

# Targeting miR-155-5p and miR-221-3p by peptide nucleic acids induces caspase-3 activation and apoptosis in temozolomide-resistant T98G glioma cells

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**Abstract.** The present study investigated the effects of the combined treatment of two peptide nucleic acids (PNAs), directed against microRNAs involved in caspase-3 mRNA regulation (miR-155-5p and miR-221-3p) in the temozolomide (TMZ)-resistant T98G glioma cell line. These PNAs were conjugated with an octaarginine tail in order to obtain an efficient delivery to treated cells. The effects of singularly administered PNAs or a combined treatment with both PNAs were examined on apoptosis, with the aim to determine whether reversion of the drug-resistance phenotype was obtained. Specificity of the PNA-mediated effects was analyzed by reverse transcription-quantitative polymerase-chain reaction, which demonstrated that the effects of R8-PNA-a155 and R8-PNA-a221 anti-miR PNAs were specific. Furthermore, the results obtained confirmed that both PNAs induced apoptosis

when used on the temozolomide-resistant T98G glioma cell line. Notably, co-administration of both anti-miR-155 and anti-miR-221 PNAs was associated with an increased proapoptotic activity. In addition, TMZ further increased the induction of apoptosis in T98G cells co-treated with anti-miR-155 and anti-miR-221 PNAs.

## Introduction

Glioblastoma multiforme (GBM) is a lethal malignant tumor accounting for 42% of the tumors of the central nervous system, with a median survival of 15 months (1-3). At present, no curative treatment is available and the most used first-line drug, temozolomide (TMZ), only moderately increases the life expectancy of the treated patients (4). In addition, a high proportion of gliomas become TMZ-resistant with time. Therefore, novel drugs and therapeutic protocols for combined treatments on TMZ-resistant glioma cells are urgently needed.

In this respect, a novel strategy for therapeutic protocols has been recently suggested, the targeting of microRNAs. MicroRNAs (miRNAs) are short non-coding RNAs that function by repressing translation or inducing the cleavage of the target mRNA transcripts, thereby regulating gene expression at the post-transcriptional level (5-7). Altered miRNA expression has been firmly demonstrated to be involved in cancer (8-13). Approaches based on the targeting of oncomiRNAs and metastamiRNAs (associated with tumor progression and metastasis, respectively) have been found to inhibit tumor cell growth and metastasis, and in some examples to reverse the resistance of tumor cells to anticancer drugs (14-17). For instance, Chan *et al* (15) have reported that the inhibition of oncomiR-138 prevents *in vitro* tumor sphere formation in malignant gliomas and suppresses *in vivo* tumorigenesis. Wagenaar *et al* (16) were able to target the transcriptional network in hepatocellular carcinomas cell

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**Abbreviations:** PNA, peptide nucleic acid; miRNA, microRNA; GMB, glioblastoma multiforme; TMZ, temozolomide; Fl, fluorescein; FBS, fetal bovine serum; BCA, bicinchoninic acid; LDB, lysate dilution buffer; RT, reverse transcription; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase-chain reaction; SDS, sodium dodecylsulphate; SDS-PAGE, SDS-polyacrylamide-gel electrophoresis; PBS, phosphate buffered saline; UPLC-MS, ultra-performance liquid chromatography-mass spectrometry

**Key words:** peptide nucleic acids, glioma, microRNAs, miR-221-3p, miR-155-5p, miRNA targeting, delivery, apoptosis, temozolomide

lines with sequence-specific antagomiR targeting miR-21, thereby inducing increased expression of miR-21-regulated genes, associated with a loss of viability. In another example, Ma *et al.* (17) reported inhibition of metastasis formation in a mouse model of mammary tumor following silencing of the oncogenic miR-10a. In this context, the use of peptide nucleic acids (PNAs) targeting oncomiRNAs might be relevant (8).

PNAs are DNA analogues described for the first time by Nielsen *et al.* (18), in which the sugar-phosphate backbone has been replaced by N-(2-aminoethyl)glycine units (18-22). PNAs are capable of forming Watson-Crick double helices following efficient sequence-specific hybridization with complementary DNA and RNA (23). Furthermore, they are able to generate triple helices with double-stranded DNA and to perform strand invasion (24-26). In virtue of these biological activities, PNAs have been demonstrated to be very efficient tools for pharmacologically-mediated alteration of gene expression, both *in vitro* and *in vivo* (27-29). In summary, PNAs and PNA-based analogues were employed as antisense molecules targeting mRNAs, triple-helix forming molecules targeting eukaryotic gene promoters, artificial promoters, and decoy molecules targeting transcription factors (26). Relevant to the present study, PNAs have been demonstrated to be able of altering miRNA functions, both *in vitro* and *in vivo* (30-37). Cheng *et al.* (37), for instance, efficiently inhibited the function of oncomiR-155-5p in a tumor mouse model by the design and synthesis of a peptide-(anti-miR)-PNA construct able to target the tumor microenvironment and to transport the anti-miR PNA across the cellular plasma membranes under the acidic conditions which characterize solid tumors. Recently, our group has reported that a PNA targeting miR-221-3p (R8-PNA-a221) (38), bearing an oligoarginine peptide (R8) enabling efficient uptake by glioma cells (30,31), was able to strongly inhibit miR-221-3p in U251, U373 and T98G glioma cells. The inhibition of miR-221-3p activity was associated with an increased expression of the miR-221-3p target p27<sup>Kip1</sup>, as analyzed by reverse transcription-quantitative polymerase-chain reaction (RT-qPCR) and by western blot analysis (38).

The present study determined the biological activity of a combined treatment of glioma cell lines with two PNAs directed against miRNAs regulating caspase-3 mRNA expression and conjugated to the octaarginine R8 peptide, allowing efficient cellular uptake. Effects on apoptosis were analyzed to determine whether additive activity was obtained by co-administration of the two PNAs to the temozolomide-resistant T98G glioma cell line, and whether combined treatments were associated with a reversion of the drug-resistance phenotype.

## Materials and methods

**Synthesis and characterization of PNAs.** The protocols for the synthesis and the characterization of the anti-miR-221 PNAs have been described in a previously published study (38). The synthesis of the new anti-miR-155 PNAs was performed using a standard Fmoc-based automate peptide synthesizer (Syro I; Biotage, Uppsala, Sweden), using a ChemMatrix-RinkAmide resin loaded with Fmoc-Gly-OH (0.2 mmol/g) as first monomer, and using commercially available monomers (Link Technologies, Bellshill, UK) with

HBTU/DIPEA coupling. After purification, the PNAs were characterized by UPLC-MS on a Waters ACQUITY System equipped with a ACQUITY UPLC BEH C18 Column (1.7  $\mu$ m; 2.1x50 mm). Gradient: 100% A for 0.9 min, then 0-50% B in 5.7 min at 0.25 ml/min flow (A, water + 0.2% formic acid; B, acetonitrile + 0.2% formic acid). R8-PNA-a155: sequence H-R<sub>8</sub>-TAT CAC GAT TAG CAT TAA-Gly-NH<sub>2</sub>; yield: 15.9% R<sub>t</sub>=2.65 min; calculated MW, 6184.3 g/mol; m/z found, 1238.2 [M+5H]<sup>5+</sup>, 1031.9 [M+6H]<sup>6+</sup>, 884.8 [M+7H]<sup>7+</sup>, 774.3 [M+8H]<sup>8+</sup>, 688.4 [M+9H]<sup>9+</sup>, 619.6 [M+10H]<sup>10+</sup>, 563.4 [M+11H]<sup>11+</sup>. R8-PNA-a155-MUT: sequence H-R<sub>8</sub>-TAT TAC GGT TAA CAT CAA-Gly-NH<sub>2</sub>; yield: 11.6% R<sub>t</sub>=2.65 min; calculated mw, 6184.3 g/mol; m/z found, 1238.0 [M+5H]<sup>5+</sup>, 1032.0 [M+6H]<sup>6+</sup>, 884.7 [M+7H]<sup>7+</sup>, 774.2 [M+8H]<sup>8+</sup>, 688.4 [M+9H]<sup>9+</sup>, 619.6 [M+10H]<sup>10+</sup>, 563.3 [M+11H]<sup>11+</sup>.

**Glioma cell lines and culture conditions.** T98G cells (39-41) were grown in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; EUROCLONE S.p.A., Pero, Italy), 100 U/ml penicillin and 100 mg/ml streptomycin, in humidified atmosphere of 5% CO<sub>2</sub>/air. In order to study possible anti-proliferative effects, the cell number/ml was monitored using a Z2 Coulter Counter (Coulter Electronics, Hialeah, FL, USA). Mycoplasma testing on T98G cells was performed prior to each experiment.

**RNA extraction.** Cultured cells were trypsinized and collected by centrifugation at 250 x g for 10 min at 4°C. Then, cells were lysed with TRI Reagent (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) and the isolated RNA was washed once with cold 75% ethanol, dried and dissolved in nuclease-free pure water prior to use (36).

**Quantitative analyses of miRNAs.** For miRNA quantification using RT-qPCR, reagents, primers and probes for hsa-miR-221-3p and hsa-miR-155-5p were obtained from Applied Biosystems (Thermo Fisher Scientific, Inc.). Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription kit, with 20 ng per sample used for the assays. qPCR was performed as described elsewhere (36), using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The relative miRNA content was calculated using the comparative cycle threshold method and U6 snRNA and has-let-7c were used as references to normalize all RNA samples, as previously described (38).

**Analysis of caspase-3 gene expression.** Gene expression analysis was performed by RT-qPCR. Total RNA (500 ng) was reverse transcribed by using random hexamers. qPCR assays were performed using gene-specific double-fluorescently labeled probes. Primers and probes used to assay caspase-3 gene expression were purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.). The relative expression was calculated using the comparative cycle threshold method and the human RPL13A (Assay ID: Hs04194366\_g1) was used as a reference gene (30,31,38).

Caspase-3 protein activity was analyzed using Bio-PlexPro RBM Apoptosis Assays (Bio-Rad Laboratories, Inc.), an

immunoassay performed on magnetic beads. Glioblastoma cells were seeded in 6-well plates, treated with the compounds and after 72 h total cell extracts were prepared. Cells were washed with cold sterile PBS, centrifuged, the pellet was suspended in LDB (lysate dilution buffer) and after 8 thermal shock cycles using dry ice, the suspension was centrifuged at 4°C for 10 min at 9000 x g. The supernatant was carefully removed and transferred into new vials, according to the manufacturer's instructions. Protein quantification was performed using BCA protein assay (Thermo Fisher Scientific, Inc.). For the analysis with Bio-PlexPro RBM Apoptosis Panel 3, samples were diluted to a final concentration of 500 µg/ml with LDB. After the reconstitution of the standard, seven serial 1:3 dilutions were prepared and the assay was performed, according with the manufacturer's instructions. Briefly, after reaction with 10 µl of Blocking buffer, 10 µl of capture beads were added, the reaction incubated, washed three times with 1X Assay buffer using Bio-Plex Pro Wash Station (Bio-Rad Laboratories, Inc.) and, finally, detection antibodies (40 µl) were added for a second incubation. Afterwards, 20 µl of diluted streptavidin-phycoerythrin (SA-PE) were added for 30 min at room temperature. After washing, the beads were suspended in 100 µl 1X Assay buffer, incubated with shaking for 30 sec and reading was performed at low PMT (photomultiplier tube) with Bio-Plex 200 Array reader (Bio-Rad Laboratories, Inc.). Data were analyzed with Bio-Plex Manager software (Bio-Rad Laboratories, Inc.).

**Analysis of apoptosis.** Annexin V and Dead Cell assay were performed on T98G cells using a Muse Cell Analyzer (Millipore Corporation, Billerica, MA, USA), as described elsewhere (38). Cells were washed with sterile PBS, trypsinized, suspended and diluted (1:2) with the one-step addition of the Muse Annexin V and Dead Cell reagent. After incubation for 20 min at room temperature in the dark, samples were analyzed. Data from prepared samples were acquired and recorded utilizing the Annexin V and Dead Cell Software Module (Millipore Corporation) (38).

**Statistical analysis.** Results are expressed as mean ± standard deviation. GraphPad Prism software version 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Comparison between groups was made by two-way analysis of variance with post-hoc Bonferroni comparison. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Identification of miRNA targets.** First, it was confirmed that T98G are resistant to TMZ treatment, by determining the cell proliferation rate and the levels of induced apoptosis. As illustrated in Fig. 1A, the T98G cell line was resistant to TMZ treatment, with only a slight inhibition of cell growth observed when TMZ was used at very high concentrations (400 and 600 µM). By contrast, an IC<sub>50</sub> inhibition was reached at 50 µM, when TMZ-sensitive U251 glioma cells were treated with TMZ (Fig. 1A). Furthermore, TMZ failed to induce a major increase in the proportion of Annexin V-positive cells when used in T98G cells (Fig.1B).

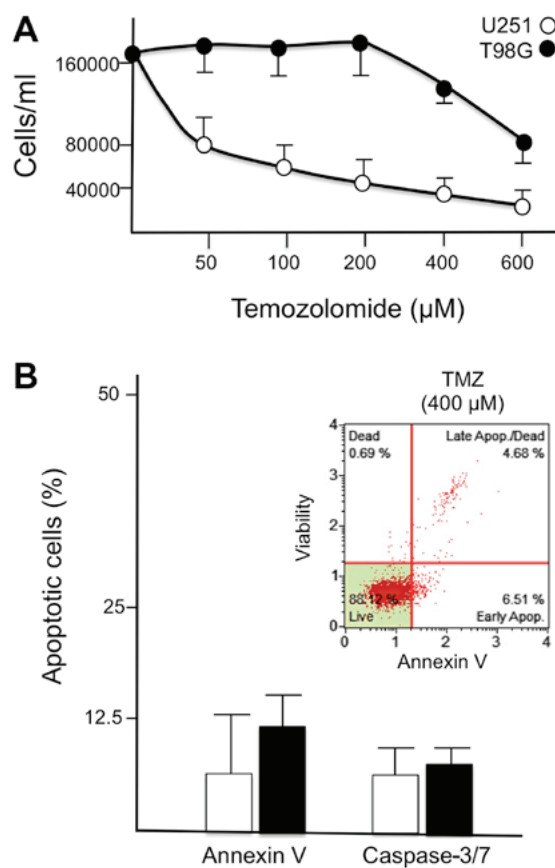


Figure 1. (A) Effects of increasing concentrations (50-600 µM) of TMZ on the viability of U251 and T98G cells. Cells were cultured for 72 h with the indicated treatments and then the number of cells/ml was counted. (B) Lack of proapoptotic effects of TMZ in resistant T98G cells. T98G cells were cultured for 48 h without (white boxes) or with (black boxes) 400 µM TMZ and then the apoptosis rate was determined by flow cytometry. A representative plot is shown in the insert. Results are presented as the average ± standard deviation from four independent experiments.

In order to identify possible miRNA targets for the present study, three sets of miRNAs were compared: miRNAs highly expressed in tissues from glioma patients; miRNAs validated in gliomas for their oncogenic properties; and miRNAs putatively interacting with the 3' untranslated region (UTR) of caspase-3 mRNA, which is deeply involved in activation of the apoptotic pathway. These lists of miRNAs are presented in the Tables SI-III. A Venn diagram for the comparison of the three lists is illustrated in Fig. 2A. Only three miRNA sequences were found in common within the three sets, miR-155-5p, miR-221-3p and miR-30a. Of note, miR-221-3p has already been demonstrated to exhibit anti-apoptotic effects in gliomas, and miR-221-3p targeting induces apoptosis and reverses TMZ-resistance (42). Similar information is available on the role of miR-155-5p, which regulates in the sensitization of glioma cell lines to antitumor drugs (43). By contrast, no report is available to date on the possible involvement of miR-30a to activation of drug resistance in gliomas. For these reasons, the present study focused on targeting miR-155-5p and miR-221-3p.

The location of the sites for these two miRNAs within the 3'UTR sequences of the caspase-3 mRNA is shown in Fig. 2B, also depicting the extent of interactions between these two microRNAs and the caspase-3 mRNA sequences. When this

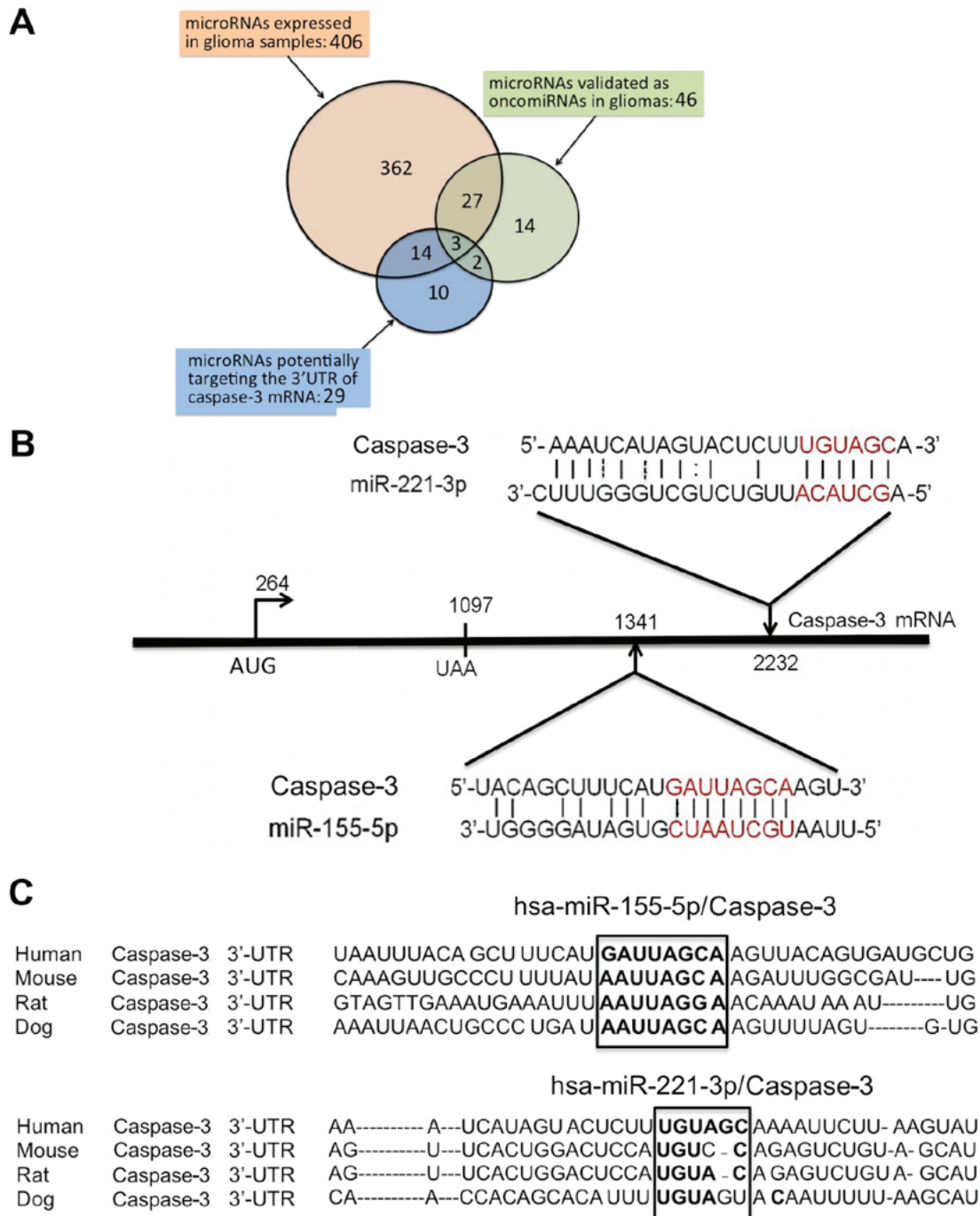


Figure 2. (A) Identification of microRNAs highly expressed in gliomas, published as oncomiRNAs and potentially recognizing the 3'UTR of the caspase-3 mRNA. The complete gene lists are included in Tables SI-III. (B) Location of the binding sites for miR-221-3p and miR-155-5p and extent of hybridization with the 3'UTR of the caspase-3 mRNA (accession no. NM\_004346.3; Human caspase-3 Isoform 1). (C) Conservation throughout evolution of the caspase 3 mRNA sequences corresponding to the miR-155-5p and miR-221-3p seed regions (bold font) of the caspase-3 mRNA throughout evolution. Human, mouse, rat and dog caspase-3 mRNA sequences are shown. The binding sites of miR-155-5p and miR-221-3p are boxed. UTR, untranslated region.

feature was compared with other miR-155-5p and miR-221-3p validated targets, it was observed that the level of interactions is similar and compatible with a true caspase-3 mRNA regulation by miR-155-5p and miR-221-3p (data not shown). This is further sustained by the finding that miR-155-5p and miR-221-3p binding sites are of caspase-3 mRNA conserved through molecular evolution (Fig. 2C).

*R8-PNA-a155 and R8-PNA-a221 exhibit inhibitory effects on miR-155-5p and miR-221-3p in glioma cells.* Firstly,

glioma cells were treated with increasing amounts of PNAs R8-PNA-a155 and R8-PNA-a221, targeting miR-155-5p and miR-221-3p, respectively. The results of the experiments are presented in Fig. 3A and B, and clearly demonstrate that 4  $\mu$ M PNAs was the optimal concentration to significantly and reproducibly inhibit miR-155-5p (Fig. 3A) and miR-221-3p (Fig. 3B) hybridization signals.

Next, we determined whether the PNA-mediated effects were sequence-specific and selective for a given miRNA molecule. To test this, glioma cells were treated with

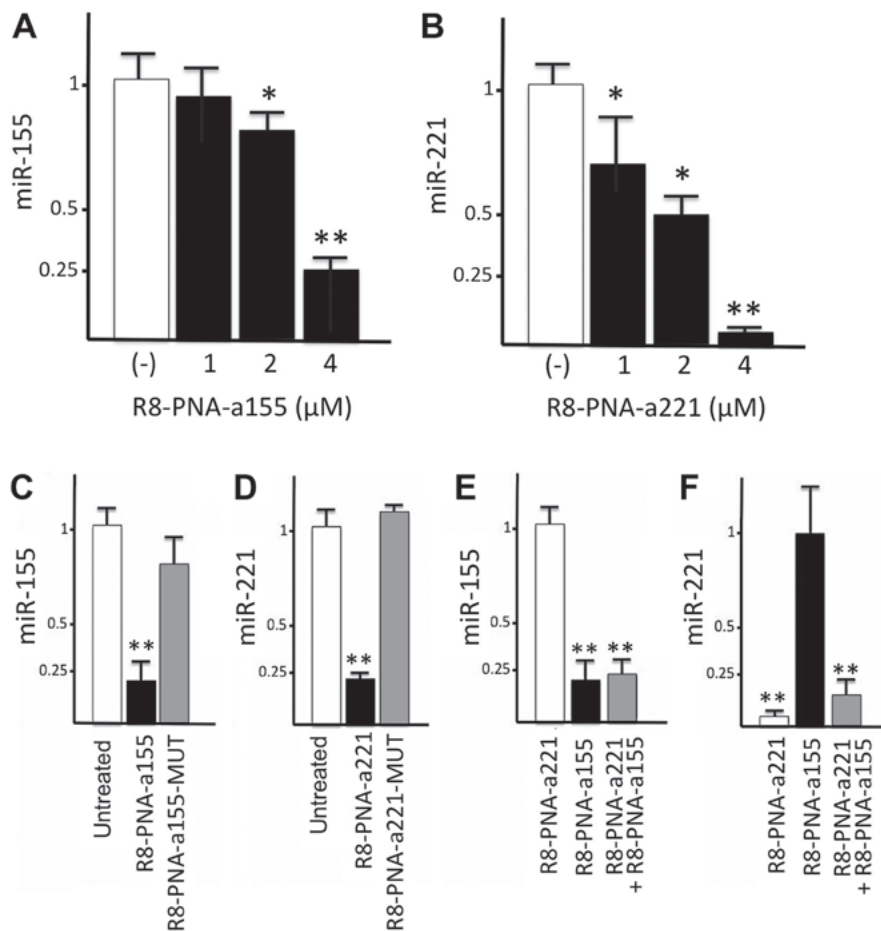


Figure 3. Effects of PNAs targeting miR-155-5p and miR-221-3p on miRNA expression in T98G glioma cells. (A and B) Effects of 48 h treatment with increasing amounts of R8-PNA-a155 and R8-PNA-a221, as indicated. \* $P < 0.05$  and \*\* $P < 0.01$  vs untreated. (C and D) Effects of 48 h treatment with 4  $\mu$ M R8-PNA-a155, R8-PNA-a221, R8-PNA-a155-MUT, R8-PNA-a221-MUT. \*\* $P < 0.01$  vs untreated. (E and F) Effects of 48 h treatment with the combination of R8-PNA-a155 and R8-PNA-a221, as indicated. \*\* $P < 0.01$  vs R8-PNA-a221 or R8-PNA-a155. Data are normalized to the miR-155-5p and miR-221-3p levels in untreated cells. Results are presented as the average  $\pm$  standard deviation from three independent experiments. PNAs, peptide nucleic acids; MUT, mutated.

R8-anti-miR155 PNA and R8-anti-miR221 PNA, and with PNAs including a mutated sequence R8-PNA-a155-MUT and R8-PNA-a221-MUT (Fig. 3C and D). Treatment of T98G cells with R8-PNA-a155 and R8-PNA-a221 resulted in a sharp and significant inhibition of miR-155-5p and miR-221-3p hybridization signals, respectively; by contrast, the mutant R8-anti-miR155-MUT PNA and R8-anti-miR221-MUT PNA displayed only minor effects (Fig. 3C and D). In a second set of experiments, results supporting the concept that the effects were specific were obtained. The results demonstrated that the miR-155-5p hybridization signal was strongly reduced only when RNA was isolated from glioma cells cultured for 48 h in the presence of R8-PNA-a155, with no major effects on miR-221-3p levels (Fig. 3E and F). Conversely, the miR-221-3p hybridization signal was significantly reduced only when RNA was isolated from glioma cells cultured for 48 h in the presence of R8-PNA-a221, while no major effects on miR-221-3p levels were observed with R8-PNA-a155 (Fig. 3E and F). Altogether these experiments support the concept that the effects of R8-PNA-155 on miR-155-5p, and of R8-PNA-a221 on miR-221-3p, are sequence-specific. As expected, miR-155-5p and miR-221-3p hybridization signals were reduced by co-administrating of R8-PNA-155 and R8-PNA-a221 (Fig. 3E and F).

*Effects of R8-PNA-a155 and R8-PNA-a221 treatment on caspase-3 expression.* Since caspase-3 mRNA is a putative molecular target of miR-155-5p and miR-221-3p (Fig. 2B), the glioma T98G cell line was treated with R8-PNA-a155 or R8-PNA-a221 and caspase-3 mRNA and protein levels were determined. To this aim, RNA was extracted for RT-qPCR analysis from an aliquot of cells, while another aliquot was used for preparing protein extracts for Bio-Plex analysis. The results demonstrated that treatment with R8-PNA-a155 or R8-PNA-a221 did not affect caspase-3 mRNA levels (Fig. 4A). By contrast, both treatments resulted in a significant concentration-dependent increase of caspase-3 protein production, as determined by Bio-Plex analysis (Fig. 4B). These results are compatible with a regulation of caspase-3 expression by miR-155-5p and miR-221-3p at the post-transcriptional level. Furthermore, analysis of caspase 3/7 function confirmed the activation of the caspase-3/7 pathway in T98G cells treated with PNAs targeting miR-155-5p and miR-221-3p (Fig. 4C and D).

*Effects of R8-PNA-a155 and R8-PNA-a221 treatment on apoptosis.* The proapoptotic effects of R8-PNA-a155 and R8-PNA-a221 were confirmed to appear in a dose-dependent manner (Fig. 5A-D). Cells were cultured for 48 h in the

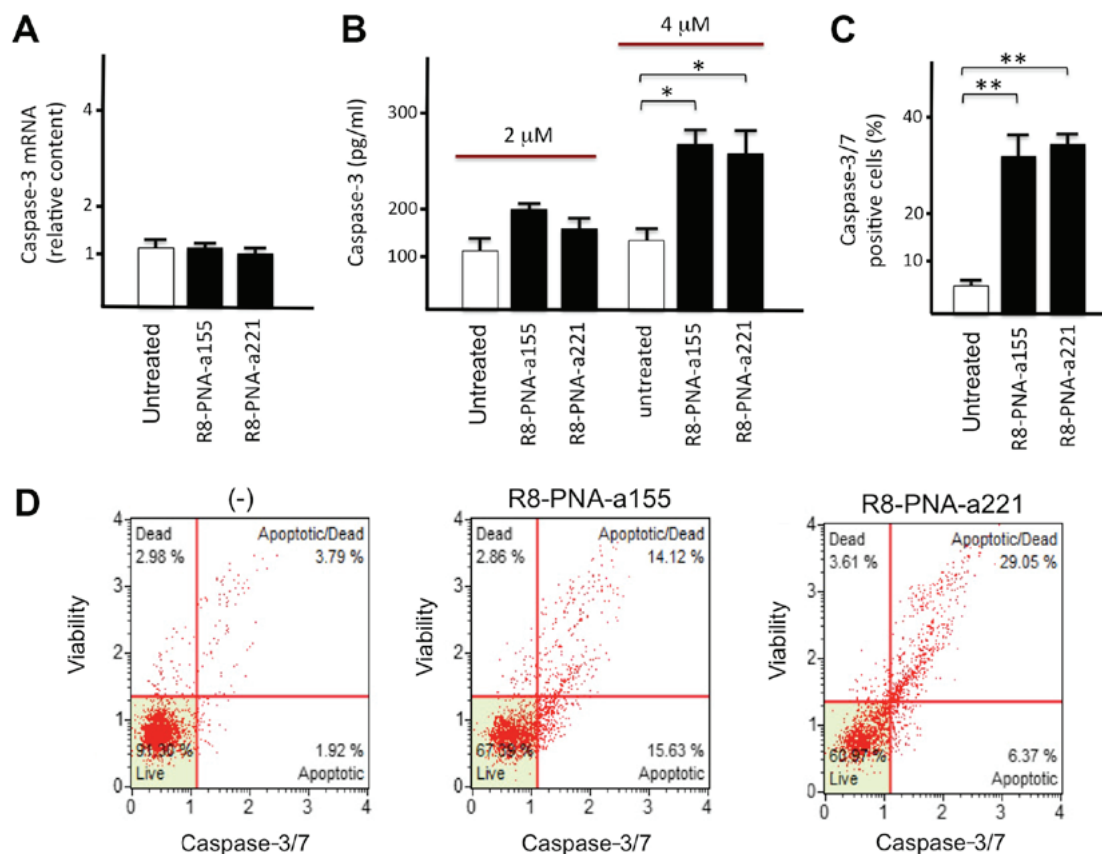


Figure 4. (A) Effects of treatment of T98G cells with 4  $\mu$ M R8-PNA-a155 and R8-PNA-a221 on production of caspase-3 mRNA, analyzed by reverse transcription-quantitative polymerase reaction. RPL13 mRNA was used as internal control and data are presented relative to untreated cells. (B) Effects of treatment of T98G cells with 2  $\mu$ M and 4  $\mu$ M R8-PNA-a155 and R8-PNA-a221, as indicated, on production of caspase-3, analyzed by Bio-Plex assay. (C and D) Effects of 4  $\mu$ M R8-PNA-a155 and R8-PNA-a221 on caspase-3/7 activity analyzed by the Muse caspase-3/7 Assay kit. Results are presented as the average  $\pm$  standard deviation from three independent experiments. (D) Representative plots from the caspase-3/7 activity experiments. \* $P$ <0.05 and \*\* $P$ <0.01 with comparisons indicated by brackets.

absence (Fig. 5A) or in the presence of 0.5, 1, 2 and 4  $\mu$ M R8-PNA-a155 (Fig. 5B) or R8-PNA-a221 (Fig. 5C) PNAs and the Annexin-V assay was performed. As clearly evident in Fig. 5D (which is in agreement with data in Fig. 3), only minor effects were observed when 0.5-2  $\mu$ M PNAs were employed, and the higher proapoptotic effects were observed with 4  $\mu$ M concentrations. When the glioma cell line T98G was cultured in the presence of singularly administered R8-PNA-a155 or R8-PNA-a221 (used at 4  $\mu$ M concentration) a significant increase of early and late apoptotic cells was found. Fig. 5E-G presents representative results obtained after treatment of T98G glioma cell lines with 4  $\mu$ M R8-PNA-a155, and 4  $\mu$ M R8-PNA-a221, confirming the proapoptotic effects of R8-PNA-a221 (as previously published by our group) (38), and demonstrating for the first time the pro-apoptotic effects of R8-PNA-a155. Notably, when TMZ was also administered in combination with R8-PNA-a155 or R8-PNA-a221, a further significant increase of the proportion of apoptotic Annexin V-positive cells was observed (Fig. 5F-H). These data suggest that treatment of the TMZ-resistant T98G glioma cells with PNAs targeting miR-155-5p or miR-221-3p resulted in a sensitization of glioma cells to TMZ.

*Effects of co-treatment with suboptimal concentrations of R8-PNA-a155 or R8-PNA-a221 and TMZ on apoptosis.* Since

dose-dependent off-target effects of antisense molecules (including PNAs) is one of the major issues in this potential therapeutic strategy, the effects of combined treatments in the presence of 2  $\mu$ M R8-PNA-a155 and R8-PNA-a221, which is half of the dose used in the experiments depicted in Fig. 5, were determined. As illustrated in Fig. 6, the levels of apoptosis induction reached by the co-administration of 2  $\mu$ M R8-PNA-a155 and 2  $\mu$ M R8-PNA-a221 in T98G cells are similar to those obtained by singular administration of 4  $\mu$ M R8-PNA-a155 or R8-PNA-a221. This indicates that the use of suboptimal concentrations of anti-miR-155 and anti-miR-221 PNAs allows reaching high levels of apoptosis (Fig. 6A). This level was further increased when TMZ was added (Fig. 6A and B). These results demonstrated that co-administration of R8-PNA-a155 and R8-PNA-a221 induced apoptosis in TMZ-treated T98G cells at levels similar to those obtained following singular administration of high doses of R8-PNA-a155 or R8-PNA-a221.

## Discussion

Because of the poor prognosis of gliomas and of the development of resistance to drugs commonly used in post-surgery antitumor protocols, novel therapeutic strategies are highly needed for GBM, some of which may already

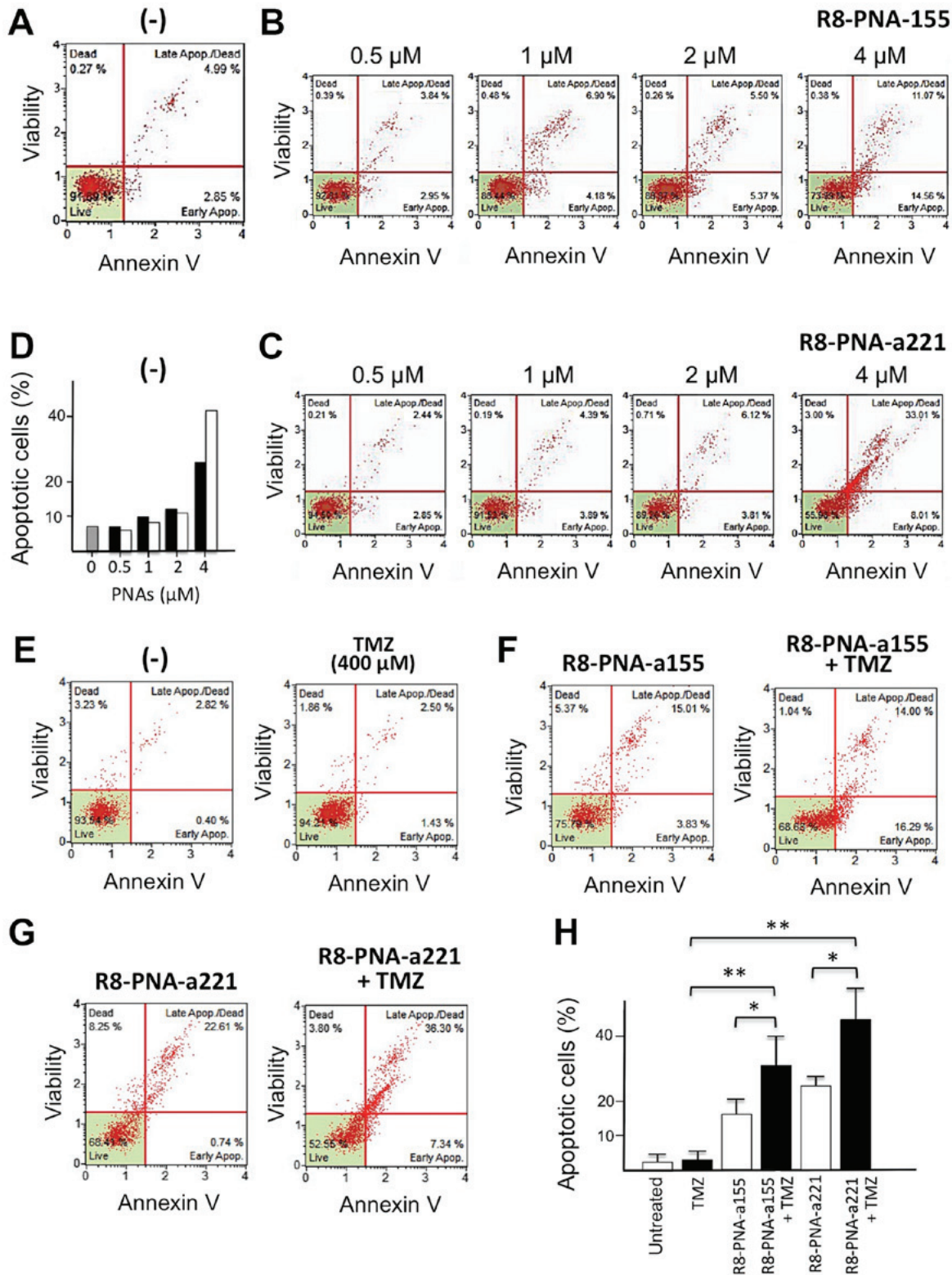


Figure 5. (A-D) Apoptosis was measured following single administration of increasing amounts of R8-PNA-a155 (A, B and D) and R8-PNA-a221 (A, C and D) in T98G cells. (E-H) Effects of single administration of R8-PNA-a155 and R8-PNA-a221 on TMZ-treated T98G cells. Panels E-G show representative plots from cells treated for 48 h as indicated and analyzed for Annexin V release. (H) Quantification of the results obtained with 4  $\mu$ M antagomiR PNAs in the presence of TMZ. T98G cells were treated with 400  $\mu$ M TMZ in the absence or in the presence of the indicated concentrations of R8-PNA-a155 and R8-PNA-a221. Results are presented as the average  $\pm$  standard deviation from three independent experiments. TMZ, temozolomide; PNAs, peptide nucleic acids. \*P<0.05 and \*\*P<0.01 with comparisons indicated by brackets.

be tested in therapeutic protocols for other tumors (44-47). MicroRNAs are putative molecular targets, based on current knowledge: glioma-associated oncomiRNAs have been

described whose expression is deeply impaired during onset and progression of these tumors (48-50); the expression of some oncomiRNAs is significantly associated with

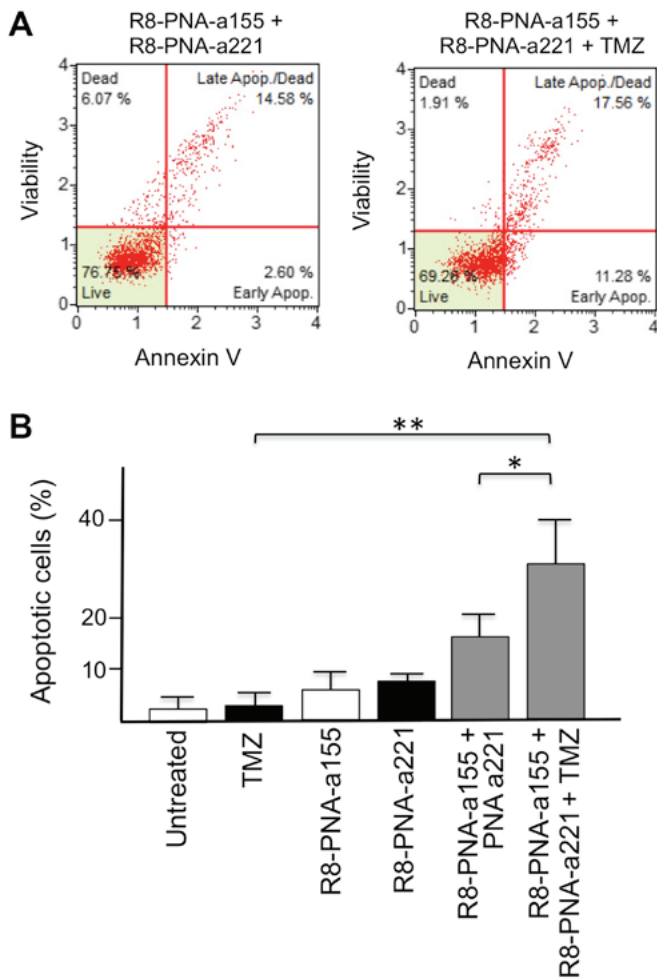


Figure 6. Effects of combined administrations of suboptimal concentrations of R8-PNA-a155 and R8-PNA-a221 on TMZ-treated T98G cells. (A) Representative plots of the proapoptotic profile obtained from cells treated as indicated for 48 h and analyzed for Annexin V release. (B) Quantification of the results obtained from three independent experiments. Results are presented as the average  $\pm$  standard deviation. TMZ, temozolomide. \* $P < 0.05$  and \*\* $P < 0.01$  with comparisons indicated by brackets.

outcome, therefore rendering them a very useful potential marker in the analysis of non-invasive liquid biopsies (51); miRNA therapeutics have been recently demonstrated to be useful in the treatment of a variety of human pathologies, including gliomas (52-55); miRNA pathways might exhibit a patient-to-patient variability, allowing the design of personalized protocols (56,57); and miRNA targeting might be employed to overcome drug-resistance, an issue of great relevance in the management of patients with glioma (58-61).

Gliomas express two microRNAs, miR-155-5p and miR-221-3p, at high levels, and these are associated with oncogenic activity and strong antiapoptotic effects (62-64). These effects are mediated by a putative targeting by these miRNAs of the 3'UTR sequence of the proapoptotic caspase-3 mRNA, possibly leading to a downregulation of caspase-3 expression (42,43,65). Therefore, miR-155-5p and miR-221-3p appear to be appealing targets for the development of therapeutic protocols for gliomas.

The use of PNAs is very promising since, from their introduction following the first description by Nielsen *et al.* (18), they have been considered for therapeutic interventions on a

variety of human pathologies, including cancer. However, several issues should be considered in proposing PNAs as therapeutic tools in miRNA therapeutics: their delivery to target cells; possible off-target effects due to the fact that a single miRNA might recognize several mRNA targets; and the presence in the 3'UTR of each target mRNA of several potential miRNA binding sites, which might require co-administration of different anti-miRNA molecules, leading to further complications of the off-targeting issue.

The major conclusion of the present study is that two miRNAs (miR-155-5p and miR-221-3p), highly expressed in gliomas and demonstrated to regulate caspase-3 gene expression (42,43,65), were the target of PNA-based induction of apoptosis in the TMZ-resistant T98G glioma cell line. Of note, TMZ was able to further increase apoptosis induced by the PNAs targeting these two miRNAs. Furthermore, combined treatment using low PNA doses and TMZ resulted in higher pro-apoptotic effects, suggesting a sensitization of glioma cells to TMZ.

The present results support the concept that anti-miRNA strategy could lead to therapeutic relevant inhibition of biological functions of miRNA-regulated mRNAs (8-11) and that PNA-based anti-miRNA molecules are very promising reagents as a tool for the development of therapeutic protocols for tumor cell growth inhibition. In this context, and considering the low uptake of PNAs (21), further research on PNA analogues is necessary with the aim of increasing delivery, improving stability, controlling the intracellular distribution and the *in vivo* tissue targeting. In addition, PNAs selectively interacting with specific mature miRNAs, pre-miRNAs or pri-miRNAs might be compared as a further step for the selection of the best candidate PNA-based drugs. The present study strongly indicated that the combined treatment of target glioma cells with PNAs inhibiting both miR-155-5p and miR-221-3p was associated with a significant improvement of the efficacy of the treatment, as evidenced by their effects on cell apoptosis. This conclusion supports the strategy of designing multifunctional PNA-containing systems or nanocarriers (67,68), enabling to perform targeting of multiple miRNA sequences. Finally, our data are compatible with a sensitization of T98G cells to TMZ, supporting previous observations indicating anti-miRNA strategy may be a potential tool to reverse drug resistance, which is one of the major unresolved issues in the therapeutic management of patients with glioma (58-60).

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### Availability of data and materials

All data generated or analyzed during this study are included within the manuscript.

### Authors' contributions

RG and RC conceived and planned all the experiments. Cell culture was performed by RM and EB. Bioinformatic analyses were conducted by EF. Design and synthesis of PNAs were performed by RC and AM. Treatments of the cells with PNAs were performed by RM and LCC. Molecular analyses were performed by AF and JG. Microarray-based analysis of miRNAs in gliomas has been conducted by GC and MCD. Analysis of apoptosis was performed by IL and MCD. RM, RG and GC contributed to the interpretation of the results. RG wrote the manuscript. All authors provided critical feedback and contributed to the final version of the manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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