

## Corilagin Induces High Levels of Apoptosis in the Temozolomide-Resistant T98G Glioma Cell Line

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Glioblastoma multiforme (GBM), a malignant tumor of the central nervous system, has a high mortality rate. No curative treatment is presently available, and the most commonly used chemotherapeutic drug, the alkylating agent temozolomide (TMZ), is only able to increase life expectancy and is often associated with drug resistance. Therefore, an urgent need does exist for novel drugs aimed at treating gliomas. In the present study, we obtained three major results using corilagin: (a) demonstrated that it inhibits the growth of U251 glioma cells through activation of the apoptotic pathway; (b) demonstrated that it is also active on TMZ-resistant T98G glioma cells; and (c) demonstrated that when used in combination with TMZ on T98G glioma cells, a higher level of proapoptotic and antiproliferative effects is observed. Our study indicates that corilagin should be investigated in more detail to determine whether it can be developed as a potential therapeutic agent. In addition, our results suggest that corilagin could be used in combination with low doses of other standard anticancer chemotherapeutic drugs against gliomas (such as TMZ) with the aim of obtaining enhanced anticancer effects.

**Key words:** Glioma; Temozolomide (TMZ); Corilagin (CORL); Apoptosis

### INTRODUCTION

Glioblastoma multiforme (GBM) is a lethal malignant tumor accounting for 42% of central nervous system tumors, with median survival of 15 months<sup>1</sup>. At present, no curative treatment is available, and the most frequently used drug, the alkylating agent temozolomide (TMZ), is not satisfactory, being only able to cause an increase in life expectancy for the treated patients<sup>2</sup>. Therefore, new drugs are urgently needed for validation and possible use in therapeutic protocols for anti-glioma treatments<sup>3</sup>. Moreover, a high proportion of gliomas become resistant to TMZ over time<sup>4</sup>, making the search for new drugs or drugs to be used in combination on TMZ-resistant glioma cells very significant, with several reports being published recently. For example, Lan et al. recently demonstrated that sulforaphane reverses chemoresistance to TMZ<sup>5</sup>, while others have shown that combined treatments with lobarstin<sup>6</sup>, bortezomib<sup>7</sup>, quercetin<sup>8</sup>, methoxyamine<sup>9</sup>, and resveratrol<sup>10</sup> sensitize resistant glioblastoma cell lines to TMZ. In addition, nucleic acid-based strategies such as

AKT3 and PI3KCA siRNAs<sup>11</sup> were developed to enhance the antitumor effects of TMZ.

One of the most interesting low-molecular weight drugs recently developed is corilagin [CORL, beta-1-*O*-galloyl-3,6-(*R*)-hexahydroxydiphenoyl-*D*-glucose]<sup>12–14</sup>, a gallotannin found in several extracts from medicinal plants (such as *Phyllanthus urinaria*), which was shown to have versatile medicinal activities, including hepatoprotective effects on male Sprague–Dawley rats induced with galactosamine and lipopolysaccharide<sup>15</sup>. Recently, it was also reported that corilagin inhibits radiation-induced microglia activation through suppression of the nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathway, suggesting that this compound is a potential agent for the treatment of radiation-induced brain injury<sup>16</sup>. These putative effects on NF- $\kappa$ B are relevant in the context of the search for novel compounds in glioma therapy<sup>17–19</sup>. In fact, NF- $\kappa$ B inhibitors are expected to exert potent apoptosis-inducing effects, which are the basis of the antitumor effects of anti-glioma agents, including TMZ<sup>20–23</sup>.

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The aims of the present study were to determine the activity of CORL on cell growth and apoptosis of the human U251 and T98G glioma cell lines, in order to verify whether CORL exerts cell growth inhibitory and proapoptotic activities. Furthermore, we aimed to determine whether CORL and TMZ can act synergistically and induce higher levels of apoptosis when administered in combination to glioma cell lines, with the objective to verify the possibility of a combined treatment protocol for gliomas.

## MATERIALS AND METHODS

### *Glioma Cell Lines and Culture Conditions*

U251 and T98G glioma cell lines<sup>24,25</sup> were cultured in a humidified atmosphere of 5% CO<sub>2</sub>/air in RPMI-1640 medium (Life Technologies, Monza, Italy) supplemented with 10% fetal bovine serum (FBS; Celbio, Milan, Italy), 100 U/ml penicillin, and 100 mg/ml streptomycin. To determine the effect on proliferation, cell growth was monitored by determining the cell number/ml using a Z2 Beckman Coulter Counter (Beckman Coulter, Pasadena, CA, USA)<sup>26</sup>.

### *Computational Studies*

All computational studies were carried out on a 4 CPU (Intel Core2 Quad CPU Q9550, 2.83 GHz) ACPI x64 Linux workstation with the Ubuntu 12.04 operating system. The 3D structure of NF- $\kappa$ B was obtained from the Protein Data Bank (PDB code: 1NFK), and the structure of CORL was obtained with MarvinSketch 5.5 software (Marvin, version 5.5.0.1, Program B; ChemAxon: Budapest, Hungary; www.chemaxon.com/products). The lowest energy conformation and the degree of protonation (pH 7.2) were determined with the OpenBabel 2.2.3 software (DOI: 10.1186/1758-2946-3-33), using the MMFF94s force field. The docking simulation was performed with AutoDock 4.2<sup>27-31</sup>. The grid box for AutoGrid was centered at  $x=-11.11$ ,  $y=18.23$ ,  $z=15.33$  (Zhang et al.<sup>31</sup>) with 0.375 grid spacing and 40×40×40 point dimensions. The Lamarckian Genetic Algorithm (LGA) was used as a conformational search engine, and 10 runs with a maximum of 2,500,000 energy evaluations were carried out. The lowest energy conformation was retained and analyzed. The images were rendered with Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC)<sup>28</sup>.

### *Analysis of Apoptosis*

Annexin V and Dead Cell assay on U251 and T98G cells, untreated and treated for 48 h with TMZ (400  $\mu$ M) and CORL (35  $\mu$ M), was performed with the “Muse” (Millipore Corporation, Billerica, MA, USA) method, following the manufacturer’s instructions. This procedure

uses annexin V to detect phosphatidylserine (PS) on the external membrane of apoptotic cells. A dead cell marker is also used as an indicator of cell membrane structural integrity. Briefly, cells were washed with sterile 1× phosphate-buffered saline (PBS), trypsinized, resuspended, and diluted (1:2) with the one-step addition of the Muse Annexin V & Dead Cell reagent. After a 20-min incubation at room temperature in the dark, samples were analyzed. Data from samples were acquired utilizing the Annexin V and Dead Cell Software Module (Millipore)<sup>26,29</sup>. The same culture conditions were followed for the caspase 3/7 assay. This analysis was performed after 48 h of treatment using the two-step Muse Caspase 3/7 reagent. Briefly, 50  $\mu$ l of cell suspension, corresponding to about  $1 \times 10^5$  cells/ml, was centrifuged (1,200 rpm×5 min) and resuspended in 25  $\mu$ l of 1× assay buffer BA. The Muse Caspase 3/7 reagent was added to each sample and incubated for 30 min at 37°C in the dark. Muse caspase 7-AAD was subsequently added, and, after 5 min, the assay was run using the Muse Cell Analyzer. Four populations of cells could be distinguished in both assays: live, apoptotic, late apoptotic, and dead cells.

### *Scratch Wound Assay*

T98G cells were seeded into a 24-well plate at a confluence of 80%; 24 h later, a vertical wound was created in the T98G cell monolayer using a 200- $\mu$ l pipette tip. After washing three times with PBS to eliminate cellular debris, fresh medium was added back, and the T98G cells were either untreated or treated with 35  $\mu$ M COR, 400  $\mu$ M TMZ, and the two drugs together. Images were captured with a camera connected to a Nikon Eclipse TS100 microscope at designated times (0, 16, 24, and 48 h) to assess the rate of gap closure<sup>30</sup>.

### *Fluorometric TUNEL Assay*

T98G apoptotic cells were detected and quantified using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) that measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using terminal deoxynucleotidyl transferase (TdT), which forms a polymeric tail. T98G cells were cultured in poly-L-lysine-coated culture slides; about  $1 \times 10^4$  cells were seeded into each slide chamber, and after 24 h they were treated with different compounds. Fluorometric TUNEL assay was performed after 48 h according to the manufacturer’s protocol. Briefly, T98G cells were fixed with formaldehyde and permeabilized, and, after an equilibration step, the TdT reaction mix (containing equilibration buffer, nucleotide mix, and TdT) was added to each sample, which was further incubated for 60 min, after which the cells were washed with PBS. The reaction was then stopped, the cells washed with PBS, and then

stained with propidium iodide. Finally, the fluorescein-12-dUTP-labeled DNA that identifies apoptotic cells could be visualized by fluorescence microscopy using a Nikon Eclipse 80i microscope.

#### Statistics

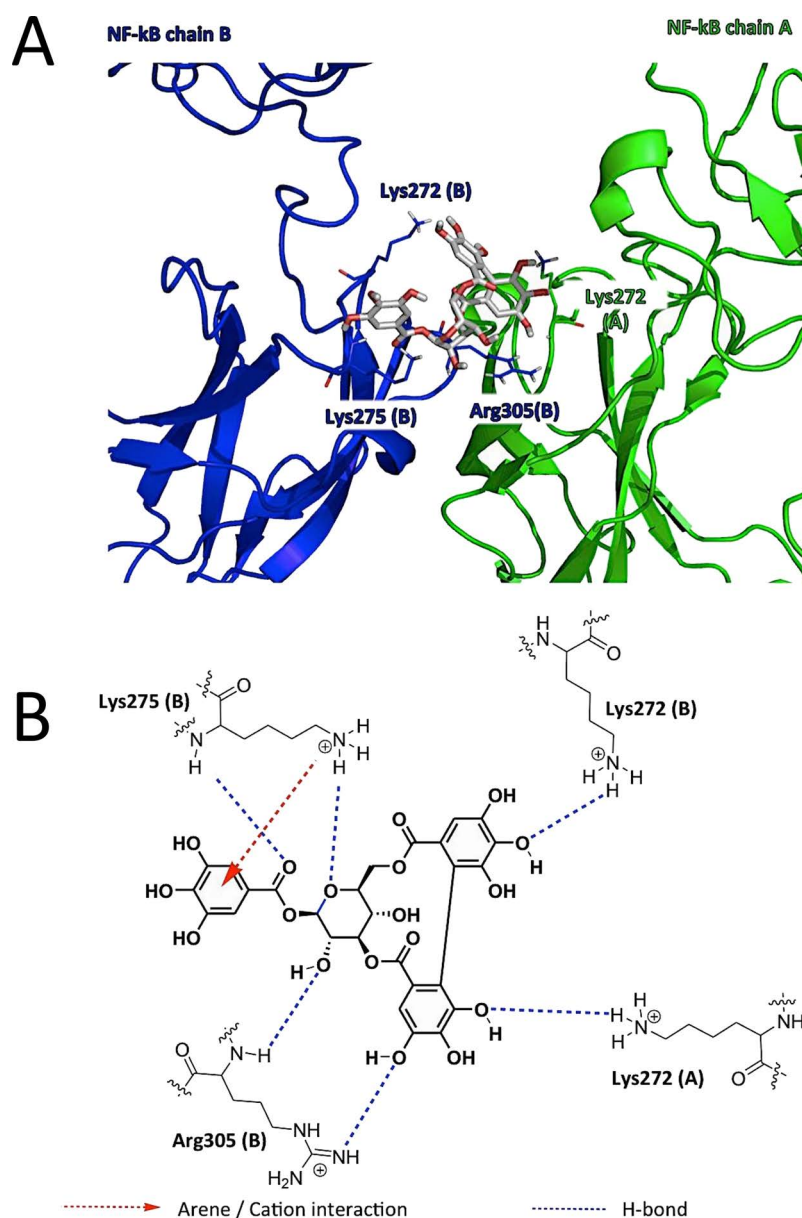
All data are presented as mean  $\pm$  SD. Statistical differences between groups were compared using one-way ANOVA (analyses of variance between groups). The  $p$  values were obtained using the paired  $t$ -test of the GraphPad Prism Software. Statistical differences were

considered significant with a value of  $p < 0.05$  and highly significant with a value of  $p < 0.01$ .

## RESULTS

#### Computational Studies

Following the hypothesis of a possible interaction with NF- $\kappa$ B, docking studies were performed to identify a possible binding mode for CORL (see the experimental section for a detailed description of the docking experiments). The CORL was proposed to establish H bonds

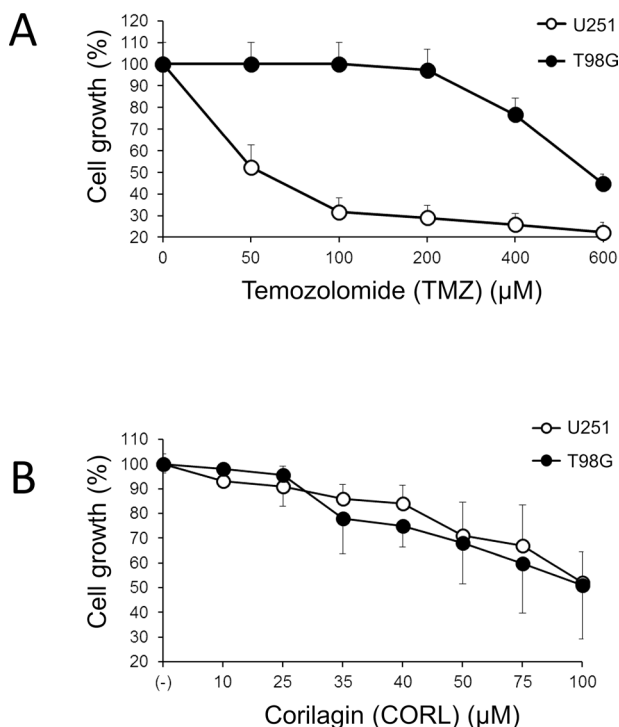


**Figure 1.** (A) Proposed binding site of corilagin (CORL) in the DNA-binding region of NF- $\kappa$ B; detail of the amino acid residues involved in the proposed binding mode of CORL (atom colored stick) with NF- $\kappa$ B (green and blue). (B) Schematic representation of the interactions revealed by molecular docking simulation between CORL and NF- $\kappa$ B.

and one arene-cation interactions with the positively charged amino acid residues (Lys and Arg), abundant in the DNA-binding region of NF- $\kappa$ B, thus impairing the interaction between the transcription factor and the nucleic acids (Fig. 1A; summarized in Fig. 1B). This possible mechanism of action of CORL is clearly different to that proposed for TMZ, which has been hypothesized to methylate the DNA<sup>31</sup>.

#### *Corilagin Inhibits Cell Growth of Glioma U251 and the TMZ-Resistant T98G Cells*

Cell lines U251 and T98G display a significantly different response to in vitro treatment with TMZ (Fig. 2A). The IC<sub>50</sub> for U251 was 25–50  $\mu$ M, while for T98G it was about 560  $\mu$ M. On the contrary, both U251 and T98G exhibited a similar response to CORL treatment, with both cell lines having IC<sub>50</sub> values close to 70–85  $\mu$ M (Fig. 2B). The results of this experiment support the concept that CORL might be proposed as an antiproliferative agent also for drug-resistant glioma cell lines.



**Figure 2.** Effects of treatment of U251 and T98G glioma cells with the indicated concentrations of (A) temozolomide (TMZ) and (B) corilagin (CORL). Cells were seeded at the concentration of  $3.5 \times 10^4$  cells/ml and cultured for 48 h in the presence of TMZ or CORL, as indicated. Each experiment was performed three times and the percent mean  $\pm$  SD of cell growth was calculated.

#### *Corilagin Induces Apoptosis of Glioma U251 and T98G Cell Lines*

Taking into consideration the fact that a possible target of CORL is the antiapoptotic factor NF- $\kappa$ B, the effects of CORL on apoptosis were studied. The antiproliferative activity of CORL was associated with the activation of the apoptotic pathway in both the U251 and T98G cell lines, as judged by Annexin V (Fig. 3A and C) and Caspase 3/7 assays (Fig. 3B and C). Only a minor difference in the dose-response activity was apparent when CORL was used on U251 and T98G cells. In both cases, a high percentage of U251 and T98G cells were positive in the Annexin V and Caspase 3/7 assays when CORL was administered. This result supports the concept that the antiproliferative effects of CORL are associated with activation of the apoptotic pathway.

#### *Effects of Combined Treatment of TMZ and CORL in the TMZ-Resistant T98G Glioma Cell Line*

A representative experiment demonstrated that combined treatment of TMZ-resistant glioma T98G cells with both CORL and TMZ leads to a significant early apoptotic effect, evaluated by the Annexin V (Fig. 4A) and Caspase 3/7 (Fig. 4B) assays. This effect is synergistic and higher than that obtained by the single administrations of either TMZ or CORL. The summary of three independent experiments is shown in Figure 5. Increased apoptosis in cells treated with both TMZ and CORL (Fig. 5B and C) is associated with a decrease in cell growth rate (Fig. 5D). The significant effects on apoptosis of the combined treatment with TMZ+CORL were also confirmed by the TUNEL assay shown in Figure 5A.

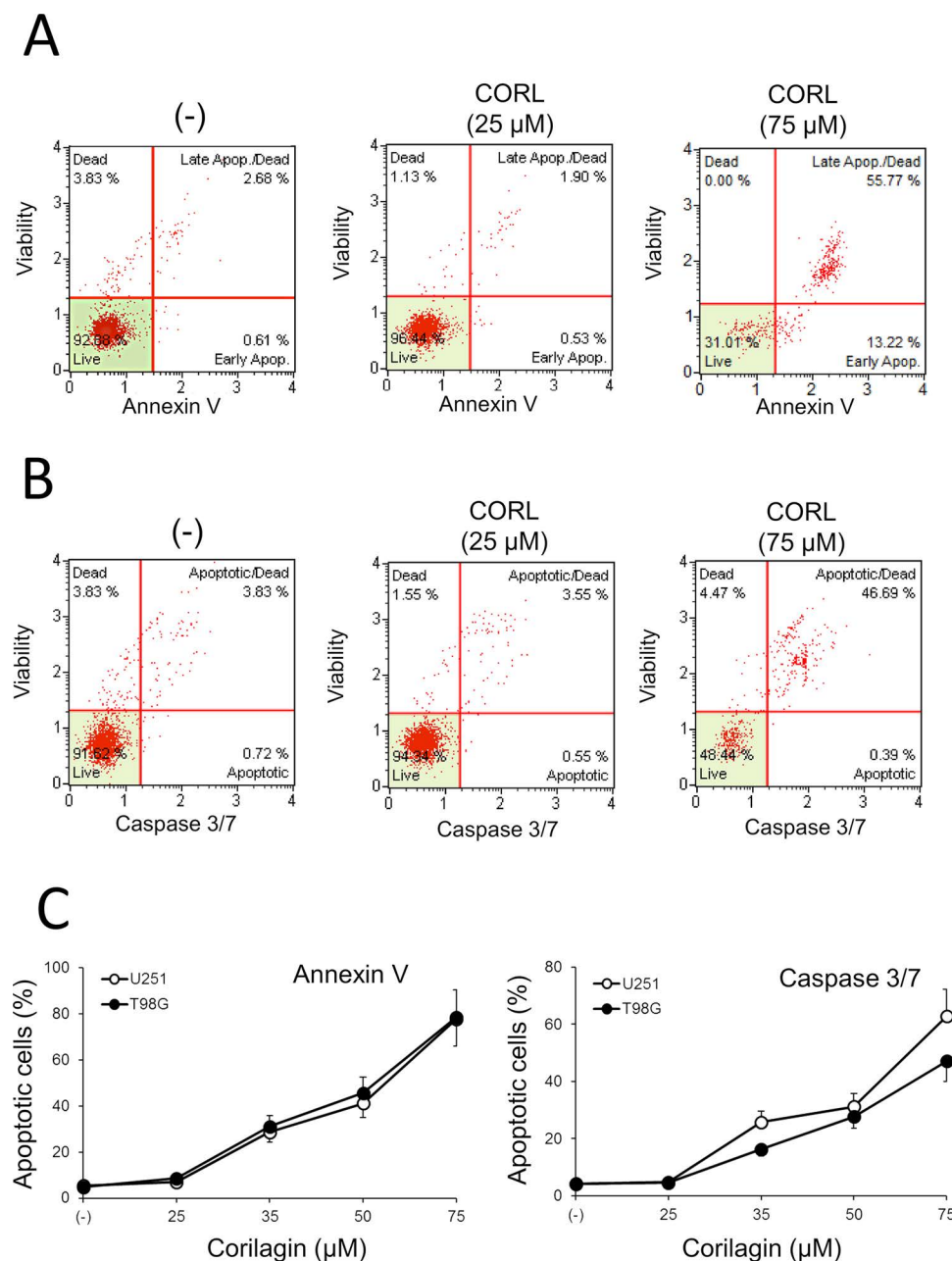
Figure 6 shows a strong inhibition of T98G cell growth when a scratch wound healing assay was performed. T98G cells were cultured in a 24-well plate in adherent monolayer. After 24 h, when T98G monolayer cells were confluent, a 200- $\mu$ l pipette tip was used to scratch the cells in the plates, and the cells were then washed three times with PBS. The cells were then placed in fresh medium and divided as follows: the control (untreated T98G cells) (Fig. 6A–H), TMZ-treated T98G cells, CORL-treated T98G cells, and cells treated with both TMZ and CORL. Samples were taken at the beginning and after 48 h of cell culture with 5% CO<sub>2</sub> at 37°C. Each treatment was compared with the control (untreated T98G cells). The experiments were repeated three times. The results obtained demonstrated that the scratch wounds were almost the same size in each experimental group at 0 h; however, the healing and cell migration rate was significantly reduced ( $p < 0.05$ ) in the CORL+TMZ-treated cells after 48 h (Fig. 6K) compared with the control

(Fig. 6H) and cells treated with singular administration of TMZ (Fig. 6J) and CORL (Fig. 6I).

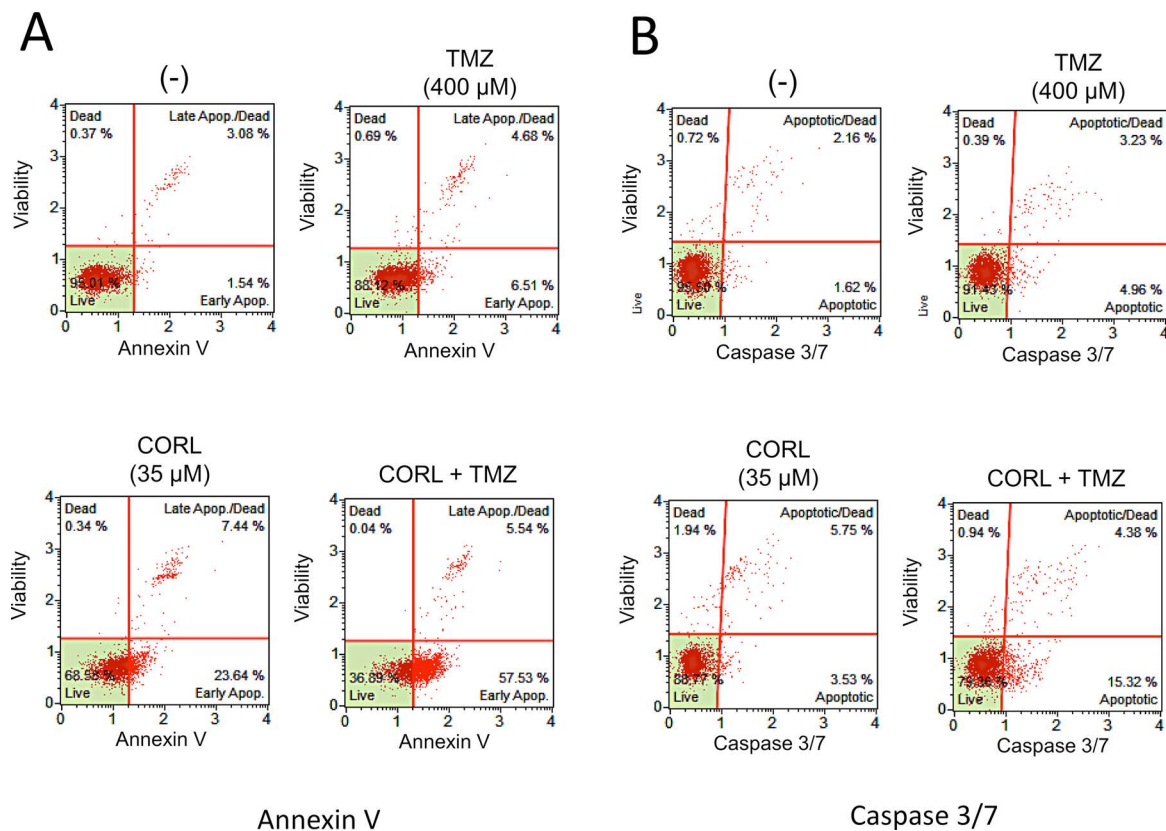
### DISCUSSION

The major conclusions of our study are the following: (a) CORL inhibits cell growth of U251 glioma cells through activation of the apoptotic pathway; (b) CORL

is active also on TMZ-resistant T98G glioma cells; and (c) when T98G glioma cells are treated with both TMZ and CORL, a higher level of proapoptotic and antiproliferative effects was obtained. Our study highlights the important biological effects of CORL and indicates that it deserves further attention, particularly in the development of effective therapeutic protocols, as suggested for



**Figure 3.** Representative analysis of apoptosis of T98G glioma cells cultured for 48 h in the absence or in the presence of the indicated concentrations of CORL. Apoptosis was assessed by the Annexin V (A) or Caspase 3/7 (B) assays. (C) Increase in apoptotic cells in U251 and T98G glioma cell lines treated with the indicated concentrations of CORL. Annexin V (left) and Caspase 3/7 (right) assays were performed after 48 h of cell culture. The results represent the average  $\pm$ SD of four independent experiments.



**Figure 4.** Representative analysis of apoptosis of T98G glioma cells cultured for 48 h in the absence or in the presence of the indicated concentrations of TMZ and CORL. Apoptosis was assessed by the Annexin V (A) or Caspase 3/7 (B) assays.

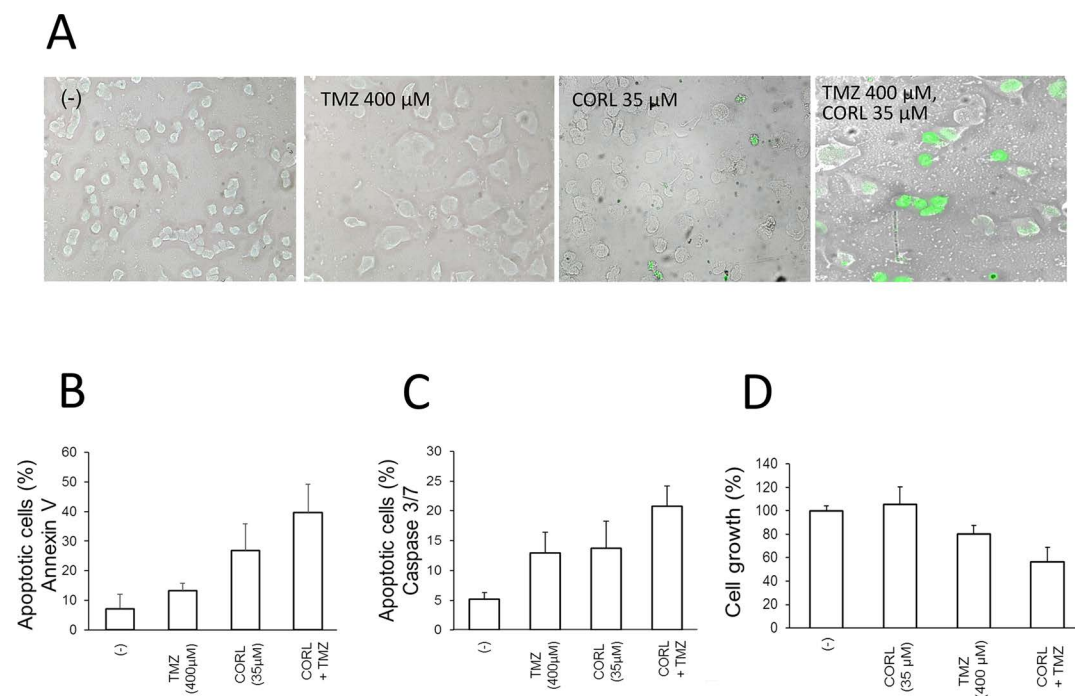
other low-molecular weight molecules demonstrated to be capable of increasing the effects of TMZ<sup>5-10</sup>.

The inhibitory activity of CORL on tumor cell growth has been the object of several investigations<sup>32-34</sup>. For instance, we have demonstrated the potential *in vivo* anti-tumor activity of CORL using the Hep3B hepatocellular carcinoma cell line and an athymic nude mice xenograft model<sup>32</sup>. CORL was administered intraperitoneally for a continuous period of 7 days at a concentration of 15 mg/kg of body weight per day. A significant inhibition of tumor growth was observed when treated mice are compared with control groups<sup>32</sup>. In addition, we have recently reported the potential sensitization of Hep3B hepatoma cells to cisplatin and doxorubicin by CORL. Our results showed that CORL is able to enhance the cytotoxicity of both cisplatin and doxorubicin on Hep3B hepatoma cells<sup>33</sup>. Other interesting effects of CORL, both *in vitro* and *in vivo*, have been reported. For example, Jia et al. demonstrated that corilagin inhibits ovarian cancer cell growth by inducing an arrest at the G<sub>2</sub>/M cell cycle stage and enhancing apoptosis<sup>13</sup>. Interestingly, weaker effects were found on normal ovarian surface epithelial cells. Furthermore, Gu et al. reported a suppression of cholangiocarcinoma (CCA) progression *in vivo* after CORL administration<sup>30</sup>.

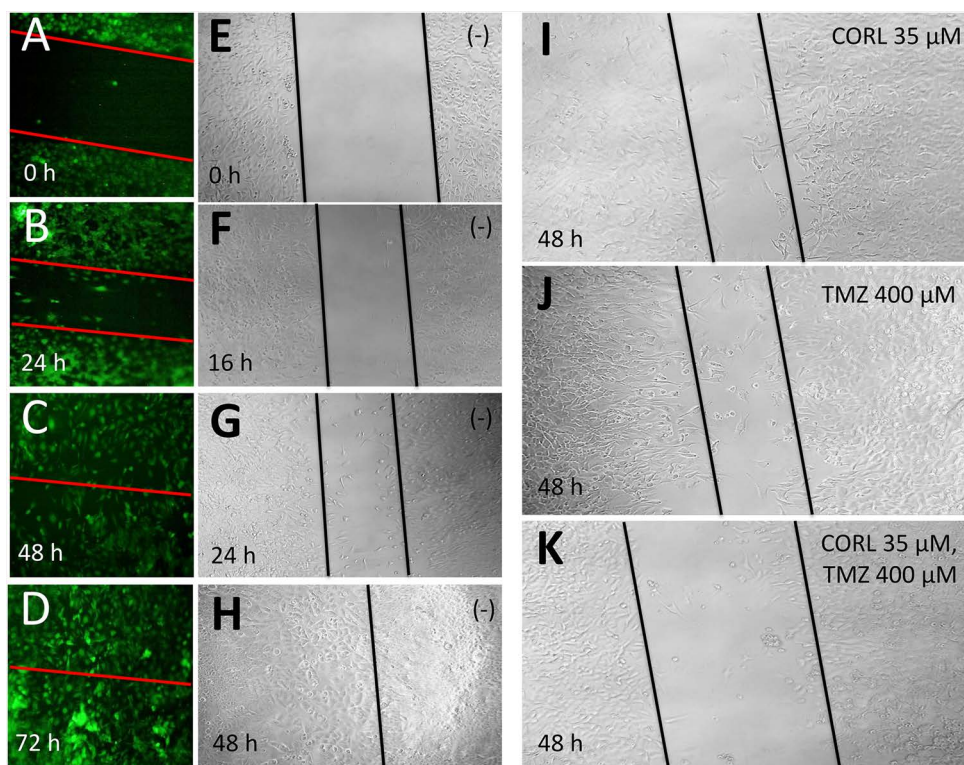
As far as mechanism of action is concerned, our results, combined with other reports, suggest that CORL induces the observed biological effects by interfering with the antiapoptotic NF- $\kappa$ B transcription factor<sup>14,35</sup>. Nevertheless, it is likely that other biochemical pathways may be operating, including the CORL-mediated block of the TGF- $\beta$  signaling pathway, as suggested by Jia et al.<sup>13</sup> The latter authors found that CORL inhibits TGF- $\beta$  secretion by ovarian cancer cell lines and blocks the TGF- $\beta$ -induced stabilization of Snail. In addition, an effect of CORL on the Notch signaling pathway was recently reported<sup>30</sup>, leading to inhibition of CCA development. In the studied experimental model, CORL significantly inhibited Notch1 and mTOR protein expression *in vivo*<sup>30</sup>.

Our present study should be considered as a proof of principle, supporting the possible use of CORL for the induction of high levels of apoptosis and inhibition of *in vitro* tumor cell growth in TMZ-resistant glioma cells.

Before proposing a possible therapeutic use of CORL in combination with low dosages of other anticancer chemotherapeutic standard drugs against gliomas (such as TMZ), further studies should be undertaken, based on the use of primary glioma cells, *in vivo* model systems mimicking gliomas, and *in vivo* pilot clinical trials on



**Figure 5.** (A) Increase in apoptotic T98G glioma cells after treatment with CORL or TMZ or a combination of both TMZ+CORL. Effects of combined CORL+TMZ treatment of T98G cells on apoptosis (B, C) and cell proliferation (D). Apoptosis was analyzed with Annexin V (B) and Caspase 3/7 (C) assays performed after 48 h of cell culture. The effects on cell growth were determined after 48 h (D). The results show the mean  $\pm$ SD of four independent experiments.



**Figure 6.** Effects of the treatments with CORL and TMZ (either added alone or in combination, as indicated in I–K) on T98G cell growth, determined by the scratch wound assay. (A–H) untreated cells (–).

selected glioblastoma patients. These studies will allow other investigators in the field to determine the real relevance and impact on clinical settings of our data.

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