressed as the mean ± SEM of viability percentage from triplicates wells from three independent experiments (24 well plates). * indicates statistically significant differences ($p \leq 0.05$) between treated and untreated cells. (B) Representative dot plots of flow cytometry analysis of gedunin-treated GL261 cells labeled with annexin V-FITC and PI after 24 and 72 h incubation. (C) Representative contour plots of SSC-H and FSC-H of gedunin-treated (6.25 - 100 µM) GL261 cells after 72 h.

3.3 Gedunin impairs GL261 cell migration and invasiveness in vitro

Tumor cell migration is an earlier step in cancer dissemination and invasion [50,51]. Scratch assay shows that gedunin (12.5 and 25 µM) reduced GL261 cell migration when compared to the untreated group within 24 h, 48 h, and 72 h (**Fig. 3A, B**). Confirming results obtained in the scratch assay, reduction of chemotaxis was also observed in the Boyden chamber migration assay, in which gedunin treatment (25 µM) impaired cell migration within 48 h (**Fig. 3C**).

Extracellular matrix remodeling by the proteolytic activity of tumor cell matrix metalloproteinases (MMPs) is a crucial step for tumor cell invasion in the surrounding tissue [52]. Gedunin also reduced the activity of MMP-2 (**Fig. 3D**), suggesting that this limonoid impairs different mechanisms associated with tumor cell invasion. In addition, we observed that gedunin treatment increased VEGF levels in the supernatant of GL261 cells (**Fig. 3E**), a phenomenon that does not contribute to its antitumoral effect since pro-angiogenic mediators contribute to tumor cell metastasis [53,54].

Figure 3. *Gedunin impairs in vitro GL261 cell migration and invasiveness.* (A) Microscopic views of *in vitro* scratch assay. GL261 migration into the scratched area 24-72 h after gedunin treatment (6.25-25 μM). (B) Percentages of GL261 cell migration calculated as the ratio of uncovered area (24-72 h) by the initial scratched area (0 h). Results are expressed as the mean ± SEM from two independent experiments (6 well plates). (C) Boyden chamber chemotaxis assay demonstrating the number of gedunin-treated or untreated cells per 15 high power fields (HPF) after 24 h migration. Results are expressed as the mean ± SEM from quadruplicate wells. (D) MMP-2 activity of GL261 cell supernatant assessed by zymography assay 48 h after gedunin treatment (6.25-50 μM). Results are expressed as the mean ± SEM from two independent experiments. (E) VEGF quantification (ng/mL) in cell supernatant by ELISA assay 24 and 72 h after gedunin treatment (6.25-100 μM). * indicates statistically significant differences ($p \leq 0.05$) between treated and untreated cells, whereas # indicates statistically significant differences ($p \leq 0.05$) between stimulated and unstimulated cells.

3.4 Gedunin modulates signaling proteins in GL261 cells

Genomic characterization of glioblastoma frequently shows amplification and phosphorylation of RTK (receptor tyrosine kinase), which ultimately leads to its autoactivation and downstream signaling pathways, resulting in constitutive cell proliferation and resistance to apoptosis [55]. PI3K/AKT and Ras/ERK are the main pro-survival pathways triggered by RTK activation in glioblastoma cells [56–58]. **Figure 4A-E** illustrates the effect of gedunin in these pathways in *in vitro*-treated GL261 cells.

Gedunin treatment reduced the amount of total cell protein within 24 h, as shown in representative immunoblots of proliferating cell nuclear antigen (PCNA) content from 8 independent experiments (**Fig. 4A**; **Suppl. Fig. 3**). Immunostaining of total proteins also decreased in gedunin-treated cells (**Fig. 4A**), which may be explained by the induction of apoptosis and cell death.

When the measurements were normalized by the constitutive protein, PCNA, no specific reduction of total STAT3 or total ERK 1/2 was observed after gedunin treatment (**Fig. 4B, C**). Total AKT protein content was reduced in GL261 cells treated with gedunin (50 μM), when compared to untreated cell values (**Fig. 4D**).

Phosphorylation of STAT3 (**Fig. 4E**) and ERK 1/2 (**Fig. 4F**) was induced by gedunin. These results suggest that apoptosis driven by gedunin is followed by a compensatory activation/phosphorylation of these survival pathways. The treatment with 17-AAG also reduced total protein content but did not induce this putative compensatory activation of

STAT3 and ERK pathways within the 24 h incubation. Even though the mean of phosphorylated AKT (pAKT) increased in gedunin-treated cells (**Fig. 4C**), it was not significantly different from untreated cells (**Fig. 4G**).

Figure 4. *Gedunin activates PI3K/AKT and STAT3/ERK 1/2 pathways.* Western blot analysis of the effect of gedunin (6.25 – 100 μM) on intracellular signaling proteins, 24 h after treatment. (A) Representative immunoblots of one experiment out of 8 independent experiments with a similar trend. Box plots of (B) STAT3/PCNA, (C) ERK/PCNA, (D) AKT/PCNA, (E) pSTAT3/STAT3, (F) pERK/ERK, (G) pAKT/AKT intracellular signaling pathways on GL261 cells. 17-AAG was used as the reference control of HSP90 activity inhibition. PCNA was used as the loading control. Results are expressed as the mean ± SEM of protein expression from eight independent experiments. * indicates statistically significant differences (p ≤ 0.05) between treated and untreated cells, whereas # indicates statistically significant differences (p ≤ 0.05) between stimulated and unstimulated cells.

3.5 Gedunin effect on glioblastoma in vivo

The orthotopic C57BL/6 mouse glioblastoma model used in the present study has been established as a standard procedure to reproduce human molecular and histological

features of glioblastoma [59,60]. Biodistribution data obtained by our group show that

^{99m}Tc-labeled-gedunin did not reach substantial levels in the brain tissue when

intravenously injected in mice (Costa et al., *manuscript in preparation*). We, therefore,

assessed whether gedunin *in vivo* treatment was effective by using osmotic pumps

placed *in situ.*

Computed tomography (CT) scans revealed solid tumor growth that occupied an

extensive area of the brain tissue of mice from all groups (white mass, top panels in

black background of **Fig. 5A,** indicated by an asterisk in the upper left image scan).

Three-dimensional reconstructions of CT images are shown in **Figure 5** bottom panels,

in which tumor mass is represented by white over gray (brain) areas. Even though

tumor volume median values (mm^3) of gedunin-treated mice were smaller than those

for untreated or DMSO-injected mice, variability within groups was high (lowest and

highest tumor volume values for each group were: untreated, 21.7 - 75.6; DMSO, 16.8

- 83.6; Ged, 11.7 - 67.4). No significant differences were observed between groups

(ANOVA, p = 0.106; **Fig. 5B, C**). **Supplemental Figure 4** shows CT of mouse brains

with and without tumors, for a better illustration of the tumor mass. Body mass (g) was

measured before GL261 injection and 28 days later and no alterations were observed

(data not shown).

Figure 5. *Brain CT analysis of gedunin-treated mice.* (A) Representative sequential coronal CT-
images and respective 3D brain reconstructions (dorsal view) from untreated, DMSO-injected

and gedunin (250 μM)-treated mice. Lighter regions in 3D reconstructions represent brain tumors. Images were acquired after 14-day *in situ* treatment (Alzet® osmotic minipumps) in GL261 cell-injected mice (1×10^5 cells/brain/2 μL). Boxplot graphs show the ratio between tumor and mouse brain volume values (B) and tumor volume values (C) from 6-8 mice per group out of 2 independent experiments. Tumor volumetry was determined by selecting contrast-enhanced regions of interest in coronal sections using InVesalius 3.1.1 software. CT: computed tomography. *White area corresponds to glioblastoma observed in all CT scans. Dashed line: coronal slice cut point.

Histological examination of coronal brain sections of untreated, DMSO- and gedunin-treated groups revealed that even though gedunin-treated mice had larger tumor-induced lesion areas, they displayed reduced vascular structures (**Table 1**). Areas of liquefactive necrosis, intratumoral hemorrhage, and mitosis were not statistically different between groups. **Figure 6** depicts representative images of GL261-injected mouse coronal brain slices stained with hematoxylin and eosin 28 days after injections. **Figure 6 (A-C)** insets represent 400× magnification of intratumoral perinecrotic regions, in which liquefactive necrosis can be observed by white spaces within the glioblastoma tissue, resulting in the loss of tissue architecture. **Figure 6 (D-F)** displays 1000 × magnification of representative images of tumor tissue showing mitosis, characterized by tumor cell proliferation, and areas of hemorrhage, defined by the presence of red blood cells.

Table 1. **Glioblastoma histology features after gedunin *in vivo* treatment**

	Untreated	DMSO	Gedunin	p
Lesion size	27%	45%	45.2%	NS
Vascular Structures	13	32	8	P< 0.05 *
Liquefactive Necrosis	9.6%	10.6%	8.8%	NS
Intratumoral Hemorrhage	+	++	++	NS
Mitosis	+	++	+	NS

Table 1. *Glioblastoma histology features after gedunin in vivo treatment.* Table shows absolute and semi-quantitative description of the histological analyses of brain slices and p-values of gedunin-treated vs DMSO-treated mice, analyzed by Tukey's post-test. NS= non-significant; += discrete; ++= moderate.

4. Discussion

Uncontrolled proliferation and apoptosis resistance are central features of tumor development, being major targets for cancer treatment [18]. HSP90 is a key factor for tumor growth, since it chaperones several essential proteins involved in oncogenic processes. In the present study, we demonstrate that gedunin, a natural occurring HSP90 inhibitor, impairs glioblastoma cell growth *in vitro*, by arresting proliferation and inducing cell death in non-toxic concentrations to primary brain cells. Gedunin also reduced glioblastoma cell migration, tumor-related signaling pathways, and extracellular metalloproteinase MMP-2 activity, features involved in glioblastoma growth. GL261-orthotopically injected C57BL/6 mice treated with gedunin showed reduced presence of vascular structures, even though no changes were observed in tumor-size.

Data shown in **Supplemental Figure 5** supports previous knowledge that both gedunin and other HSP90-binding agents induce the expression of HSP70 in cancer cells due to

HSP90 inhibition [61–63]. The antiproliferative and cytotoxic effects of gedunin on
GL261 cells are also in accordance with previous *in vitro* results showing similar
antitumoral effects of gedunin against different cell lines [36,38–41]. Worthy of note,
gedunin toxicity against GL261 cells was achieved at a concentration lower than half
the one required to affect primary glial cells, with a selectivity index of 2.3 [64]. This
might be explained by the increased affinity of HSP90 inhibitors to HSP90 machinery
expressed by tumor cells [17], or by biological and metabolic differences between
primary and glioblastoma cells [65]. Gedunin-triggered apoptosis, detected by
phosphatidylserine exposure on the cell surface, was confirmed by flow cytometric
analysis of cell physical criteria that showed the reduced size and increased granularity
of apoptotic cells, due to the formation of apoptotic bodies and plasma membrane
blebbing [49]. Our results clearly show that, as apoptosis progresses, GL261 cell FSC
becomes progressively lower, likely due to cell dehydration and shrinkage. On the other
hand, enhancement of SSC occurs due to cytoplasm and nuclear condensation [49,66].

Glioblastoma is a highly invasive and diffuse tumor that requires cell migration and
extracellular matrix degradation to spread to adjacent tissue [67,68]. Scratch and
chemotaxis assays showed that gedunin diminished GL261 migration, in concentrations
and time points lower than the ones that induced cytotoxicity. Another way by which
gedunin controls tumor invasiveness is by inhibiting MMP-2 activity, with a higher
effect than 17-AAG. Indeed, it has been shown that HSP90 stabilizes and promotes the
activation of extracellular MMP-2, being crucial for GL261 and HT-1080 tumor cell
invasion [69,70].

Our results show that gedunin induced a reduction of total protein in GL261 cells,
which is compatible with the HSP90 inhibition. PI3K/AKT and STAT3 are signaling
pathways that function as pro-survival factors in a wide range of primary tumors,
including glioblastoma [56,71]. Reduced expression of total PI3K and AKT by gedunin
suggests that this limonoid impairs either PI3K/AKT network or an upstream protein,
such as epithelial growth factor receptor (EGFR). In accordance, it has been shown that
gedunin also impaired the PI3K/AKT/mTOR pathway in *in vivo* models of hamster oral
carcinoma [41] and murine pancreatic cancer [38]. Even though PI3K/AKT is the
preferential signaling pathway in glioblastoma, it has been reported that glioblastoma
cells are not exclusively dependent on this pathway and that PI3K/AKT/mTOR-specific

inhibitors might not be effective against this brain tumor [56]. Hence, we evaluated the

effect of gedunin in AKT, ERK 1/2, and STAT3 signaling, which are also altered in

glioblastoma cells and are involved in the cell cycle [72,73]. It is important to note that

even though gedunin treatment reduced total ERK content, it did not inhibit ERK1/2

phosphorylation. On the contrary, ERK was strongly phosphorylated/activated in cells

treated with gedunin. Indeed, Duron et al. showed that HSP90 inhibition can also

prompt the phosphorylation/activation of ERK [74,75]. There might be a compensatory

survival mechanism in GL261 cells that activate ERK when HSP90 is inhibited. This

result must be further explored and points to the perspective of combining gedunin with

ERK inhibitor anti-tumoral drugs.

It has been previously reported in glioblastoma that constitutive activation of STAT3

results from the fusion of the RTK epidermal growth factor receptor (EGFR) with the

GTP-binding protein Septin 14 (SEPT14) [58]. STAT3 signaling promotes glioblastoma

cell survival, and suppresses apoptosis [58]. Abnormal STAT3 activation in

glioblastoma is correlated with the control of several events involved in tumor growth

[71]. The late (24 h) activation of STAT3 by gedunin might also correspond to survival

feedback following the induction of apoptosis. Therefore, gedunin may inhibit GL261

cell proliferation by acting on different pathways that induce apoptosis. Activation of

STAT3 is required for VEGF production [76,77]. Even though we observed that

gedunin impaired signaling pathways (which was expected to impair both VEGF

synthesis and signaling pathway), this phenomenon did not inhibit VEGF release by

GL261 *in vitro*.

As previously mentioned in the results section, even though our findings indicate that

gedunin fits the predicted ADMET criteria for druggability and BBB permeation,

systemic (i.v.) administration of [99m]TC-labeled gedunin failed to reach the brain tissue

at substantial levels (Costa *et al.*, manuscript in preparation). Hence, we used gedunin-

filled Alzet® minipumps to perform *in situ* treatment (resembling the FDA-approved

Gliadel® [carmustine] implants) to ensure that gedunin would be available at the tumor

site. It is important to note that the residual volume of gedunin inside the minipumps

was measured at the end of treatment to ensure that gedunin was released *in situ*, (values

shown in **Suppl. Table 1**). We observed that the number of mice bearing reduced-

volume tumors was higher in gedunin-treated group when compared to DMSO-treated

group, even though this treatment did not significantly reduce tumor volumes detected by CT imaging. Regarding our CT imaging results, it is important to emphasize the limitation of the technique, since the limited spatial resolution of clinical CT scanners might not be precise to analyze such a small brain [78,79]. While our findings reinforce the feasibility of using clinical CT scanners for *in vivo* brain tumor imaging in mice, their use for detecting treatment responses in tumor volume requires further investigation, since clinical CT scans are made for imaging humans that have brain volumes roughly 3,000 times greater than mice [80–82]. Another limitation is that mouse tumor sizes were not evaluated over the days, since CT scan was only feasible in mice with no minipump implants, due to the stainless steel of brain infusion cannulas.

As expected, GL261-injections triggered the development of mouse glioblastoma in orthotopically-injected C57BL/6 mouse with liquefactive necrosis, hemorrhage, mitosis and increased vascular structures [59]. *In situ* treatment with gedunin reduced the presence of vascular structures, which might decrease tumor nutritional supply. Considering that gedunin increased the production of VEGF *in vitro*, this finding might suggest that gedunin impairs angiogenesis in GL261 tumors by a mechanism independent of VEGF *in vivo* as previously described [83]. Despite the reduction of vascular structures, there was no evidence of reduction in tumor size. Moreover, the impact of the reduction in vascular structures on the survival rate of orthotopically-injected C57BL/6 mice was not evaluated. Therefore, whether gedunin *in vivo* treatment impairs mouse glioblastoma growth needs to be further investigated, including *in situ* measurement of gedunin release from minipumps and VEGF contents. Noteworthy, both DMSO- and gedunin-treated mice showed increased lesion areas when compared to untreated mice, which might be a consequence of the surgical procedure required for minipump implantation. It is possible that an optimized treatment protocol including a postoperative treatment (as generally performed in glioblastoma patients) and/or association with other drugs result in a better outcome.

The severity and the poor prognosis of patients bearing glioblastoma urge for new therapeutical targets and drugs. Data presented in this paper sheds light on alternative treatments for this type of tumor by revealing GL261 dependency of HSP90 and gedunin as a potential lead compound.

Author contributions

CP, TEMMC, and TEK conceived and designed the work. TEMMC, CP, TEK, ACM,
and MGH designed experiments. TEMMC, TAP, LNS, EMC, CCF, CMMM, JXP,
VGR, and TEK performed the experiments, acquired, and/or analyzed data. CP, TEK,
ACM, YAV, JXC, and MGH contributed with funding and resources. CP supervised the
work. CP and TEMMC wrote the original draft. CMMM and TEK wrote and revised
the manuscript. All authors revised and edited the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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