dl922-947 Adenovirus and G-Quadruplex Binder Combination Against Breast Cancer and Modulation of Monocyte Activity

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Research

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Abstract

Background: G-quadruplex (G4) are nucleic secondary structures characterized by G-tetrads. G4 motif stabilization induces DNA damage and cancer cell death; therefore, G4-targeting small molecules are the focus of clinical investigation. DNA destabilization induced by G4 ligands might potentiate the anticancer activity of agents targeting DNA or inhibiting its repair like oncolytic viruses. This study represents the first approach combining G4 ligands and the adenovirus *dl922-947* in MDA-MB-231 and MCF-7 cell lines.

Methods: We used G4 binders, BRACO-19 (B19), pyridostatin (PDS) and *dl922-947* or their combination in cytotoxicity assays. G4 motif formation and distribution, cell cycle and viral entry were evaluated using agents alone or in combination by flow cytometry. Viral replication was evaluated by RT-PCR. Cell senescence was assessed by microscope observation and count of blue SA-b-Gal blue-stained cells. Phagocytosis of THP-1 cells was evaluated by flow cytometry.

Results: G4 binders and *dl922-947* induce cytotoxicity in MDA-MB-231 and MCF-7 cells. G4 binders induce G4 motifs mainly distributed in the S and G2/M phases of the cell cycle in MCF-7 cells. The combination G4 binders/*dl922-947* increased viral entry, replication and cell cytotoxicity. The combinatory treatments increased the subG0/G1 phase of the cell cycle, whereas the agents used singularly or in combination similarly enhanced cell senescence. Noteworthy, *dl922-947* induces G4 motifs and its combination with PDS potentiates this effect. The conditioned media of MCF-7 cells increased phagocytosis whereas the combination *dl922-947*/PDS restored it to the control values and did not affect monocyte proliferation.

Conclusions: We present the first evidence of a new mechanism of oncolytic viruses able to induce senescence and G4 structures in breast cancer cells. We suggest a novel strategy based on the use of G4 binders/virotherapy combination to enhance anti-cancer activity. Indeed, oncolytic viruses and G4 ligands are already singularly employed in clinical settings, thus their combination may be envisioned for translational studies.

Background

Several studies suggest the anticancer potential of small molecules, which by selectively targeting nucleic acid G-quadruplex (G4) motifs, are capable of inducing a therapeutic response(1, 2). G4 secondary structures are characterized by stacking of planar cyclic G-tetrads in which four guanine bases are connected by Hoogsteen hydrogen bonds (3). The distribution of G4-forming sequences in the human genome is not random: they are detectable at the telomeres and in the promoter regions of several proto-oncogenes (4). The anticancer potential of numerous G4 binders able to stabilize/induce G4 motifs has been investigated in different tumor cell lines and the obtained data support their use in clinical trials (5). Their anticancer activities include inhibition of cell growth, cell cycle arrest, DNA damage, induction of senescence and apoptosis. We have recently demonstrated that BRACO-19 (B19) and C066-3108 (C066), two G4-targeting ligands endowed with high affinity and good selectivity toward telomeric G4 motifs,

exert anticancer effects in prostate and breast cancer cell lines (6). We observed that in the less aggressive MCF-7 cell line both compounds induced modest DNA damage associated to apoptosis induction, whereas in the triple negative MDA-MB-231 cell line high DNA damage was associated to induction of immunogenic cell death (ICD) and consequent human T cell activation. These effects were correlated to the G4 motifs; we observed that both compounds induced G4 structures in MDA-MB-231 cells, whose content was detectable in dying cells (6). Among highly selective G4 binders, pyridostatin (PDS) showed reduction of the proto-oncogene tyrosine-protein kinase (SRC) levels and impaired in a SRC-dependent manner MDA-MB-231 cell motility, thus validating SRC as a relevant target and its interaction with non-telomeric DNA loci (7). In this study, we hypothesized that G4 motif formation and stabilization in cancer cells might be suitable to favor and potentiate the antitumor activity of other DNA damage inducers, like oncolytic viruses (OVs). Anticancer therapies based on the use of OVs represent promising strategies, and virotherapy-based approaches have successful outcomes in clinical setting (8). Along with a direct effect on cancer cells, OVs elicit indirect effects such as the induction of innate and adaptive anti-tumor immune response accompanied by a re-shape of the tumor microenvironment (TME) (9). OVs modulate TME toward an antitumor phenotype constituted by cytokines released by dying cells, tumor-associated antigens (TAAs), damage-associated and pathogen-associated molecular patterns (DAMPs, PAMPs), able to induce ICD and an anti-cancer immune response (10, 11). Among OVs, we have extensively studied the adenoviruses, in particular the adenoviral mutant *dI922-947* bearing a 24 bp deletion in the E1A-Conserved Region 2 (CR-2) (21–23). This deletion allows viral replication only in cells presenting a defective retinoblastoma (RB) pathway that is an abnormality observed in almost all the human malignancies. We have proved the anticancer efficacy of *dI922-947* and its combination with other therapeutic agents in several tumor models (12–16). We showed that *dI922-947* exerted antiangiogenic effects and mediated the reduction of tumor-associated macrophages (TAM) in anaplastic thyroid carcinoma (11). Recently, we observed that *dI922-947* inhibited cell growth and cycle, induced ICD, and modulated angiogenic signals in malignant mesothelioma cells, also *dI922-947* combination with cisplatin potentiated the cytotoxic effect (6). Additionally, we used *dI922-947* in combination with AZD1775, an inhibitor of the G2/M DNA damage checkpoint kinase WEE1 and observed synergism in the induction of malignant mesothelioma cell death (13). Indeed, *dI922-947* and/or AZD1775 activated the DNA damage response (DDR) increasing the levels of phosphorylated histone H2AX (γ -H2AX), phospho-replication protein A (RPA)32 and phospho-checkpoint kinase 1 (CHK1) (12). Furthermore, we observed that *dI922-947* increased Tyr-15-phosphorylated cyclin-dependent kinase 1 (CDK1), a key WEE1 substrate indicative of G2/M checkpoint activation, and AZD1775 suppressed this increase. In the present study, we investigated the combinatory activity of the adenovirus *dI922-947* and G4 binders to establish *dI922-947* antitumor effects in breast cancer cells and if G4s induction/stabilization potentiated such effects. We chose breast cancer as model since we have already described that G4 binders are highly active in these cells.

Methods

Cells and Adenovirus

MCF-7 and MDA-MB-231 breast cancer cell lines were cultured in DMEM (GIBCO, Paisley, UK) supplemented with 2 mM L-glutamine, 50 ng/mL streptomycin, 50 units/mL penicillin, and 10% heat-inactivated fetal bovine serum (FBS) (GIBCO). THP-1 human cell line was cultured in RPMI 1640 medium (GIBCO, Paisley, UK) containing 2 mM L-glutamine, 50 ng/mL streptomycin, 50 units/mL penicillin, and 10% heat-inactivated fetal bovine serum (FBS). Cells were maintained in a humidified atmosphere with 5% CO₂ at 37 °C. Cells reaching 70–80% of confluence were harvested with 0.25% trypsin (Sigma-Aldrich, St Louis, MO, USA) and used for the experiments. *dI922-947* viral stocks were expanded in the human embryonic kidney cell line HEK-293, purified, stored and quantified (1.22×10^8 p.f.u./ml).

Sulforhodamine B (SRB) cytotoxicity assays

MCF-7 and MDA-MB-231 cells were seeded in triplicates in 96-well plates (500 cells/well) and allowed to adhere for 24 h. G4 ligands were added at concentrations ranging from 0.1 to 10 µM. *dI922-947* was added to the cell cultures in a range from 0.1 to 10 pfu x cell. After 6 days of incubation, cells were fixed with 50% v/v trichloroacetic acid for 2 h under stirring at 4 °C. Cells were washed with distilled water, dried overnight and stained with 0.4% w/v SRB in 1% v/v acetic acid at room temperature for 30 min on shaker. Washes were made with 1% acetic acid, until the removal of the unbound dye and the plates were left to dry. The dye was solubilized in TRIS-HCl 10 mM as previously described (17, 18). The reading was performed at 495 nm by Glomax® Discover Microplate Reader (Promega). The doses of *dI922-947* or G4 ligands required to inhibit 50% of cell viability (half maximal inhibitory concentration, IC₅₀) was determined by GraphPad Prism 7 Software.

G4 motif formation and cell cycle analyses

MCF-7 and MDA-MB-231 cell lines were cultured in 24-well plates (8000 cells/well) in the presence and absence of G4 ligands or *dI922-947* at IC₅₀ doses for six days. The combination *dI922-947*/G4 binders was also settled. After the incubation, cells were detached with trypsin, washed twice with PBS, fixed in 70% (v/v) ethanol and stored at – 20 °C at least overnight for the determination of the cell cycle (19, 20) and G4 motifs (6). The cell pellet, washed with PBS/Tween buffer (PBT) (0.5% w/v BSA and 0.1% v/v Tween 20 in PBS) was re-suspended in PBT and stained with BG4 monoclonal antibody (Millipore, MABE 917) for 1 h at room temperature. Samples were washed with PBT and stained with Alexa Fluor 488 anti-mouse (Invitrogen, USA, #A11001, 1:100) in PBT at darkness. After 30 min, cells were washed with PBT and re-suspended in PBS containing RNaseA (Roche) (0.4 U) and PI (0.015 mol/L). Flow cytometry acquisition was performed for the emission in FL1 and FL3 channels for the determination of G4 structures and in the FL3 channel for the acquisition of the cell cycle with a BD LSRFortessa (BD Biosciences, San Jose, CA, USA) and analyses were performed using Flowlogic Software (MACS, Milteny Biotech). To remove artifacts, doublets and aggregates, an electronic doublet discrimination was performed using the area versus width of the fluorescence (FL3) pulse.

AdGFP infection

MCF-7 and MDA-MB-231 cells were seeded (12.500 cells/well) in 24-well-plates and after 24 or 48 h infected with AdGFP (30 pfu/cell). G4 binders were added at their IC₅₀ values simultaneously (24 h after the adherence of the cells to the plastic) with the virus, or 24 h after virus infection, or 24 h before virus infection. After 48 h of incubation, cells were detached with trypsin and the pellet was re-suspended in PBS to read GFP emission by flow cytometry.

Evaluation of dI922-947 DNA amplification

MCF-7 and MDA-MB-231 cells (10.000 cells/well) were seeded in duplicate in 12-well plates and 24 h later were infected with *dI922-947* at the IC₅₀ value (or AdGFP at 30 pfu x cell, used as control) and treated with G4 ligands at their IC₅₀ values. Cells and supernatants were separately collected 24 and 48 hpi. Cell pellets were disrupted by three freeze–thaw cycles to favor the release of the virus and centrifuged at 1,000 g for 5 min, the supernatants were collected. Viral DNA was extracted from supernatants and cell pellets by High Pure Viral Nucleic Acid Kit (Roche) and quantified by Real-Time PCR using the primers for viral HEXON gene expression.

Induction of cell senescence

MDA-MB-231 (30.000 cells/plate) and MCF-7 (40.000 cells/plate) cells were seeded in 6-well plates and 24 h later were infected with *dI922-947* at the IC₅₀ value, or treated with G4 ligands. Virus/G4 ligand combinations were also examined. After 72 h, we collected conditioned medium (CM) and assessed the senescence using the SA-b-gal Staining Kit (Cell BioLabs). Cells were washed twice with PBS (1x) and incubated at room temperature for 5 min with 1X Fixing Solution. Cells were washed three times with PBS, the final wash was aspirated, and the cells were completely covered with freshly prepared Cell Staining Working Solution in the dark for 4 h at 37°C. After the incubation, the solution was removed, the cells were washed twice with PBS and blue-stained senescence cells were observed by a light microscope and counted at 490 nm by Glomax® Discover Microplate Reader (Promega).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assays

THP-1 cells (200.000 cells/well) were incubated in 96-well plates with CM derived from MDA-MB-231 and MCF-7 cells infected with *dI922-947* or treated with G4 ligands alone and/or in combination with the virus at the IC₅₀ values. Doxorubicin was used as a positive control. CM were collected after 72 h of cultures and diluted 1:2 with the growing culture medium of THP-1 cells. After 48 h of incubation, cell viability was assessed by the addition of MTT reagent (10 µl) and after 3 h of incubation MTT solvent was added to the wells and absorbance was read at 590 nm as previously described (21, 22).

Phagocytosis assay

THP-1 cells (200.000 cells/tube) were incubated in eppendorf punched with a needle in the lid with the CM derived from MDA-MB-231 and MCF-7 cells infected with *dI922-947*, or treated with G4 ligands alone and/or in combination with the virus, and then collected after 6 days of culture. CM was diluted 1:2 with

the growing culture medium of THP-1 cells. Latex beads-rabbit IgG-PE complex (Phagocytosis Assay Kit, Cayman Chemicals) was added to the cells followed by incubation for 3 hours at 37°C. To assess phagocytosis, cells were centrifuged for 5 min at 400xg, re-suspended in assay buffer and fluorescent bead incorporation was assessed by flow cytometry.

Statistical analysis

Statistical analysis was performed by Prism 7 (GraphPad Software). Statistical significance of differences among groups was analyzed by ANOVA corrected for multiple comparisons (as indicated in the figure legends) and p values ≤ 0.05 were considered statistically significant.

Results

Anti-proliferative effects of PDS and B19 in breast cancer cells

Recently, we reported the anti-proliferative effect of B19 in breast cancer cells (6). Herein, we compared the inhibitory activity of B19 with PDS in MDA-MB-231 and MCF-7 cells. After 6 days of incubation, both compounds dose-dependently inhibited cell proliferation (Fig. 1A, B) in both cell lines and we calculated the IC₅₀ values of the drugs (see Fig. 1 legend). We observed that B19 and PDS achieved a similar IC₅₀ value in MDA-MB-231 cells, whereas the values were higher in MCF-7 cells, particularly in the case of B19.

G4-binder induces G4 motifs in breast cancer cells

We investigated the induction of G4 motifs by PDS and B19 (at their IC₅₀ values) in MDA-MB-231 and MCF-7 cells by flow cytometry. As reference compound, we used B19 already tested for similar assays in MDA-MB-231 cells (6). The analyses were restricted to the DNA gating PI positive cells (dot plot PI-H vs. PI-W). Gated populations were selected to analyze G4 content using the specific BG4 antibody (Fig. 2A, B). As previously observed, we detected low basal levels of G4 motifs in both the cell lines (6). The treatment with B19 induced a significant increase of G4 motifs in MCF-7, whereas a slight statistically non-significant increase was observed in MDA-MB-231 cells. On the other hand, the induction of G4 motifs by PDS at the IC₅₀ was similar and statistically significant both in MCF-7 and MDA-MB-231 cells (Fig. 2A, B). As previously done with B19 (6), we used PDS at increasing concentrations (2.5 and 5 μ M) and observed a dose-dependent effect in both cell lines (Fig. S1). Additionally, we analyzed at the IC₅₀ values the percentage of G4 motifs associated to cell cycle phases. In the same selected regions of the dot-plot described above, we gated sub-regions within BG4-FITC positive cells corresponding to the subG0/G1, G0/G1, S and G2/M phases of the cell cycle (Fig. 2C). B19 treatment induced in MCF-7 cells an increase in the subG0/G1, S and G2/M phases with respect to the control, while in MDA-MB-231 cells G4 motifs were detected in G2/M phase as in the control, with a non-significant increase in the subG0/G1 and S phase. The distribution of G4 motifs following the treatment with PDS, showed an increased G4 content in S and G2/M phases in the case of MCF-7 cells, and in S phase in MDA-MB-231 cells (Fig. 2C).

dl922-947 infection induces cytotoxic effects in breast cancer cells

Previous studies reported in breast cancer cells the higher cytotoxic activity of *dl922-947* with respect to other adenoviruses (23). We confirmed the inhibitory effect of *dl922-947* by SRB assay, using serial dilutions of the adenovirus ranging from 0.1 to 6.0 p.f.u./cell. We observed a dose-response cytotoxic effect in MDA-MB-231 and MCF-7 cell lines infected with *dl922-947* with IC_{50} values of 0.8 and 0.3 p.f.u./cell, respectively. Dose-response curves of *dl922-947* infection are reported in Fig. 1C for both the cell lines. The following assays were performed to address whether the combination of G4-binders and *dl922-947* might improve the anticancer efficacy.

G4-binders enhance dl922-947 entry in breast cancer cells

A non-replicating reporter adenovirus transducing GFP (AdGFP) was used to evaluate adenovirus entry in MDA-MB-231 and MCF-7 cells by flow cytometry assessment of GFP emission. We observed viral entry in MDA-MB-231 cells and the effect reached the statistical significance after 24 h of treatment. In MCF-7 cells, we did not detect AdGFP entry with respect to the control (Fig. 3A, B, C). Furthermore, we explored if the presence/induction of G4 motifs in these cells might enhance viral entry. To assess the optimal combination timing, we performed several combinatory assays evaluating GFP emission after 48 h of co-incubation of AdGFP and G4 binders. Cells were left to adhere for 24 h, afterwards, AdGFP and G4 binders were administrated. The following combination scheme was adopted: i) AdGFP and G4 binders were added concomitantly (24 h after seeding the cells, Fig. 4A,B); ii) AdGFP was added 24 h after G4 binders (AdGFP was added 48 h after seeding the cells, Fig. 4A,B); iii) G4 binders were added 24 h after AdGFP (AdGFP was added 24 h after seeding the cells). In MDA-MB231 cells, we observed that in the case i) (Fig. 3A) and iii) (Fig. 3C), viral entry was enhanced with respect to AdGFP used alone. Using this scheme, B19 and PDS exerted a similar effect. In the case ii) (Fig. 3A gray bars, AdGFP 48 h), namely AdGFP added after G4 binders, in MDA-MB-231 cells viral entry was similar to AdGFP alone. In MCF-7 cells, we observed that G4 binders enhanced viral entry in all cases, particularly in the cases i) and ii) (Fig. 3B). In the case iii) indicating G4 binders added 24 h after AdGFP, B19 and PDS similarly enhanced viral entry with respect to AdGFP alone, however, the effect observed with B19 was lower with respect to the cases i) and ii) (Fig. 3C). Thus, the following combination assays were performed adding the virus and G4 binders simultaneously.

Evaluation of dl922-947 DNA amplification in combination with G4 binders

The efficacy of *dl922-947* amplification was evaluated in breast cancer cell lines in combination with B19 and/or PDS (using *dl922-947* and G4 binders at their corresponding IC_{50} values determined above). At 24 h post infection (hpi) the virus was equally distributed in the intracellular and extracellular fractions of breast cancer cells. 48 hpi, the intracellular amplification resulted drastically enhanced with respect to the extracellular fraction in both MDA-MB-231 and MCF-7 cells (Fig. 3D).

We used as control AdGFP alone and in combination with B19 and/or PDS. As expected we did not observe DNA amplification after 48 h of treatment.

Cytotoxic effects of dl922-947/G4 binder combination

The combination *dl922-947*/G4 binders was investigated to evaluate a potential increase of cytotoxicity in breast cancer cells. We settled several combinations varying the number of p.f.u./cell of *dl922-947*, in particular, we selected the IC₂₅ and IC₅₀ values of the virus in combination with the IC₂₅ and IC₅₀ concentrations of G4 binders above calculated (data not shown). The virus and G4 binders were added simultaneously, since data above described showed that the concomitant administration improved viral entry in both cell lines. We observed improved effects when we fixed *dl922-947* at the IC₅₀ dose varying the concentration of G4 binders (IC₂₅ and IC₅₀ values). We observed a statistically significant inhibition of cell proliferation using *dl922-947* at the IC₅₀ dose with respect to the control in MDA-MB-231 and MCF-7 cells (Fig. 4A). Additionally, we obtained a dose-response inhibitory effect using B19 and PDS at their IC₂₅ and IC₅₀ concentrations (Fig. 4A). The analysis of adenovirus/G4 binder combinations in MDA-MB-231 cells showed that *dl922-947* (at the IC₅₀ dose) and PDS at the IC₂₅ dose significantly increased cytotoxicity with respect to PDS used alone, whereas the combination with PDS at the IC₅₀ dose significantly potentiated the cytotoxic effect with respect to the virus and PDS (at the same concentration) used separately. Conversely, the combination *dl922-947*/B19 did not enhance cytotoxicity with respect to the single components. In MCF-7 cells, the combination of PDS and B19 with *dl922-947* (at the IC₅₀ value) showed an increased inhibition of cell proliferation when both drugs were used at their IC₅₀ concentration; however, the effect was significant only with respect to the virus used alone (Fig. 4A).

dl922-947/G4 binder combination interfere with cell cycle progression

To correlate the observed cytotoxicity with the cell cycle profile, we treated breast cancer cells with G4 binders and/or the virus or their combinations. The treatment with B19 at the IC₅₀ value in both cell lines did not alter cell cycle profile with respect to the control, as well as PDS in MCF-7 cells. Notably, PDS induced cell accumulation in the subG0/G1 phase of the cell cycle in MDA-MB-231 cells (Fig. 4B). The treatment with *dl922-947* induced significant alterations in the cell cycle profile, we observed a decrease of G0/G1 phase and an increase of G2/M phase with respect to the control in MCF-7 cells (Fig. 4C), while an increase of subG0/G1 phase and decrease of G1 phase was observed in MDA-MB-231 cells (Fig. 4B). The combination *dl922-947*/G4 binders altered significantly cell cycle progression in both cell lines. In MCF-7 cells, we observed an increase of the subG0/G1 and a decrease of G0/G1 phases with respect to the control (Fig. 4C). In MDA-MB-231 cells, virus/G4 binder combination produced an effect similar to that elicited by the virus alone, such as an increase of subG0/G1 phase and decrease of G1 phase (Fig. 4B). To better characterize the effect of PDS, we used it at increasing concentrations and observed a dose dependent decrease of the G0/G1 phase of the cell cycle both in MDA-MB-231 (Fig. S3) and MCF-7 cells, also in these latter cells an increase of the subG0/G1 phase at the highest PDS concentration was observed.

Induction of senescence in breast cancer cells

Early cell modifications due to single agents or their combinations were assessed analyzing cell senescence after 72 h of incubation. G4 binders are well-known inducers of cell senescence, thereby, as expected, both B19 and PDS induced senescence in both cell lines analyzed. Surprisingly, *dl922-947* elicited a similar effect to that reported for G4 binders. However, the combination of the virus with G4 binders did not showed increased effects (Fig. 5A). As reference compound, we used doxorubicin previously described to induce senescence in breast cancer cells (24). The observed effects were similar to those elicited by doxorubicin. Notably, the induction of senescence was higher in MDA-MB-231 than in MCF-7 cells (Fig. 5A).

DI922-947 and its combination with PDS induce G4 motifs in MCF-7 cells

The induction of cell senescence by *dl922-947* prompted us to investigate its ability to elicit G4 structures in breast cancer cells. To this aim, we evaluated the induction of G4 motifs by *dl922-947* as above described for G4 binders. Surprisingly, we observed that *dl922-947* was a good inducer of G4 motifs, even better than PDS in MCF-7 cells. Additionally, the combination *dl922-947*/PDS at the IC₅₀ values significantly enhanced G4 structure induction with respect to the agents used separately. Conversely, no induction of G4 motifs was observed in MDA-MB-231 cells (Fig. 5B, C). Next, we evaluated in MCF-7 cells the distribution of G4 motifs in the phases of the cell cycle. We observed a similar distribution of G4 motifs in the cells treated separately with *dl922-947* and PDS with no statistically difference with respect to the control. The combination *dl922-947*/PDS showed a statistically significant increase of G4 motif in G2/M phase of the cell cycle with respect to the control and to the adenovirus and PDS used separately (Fig. 5D).

Monocyte proliferation and phagocytosis

The effects of the virus and G4 ligands or their combination in TME was assessed evaluating monocyte activity. Using CM (1:2 dilution) of breast cancer cells collected from above senescence experiments, we investigated potential changes in THP-1 cell proliferation. THP-1 cells were incubated for 48 h with the CM of treated or untreated MDA-MB-231 and/or MCF7 cells. We observed that any CM affected THP-1 cell growth (Fig. 6A). Additionally, we investigated the effect on phagocytosis of THP-1 cells. CM were added to THP-1 cells for 24 h and latex-beads were added in the last three hours of incubation. Latex-beads incorporation was evaluated by flow cytometry. CM of MDA-MB-231 cells did not affect phagocytosis, whereas CM of MCF-7 cells without treatment, enhanced phagocytic activity of THP-1 cells with respect to cells incubated without CM. Notably, the presence in the CM of the combination *dl922-947*/PDS reduced phagocytosis to the control levels (Fig. 6B).

Discussion

The standard B-DNA conformation is canonically adopted by the genomic DNA, however, DNA can also fold into alternative structures such as hairpin, holliday junction, triplex, i-motif or G4 (25). Although the importance of G4 motifs in living cells was controversially discussed, accumulating evidence now support the existence and relevance of these structures in living cells (26). Studies in the last years discussed G4 structure stabilization by G4 ligands as a potential therapeutic tool against cancer (27). We previously showed that G4-targeting ligands, B19 and C066 reduced breast cancer cell survival and induced DNA damage. We reported the first evidence that these ligands induce the release of danger signals associated with ICD and T cell activation (6). In the present study, we compared the anticancer efficacy in breast cancer cells of B19 and PDS, this latter being able to target, among others, the G4 motifs in the *SRC* proto-oncogene (7). The *SRC* gene encodes for a tyrosine kinase involved in cell motility, adhesion, and invasion, as well as in cell proliferation and survival. Numerous malignant neoplasia including breast cancer show Src overexpression and activation, supporting increased invasiveness and metastasis (28). We have previously observed the inhibitory effect of B19 in MDA-MB-231 and MCF-7 cell proliferation (6). In this study, we observed that B19 and PDS at similar IC₅₀ doses elicit cytotoxicity in MDA-MB-231 cells. The IC₅₀ doses of both drugs were generally highest in MCF-7 cells, with lower IC₅₀ values in the case of PDS with respect to B19. These data suggest that MDA-MB-231 cells might be more sensitive to G4 binder treatments. We evaluated potential differences in G4 motif induction by the two drugs and between the cell lines. In MDA-MB-231 cells, PDS was more efficient with respect to B19 in inducing G4 motifs, while in MCF-7 cells both the drugs similarly induced G4s. The evaluation of G4 motif distribution in the different cell cycle phases showed in MDA-MB-231 cells a prevalence of G4 motifs in the S and G2/M phases with a statistical significance only for PDS in the S phase with respect to the control. In MCF-7 cells, we observed an increase of S and G2/M phases for both the agents and a significant increase also in the subG0/G1 phase by B19. In a previous study, we observed that MDA-MB-231 cells treated with B19 exhibited G4 motifs only in the subG0/G1 phase, however, in that study highest concentrations of B19 and a less specific G4-detecting antibody were used (6). To consolidate our data, we analyzed the induction of G4 motifs by increasing PDS concentrations, observing a drug-dose dependent increase of G4 motifs (Fig. S1). In the light of the known genome instability induced by G4 ligands and the consequent DNA damage (6), we believe plausible that DNA destabilization might favor or potentiate the anticancer activity of other agents that, by targeting DNA, may better elicit their antineoplastic activity or cooperate with G4 ligands to counteract cancer cell growth and diffusion. To this aim, we hypothesized that OVs, in particular the adenovirus *d/922-947*, could represent a good candidate for this approach. In breast cancers characterized by frequent mutations of genes in retinoblastoma (Rb) and p53 pathways (29), the tumor-selective replication activity of OVs with corresponding mutations in the E1A and E1B55K is desired. The deletion of 24 bp in the CR-2 of E1A, normally involved in the inactivation of pRb, restricts viral replication to cancer cells with a dysfunctional G1-S checkpoint. Previous reports described high anticancer activity of *d/922-947* in breast cancer cells that was considered along with AdEH2EF, the adenovirus with the greatest cytotoxicity in vitro against a panel of breast cancer cells. Its efficacy was validated in MDA-MB-231 carcinoma xenograft in vivo (23). Nonetheless, we demonstrated in the malignant mesothelioma that *d/922-947* was able to induce ICD and synergize with cisplatin (10), which is known to target duplex DNA but also to react with G4 motifs

(30). Herein, we determined by a dose dependent titration of *dI922-947* the cytotoxicity (IC_{50}) induced by the virus in both MDA-MB-231 and MCF-7 cells. We addressed whether G4 induction might promote viral entrance in these cell systems. We observed that the virus is able to entry MDA-MB-231 cells efficiently, whereas the entrance is less effective in MCF-7 cells. This difference might be explained considering that MDA-MB-231 cells differ from MCF-7 cells because of a mutation in the p53 and pRb genes (31). Interestingly, the co-administration of the virus with G4 ligands significantly increased viral entrance in both cell lines, thus suggesting that induction of genome instability or DNA damage in cancer cells favor the adenovirus entrance. Additionally, 48 hpi the virus actively replicated in cancer cells since almost exclusively present in the intracellular fraction. Furthermore, we assessed if the combination *dI922-947*/G4 ligands might enhance cytotoxic effects and improve anticancer efficacy. We used different combinations varying virus pfu and G4 ligand concentrations (data not shown). Finally, we fixed the dose of the virus at the IC_{50} value and combined it with B19 and PDS at the IC_{25} and IC_{50} values. We observed in MDA-MB-231 cells that PDS at both IC_{25} and IC_{50} concentrations enhanced the cytotoxic effect with respect to *dI922-947* alone, furthermore the combination with PDS at the IC_{50} dose enhanced cytotoxicity with the respect not only to the virus but also to PDS used alone. No significant difference using the combination *dI922-947*/B19 with respect to the agents used alone was observed. In MCF-7 cells, the combination of both the G4 ligands with *dI922-947* (at IC_{50} values) enhanced cytotoxicity with respect to the virus used alone. These results were confirmed by the increase of the subG0/G1 phase of the cell cycle using virus/G4 binder combinations in both MCF-7 and MDA-MB-231 cells. Nevertheless, both the virus and PDS used alone increased significantly the subG0/G1 phase in MDA-MB-231 cells. In a previous study, we demonstrated that B19 induced apoptosis in MCF-7 cells, whereas in MDA-MB-231 cells induced ICD (6). We have also demonstrated the ability of *dI922-947* to stimulate ICD in the malignant mesothelioma (10). In order to investigate earlier modifications leading to cell death, we analyzed the cell senescence. G4 ligands are known to induce senescence in cancer cells, thus we examined whether the combination of *dI922-947* might potentiate ligand-induced senescence. Surprisingly, *dI922-947* alone induced cell senescence and its effect was similar to that elicited by G4 binders. The combination virus/G4 binders was not different with respect to the agents used alone, or to doxorubicin used as positive control. Our data represent the first evidence that the adenovirus *dI922-947* induces senescence in cancer cells. Thus, we investigated the ability of *dI922-947* to induce G4 motifs. Surprisingly, we detected G4 motif formation following virus treatment, and this effect turned out to be enhanced in the presence of PDS in MCF-7 cells. G4 motif distribution upon *dI922-947* or *dI922-947*/PDS combination was significantly enhanced in the G2/M phase of the cell cycle. Finally, as functional read out we evaluated the effect of the combinatory treatment on the cells of TME, particularly focusing on the monocyte activity. We observed that CM of MDA-MB-231 and MCF-7 cells in the presence of G4 binders or virus and/or their combination did not affect THP-1 cell proliferation. On the other hand, the CM of MCF-7 cells increased monocyte phagocytosis with respect to the control without CM, and notably, the combination *dI922-947*/PDS restored phagocytosis levels to the control values. These data might suggest that the CM of MCF-7 cells induces potential changes in monocytes, likely similar to TAM phenotype, and the combinatory treatment seems to decrease such effect. Further studies are currently

ongoing in our laboratory to assess monocyte/macrophage phenotype. In conclusion, our results suggest that G4 binders may be good candidates used as single therapy against breast cancer, or to side virotherapy in types of tumors suitable or insensitive to OV. Additionally, our data might open the way to further investigation aimed to evaluate potential advantages of a combined use of G4 binders and virotherapy to evoke enhanced antitumor activity.

Conclusions

These data propose a never investigated anticancer approach that combines virotherapy and G-quadruplex (G4) motifs. Both anticancer therapies separately applied, virotherapy based on the use of oncolytic viruses (OVs) and the use of G4-targeting molecules, revealed interest in clinical setting. In breast cancer cells, the combination of OVs and G4 binders enhances the cytotoxic effects, viral entry and replication. G4 ligands and the adenovirus dl922-947 induce breast cancer cell senescence and surprisingly dl922-947 is a G4 motif inducer, effect potentiated by pyridostatin. dl922-947/pyridostatin combination decreases phagocytosis induced by the conditioned media of MCF-7 cells. These data provide new knowledge about the mechanism of action of the dl922-947 and propose that its combination with G4 ligands might promote the use of virotherapy also in types of tumours not sensitive to OV treatment. Our approach based on already known antitumor agents highlights that this novel combinatory strategy might likely find a translation to the clinical setting.

Declarations

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests

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Authors' contributions

F.N., S.D.S. performed the experiments, J.A., B.P. and A.R. provided G4 ligands and rationale for G4 ligand use and interpreted the data, G.P. and A.M.M. interpreted the data and wrote the manuscript.

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Figures

Fig 1

Figure 1

Cytotoxic effects of G4 ligands and dl922-947 in breast cancer cells. Dose-response curves obtained through SRB assay assessing cell viability in MCF-7 and MDA-MB-231 cells with different concentrations of PDS (A) and B19 (B) and serial dilution of dl922-947 (C) after six days of treatment. Data are reported as percentages of cell viability calculated with respect to the control, and are the means $\pm$ SD of three independent experiments in triplicate. IC50 values were calculated by GraphPad Prism 7. IC50 values are 1.0 and 1.5 μ M for PDS, and 1.1 and 2.5 μ M for B19 in MDA-MB-231 and MCF-7 cells, respectively.

Figure 2

G4 motif content and distribution in cell cycle phases. (A) MDA-MB231 and MCF-7 cells were treated with G4 ligands at the IC50 values. The dot plot profiles in the upper fluorescein isothiocyanate (FITC) panel indicate the amount of positivity for G4 motif. The x axis reports PI positivity to specifically stain cell DNA. The dot plots reported are representative of a single experiment, whereas the histograms (B) represent the mean $\pm$ SD of at least three independent experiments. The statistical analysis was performed by GraphPad Prism 7 by two-way ANOVA using Dunnet's multiple comparisons test. In the panel (C), G4 motif distribution is reported by evaluation of PI positive cells (PI-W versus PI-H dot plot) in each phase of the cell cycle as the percent of G4 motif positivity. The statistical significance was calculated by GraphPad Prism 7 with two-way ANOVA using Tukey's multiple comparisons test. The calculated p values are the following: ***p < 0.0001, ** p < 0.005, * p < 0.05.

Figure 3

Adenovirus AdGFP entry and dl922-947 replication in breast cancer cells. MDA-MB231 (A) and MCF-7 (B) cells were infected with the non-replicating AdGFP and treated with G4 binders at different times. AdGFP and G4 binders were added concomitantly (24 h after seeding the cells); AdGFP was added 24 h after G4 binders (AdGFP was added 48 h after seeding cells); (C) G4 binders were added 24 h after AdGFP (AdGFP was added 24 h after seeding the cells). GFP emission was evaluated by flow cytometry. The statistical significance was calculated by GraphPad Prism 7 with two-way ANOVA using Tukey's multiple comparisons test (**p < 0.0001, *p < 0.05). MDA-MB-231 and MCF-7 cells (D) were treated with dl922-947 (at the IC50 value) or AdGFP (30 p.f.u. x cell, used as control at 48 hpi) in combination with G4 ligands (at

their IC50 values). After 24 and 48 h supernatant and adherent cells were collected separately to evaluate extracellular release and intracellular viral particles, respectively. Viral DNA was extracted and used to quantify viral titer by Real-Time PCR. The data represent the mean of three different experiments (D).

Figure 4

Cytotoxic effects of G4 ligands/dl922-947 combination and regulation of the cell cycle progression. Cytotoxic effect of G4 ligands with dl922-947 determined by SRB assays in MDA-MB231 and MCF-7 cells after six days of treatment (A). Figures report cell viability (absorbance, Abs) as mean of three independent experiments generated with IC25 and IC50 values of PDS and B19 and IC50 value of dl922-947. Statistical significance was calculated by GraphPad Prism 7 with two-way ANOVA using Tukey's multiple comparison test. *calculated versus CTR (0 μM); \$ calculated versus CTR (PDS 0.3 μM); & calculated versus dl922-947 IC50 (0 μM); + calculated versus CTR (PDS 1.0 μM) (*&+ p < 0.05, ** p < 0.005, *** p < 0.0005, ****, \$p < 0.0001). MDA-MB-231 (B) and MCF-7 (C) cells were treated with dl922-947, G4 ligands or their combination at the IC50 values. After 6 days of treatment, cells were stained with PI. A representative flow cytometry profile of the cell cycle is shown for both cell lines, and the percent of cells in each phase of the cell cycle is indicated for the single experiment, whereas the bars in the histograms represent the mean ± SD of the least three independent experiments. The statistical significance was calculated by GraphPad Prism 7 with two-way ANOVA using Tukey's multiple comparisons test (&p<0.0001, **p<0.005, *p<0.05).

Fig 5

Figure 5

Induction of cell senescence and G4 motif content and distribution in cell cycle phases MDA-MB-231 and MCF-7 cells were treated for 72 h with dI922-947, G4 binders or their combination. Senescent cells are indicated in the images by the arrows as blue SA-b-Gal positive stained cells. The histograms indicate the mean of three independent experiments (A). The statistical significance was calculated by GraphPad Prism 7 with two-way ANOVA with Tukey's multiple comparisons test (**** $p < 0.0001$, *** $p < 0.0005$,

**p<0.05, *p<0.05). G4 motif induction was evaluated by flow cytometry. MCF-7 and MDA-MB231 cells were treated with dl922-947, PDS or their combination at the IC50 values for 6 days. The histograms represent the mean ± SD of at least three independent experiments (B). Flow cytometry profile representative a single experiment is reported (C). Gating strategies were adopted to select in each phase of the cell cycle the percent of G4 structure positivity in MCF-7 cells (D). The statistical significance was calculated by GraphPad Prism 7 with two-way ANOVA using Sidak's multiple comparisons test (*&p<0.0001, £ p<0.05; *=statistical significance calculated vs. ctr, &= statistical significance calculated vs. pyridostatin, £=statistical significance calculated vs. dl922-947).

Fig 6

Figure 6

Monocyte viability and phagocytosis. THP-1 cells were treated with CM derived from MDA-MB-231 and MCF-7 cells treated with dl922-947, G4 ligands or their combination at the IC50 values, or with doxorubicin (positive control). THP-1 cell viability was assessed by MTT assay after 48 h of treatment (A). Phagocytic activity of monocytes was assessed after 24 h of treatment with the CM of MDA-MB-231 and MCF-7 cells treated with dl922-947, G4 ligands or their combination. Phagocytic activity was measured as latex bead fluorescent complex (LB PE complex) by cytofluorimetric analysis (B). The statistical significance was calculated by GraphPad Prism 7 with two-way ANOVA with Sidak's multiple comparisons test (**p<0.001,*p<0.01).

Supplementary Files

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