Glioma Regression In vitro and In vivo by a Suicide Combined Treatment

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Abstract

We present here a suicide therapy against malignant gliomas based on the transfer to tumor cells of a gene encoding a β-glucosidase, linamarase (lis), which in the presence of the innocuous substrate linamarin (lin) produces cyanide, blocking the mitochondrial respiratory chain. Dog glioma cells carrying the lis gene are thus sensitive to lin (IC50 of 250 μg/mL at 48 hours) and cell death is accompanied by mitochondrial fission and ATP depletion. The combination of lis/lin with an otherwise nontoxic level of glucose oxidase (GO) enhances the therapeutic potential (IC50 of 50 μg/mL at 48 hours). GO produces hydrogen peroxide, inducing oxidative damage and increasing cellular stress. We show here the antitumoral effect of the lis/lin/GO therapy in a canine glioma cell line and in a xenograft glioma model in nude mice. The synergetic combination causes mitochondrial membrane depolarization and phosphatidylserine externalization and accelerates death by 48 hours. The lethal process is caspase independent; poly(ADP-ribose) polymerase 1 is not implicated; and there is no apoptosis-inducing factor translocation to the nucleus. The combined system induces autophagic cell death that can be rescued by 3-methyladenine and is characterized by the presence of double-membrane vesicles and punctate LC-3 pattern. (Mol Cancer Res 2008;6(3):407–17)

Introduction

Malignant gliomas are very invasive and tumors generally recur within a year despite aggressive treatment using radical surgery, radiation, and chemotherapy (1); therefore, novel strategies are essential to improve the prognosis. We are here describing a system based on the linamarase plant gene (lis) that encodes a cyanogenic β-glucosidase that hydrolyses linamarin (lin; 2-hydroxy isobutryronitrile-β-d-glucopyranoside) to acetone, glucose, and cyanide (2). When expressed in cells, linamarase is exported and cyanide is produced in the extracellular environment. The toxic molecule diffuses freely across cellular membranes, inducing a potent bystander effect that amplifies the therapeutic potential and counteracts the poor transduction efficiencies attained in vivo (3). Cyanide inhibits the cytochrome c oxidase of the mitochondrial respiratory chain, blocking the oxidative phosphorylation. Targeting the main energy source of the cell, cyanide induces a rapid ATP depletion and a drop in the mitochondrial ΔΨ, causing DNA fragmentation and cell death (4, 5). The uncoupling of the respiratory chain at complex IV level by cyanide also induces electron escape, especially through complex I and III, which could react with oxygen molecules generating reactive oxygen species, mainly superoxide radicals and hydrogen peroxide (H2O2; ref. 6). The Aspergillus niger glucose oxidase (GO) enzyme catalyzes the conversion of glucose to gluconic acid, generating hydrogen peroxide (7), which can diffuse freely through the cellular membranes generating oxidative damage (8). Some studies have used GO with therapeutic purposes by inducing oxidative stress in tumor cells (9, 10). In this study, we have combined a subtoxic amount of GO with the lis/lin system to increase the cellular stress to induce neoplastic cell death.

Autophagy is a mechanism for the degradation and recycling of cellular components in the cytoplasm. The process implies sequestering of cytoplasmic cargo within double-membrane vacuoles called autophagosomes, followed by their fusion with lysosomes for degradation. Although autophagy promotes a cell survival response, morphologic features of this process have also been observed in dying cells in cases in which caspases, the most important enzymes of apoptosis, were suppressed or not sufficiently activated (11-13). Inducing autophagic death seems a good strategy to be used as cancer therapy because the pathway is conserved in tumors conferring to neoplastic cells an adaptive advantage to survive under the stress conditions usually present within the tumor mass (14, 15). Additionally, it has been described that autophagy mediates cell death in malignant glioma cells treated with chemotherapeutics such as arsenic trioxide and temozolomide (16, 17).

Results

The lis/lin and lis/lin/GO Death Induction

The dog glioblastoma cell line (W&W) was stably transfected with a plasmid carrying lis, generating W&Wis cells. When linamarin is added to these cells, they die proportionally...
to the linamarin concentration (Fig. 1A) due to cyanide production (Supplementary Fig. S1) with an estimated IC50 of 250 µg/mL at 48 hours. Cyanide toxicity has double components: the ATP depletion caused by the oxidative phosphorylation blockage and the reactive oxygen species production. The lethal effect of the system is improved by its combination with a subtoxic amount of the enzyme GO [5 enzymatic milliunits (mEU)/mL], which reacts with glucose to produce gluconic acid and hydrogen peroxide. In the absence of GO, lin led to the total W&Wis cell culture death in 96 hours. When these cells were treated with both lin and GO, cell death was completed 48 hours earlier and the IC50 at 48 hours was 50 µg/mL lin. The GO addition improved the cell sensitivity to linamarin, having no noticeable killing effect when used alone. This increase in sensitivity was not associated with a significant increment in cyanide production or with a putative glucose level depletion originated by the GO reaction (Fig. 1B and Supplementary Fig. S1). We may thus conclude that the hydrogen peroxide production is the determinant element that accelerates cell death. To confirm this hypothesis, cell survival percentages were studied in W&Wis cells in the presence of the thiol-containing antioxidant N-acetyl-L-cysteine. The lis/lin system was not significantly affected by the presence of the antioxidant whereas N-acetyl-L-cysteine delayed cell death in the combined lis/lin/GO system (Fig. 1C). A similar result was obtained using tiron, another antioxidant (Fig. 1D). The results suggest that the oxidative stress generated as a result of the peroxide production by GO is responsible for the observed cell death precipitation. Additionally, we have measured oxidative stress, mainly superoxide anion, with dihydroethidium, showing that, although GO or lin separately significantly increased the cellular oxidative stress with respect to the control, the combination of both insults caused a large synergism in reactive oxygen species production (Fig. 1E).

Tumor Gene Therapy in Nude Mice

Nude mice were first inoculated with cells that already expressed linamarase (W&Wis) to optimize the therapy doses, showing the best therapeutic effect with the combination of 0.25 mg of lin and 0.1 enzymatic unit (EU) of GO daily per gram of animal weight (Supplementary Fig. S2). In an attempt to make a closer approach to a putative human therapy, we have then used adenovirus as the linamarase therapy vector. This vector infects W&W cells very efficiently, inducing high sensitivity to linamarin addition due to cyanide production (Supplementary Fig. S3). W&W cells were injected in nude mice, which, once their tumors reached ~50 mm3, were treated with a local injection of adenolis, lin, and GO (Fig. 2A). The results showed that the difference between treated and untreated tumors was statistically significant from the 11th day of treatment. These results are comparable to the ones obtained in animals inoculated with W&Wis cells and daily treated with lin and GO (Fig. 2B). In those cases, significant differences were obtained from the 9th day of treatment. Control treatments only with adenolis [up to 5 x 109 infective units (IU)] or GO (up to 0.25 EU/g) did not have any therapeutic effect (data not shown). These results show both the antitumor effect of the combination lis/lin/GO and the effectiveness of using adenolis as therapeutic vectors in vivo.

Mitochondrial Morphologic Changes in lis/lin and lis/lin/GO Cell Death

Most cell death stimuli have a mitochondrial step involving morphologic as well as outer membrane permeabilization changes; therefore, we studied the morphologic changes in this organelle. Representative images of W&W cells transfected with the plasmid pDsRed2-Mito showed the disruption of the filamentous mitochondria pattern in lin-treated cells after 48 hours, becoming more pronounced at 72 hours (Fig. 3A). This was characterized by a change in the shape of the organelle, its filament fission, plus the appearance of small round bodies and slight swelling. In addition, whereas cells treated only with GO did not show any detectable morphologic changes in mitochondrial structure, the combination lin/GO again induced fission and pronounced swelling from 24 hours onward. At 48 hours, a perinuclear pattern of extremely swollen mitochondria was visible. The time course of this process during the first 24 hours (Fig. 3B) showed that, in both lin- and lin/GO–treated cells, mitochondrial disruption appeared in the first 3 hours, affecting up to about half of the cellular population by 12 hours (data not shown). After 24 hours, the entire cell culture showed a mitochondrial fragmented pattern, being highly swollen in the case of the lin/GO combined treatment. We may thus conclude that the mitochondrial fragmentation observed is primarily due to the oxidative phosphorylation blockage caused by the cyanide produced by lis/lin, and that the presence of GO increases swelling and clusters mitochondria to a perinuclear pattern.

Determination of Intracellular ATP Levels

Depletion of cellular ATP predisposes to necrotic death (18, 19). W&Wis cells showed a rapid decrease in ATP levels by lin treatment, which was totally accomplished in 48 hours (Fig. 3C). In the combined lin/GO treatment, we observed a behavior parallel to the lin treatment, which was slightly more pronounced. These results show that the determinant element for the observed ATP depletion is mainly the oxidative phosphorylation inhibition caused by cyanide. Thus, the observed advance in the time of death in the combined lis/lin/GO does not seem to be due to a faster depletion in ATP levels caused by the contribution of GO.

Loss of Mitochondrial Membrane Potential

We also studied the changes in the mitochondrial potential (∆Ψm) as a marker of the mitochondrial function. Cyanide (lis/lin) did not induce depolarization of the mitochondrial membrane during the first 48 hours (Fig. 3D), despite the severe mitochondrial fragmentation and complete ATP depletion described above by that time. On the other hand, the combination with GO produced a total loss of mitochondrial membrane potential that decreased progressively from 24 hours onward. As expected, N-acetyl-L-cysteine addition to the lis/lin/GO system partially protected ∆Ψm, suggesting that the oxidative stress generated by GO provides an extra death signal responsible for the potential loss.

We may conclude that the lis/lin system induces mitochondrial fragmentation and total ATP depletion in the first 48 hours but mitochondria still maintains its ∆Ψm. The GO addition to the system creates an extra stress signal that induces total mitochondrial potential loss, mitochondrial swelling, and rapid cell death.
FIGURE 1. The lis/lin and lis/lin/GO systems. A. Survival percentages of W&W lis cells treated with different concentrations of linamarin (lin 0, 50, 200, or 500 μg/mL) in the absence or presence of GO (5 mEU/mL) at different times. B. Survival percentages of W&W lis cells in the absence or presence of lin (500 μg/mL; lis) or the cells also treated with GO (lis/GO) at 48 h. Cyanide production and glucose concentration in the medium in these conditions. C. Survival percentages of W&W lis cells at different times in the presence of lin (0 or 500 μg/mL) and in the absence or presence of N-acetyl-L-cysteine (NAC; 10 mmol/L; left), and the same analysis when the cells were also treated with GO (right). D. Survival percentages of W&W lis treated with lin (500 μg/mL) and GO (5 mUE/mL) in the absence or presence of tiron (1 mmol/L) at 30 h. E. Reactive oxygen species (ROS) measurements of W&W lis treated with lin (500 μg/mL), GO (5 mUE/mL), and NAC (10 mmol/L) with 4 μmol/L dihydroethidium at 36 h. Points and columns, mean of three independent samples; bars, SD. *, P < 0.05; **, P < 0.01.
Plasmatic Membrane Features of Cell Death

Cell death was analyzed by flow cytometry labeling with Annexin V-FITC and propidium iodide. We examined the features of cell death in W&Wiis cells treated with different concentrations of lin or with lin and GO (data not shown). Cyanide caused a type of cell death characterized by loss of plasmatic membrane integrity, showing double Annexin V and propidium iodide label in lin-treated cells (Fig. 4A). This necrosis-like feature was also supported by the finding that the high mobility group box 1 protein, which has a nuclear localization in control cells, is lost in the lis/lin–treated cells, characteristic of a necrosis cell death (ref. 20; Supplementary Fig. S4). The hydrogen peroxide generated by GO itself did not cause any significant change with respect to the untreated cells. The combined lis/lin/GO system is characterized by phosphatidylserine externalization and maintenance of membrane integrity, shown in 82.5% of cells in the presence of 500 μg/mL lin at 48 hours. In addition, we followed the death progress in the presence of N-acetyl-L-cysteine (Fig. 4A) and found that, in accordance with the previous survival analysis, in this condition, cyanide-induced death was not affected; however, there was a significant reduction of the dead fraction in the cells treated with the combination of lin and GO, suggesting that the observed death pattern is due to the oxidative stress component of the system.

Involvement of Caspase-Dependent and Caspase-Independent Mechanisms in the Combined Therapy

The mitochondrial potential loss and the membrane features of cell death, showing phosphatidylserine externalization and the maintenance of membrane integrity, were compatible with an apoptotic type of death; thus, we wanted to investigate whether the main apoptotic proteins were involved in the lethal process. The caspase family of cysteine proteases plays an important role in apoptosis (21), but the relationship of these proteases to necrosis is not clear (22). We evaluated caspase-3 pattern by Western blot when increasing linamarin concentrations were added to W&Wiis cells in the absence and presence of GO (Fig. 4B). There was no significant caspase-3 activation in the cells treated with the lis/lin system with respect to the control cells. The presence of GO hardly activated caspase-3, which progressively decreased to zero as the concentration of lin was increased in the combined system, in accordance with what would be expected of a progressive ATP depletion caused by cyanide. In a similar way, caspase-12 showed a very weak increment in lin- or GO-treated cells. As the combination lin/GO did not show an increase above control level, we rejected a possible implication of this caspase. Additionally, caspase-8 was not cleaved either in the combined system. To confirm this result, as well as analyzing any other caspase implication in the therapy, death of the combined system was studied in the presence of a pan-caspase inhibitor, z-VAD-fmk (100 μmol/L; Fig. 4C). We may conclude that lis/lin/GO death is mediated by a caspase-independent mechanism because the addition of z-VAD-fmk did not prevent or significantly reduced death, although it could totally rescue a typical caspase-dependent apoptosis such as UV irradiation. We used 100 μmol/L z-VAD-fmk to ensure caspase total inhibition (23) and we did not observe any toxic effect in the untreated cells (data not show).

Once caspase implication in the combined therapy was rejected, we wanted to investigate whether caspase-independent pathways were involved. We studied the putative role poly (ADP-ribose) polymerase 1 (PARP-1) in lis/lin/GO–induced death due to its relevance as an oxidative stress sensor. PARP-1 is a nuclear protein that, in response to DNA damage, translocates to the mitochondria where it activates apoptotic proteins like apoptosis-inducing factor (AIF; ref. 24). To determine whether PARP-1 was implicated in the combined system, we studied cell death in the presence of 1,5-isoquinolinediol, which is a specific PARP-1 inhibitor (25). Nevertheless, no difference in the apoptotic pattern was observed when...
1,5-isoquinolinediol was added to the lis/lin/GO system, indicating that PARP-1 is not implicated in the combination-induced death (Fig. 5A). Additionally, we analyzed PARP status by Western blot showing that full-length PARP p116 is degraded in the combined therapy. No increase of p85 fragment, which is a product of PARP inactivation by caspase-3 cleavage, was observed (Supplementary Fig. S5A). However, some studies have shown that AIF activation could be PARP-1 independent (26). AIF is a mitochondrial protein that, when activated, is translocated to the nucleus where it associates with cyclophilin A, inducing DNA condensation and large-scale degradation (27). We studied subcellular localization of this protein by confocal microscopy, which clearly revealed that AIF did not translocate to the nucleus after lin/GO treatment of W&W lis cells, despite the severe mitochondrial fragmentation (Fig. 5B).

To confirm these results, we studied cell survival in the system when AIF was knocked down. Two AIF targeting RNAis were designed: one (AIFi1) was unable to diminish AIF levels and the other RNAi was designed against a noneukaryotic sequence (Fig. 5C). The AIFi1 RNAi was used as a control. The second RNAi (AIFi2) caused an important reduction of AIF levels. Both AIF RNAis were compared showing that neither lis/lin– nor

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**FIGURE 3.** Mitochondrial morphology after the treatment. A. Representative confocal images of W&W lis cells expressing DsRed2-mito and treated with 500 μg/mL linamarin (lis/lin system) or with 5 mEU/mL GO and linamarin (lis/lin/GO system) at the indicated times. B. Evolution of the mitochondrial pattern of the cells at different times. Mitochondria are shown in red and nuclei are shown in blue due to To-Pro-3 staining. Bar, 10 μm. C. Percentage of intracellular ATP levels at different times in W&W lis cells treated with lin (500 μg/mL) and GO (5 mEU/mL) as indicated. Points, mean of three independent samples; bars, SD. D. Percentage of mitochondrial membrane potential in the same conditions and in the presence of N-acetyl-L-cysteine (10 mmol/L).
AIF-induced death was significantly blocked when AIF was knocked down (AIFi2) with respect to the control (AIFi1). We may thus conclude that AIF is not involved in the lis/lin/GO system death.

**Autophagy Mediates lis/lin/GO Therapy**

We then evaluated the possible generation of death by autophagy by determining if death was inhibited by 3-methyladenine (10 mmol/L), an inhibitor of class III phosphatidylinositol 3-kinase, which is known to inhibit cytoplasmic content sequestration into autophagosomes. We could observe that 3-methyladenine addition to the lin/GO-treated W&W lis cells completely rescued cell death (Fig. 6A).

The conjugation of microtubule-associated protein 1 light chain 3 (LC-3) is essential for double-membrane vacuole formation. Therefore, we wanted to evaluate LC-3 pattern in the system by transfecting W&W lis cells with a plasmid carrying the LC-3 gene fused to the green fluorescent protein (GFP) gene. Whereas untreated cells showed a mainly diffuse LC-3 pattern, lin- and lin/GO–treated cells increased the punctate pattern in the transfected cells up to 15% and 18%, respectively (Fig. 6B and C), suggesting an increase in cell vacuolization. As expected, this pattern is partially reverted by 3-methyladenine (Supplementary Fig. S5B). Additionally, we analyzed the ultrastructure of the W&W lis cells treated with lin and GO by electron microscopy (Fig. 6D). Double-membrane vesicles...
with cytoplasmic cargo inside were observed (arrows and bottom images) whereas untreated control cells did not show such features. Some of these vesicles showed mitochondria within double membranes (Fig. 6D, bottom right). In agreement with Gomez-Santos et al. (28), we found a certain implication of c-Jun NH2-terminal kinases in the process because a c-Jun NH2-terminal kinase inhibitor (SP600125) partially rescued (~20%) death mediated by lis/lin/GO and a clear independence from p38 (Fig. 6E). Although a c-Jun NH2-terminal kinase implication in autophagic death is well established, direct molecular targets are still unknown (29). These findings suggest a direct implication of autophagy in dog glioma cell death when cells are treated with the combined therapy lis/lin/GO.

Discussion

In the present study, we have shown the antitumoral effect generated by the lis/lin/GO treatment. The linamarase/linamarin system induces cell death in a proportional manner to the linamarin concentration, destroying the entire cell culture in ~96 to 120 hours. Cyanide mainly inhibits oxidative phosphorylation, the most important cellular source of energy. However, several studies had reported that the mitochondrial function is impaired in tumor cells, resulting in an elevated rate of glycolysis (30-32), which would circumvent the described therapy. In this context, we tried to combine lis/lin system with subtoxic amounts of the glycolysis inhibitor 3-bromo-pyruvate and we did not find any improvement of the therapy (data not shown).

We have nevertheless investigated a combination that increases the system aggressiveness by combining a cyanide-inducing process with a nontoxic amount of GO that accelerates death by 48 hours. In the conditions we have used, GO advances cell death without causing any detectable toxic effect.

Our results also show the efficiency of the adenoviral vector adeno-lis. Even at very low multiplicity of infection (0.2), the entire cell culture can be eliminated in 96 hours, confirming the potent bystander effect associated with the system, and affecting not only the enzyme producer cells but also their neighboring ones (3). We have shown the therapeutic potential of the lis/lin and lis/lin/GO approaches in nude mice inoculated with linamarase-expressing tumor cells, where the therapeutic window was established, as well as the use of adenolis as a therapy vector in vivo. The choice of a canine glioma system lies in the importance of developing an immunocompetent mammal model in which brain size was more similar to human than murine models for future preclinical studies. Additionally, W&W model was established in neonates showing many of the histologic characteristics of human glioblastomas like capillary proliferation, pseudopallisading, frequent mitotic figures, and multinucleated giant cells (33).

The lis/lin/GO therapeutic model is especially attractive because it does not require infection of a large percentage of tumor cells to produce a concentration of cyanide sufficient to cause severe respiratory damage to the cells within the tumor. The rest of the components, lin and GO, can be delivered to the tumor by direct injection, as we have done, but could be also given by other procedures, such as i.v. or controlled delivery.
compounds (34), where lower but more constant cyanide levels could presumably be obtained, which may be improving the therapeutic results and limiting possible toxicity. This is something we are exploring at the moment. The system as described here is not specific for tumoral cells versus normal cells; the local treatment and the dosage used limit the putative side effects. We have not observed signs of toxicity in the animals at the therapeutic conditions used here, and we are at present carrying out a more exhaustive toxicology study. The vectors could be also improved using, for example, tumor conditional replicating adenovirus or driving linamarase expression by tumor-specific promoters like survivin promoter. Retroviruses that only infect dividing cells have also been considered, although with less success than adenovirus (data not shown).

Mitochondria play a crucial role in death mechanisms, defined by their outer membrane permeabilization (35). We have analyzed how these organelle features are altered during the different treatments. The cyanide produced in the lis/lin system induces severe mitochondrial damage, blocking oxidative phosphorylation that causes a fast ATP depletion and promoting the disruption of the mitochondrial filament pattern. In the conditions used in this study, the lis/lin system induces necrosis according to what would be expected from the conditions of a complete ATP depletion (19), in agreement with previous studies in which linamarase-conjugated antibodies were used for tumor therapy (36). The observed death acceleration by the lis/lin/GO combination is not a consequence of a faster mitochondrial fragmentation or ATP depletion, confirming that the cyanide oxidative phosphorylation blockage is responsible for both effects. Nevertheless, mitochondrial function, reflected in its membrane potential, is totally lost in the first 48 hours, in agreement with the observed mitochondrial

**FIGURE 6.** The lis/lin/GO system induces autophagy in W&Wlis.

A. Survival percentage of cells treated with lin (500 μg/mL) and GO (5 mEU/mL) in the absence or presence of 3-methyladenine (3MeA; 10 mmol/L) at 48 h. B. LC-3 punctate pattern percentage in treated cells at 24 h. Columns, mean of three independent samples counting ~200 to 300 GFP-positive cells per sample; bars, SD. C. Representative images of LC-3-GFP pattern of cells treated, as labeled, at 24 h. Last image shows a magnified detail of a cell with punctate pattern. White arrows, punctated pattern cells.

D. Electron microscope images of control cells and treated with lin and GO showing double-membrane vesicles (black arrows; bar, 2 μm), some of which are magnified in bottom images (bar, 0.5 μm). E. Survival percentage of W&Wlis cell treated with lin, GO, or the combination in the presence of p38 inhibitor (SB203580, 30 μmol/L) or c-Jun NH2-terminal kinase (JNK) inhibitor (SP600125, 10 μmol/L) at 30 h. Columns, mean of three different samples; bars, SD. *, *P < 0.05; **, **P < 0.01.
swelling. In W&W\textit{lis} cells, death is characterized by phosphatidylserine externalization and maintenance of the membrane integrity. Analysis of death mediators showed a caspase-independent mechanism because there is no activation of the main caspases, and death is not blocked in the presence of a pan-caspase inhibitor (z-VAD-fmk). This correlates well with what would be expected from a mechanism of death that takes places with negligible energy levels due to ATP depletion, in contrast with energy-controlled apoptosis activation necessary for caspase-dependent pathways \cite{19, 37}. It is important to note that several effective antitumor treatments induce a caspase-independent death, arsenic trioxide or temozolomide \cite{16, 17} among others. Developing new strategies against cancer, implying caspase-independent death mechanisms, may be important because resistance developed in nonresponsive treated tumors is mainly due to mutations or down-regulations in caspase pathways \cite{38}. A different mechanism that has been described to have implications in oxidative stress-induced death is the one involving mitochondrial to nuclear translocation of AIF \cite{25}. In the \textit{lis}/lin/GO treatment, we did not observe the activation of one of the mediators of AIF liberation, PARP-1, because death is not blocked in the presence of 1,5-isoquinolinediol. Accordingly, we did not observe any nuclear translocation of AIF.

We are showing here for the first time that the \textit{lis}/lin/GO treatment induces autophagy in malignant dog glioma cells. We have observed autophagosomes by electron microscopy and we have reversed cell death by the autophagy inhibitor 3-methyladenine. Additionally, an increase in the generation of autophagic vesicles by both \textit{lis}/lin and \textit{lis}/lin/GO systems can be followed by the punctate pattern of LC-3, which is also reverted by 3-methyladenine. All these features determine a type II cell death \cite{39}. It has recently been shown that autophagic death shows phosphatidylserine externalization \cite{40}, as we have seen, being important for the clearance of death cells during development. Moreover, the PARP degragation observed at the combined treatment conditions may be explained by an increment in protein degradation that takes place during autophagy. The death synergism observed in the combined system could be due to the mitochondrial network fragmentation caused by cyanide production, facilitating the engulfment of this organelle in autophagic vesicles and favoring mitochondrial potential loss leading to cellular death \cite{41}.

Autophagy-mediated antitumor strategies, such as the one we present here, are presumably going to play an important role in the future design of cancer therapies. It is known that the majority of the cancers preserve their capacity to generate macroautophagy because it confers an advantage to survive in the starving conditions usually found in the tumor mass \cite{14}. This is reflected in the fact that many tumors conserve at least the starving conditions usually found in the tumor mass \cite{14}, 3-methylautophagy because it confers an advantage to survive in the tumor mass \cite{14}. This is reflected in the fact that many tumors conserve at least the starving conditions usually found in the tumor mass \cite{14}.

In conclusion, we present here evidence supporting that the \textit{lis}/lin/GO treatment is very efficient \textit{in vitro} and \textit{in vivo} against dog malignant brain tumors and that death is mediated by autophagy.

### Materials and Methods

#### Cell Lines and Culture

Wodinsky and Walker cell line from canine glioblastoma (W&W) was cultured in DMEM supplemented with 10% FCS at 37\(^\circ\)C, 7% CO\(_2\), and 97% relative humidity. W&W\textit{lis} cells were obtained by stable transfection (puromycin, 1 \(\mu\)g/mL) of W&W cells with the linamarase gene inserted in the pIRES-P \cite{42}.

#### Cell Survival Determination

Cells were treated with linamarin (50, 200, or 500 \(\mu\)g/mL; Toronto Research Chemicals), GO (5 mEU/mL), N-acetyl-l-cysteine (10 mmol/L), tiron (1 mmol/L), 3-methyladenine (10 mmol/L; all of them from Sigma), and p38 inhibitor (SB203580; 30 \(\mu\)mol/L) or c-Jun NH\(_2\)-terminal kinase inhibitor (SP600125, 10 \(\mu\)mol/L; both from Tocris) as indicated. Thereafter, the survival percentage was determined with 3-[4,5-dimethylthiazolo-2-yl]-2,5-diphenyltetrazolium bromide (200 \(\mu\)g/mL; Sigma).

#### Cyanide Detection in the Culture Medium

Cells were grown and treated as required in DMEM without phenol red. Culture medium samples were diluted in 0.1 mol/L sodium acetate (pH 7.4) and the amount of free cyanide present in each sample was determined as described \cite{43}.

#### Determination of Glucose Concentration in the Culture Medium

Medium samples were diluted in 1 mL of phosphate buffer 0.1 mol/L (pH 7.4), then 2 EU of GO, 3.7 EU of peroxidase (Roche), and 0.1 mg of \textit{ortho}-dianisidine (Sigma) were added. Absorbance at 562 nm was determined and compared with glucose calibration curves.

#### Adenosil Infection \textit{in vitro}

Adenoviruses, deleted in E1 gene and carrying the plant \textit{lis} gene under the transcriptional control of the cytomegalovirus promoter (adeno\textit{lis}), were used as the therapy vector (Crucell). W&W cells were infected at different multiplicities of infection (0, 0.2, 1, or 10) with adeno\textit{lis} (1.6 \(\times\) \(10^{11}\) IU/mL). One day after infection, lin (500 \(\mu\)g/mL) was added to the tubes. After 96 h, the survival percentage and cyanide concentration in the medium were measured as described above.

#### Statement

For animal care and handling, we have strictly followed the legislation and guidelines in our country (Spanish Royal Decree 1201/2005 BOE published October 21st 2005), as well as the guidelines from the British UK Coordinating Committee on Cancer Research for the welfare of animals in experimental neoplasia (revised version of July 1997) and those from the European Union (2003/65/CE from the European Parliament and Council July 2003).

#### Determination of Animal GO Toxicity

Swiss mice, 6 wk old, weighing \(\sim\) 20 g, were injected with GO i.p. (1, 0.5, 0.25, or 0.1 EU/g of body weight/d) or i.v. (0.25 or 0.1 EU/g of body weight/d). The mice were sacrificed after 5 d of treatment or till they developed intoxication symptoms.
Treatment of Dog Tumor Xenografts in Nude Mice

Nude mice, 7 to 9 wk old, BALB/c nu/nu, weighing ~20 g, were s.c. injected with 10⁶ cells from either W&Wlis or W&W in both side flanks. Tumor volume was estimated [π/6 (length × width × height)]. The intratumoral treatment started daily once the tumor size reached ~50 mm³. The mice with W&Wlis tumors were locally inoculated with linamarin (0.1, 0.25, 0.35, or 0.5 mg/g of body weight/d) with or without GO (0.1 EU/g of body weight/d) during a 10- to 13-d period. The mice with W&W tumors were treated with 10⁷ IU adenosin followed by 2-d treatment with lin and GO. This 3-d cycle was repeated four times. These assays were finished and the animals were sacrificed when any of the untreated tumors reached 10% volume of animal weight, which is ~2,000 mm³. Several controls were done: W&Wlis tumors were treated only with 0.25 or 0.1 EU GO/g of body weight/d, and W&W tumors were treated only with 5 × 10⁷ IU adenosin.

Structural Changes in Mitochondria

W&Wlis cells were stably transfected with the plasmid pDsRed2-Mito (Clontech). After treatment, the cells were fixed with 4% paraformaldehyde and treated with To-Pro-3 (Molecular Probes). The cells were analyzed with a Radiance 2000 confocal system (Bio-Rad) coupled to an Axiovert S100 TV inverted microscope (Zeiss). The deconvolution process, if necessary, was done using the classic MLE-TIME algorithm with Huygens Professional 2.5.1a.<2> software.

Intracellular ATP Measurements

After the indicated treatment, the cells were collected and processed as indicated by the manufacturer (CSL HATP Bioluminescence Assay Kit, Roche). Luminescence was analyzed with a Monolight 2010 lumimeter (Analytical Luminescence Laboratory) and expressed in relative light units.

Flow Cytometry Analysis

For reactive oxygen species measurement, cells were treated as indicated, harvested, and washed with PBS. The pellet was resuspended in mitochondria incubation buffer [68 mmol/L sucrose, 10 mmol/L HEPES-KOH (pH 7.4), 70 mmol/L KCl, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 2 μmol/L aprotinin] and incubated with 4 μmol/L dihydroethidium (Calbiochem) for 30 min at 37°C in the dark. Flow cytometry analysis was then done (FACSCalibur, BD Biosciences).

For mitochondrial potential measurement, the treated cells were incubated with 50 mmol/L DiOC₆(3) (Molecular Probes) for 30 min at 37°C and analyzed by flow cytometry.

The W&Wlis cells were treated as explained. When specified, culture medium was supplemented with 10 mmol/L N-acetyltcysteine, 300 μmol/L 1,5-isoquinolinyliodol (Sigma), or 100 μmol/L z-VAD-fmk (Tocris). The cells were stained with propidium iodide and Annexin V-FITC (BD Biosciences) and analyzed by flow cytometry.

Immunofluorescence Assays

In one set of experiments, W&W cells were infected with adenosin and, after 2 d, were fixed with methanol (~20°C) and incubated with anti-linamarase antibody (1/200; kindly provided by Monica Hughes, Department of Biochemistry and Genetics, The University of Newcastle upon Tyne, England), followed by a second incubation with fluorescein donkey anti-rabbit IgG antibody (Amersham). In another set of experiments, W&Wlis cells treated with lin and GO, as detailed, were fixed with 4% paraformaldehyde, permeabilized with PBS-0.1% SDS, and incubated with anti-high mobility group box 1 antibody (Abcam) or anti-AIF antibody (Cell Signaling), followed by a second incubation with Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen), and treated with To-Pro-3 (Invitrogen).

Staining of Autophagosomes

W&Wlis cells were transfected with a GFP-LC-3 expression plasmid (44). After 24 h, the cells were treated with lin, GO, and lin/GO, and the GFP-LC-3 pattern was observed under an Axiovert200 (Zeiss) fluorescence microscope.

Western Blot Analysis

The cells were collected 30 h after lin and GO treatment, washed, and resuspended in lysis buffer [50 mmol/L HCl-Tris (pH 7.5), 300 mmol/L NaCl, 0.5% SDS, and 1% Triton X-100]. Additionally, cells were irradiated with 16.7 J/m² and similarly processed 24 h later. The primary antibodies used were anti-caspase-3 and anti-AIF (Cell Signaling), anti-caspase-12 (sc-5627), anti-caspase-8 (sc-7890; Santa Cruz Biotechnology), anti-actin, and anti-PARP (Sigma). The secondary antibodies used were horseradish peroxidase–conjugated antirabbit or antimouse IgGs (DAKO). Luminescence was enhanced with an enhanced chemiluminescence–based detection kit (Pierce).

RNAi Constructions

Two synthetic oligonucleotides containing targeting sequences homologous to mouse AIF (DQ 016497) and human AIF (NM 004208) genes were cloned in the plasmid pSuper.neo (Oligoengine) following commercial indications. Targeting sequences were AIFt1, 5’GCGTACTGGCATCGTCA3’; AIFt2, 5’GGTGAAGA AACTGACCACA T3’; and noneukaryotic sequence, 5’TCAAGGAGGTTGATCCTA3’.

Electron Microscopy

W&Wlis cells treated with lin/GO were fixed in situ with 2% glutaraldehyde in Sörensen phosphate Na/K buffer (pH 7.4). Cells were embedded in Epoxy, TAAB 812 Resin (TAAB Laboratories). Postfixation was done with a mixture of 1% osmium tetroxide and 0.8% potassium ferricyanide. Samples were incubated with 0.15% tannic acid in buffer, followed by 2% uranyl acetate. After dehydration with increasing ethanol concentrations, the samples were infiltrated in the resin in increasing concentrations of ethanol/Epon. Ultrathin sections were stained with saturated uranyl acetate and lead citrate and examined at 80 kV under a Jeol JEM-1010 electron microscope.

Statistical Analysis

Statistical comparison of the data sets was done by the Student t test. The differences are presented with their corresponding statistical significance or P value, which is the probability that the observation in a sample occurred merely by chance under the null hypothesis.
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References


# Molecular Cancer Research

**Glioma Regression *In vitro* and *In vivo* by a Suicide Combined Treatment**

Vega García-Escudero, Ricardo Gargini and Marta Izquierdo


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