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Efficient Delivery of MicroRNA and AntimiRNA Molecules Using an Argininocalix[4]arene Macrocycle

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MicroRNAs (miRNAs) are short non-coding RNA molecules acting as gene regulators by repressing translation or by inducing degradation of the target RNA transcripts. Altered expression of miRNAs may be involved in the pathogenesis of many severe human diseases, opening new avenues in the field of therapeutic strategies, i.e., miRNA targeting or miRNA mimicking. In this context, the efficient and non-toxic delivery of premiRNA and antimiRNA molecules might be of great interest. The aim of the present paper is to determine whether an argininocalix[4]arene is able to efficiently deliver miRNA, pre-miRNA, and antimiRNA molecules to target cells, preserving their biological activity. This study points out that (1) the toxicity of argininocalix[4]arene 1 is low, and it can be proposed for long-term treatment of target cells, being that this feature is a pre-requisite for the development of therapeutic protocols; (2) the delivery of premiRNA and antimiRNA molecules is efficient, being higher when compared with reference gold standards available; and (3) the biological activity of the premiRNAs and antimiRNAs is maintained. This was demonstrated using the argininocalix[4]arene 1 in miRNA therapeutic approaches performed on three well-described experimental model systems: (1) the induction of apoptosis by antimiR-221 in glioma U251 cells; (2) the induction of apoptosis by premiR-124 in U251 cells; and (3) the inhibition of pro-inflammatory IL-8 and IL-6 genes in cystic fibrosis IB3-1 cells. Our results demonstrate that the argininocalix[4]arene 1 should be considered a very useful delivery system for efficient transfer to target cells of both premiRNA and antimiRNA molecules, preserving their biological activity.

INTRODUCTION

MicroRNAs (miRNAs, miRs) are short non-coding RNA molecules that act as gene regulators by repressing translation or by inducing degradation of the target RNA transcripts.[1–4] Emerging evidence suggests that the altered expression of miRNAs may be involved in the pathogenesis of several human diseases, such as Alzheimer's disease, type 1 diabetes, cancer, cardiovascular, inflammatory, and genetic diseases.[5–11] The involvement of miRNAs in human pathologies is docu-mented by an increasing nomenclature describing miRNA classes such as oximiRNAs (i.e., miRNAs involved in the oxidative stress),[12] apoptomiRNAs (i.e., miRNAs involved in apoptosis),[13] oncomiR-NAs, and metastamiRNAs (i.e., miRNA associated with tumor pro-gression and metastasis, respectively).[14,15] This field of biological investigation is important not only for basic research, but also in applied biomedicine. In fact, approaches based on miRNAs targeting (miRNA therapeutics)[16–25] can be designed to inhibit tumor cell growth and metastasis and to counteract the resistance of tumor cells to anti-cancer drugs.[15,17]

In the case of miRNAs directly or indirectly causing pathologies (such as oncomiRNAs and metastamiRNAs in cancer), their functions can be inhibited or fully suppressed with antagomiR molecules,[17,21,26] while in the case of miRNAs downregulated in diseases and, for this reason, contributing to clinical complications caused by their dys-regulated expression, their biological activity can be rescued with the use of miRNA mimics (for instance, transfection with premiRNA molecules).[17,22,23,27] For instance, Chan et al.[28] demonstrated that sequence-specific functional inhibition of oncomiR-138 in malignant gliomas prevents tumor sphere formation *in vitro* and impedes tumorigenesis *in vivo*. In addition, Wagenaar et al.[29] were able to deregulate the transcriptional network in hepatocellular carcinomas by targeting miR-21 with sequence-specific antagomiR, thus inducing a significant de-repression of direct targets of miR-21, which led to loss of viability in the majority of hepatocarcinoma cell lines tested. In another example, Ma et al.[30] reported that therapeutic silencing of the oncogenic miR-10b inhibits metastasis in a mouse model of mammary tumor.
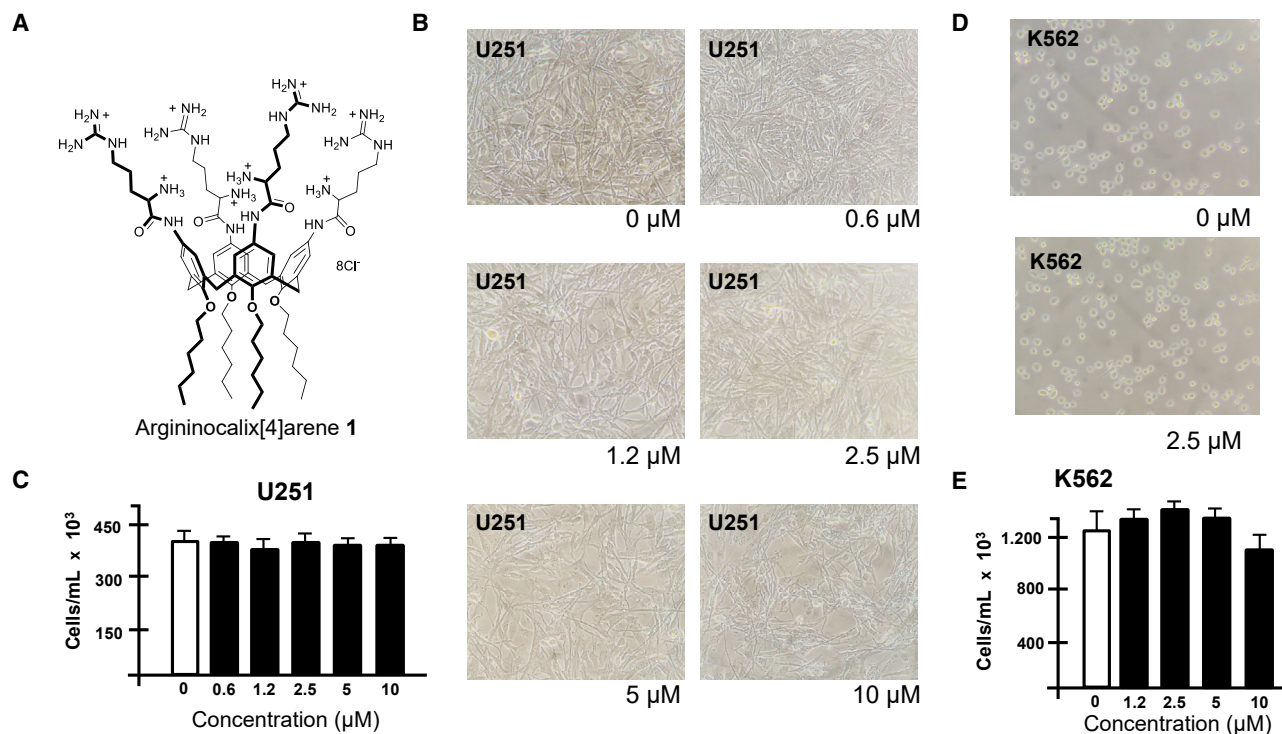
Figure 1. Effects of Argininocalix[4]arene 1 on Cell Morphology and Cell Growth

(A) Molecular structure of argininocalix[4]arene **1**. (B–E) Effects of argininocalix[4]arene **1** on morphology (B and D) and cell proliferation (C and E) of U251 glioma (B and C) and leukemic K562 (D and E) cells, analyzed after 48 (B and D) and 72 (C and E) h of cell culture in the presence of the indicated concentration of argininocalix[4]arene **1**. The average ± SD of three indipendent experiments are shown in panels C and E.

As far as miRNA targeting is concerned, the vehiculation of miRNAs and antagomiRNAs to target cells is a key issue.[25,31–34] In fact, miRNA vehicles not only are essential to transfer miRNA-based molecules into target cells, but also have a key role in avoiding their degradation. A large number of vehicles were tested both *in vitro* and *in vivo*, but most of them present several issues, such as inappropriate biodistribution, disruption, and saturation of endogenous RNA machinery and high toxicity.[31]

Concerning the delivery of nucleic acids, we reported[35–38] on the use of macrocyclic vectors based on calix[4]arene amphiphiles for the transport of plasmid DNA into cells. In particular, the arrangement of only four units of a basic amino acid,[37] such as arginine and lysine, linked at the aromatic para position of the calixarene scaffold, gave rise to new, potent, non-viral delivery systems. The parallel spatial organization of the amino acid units characterizing these derivatives was particularly effective on the transfection process, perhaps for the significant presence of available primary α-amino groups with respect to the usual oligoarginine and oligolysine peptides used for these purposes where these groups are involved in amide bonds. These features make in particular argininocalix[4]arene **1** (Figure 1A), the progenitor of a new class of efficient synthetic cationic surfactants, which could constitute a valuable alternative to commercially available formulations in transfection protocols.[37,38]

The remarkable properties of the calixarene-based molecule argininocalix[4]arene **1** as DNA vector seemed correlated also to the length of the long nucleic acid filament used in that case, in particular for the condensation process,[35,38] so that the possibility of the use of argininocalix[4]arene **1** for the transfection of oligonucleotides and miRNAs was not easily expected. Nevertheless, starting from the evidence that this molecule showed an impressive ability to vehicle DNA with respect to a larger library of calixarene-based molecules, it was chosen to initiate the investigation of miRNA delivery.

The U251 glioma cell line,[39] the cystic fibrosis IB3-1 cell line,[40] and the chronic myelogenous leukemia K562 cell line[41] were chosen as cellular model systems. U251 and IB3-1 cell lines grow attached to the flasks,[39,40] while K562 cells grow in suspension.[41] For miRNA targets, miR-221-3p was considered as a model for antimiRNA therapy, while miR-210-3p, miR-124-3p, and miR-93-5p were considered as model systems for miRNA replacement therapy. The effects of miRNA targeting and/or mimicking on the proposed cellular model systems are well known.[42–48] Targeting of miR-221-3p facilitates apoptosis of U251 glioma cells,[42,43] miR-210-3p mimicking causes inhibitory effects on the target mRNA BCL11A,[44] miR-124-3p mimicking is associated with induction of apoptosis of U251 glioma cells,[45,46] and miR-93-5p mimicking leads to downregulation of the
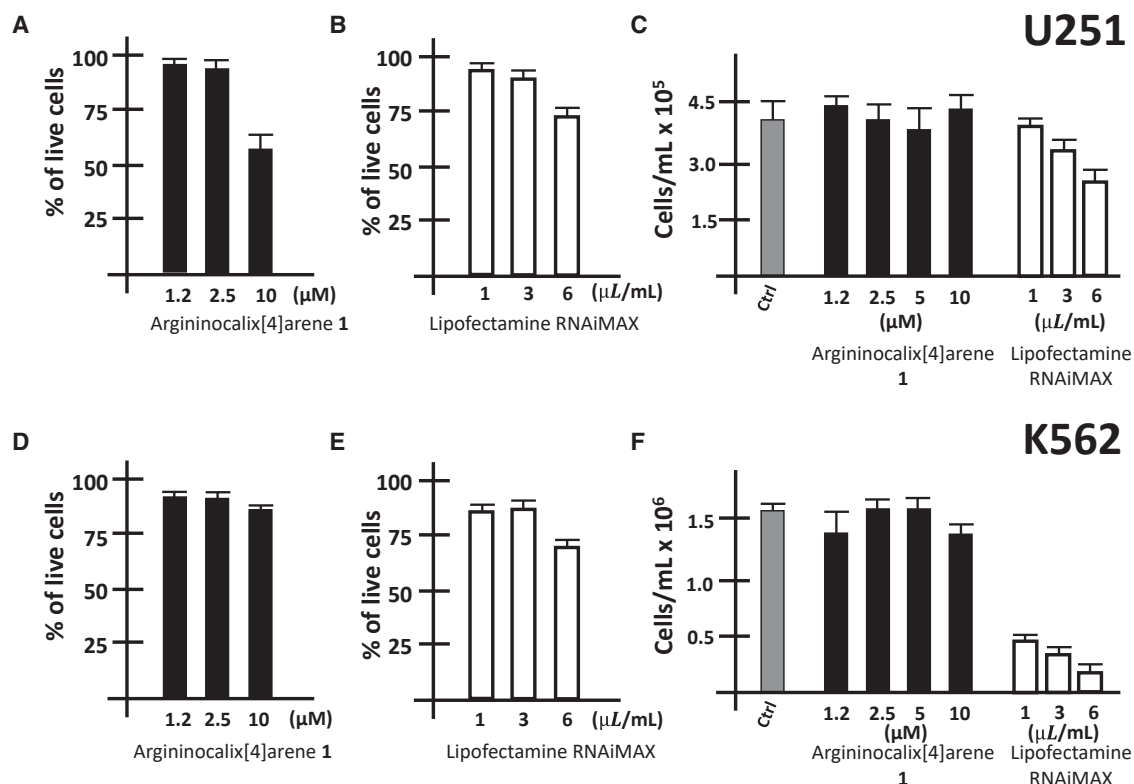
Figure 2. Effects of Argininocalix[4]arene 1 on Cell Morphology and Cell Growth, Comparison with Lipofectamine RNAiMAX

(A–F) Effects of argininocalix[4]arene **1** (A, C, D, and F) and lipofectamine RNAiMAX (B, C, E, and F) on vitality (A, B, D, and E) and cell proliferation (C and F) of U251 glioma (A–C) and leukemic K562 (D–F) cells, analyzed after 48 h of cell culture in the presence of the indicated concentration of argininocalix[4]arene **1** or Lipofectamine RNAiMAX. The average ± SD of three indipendent experiments are shown.

expression of interleukin-8 (IL-8) and interleukin-6 (IL-6) in several cellular model systems.[47,48]

RESULTS

Effects of Argininocalix[4]arene 1 on Cell Morphology and Cell Growth

One of the major issues associated with the use of commercially available vehicles for the delivery of bioactive RNA- and DNA-based molecules is the high toxicity and anti-proliferative effects of these compounds. Of course, in the case of any proposed protocol for miRNA therapeutics, the possibility of performing long-term treatment is required. As the paper by Bagnacani et al.[37] just considered short-term (5 h exposure) treatments, the first set of experiments were designed to verify the possibility to perform exposure to argininocalix[4]arene **1** for longer periods of time. Effects on cell morphology and cell growth were determined at 48 and 72 h after the transfection, testing different concentrations of calixarene-based molecule (0.6, 1.2, 2.5, 5, and 10 μM). The argininocalix[4]arene **1** was maintained in contact with the cells at the indicated concentrations for all the treatment time. As shown in Figure 1B, 48 h after the transfection no major differences in morphology were found in the presence of the argininocalix[4]arene **1**, while only a slight alter-

ation of cell morphology was detected when 10 μM of argininocalix[4]arene **1** was used.

In addition, Figure 1C demonstrates that argininocalix[4]arene **1** does not show antiproliferative effects. Lack of inhibitory effects of argininocalix[4]arene **1** on morphology and cell growth were confirmed using the K562 cell system (Figures 1D and 1E). Comparison with Lipofectamine RNAiMAX is shown in Figure 2, which analyzes vitality (Figures 2A, 2B, 2D, and 2E) and cell growth (Figures 2C and 2F) after 48 h in U251 and K562 cells. A slight decrease of viability was observed for argininocalix[4]arene **1** and Lipofectamine RNAiMAX when used at high concentrations. The decrease of the proportion of live U251 and K562 cells is not unexpected, since it is well established that most of the transfection reagents are to some extent cytotoxic to the cells. However, inhibition of cell growth was detected using Lipofectamine RNAiMAX at the optimal concentration for RNA transfection, in agreement with other published studies[49–53] (Figures 2C and 2F).

On the basis of the results shown in Figures 1 and 2, the experiments presented in this study and related to the analysis of the biological effects of transfection protocols were conducted at 2.5 μM, unless otherwise indicated.
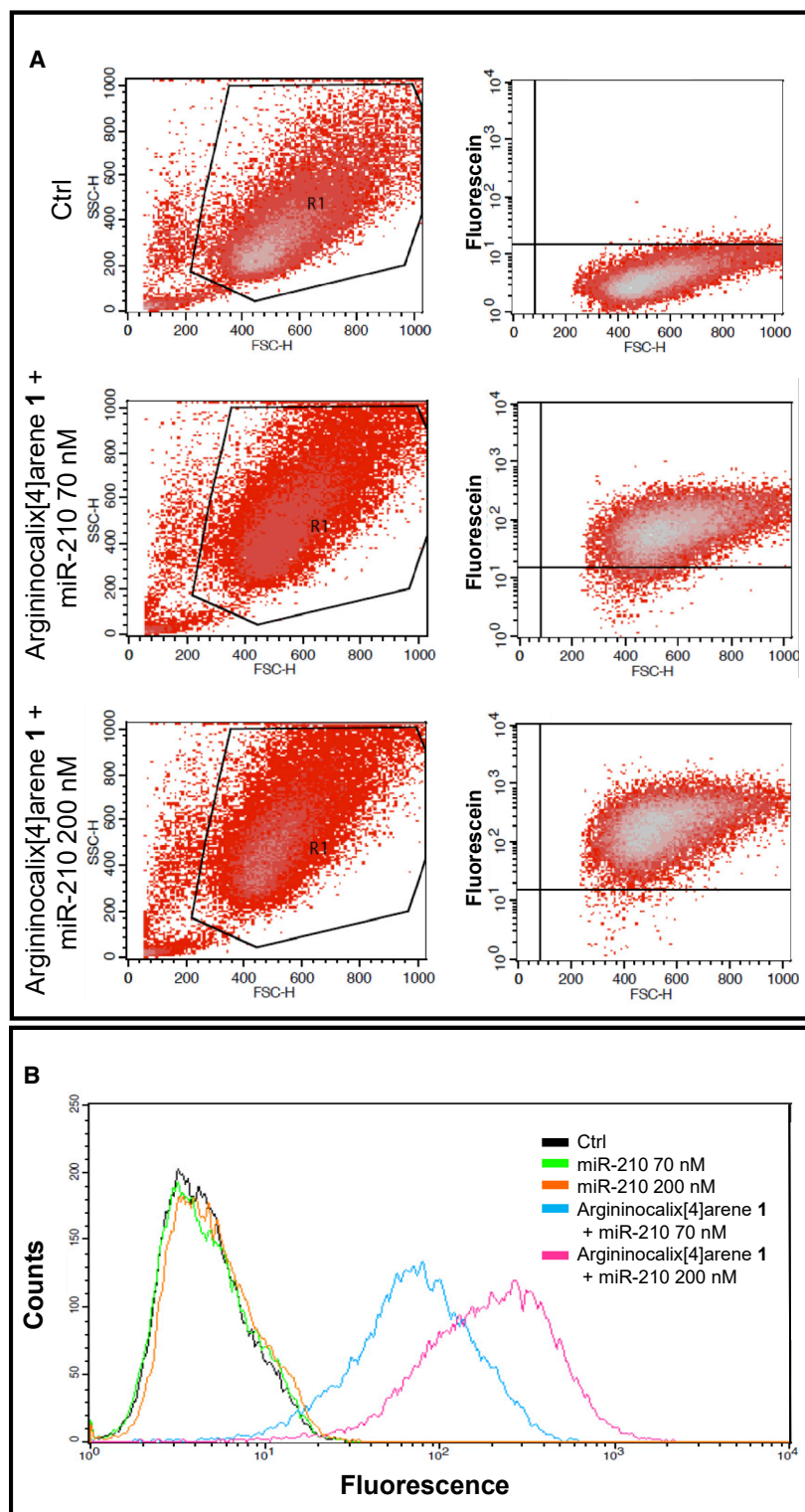
Figure 3. Transfection of Mature Fluorescent miRNA

Human glioma U251 cells were cultured in absence (Ctrl) or in the presence of the indicated concentrations (70 and 200 nM) of fluorescein labelled mature miR-210, either free or vehiculated with 10 μM of argininocalix[4]arene **1**. Mature miRNA internalization was verified using FACS analysis. (A) Representative fluorescence plots. (B) Overlay plot.

ployed, allowing verifying miRNA internalization using rapid methods, as fluorescence microscopy and FACS (fluorescence-activated cell sorting) analysis.

As indicated in Figure 3, the adherent U251 cell line displayed a sharp change in fluorescence intensity when the cells were transfected with the fluorescent mature miR-210, using argininocalix[4]arene **1** at final concentration of 10 μM. For this preliminary experiment, the same argininocalix[4]arene **1** concentration previously reported by Bagnacani and colleagues[37] for DNA transfection was used. U251 cells transfected with two different concentrations of miR-210 (70 nM and 200 nM) were analyzed by FACS (Figures 3A and 3B), and fluorescence variation with respect to the control cells was reported. As indicated in Figure 3B, when miR-210 was added with no vehicle, no variation of fluorescence was detected, while the transfection of miR-210 with argininocalix [4]arene **1** lead to a shift in fluorescence proportional to the miR-210 concentration.

The data obtained by fluorescence analysis were then confirmed by the quantification of intracellular levels of miRNAs after the transfection using qRT-PCR (Figures 4A–4D). Comparison with the cell line K562 was also considered, since K562 cells are known to be very difficult to be transfected. Moreover, an additional mature miRNA (miR-221) was transfected to exclude that the interaction between argininocalix[4]arene **1** and mature miRNA was limited to a specific miRNA sequence. As reported in Figures 4A and 4C, CT (cycle threshold) values before (white rhombuses) and after (black squares) transfection were determined for both miRNAs miR-210 and miR-221 and for both U251 and K562 cell lines. In all cases, a CT decrease was observed in the presence of vector argininocalix [4]arene **1**, indicating that the miR-221 and miR-210 content was sharply increased. Concerning the starting levels of miR-221 and miR-210 in U251 and K562 cell lines, while miR-210 seems to have similar CT values in the two cells lines, miR-221 is more expressed in the U251 cell line with respect to K562 cells. Moreover, miRNA accumulation in transfected cells
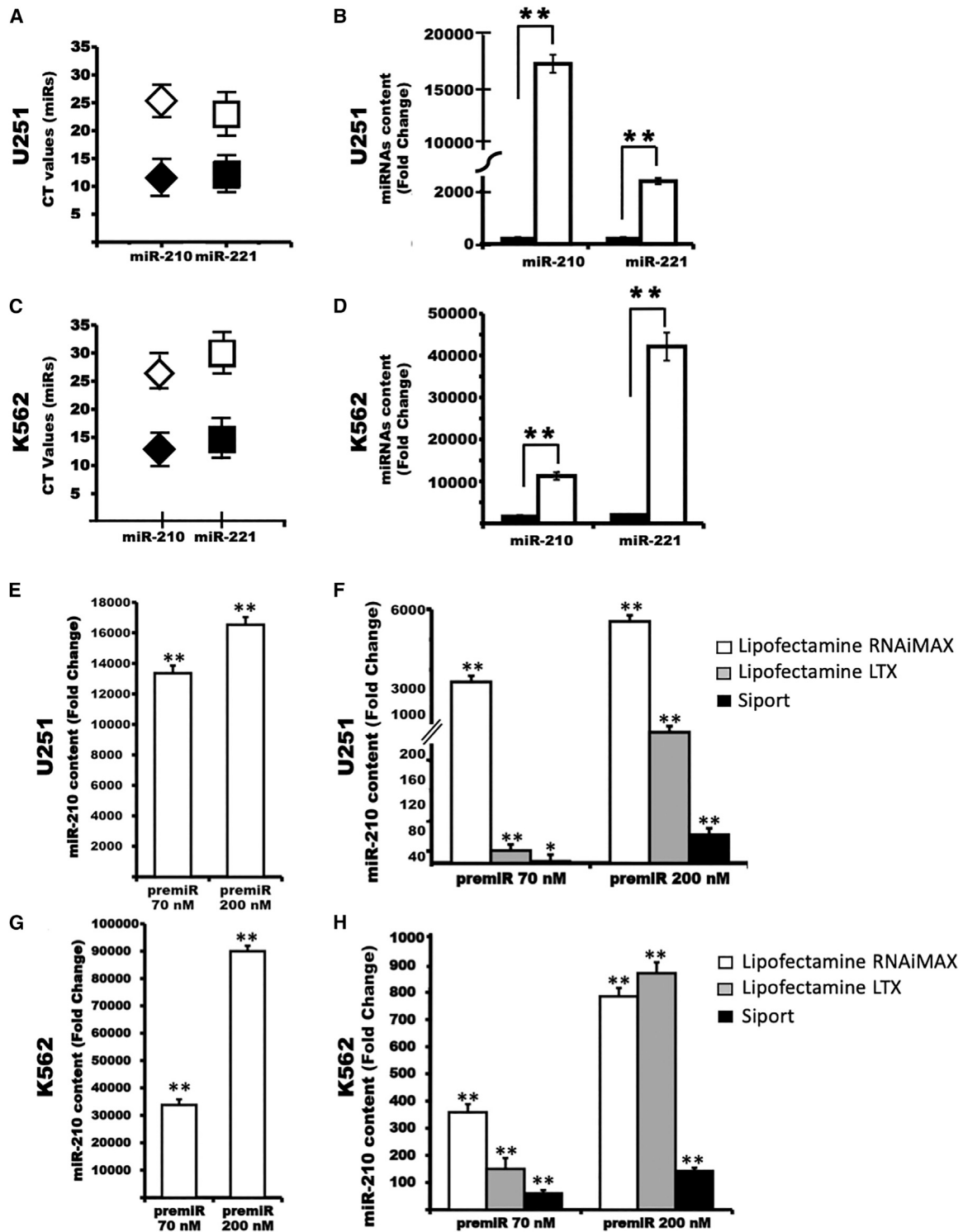
Transfection of Mature miRNAs Using Argininocalix[4]arene 1

In order to evaluate the transfection ability of argininocalix[4]arene **1**, a synthetic mature miRNA conjugated with fluorescein was first em-

Figure 4. Transfection of Mature miRNAs and premiRNAs with Argininocalix[4]arene 1

(A–D) Mature miRNA transfection. Two different cell lines, U251 (A and B) and K562 (C and D), were employed. Cells were characterized for their starting miR-210 (white rhombuses) and miR-221 (white squares) levels, comparing CT (cycle threshold) values. Moreover, measured CT values after transfection were reported: black rhombuses (miR-210) and black squares (miR-221) (A and C). In (B) and (D), the fold change in transfected cells (white boxes/bars) with respect to control samples (black boxes/bars) was

(open bars) was compared with those found in the no-transfected sample (black bars), and the results are depicted in Figures 4B and 4D. For both miRNAs and in both cell lines, significant (p < 0.001) increase of miRNA levels after transfection was detected, in agreement with CT changes.

In order to increase miRNA levels into target cells, one of the most useful methods is to deliver synthetic premiRNA molecules into them. As for miRNAs, cationic liposomes are exploited, interacting with the negative charges present in the premiRNA structure and forming a complex that is internalized by the cells. Lipofectamine (Invitrogen) or siPORT (Ambion) are two examples of reagents commonly used for this purpose.[53] Generally, these reagents exhibit high toxicity and a very variable transfection efficiency, dependent on the employed cell line. In order to increase miRNA levels, in the common laboratory practice premiRNAs are usually preferred to mature miRNA, for functional issues. In fact, premiRNA is partially processed by the cellular miRNA processing machinery that makes it more similar to 'physiological' miRNA.

For this reason, after the achievement of the proof-of-principle data shown in Figures 1, 2, 3, and 4A–4D, we tried to transfect premiRNA molecules with argininocalix[4]arene 1. As a representative example, the data obtained using premiR-210 are shown.

Transfection of PremiRNA Using Argininocalix[4]arene 1

In Figures 4E–4H, both cell lines were transfected with two different premiR-210 concentrations, commonly used with commercially available transfection agents.[44] The final concentration of argininocalix[4]arene 1 was 10 μM. The increase of miR-210 intracellular concentration with respect to untransfected samples was determined using qRT-PCR and let-7c and small nuclear RNA (snRNA) U6 as reference. In both cell lines, the levels of miR-210 were significantly increased after 24 h transfection. Moreover, when a comparative analysis was performed between argininocalix[4]arene 1 and a limited panel of the most efficient commonly used commercially available transfection agents, Lipofectamine LTX, Lipofectamine RNAiMAX, and siPORT, at the same premiR-210 concentration, the argininocalix[4]arene was found at least 10-fold more active than all of these (Figures 4F and 4H).

The experiments described in Figures 5A–5D were designed to find the optimal transfection conditions, when argininocalix[4]arene 1 was used as vehicle and premiR as the molecule to be carried. First of all, we tried to set up the optimal premiRNA concentration, using the same vehicle concentration (10 μM) for each point (Figures 5A and 5C). The collected results demonstrated that using argininoca-

lix[4]arene 1 at 10 μM, 70 nM and 15 nM premiR-210 should be used to obtain efficient uptake by U251 and K562 cells, respectively. After this set of experiments, concentration of argininocalix[4]arene 1 was progressively reduced to verify whether at low values efficient premiR-210 transfection was still supported. Four different decreasing concentrations of argininocalix[4]arene 1 were investigated (from 10 μM to 1.25 μM), and the miR-210 fold increase with respect to control was determined by qRT-PCR. For both cell lines, vector argininocalix[4]arene 1 at 1.25–2.5 μM concentrations was sufficient to efficiently deliver premiR-210. From the qualitative point of view, since the qRT-PCR quantifies the mature miR-210, the experiments shown in Figure 5 demonstrate that premiR-210 processing to mature miR-210 occurs within both the U251 and K562 transfected cells.

In all the experiments depicted in Figures 3, 4, and 5A–5D, the transfection of miRNA/argininocalix[4]arene 1 formulations were performed using the protocol indicated by Bagnacani et al.[37] and described in detail in the Materials and Methods section. In brief, a mix containing culture medium, without FBS, argininocalix[4]arene 1, and the molecule that needs to be carried is prepared and after 20 min of incubation at room temperature is transferred to cells. After 5 h contact, the miRNA/argininocalix[4]arene 1 formulation is removed and replaced with fresh culture medium. While this protocol is well suited for biotech experiments (i.e., highly efficient transfection to target cells in vitro), it is clearly not suited when protocols are designed for pre-clinical therapeutic approaches, when the exposure to the putative therapeutic treatment should be performed in continuous, and not in "pulse" fashion. For this reason, we evaluated the possibility to maintain the transfection mix in contact with cells for all the treatment time, as anticipated in Figure 1. Starting from the hypothesis that in the case of a continuous contact, a smaller amount of argininocalix[4]arene 1 is required, we tested three different concentrations of argininocalix[4]arene 1 (1.25, 2.5, and 10 μM) and compared miR-210 increase obtained after 5 h contact with that obtained after continuous contact (24 h). As expected from the data demonstrating no toxicity of long-term cultures, the results obtained show that transfection dependent on argininocalix[4]arene 1 was more efficient in continuous cultures (Figures 5E and 5F).

Transfection of AntagomiRNA Molecules with Argininocalix[4]arene 1

Not only miRNA and premiRNA, but also antagomiRNA molecules, which are usually used to reduce miRNA levels, require an adequate delivery system. Normally, commercially available delivery systems are able to vehicle both premiRNA and antagomiRNA molecules, using similar transfection conditions. Starting from this consideration,

calculated using qRT-PCR. To calculate fold change and CT values, three independent experiments were performed, and average ± SD values are shown. (E–H) PremiRNA transfection. (E and G) two different concentrations (70 and 200 nM) of premiR-210 were transfected in both cell lines: U251 (E) and K562 (G) and miR-210 fold change was determined 24 h after premiRNA transfection, using qRT-PCR. (F and H) Three commercial available transfection agents, Lipofectamine RNAiMAX (white boxes), Lipofectamine LTX (gray boxes), and siPORT (black boxes) were compared with argininocalix[4]arene 1 using U251 (F) and K562 (H) as experimental model systems. All data were expressed as miR-210 fold change in transfected samples with respect to untransfected samples. The average ± SD values of three independent experiments are shown ±. p < 0.05 (*, significant), p < 0.01 (**, high significance).
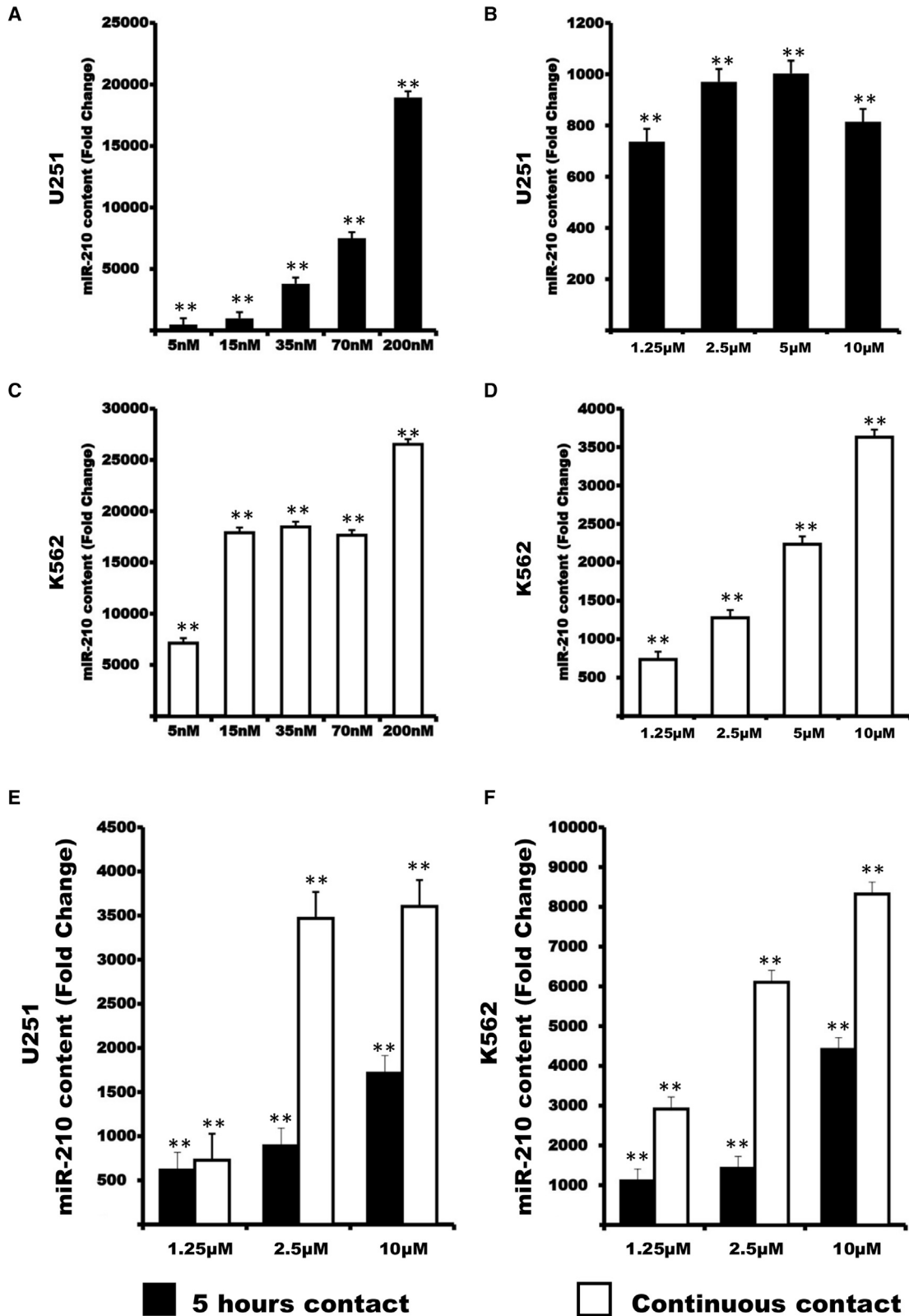
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we verified the ability of argininocalix[4]arene **1** to carry antago-miRNA molecules into cells (Figures 6A and 6B). We tested two different antagomiRNA molecules, antimiR-210 (Figure 6A, white bars), which was transfected into K562 cells, and antimiR-221 (Figure 6B, white bars), transfected into U251 cells which express miR-221 at high levels. Moreover, a comparison between argininocalix[4]arene **1** (white bars) and one of the most efficient commercially available transfection agents was performed (black bars). Similar miRNA level decreases were observed for both antimiR-210 and antimiR-221 delivered with the calixarene. Furthermore, miRNA decrease seems to be greater when argininocalix[4]arene **1** was used as transfection agent with respect to Lipofectamine RNAiMAX.

PremiRNA and AntimiRNA Molecule Transfection Using Argininocalix[4]arene 1 to Erythroid Precursor Cells (ErPCs)

Since all the previously shown experiments were performed in immortalized cell lines, to further investigate the potentiality of argininocalix[4]arene **1**, we also employed a primary cell model, the erythroid precursors isolated from peripheral blood, which also are generally poorly transfected (Figure 6). The employment of ErPCs[54] allowed us to verify the behavior of argininocalix[4]arene **1** in a more "physiological" model. Transfection was performed according to the "standard" protocol, 10 μM of vector and 5 h mix contact, using both premiR-210 (Figure 6, panel C) and antimiR-210 (Figure 6, panel D). Intracellular miR-210 levels in ErPCs, isolated from three different donors, were quantified using qRT-PCR, miR-210 fold change was calculated with respect to the control sample (no transfected sample), and snRNA U6 was employed as internal reference. This experiment demonstrates that argininocalix[4]arene **1** is an excellent transfection reagent for primary cells (Figures 6C and 6D).

MicroRNAs are intensely studied because of their ability to modulate target mRNA, so the alteration of intracellular miRNA content is actually only the first step to verify the effectiveness of the transfection experiments. In fact, is important to verify if miRNA modulation is able to modify the expression of its target mRNA; that represents the real goal of miRNA-based therapy. It was then essential for us verify if premiRNA or antimiRNA molecules carried by argininocalix[4]arene **1** were able to generate the final biological effect. Regarding premiR-210, we started with what was previously demonstrated by Gasparello et al.[44] In brief, miR-210-3p is able to target mRNA of BCL11A, one of the most studied gamma globin gene repressors. For this reason, we transfected ErPCs cells, the most suited cellular model for this kind of experiment, with premiR-210 using argininocalix[4]arene **1** (Figure 6E). BCL11A-XL mRNA expression was analyzed using qRT-PCR 48 h after the transfection, and fold change with respect to the control sample was calculated. This experiment demonstrated that, as expected, the formulation premiR-210/argininocalix[4]arene **1** is able to induce a decreased BCL11A mRNA content.

Therapeutic Potential of Argininocalix[4]arene 1 as Vehicle of antimiRNA Molecules: Delivery of AntimiR-221 to U251 Glioma Cells

As demonstrated in several studies,[42,43,55–57] miR-221-3p is involved in the apoptotic pathway, and its downregulation causes the increase of apoptotic mechanisms in several cell lines. Consequently, if antimiR-221 delivered by argininocalix[4]arene **1** is able to decrease miR-221 levels in cells, an increase of apoptotic cells should be detected. U251 cells were transfected with antimiR-221 at a final concentration of 70 nM, using 2.5 μM argininocalix[4]arene **1**.

The mixture argininocalix[4]arene **1**/antimiR-221 was maintained in contact with cells for 72 h, and then the percentage of apoptotic cells was determined (Figure 7). In order to evaluate the percentage of apoptotic cells, annexin V assay was performed, and cell population plots are reported in Figure 7A. As it is possible to see in Figure 7A, cell population, on the base of the fluorescence levels, is divided into four groups: live cells, early apoptotic cells, late apoptotic cells, and dead cells. Mean values for each sample are reported in Figure 7B, while the percentage of apoptotic cells in each representative sample is reported in the plots in Figure 7A. When cells are transfected with antimiR-221 delivered by argininocalix[4]arene **1**, a significant increase of the percentage of apoptotic cells is detected. Moreover, when only argininocalix[4]arene **1** was employed, an increase of apoptotic cells was noticed, even if lower than the value observed for antimiR-221 delivered with argininocalix[4]arene **1**. Interestingly, the apoptotic profile observed is significantly different between sample treated with only argininocalix[4]arene **1** and cells treated with antimiR-221 transfected with argininocalix[4]arene **1**; in fact, while the percentage of early apoptotic cells in the two samples are very similar, a marked difference is reported in the case of late apoptotic cells. Percentage of late apoptotic cells in the calixarene sample is marked lower than the percentage reported for

Figure 5. PremiRNA Transfection with Argininocalix[4]arene 1

(A–D) Setup of the experimental conditions. The optimal transfection protocol was setup for both U251 (black boxes) and K562 (white boxes) cells. In histograms, miR-210 fold change in transfected cells with respect to control (untransfected cells) was calculated 24 h after the transfection. In (A) and (C), incremental final concentration of premiR-210 (from 5 to 200 nM) were tested using for each point the same argininocalix[4]arene **1** concentration (10 μM), while in (B) and (D), four different concentrations of argininocalix[4]arene **1** (1.25, 2.5, 5, and 10 μM) were tested using for each point the same premiR-210 concentration (35 nM). All data are presented as the average of three independent experiments ± SD. (E and F) PremiRNA transfection with argininocalix[4]arene **1**: protocol setup. Two different transfection protocols were tested. In the first protocol, a mix containing premiR-210 and argininocalix[4]arene **1** was maintained in contact with cells for 5 h and then was removed and replaced with new fresh medium (black bars). In the second case, a premiR-210/argininocalix[4]arene **1** mixture was maintained in contact with cells for all the treatment time (white bars). The two protocols were tested in U251 (E) and K562 (F) cell lines. Experiment was performed in triplicate and the average values ± SD was reported. $p < 0.05$ (*, significant), $p < 0.01$ (**, high significance).
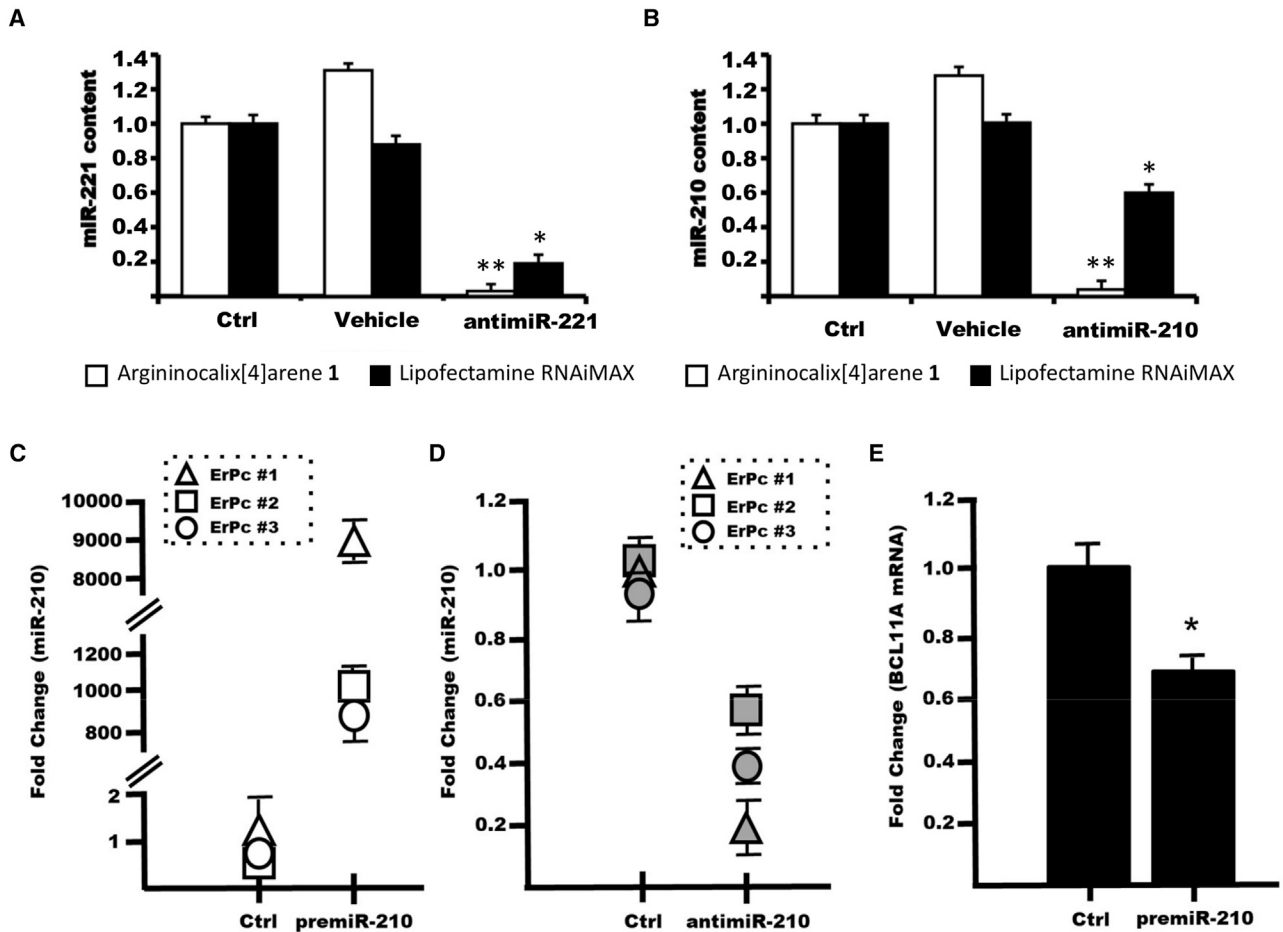
Figure 6. Transfection of AntagomiRNA Molecules with Argininocalix[4]arene 1

(A and B) AntimiRNA molecules transfection with argininocalix[4]arene **1**. Two different antagomiRNA molecules were transfected using argininocalix[4]arene **1** (white boxes): antimiR-221 was transfected in U251 cells (A), while antimiR-210 was transfected in the K562 cell line (B). Moreover, the argininocalix[4]arene **1** transfection efficiency was compared with Lipofectamine RNAiMAX (black boxes), used according to the manufacturer's instructions. Fold changes of miR-221 and miR-210 in transfected samples with respect to control samples (untransfected cells and cells treated with vectors alone) were calculated, and the average values ± SD was reported. (C and D) PremiRNA and antimiRNA molecules transfection with argininocalix[4]arene **1** in erythroid precursor cells (ErPCs). ErPCs isolated from three different donors were transfected with both premiR-210 (C) and antimiR-210 (D), using argininocalix[4]arene **1** as vehicle. Content of miR-210 in samples was determined using qRT-PCR, and the expression change of miR-210 was calculated using $2^{-\Delta\Delta CT}$ method considering untransfected cells as reference sample. The average ± SD of three independent experiments was reported. (E) Biological effect of premiR-210 in ErPcs. Erythroid precursor cells isolated from three different donors were transfected with premiR-210 (final concentration 200 nM) and argininocalix[4]arene **1** (final concentration 2.5 μM). 48 h after the transfection RNA was extracted and BCL11A-XL expression was analyzed using qRT-PCR. p < 0.05 (*, significant), p < 0.01 (**, high significance).

antimiR-221 delivered with argininocalix[4]arene **1**, and also comparable with the average values observed in the control sample. The extent of apoptosis induction was somehow low, considering the strong inhibition of miR-221-3p caused by the antimiR-221 delivered with argininocalix[4]arene **1**. This is not surprising, considering the fact that other anti-apoptotic miRNAs have been reported to play a role in glioma cell line, such as miR-222-3p and miR-155-5p. In this case, a combined therapy based on the co-delivery of antagomiRNAs targeting different anti-apoptotic miRNAs can be considered to maximize apoptosis induction, as reported by Brognara et al.[43] and, more recently, by Milani et al.[57]

Therapeutic Potential of Argininocalix[4]arene 1 as Vehicle of PremiRNAs: Delivery of miR-124-3p to U251 Glioma Cells

The miRNA miR-124-3p is a well-established oncosuppressor miRNA.[45,46] Accordingly, forced miR-124 expression in glioma cell lines and in other *in vitro* tumor cellular systems leads to deep changes, including inhibition of cell growth, increased chemo- and radio-sensitivity, and induction of apoptosis. Mimicking miR-124 has been proposed as a miRNA replacement therapy to exert anti-tumor effects both *in vivo* and *in vitro*. We have elsewhere reported induction of apoptosis by transfection of premiR-124.[17,45,46] For these reasons, we delivered premiR-124 with the argininocalix[4]arene **1** (Figure 8). We demonstrated first that the
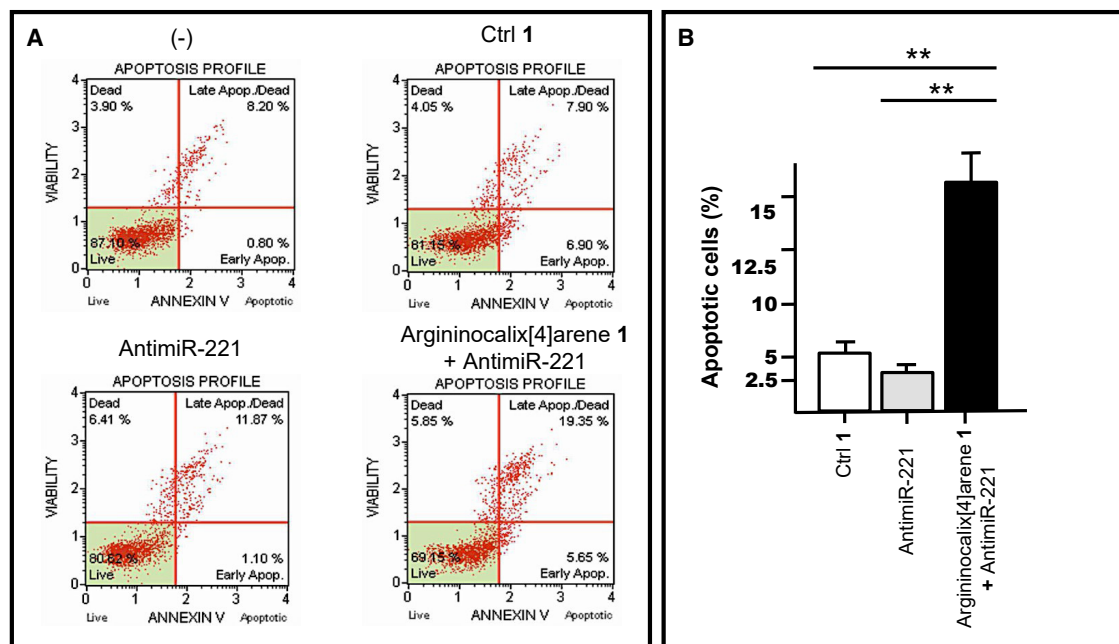
Figure 7. Biological Effects of AntimiR-221 Delivered to U251 Cells by Argininocalix[4]arene 1

Glioma U251 cells were untreated (−) or cultured in the presence of compound argininocalix[4]arene **1** (ctrl **1**), antimiR-221, or argininocalix[4]arene **1** + antimiR-221, as indicated. (A) Representative apoptosis profile plots. (B) Quantitative data representing the average ± SD of three independent quantifications (values of untreated cells subtracted) $p < 0.01$ (**, high significance).

argininocalix[4]arene **1** was able to transfect to the glioma U251 cells large amounts of premiR-124 (Figure 8A). Notably, addition of argininocalix[4]arene **1** does not increase miR-124 levels, demonstrating no induction effects of argininocalix[4]arene **1** on endogenous miR-124 production. Furthermore, addition of miR-124 in the absence of argininocalix[4]arene **1** was unable to enter the cells. By contrast, large amounts of miR-124 were internalized by the treated U251 cells (Figure 8A), leading to significant increase of apoptosis (Figures 8B and 8C).

Therapeutic Potential of Argininocalix[4]arene 1 as Vehicle of PremiRNAs: Delivery and Biological Effects of miR-93-5p to IB3-1 Cystic Fibrosis Cells

In order to further demonstrate that the biological activity of delivered premiRNA molecules is maintained when argininocalix[4]arene **1** is employed, we have used the well-characterized premiR-93, which has been elsewhere reported to exert anti-inflammatory properties, since it inhibits the expression of IL-8 and IL-6.[47,48]

The experiment performed is shown in Figure 9, which demonstrates the increase of cellular miR-93-5p content only in cystic fibrosis IB3-1 cells treated with premiR-93 delivered with compound argininocalix[4]arene **1** (Figure 9A). This was associated with significant inhibition of IL-8 and IL-6 mRNAs (Figure 9B) and, more importantly, for a therapeutic point of view, decrease of the release of IL-8 and IL-6 (Figure 9C). This demonstrates that the argininocalix[4]arene **1** is able to deliver premiRNA molecules that maintain the expected biological

functions (in this case a sharp inhibition of release of the pro-inflammatory IL-8 and IL-6 proteins).[47,48]

DISCUSSION

miRNAs (or miRs) are short non-coding RNA molecules, which act as gene regulators by repressing translation or by inducing the cleavage of target RNA transcripts.[1,3,4] Emerging evidence suggests that the altered expression of miRNA may be involved in the pathogenesis of several severe human diseases.[8–15] This opens new avenues in the field of therapeutic strategies, i.e., miRNA targeting or miRNA mimicking.[18,19] In the case of antimiRNA strategy, the cells can be transfected with a variety of antimiRNA molecules and the target miRNA inhibited.[21] In these conditions, upregulation of mRNA due to the targeted miRNA is obtained. For instance, Dhanasekaran et al.[58] demonstrated that antimiR-17 therapy delays tumorigenesis in MYC-driven hepatocellular carcinoma (HCC). Broderick and Zamore[59] found that antimiRNA-23a oligonucleotide suppresses glioma cells growth by targeting apoptotic protease activating factor-1. Feng et al.[60] were able to target the seed region of miR-155 with antimiR molecules, demonstrating that this approach was associated with inhibition of malignant progression of multiple myeloma cells.

In the case of miRNA mimicking strategy, cells can be transfected with premiRNA molecules, causing a downregulation of miRNA-regulated mRNAs. For instance, Zhu et al.[61] found that elevated expression of miR-137 reduces the protein expression levels of
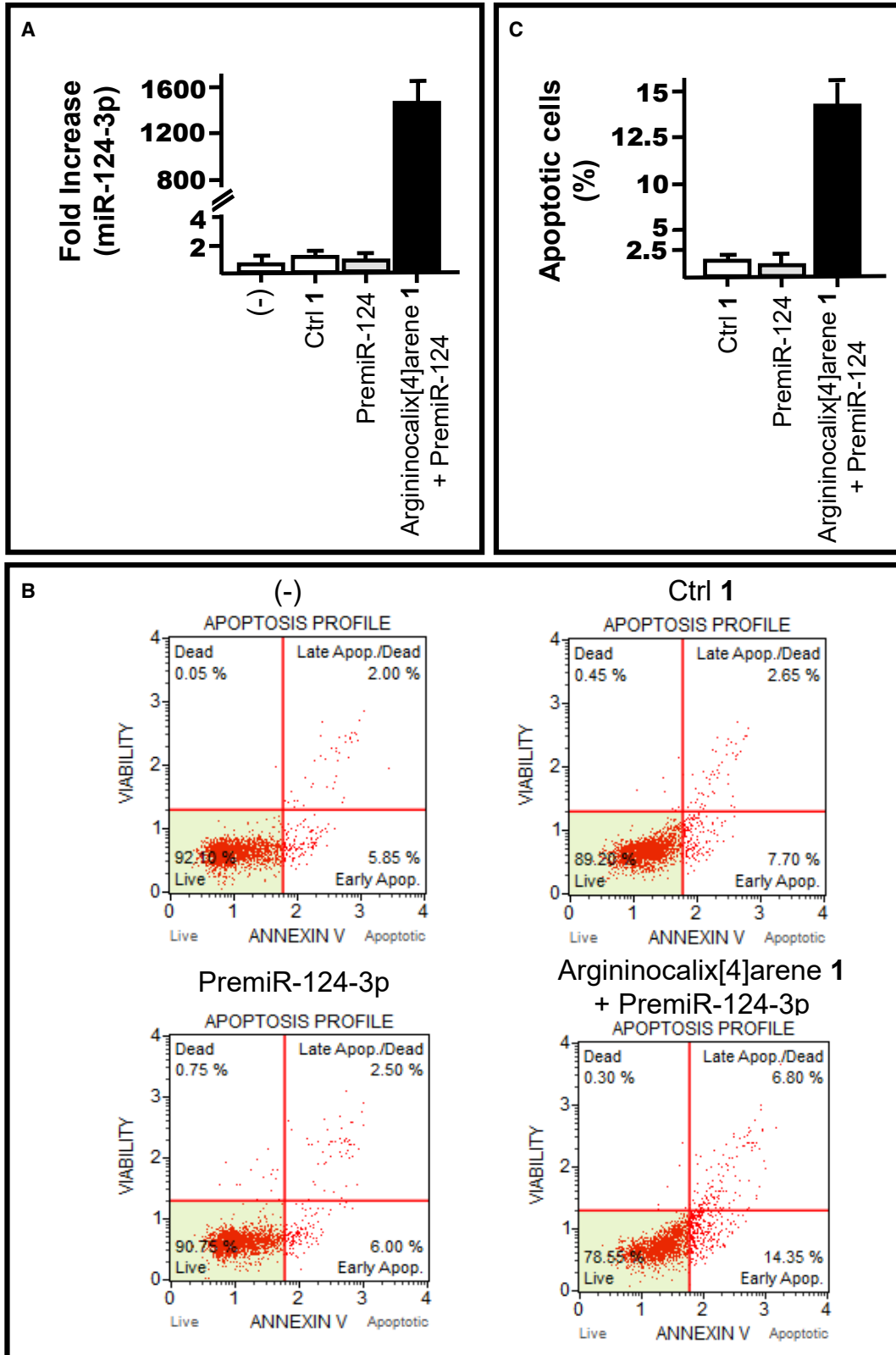
(legend on next page)

YB-1 and P-gp, mimicking the effect of YB-1 knockdown in the sensitivity of MCF-7/ADM cells to anticancer agents, whereas restoration of YB-1 diminished this effect. More recently, Xu et al.[62] demonstrated that miR-19b attenuates H_2O_2-induced apoptosis in rat H9C2 cardiomyocytes via targeting the PTEN network. These data indicate that miR-19b overexpression might be a novel therapy for myocardial I-R injury. Finally, Koo and Kwon[63] found that miR-4779 suppresses tumor growth by inducing apoptosis and cell cycle arrest through direct targeting of PAK2 and CCND3.

In this context, the efficient and non-toxic delivery of premiRNA and antimiRNA molecules might be of great interest.[8–15] This work should be considered an extension of the study published by Bagnacani et al.,[37] who recently reported the very interesting example of the delivery of plasmid DNA using a macrocyclic cone calix[4]arene as scaffold for non-viral vectors, displaying two spatially well-defined regions, one apolar at the lower rim and one polar at the upper rim.

The aim of the present paper was to determine whether the argininocalix[4]arene 1 is able to efficiently deliver miRNA, premiRNA, and antimiRNA molecules to target cells preserving their biological activity. The following conclusions can be drawn from the obtained results: (1) since the toxicity of argininocalix[4]arene 1 is low (see Figure 2), it can be proposed for long-term treatment of target cells, this feature being a pre-requisite for the development of therapeutic protocols; (2) the delivery of premiRNA and antimiRNA molecules is efficient, being higher when compared with the reference gold-standard available; (3) the biological activity of the premiRNAs and antimiRNA is maintained (see Figures 6, 7, 8, and 9), suggesting possible applications in the field of miRNA therapeutics.

This was demonstrated using the argininocalix[4]arene 1 in miRNA therapeutic approaches performed on three well-described experimental model systems, (1) the induction of apoptosis by antimiR-221 in U251 cells,[42,43] (2) the induction of apoptosis by premiR-124 in U251 cells,[45,46] and (3) the inhibition of pro-inflammatory IL-8 and IL-6 genes in cystic fibrosis IB3-1 cells.[47,64,65] Our results demonstrate that the argininocalix[4]arene 1 should be considered a very useful delivery system for efficiently transferring to target cells both premiRNAs and antimiRNA molecules, preserving their biological activity. The argininocalix[4]arene 1 was found to be not toxic under the employed experimental conditions.

Conclusions
The major conclusion of this study is that the argininocalix[4]arene 1 is able to efficiently deliver miRNA, premiRNA, and antimiRNA molecules to target cells preserving their biological activity. In addition, our study shows that this delivery activity is accompanied by absence of cytotoxicity effects and anti-proliferative activity. Therefore, argi-

ninocalix[4]arene 1 and analogs can be proposed for long-term treatment of target cells, this feature being a pre-requisite for the development of therapeutic protocols. In comparison with well-established transfection reagents for RNA delivery, argininocalix[4]arene 1 was found to be more efficient. When considered together, our results demonstrate that the argininocalix[4]arene 1 should be considered a very useful delivery system for efficiently transferring to target cells both premiRNAs and antimiRNA molecules, maintaining their biological activity. While this manuscript is focused on in vitro delivery of RNA-based biodrugs, the experimental systems described in this manuscript might be in the future proposed for in vivo validation, which will clarify the possible employment of argininocalix[4]arene 1 and analogs in therapeutic protocols.

MATERIALS AND METHODS
Synthesis and Characterization of Argininocalix[4]arene 1
The synthesis and characterization of argininocalix[4]arene 1 were previously reported.[37] Argininocalix[4]arene 1 employed for miRNA delivery experiments was re-suspended in a solution of water:ethanol:DMSO (2:2:1) in sterile conditions.

Cell Lines and Culture Conditions
The human glioma U251,[39] the cystic fibrosis IB3-1,[40] and chronic myelogenous K562[41] cells were cultured in a humidified atmosphere of 5% CO_2/air. U251 and K562 cells were maintained in RPMI-1640 medium (Gibco, Life Technologies, Monza, Italy) supplemented with 10% (v/v) fetal bovine serum (FBS; Biowest, Nuaillé, France), 100 units/mL penicillin, and 100 μg/mL streptomycin (pen-strep; Sigma-Aldrich, St. Louis, MO, USA); while IB3-1 cell line was cultured in LHC-8 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% of FBS. Erythroid precursor cells (ErPCs) were isolated from peripheral blood using a two-phase liquid medium selection procedure.[54] The use of human material was approved by the Ethics Committee of Ferrara's District, document number 06/2013, approved on 20 June 2013. All samples of peripheral blood have been obtained after receiving written informed consent.

Transfection Procedures
Two different protocols were employed to transfect miRNA-based molecules with argininocalix[4]arene 1: (1) short-term transfection protocol (similar to that proposed by Bagnacani et al.[37] for plasmidic DNA); (2) continuous contact protocol. In brief, a mixture containing RPMI-1640 medium, argininocalix[4]arene 1 at appropriate concentration, and miRNA-based molecules (premiRNA, antimiRNA, or mature miRNA) was prepared and incubated for 20 min at room temperature, without serum. After the incubation, 10% (v/v) of FBS was added. Cell culture medium was removed and replaced with the transfection mixture. Transfection mixture was maintained

Figure 8. Biological Effects of PremiR-124 Delivered to U251 Cells by Argininocalix[4]arene 1
Glioma U251 cells were cultured for 3 days without additions (−) or in the presence of argininocalix[4]arene 1 (ctrl 1), premiR-124, or argininocalix[4]arene 1 + premiR-124, as indicated. (A) Content of miR-124-3p following the described treatments. (B) Representative apoptosis profile plots. (C) Quantitative data representing the average ± SD (n = 3).
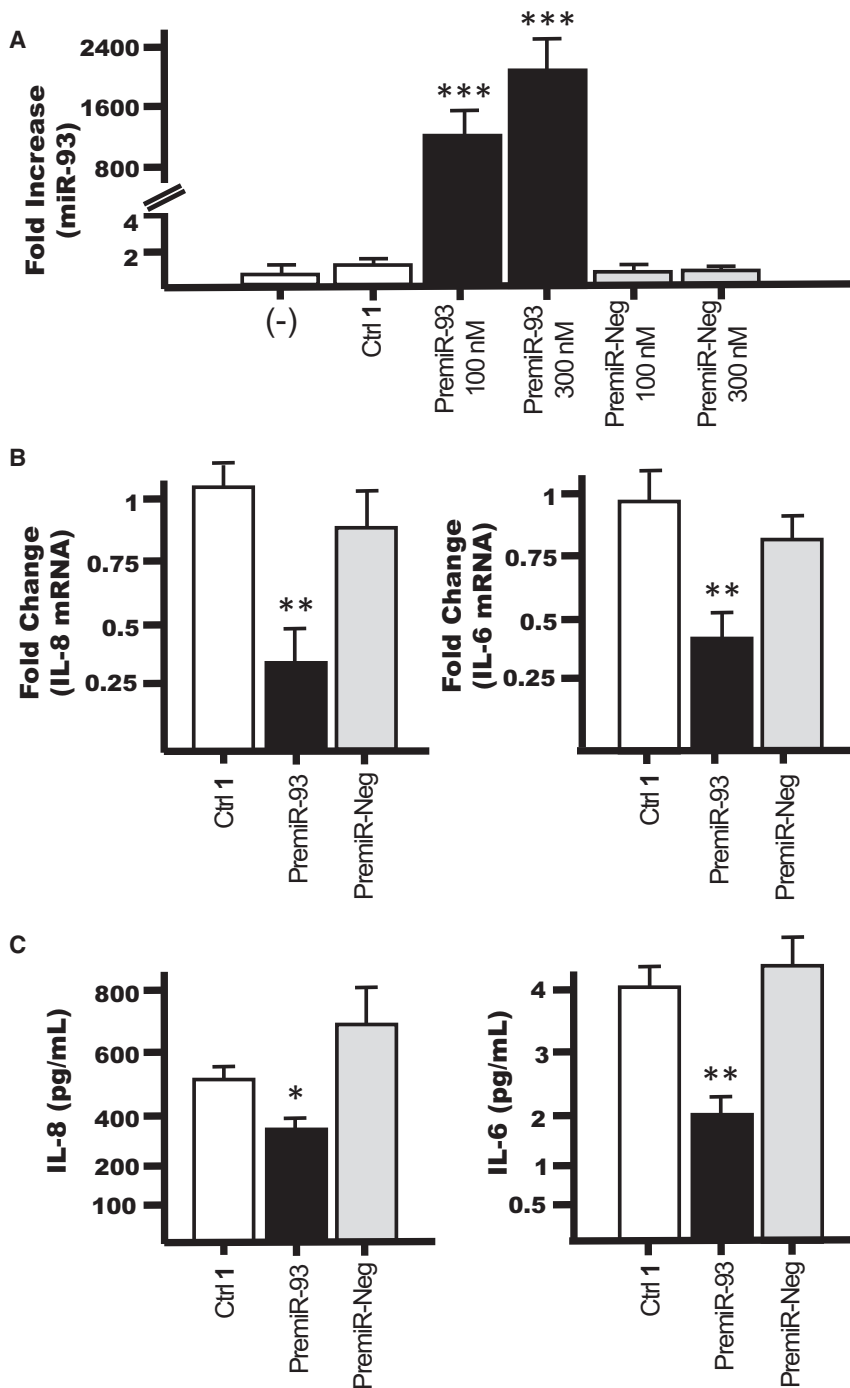
Scientific, Waltham, MA, USA), while mature miRNAs were synthesized by IDT (Integrated DNA Technology, Coralville, IA, USA) according to miRBase sequence. All transfections with commercially available reagents were performed according to manufacturer's instructions.

Effects on Cell Morphology and Cell Growth
The effects on cell morphology and cell growth were determined 48 and 72 h after transfection, testing different concentrations of calixarene-based molecule. The effects on cell growth were studied by determining the cell number/mL using a Z2 Coulter counter (Coulter Electronics, Hialeah, FL, USA).

FACS Analysis
Uptake of a fluorescent, mature miRNA (miR-210, IDT) was evaluated using FACScan (BD, Franklin Lakes, NJ, USA). Cells were detached, washed twice with Dulbecco's phosphate-buffered saline (DPBS) 1×, resuspended in 150 μL of DPBS 1×, and analyzed by FACS analysis for fluorescein isothiocyanate (FITC) fluorescence. For each sample, 30,000 events were acquired, and data analysis was performed using CellQuest Pro software (BD, Franklin Lakes, NJ, USA).

RNA Extraction
Cultured cells were collected by centrifugation at 1,200 rpm for 10 min at 4°C, washed with cold DPBS 1×, and lysed with Tri-reagent (Sigma Aldrich, St. Louis, MO, USA), according to manufacturer's instructions. Isolated RNA was washed once with cold 75% ethanol, air-dried, and dissolved in nuclease-free water before use.

Quantification of miRNAs
For miRNA quantification, obtained RNA was reverse transcribed using a TaqMan microRNA reverse transcription kit (Applied

in contact with cells for 5 h and then replaced with fresh culture medium in case of transfection protocol (1) while the mixture was maintained until the end of the treatment, when transfection protocol (2) was performed. PremiRNA (premiR-210, PM10516; premiR-124, PM10691; premiR-93, 10951; premiR negative control #1, AM17110) and antimiRNA (antimiR-210, AM10516; antimiR-221, AM10337) molecules were purchased from Ambion (Thermo Fisher

Table 1. Employed TaqMan Assays

Target Name	Company	Assay ID
B cell lymphoma/leukemia 11A (BCL11A) mRNA[a]	Applied Biosystems	Hs00256254_m1
B cell lymphoma/leukemia 11A (BCL11A) mRNA[b]	Applied Biosystems	Hs00250581_s1
Interleukin-8 mRNA	IDT	Hs.PT.58.38869678.g
Interleukin-6 mRNA	IDT	Hs.PT.58.40226675
Ribosomal protein L13a (RPL13A)	Applied Biosystems	Hs03043885_g1
18S ribosomal RNA	Applied Biosystems	4310893E

[a]Assay employed for amplification of BCL11A transcripts in K562 cells.
[b]Assay used for amplification of BCL11A transcripts in erythroid precursor cells.

Biosystems, Foster City, CA, USA) and specific stem-loop primers (hsa-miR-210-3p, ID: 000512; hsa-miR-221-3p, ID: 000524; hsa-miR-124-3p, ID: 001182; hsa-miR-93-5p, ID: 001090). qRT-PCR was performed according to the manufacturer's protocols and as indicated elsewhere.[42,44] All RT reactions, including RT-minus controls and no-template controls, were run in duplicate using the CFX96 touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The relative expression was calculated using the comparative cycle threshold ($\Delta\Delta CT$) method and U6 snRNA (hsa U6 snRNA, ID: 001973) and hsa-let-7c (hsa-let-7c, ID: 000379) as endogenous controls.

Quantification of mRNA by qRT-PCR

BCL11A mRNA expression was analyzed using qRT-PCR. 500 ng of RNA were reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems) and random hexamers. qRT-PCR experiments were carried out using a 5′ nuclease assay with primers and probes. The cDNA (1 μL) was amplified for 40 PCR cycles using TaqMan universal PCR master mix 2× (Applied Biosystems). Relative expression was calculated using the $\Delta\Delta CT$ method and the endogenous controls human 18S rRNA and human RPL13A were used as normalizer genes. Employed TaqMan assays are reported in Table 1.

Analysis of Apoptosis

Analysis of apoptotic profile on U251 cells, transfected with antimiR-221 or premiR-124, was performed using Muse annexin V and dead cell kit (Millipore, Billerica, MA, USA) as indicated elsewhere.[42,43] In brief, 100 μL of suspension cells was added to 100 μL of Muse annexin V and dead cell reagent, incubated at room temperature, protected from the light for 20 min and analyzed using Muse cell analyzer (Millipore, Billerica, MA, USA).

Bio-Plex Analysis

Cytokines released in culture medium by IB3-1 cells were measured by Bio-Plex cytokine assay (Bio-Rad, Hercules, CA, USA) as described elsewhere.[64,65] The Bio-Plex cytokine assay is designed for the multiplexed quantitative measurement of multiple cytokines in a single well using as little as 50 μL of sample. The pre-mixed multiplex beads of the Bio-Plex human cytokine human 27-plex panel (Bio-Rad, cat. # 171-A11127) were used, which included 27 cytokines (IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-10, IL-12 [P70], IL-13, IL-15, IL-17, fibroblast growth factor-basic [FGF-basic], eotaxin, granulocyte-colony stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF], interferon-γ [IFN-γ], interferon gamma-induced protein 10 [IP-10], monocyte chemoattractant protein-1 [MCP-1] [chemotactic and activating factor, MCAF], macrophage inflammatory protein-1 alpha and beta [MIP-1α, MIP-1β], platelet-derived growth factor-BB [PDGF-BB], RANTES, tumor necrosis factor alpha [TNF-α], vascular endothelial growth factor [VEGF]). In brief, 50 μL of cytokine standards or samples (supernatants from treated cells) were incubated with 50 μL of anti-cytokine conjugated beads in 96-well plates for 30 min at room temperature with shaking. Plates were then washed three times with 100 μL of Bio-Plex wash buffer using the Bio-Plex pro wash station (Bio-Rad, Hercules, CA, USA), 25 μL of diluted detection antibody was added, and plates were incubated for 30 min at room temperature with shaking. After three washes, 50 μL of streptavidin-phycoerythrin was added, and plates were incubated for 10 min at room temperature with shaking. Finally, plates were washed three times, beads were suspended in Bio-Plex assay buffer, and samples were analyzed on Bio-Rad 96 plate reader using the Bio-Plex suspension array system and Bio-Plex manager software (Bio-Rad, Hercules, CA).

Statistical Analysis

Results are expressed as mean ± SEM. Comparisons between groups were made by using paired Student's t test. Statistical significance was defined with $p < 0.05$ (*, significant), $p < 0.01$ (**, high significance), and $p < 0.001$ (***, very high significance).

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