Cytotechnology (2018) 70:1607–1618
https://doi.org/10.1007/s10616-018-0253-1

ORIGINAL ARTICLE

Silver/silver chloride nanoparticles inhibit the proliferation of human glioblastoma cells

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Received: 23 March 2018 / Accepted: 24 August 2018 / Published online: 10 September 2018

Abstract Glioblastomas (GBM) are aggressive brain tumors with very poor prognosis. While silver nanoparticles represent a potential new strategy for anticancer therapy, the silver/silver chloride nanoparticles (Ag/AgCl-NPs) have microbicidal activity, but had not been tested against tumor cells. Here, we analyzed the effect of biogenically produced Ag/AgCl-NPs (from yeast cultures) on the proliferation of GBM02 glioblastoma cells (and of human astrocytes) by automated, image-based high-content analysis (HCA). We compared the effect of 0.1–5.0 μg mL^{-1} Ag/AgCl-NPs with that of 9.7–48.5 μg mL^{-1} temozolomide (TMZ, chemotherapy drug currently used to treat glioblastomas), alone or in combination. At higher concentrations, Ag/AgCl-NPs inhibited GBM02 proliferation more effectively than TMZ (up to 82 and 62% inhibition, respectively), while the opposite occurred at lower concentrations (up to 23 and 53% inhibition, for Ag/AgCl-NPs and TMZ, respectively). The combined treatment (Ag/AgCl-NPs + TMZ) inhibited GBM02 proliferation by 54–83%. Ag/AgCl-NPs had a reduced effect on astrocyte proliferation compared with TMZ, and Ag/AgCl-NPs + TMZ inhibited astrocyte proliferation

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10616-018-0253-1) contains supplementary material, which is available to authorized users.

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by 5–42%. The growth rate and population doubling time analyses confirmed that treatment with Ag/AgCl-NPs was more effective against GBM02 cells than TMZ ($\sim$ 67-fold), and less aggressive to astrocytes, while Ag/AgCl-NP + TMZ treatment was no more effective against GBM02 cells than Ag/AgCl-NPs monotherapy. Taken together, our data indicate that 2.5 μg mL^{-1} Ag/AgCl-NPs represents the safest dose tested here, which affects GBM02 proliferation, with limited effect on astrocytes. Our findings show that HCA is a useful approach to evaluate the antiproliferative effect of nanoparticles against tumor cells.

Keywords Cancer · Glioblastoma · Antiproliferative effect · Metallic nanoparticles · Silver/silver chloride nanoparticles · High-content analysis

Introduction

Glioblastoma (GBM) is the most common and aggressive type of primary brain tumor, representing $\sim$ 50% of all brain tumors. GBMs are fast-growing tumors classified as grade IV by the World Health Organization (WHO) (Louis et al. 2007), and considered incurable, with unfavorable patient prognosis. GBM originates from the unregulated replication of glial cells, non-neuron-type central nervous system cells that surround nerve cells, providing support to neurons (Alves et al. 2011). The current first-line treatment for GBM patients (in addition to surgical resection) is a combination of radiotherapy and chemotherapy with temozolomide (TMZ) (brand names Temodar® or Temodal®) (Goldlust et al. 2008; Stupp et al. 2009). This approach provides a 24-month average survival for GBM patients after diagnosis, although only 5–15% of patients live for more than 5 years (Arvold and Reardon 2014; Raizer et al. 2015). In addition, 95% of GBM patients exhibit recurrence at the primary site after treatment, highlighting the need for more effective treatment options against GBM (Wen and Kesari 2008; Ramirez et al. 2013).

Metallic nanoparticles have attracted particular attention in the biomedical sciences due to their more favorable intrinsic properties when compared with those of their micro-sized counterparts. AgNPs are the most studied silver-based nanoparticles, and have effective microbicidal activity against a broad spectrum of pathogenic bacteria—including antibiotic-resistant strains—as well as against fungi, protozoa and viruses (Allahverdiyev et al. 2011; Durán et al. 2016a). In addition, AgNPs have antitumor activity in vitro and in vivo. The treatment of MCF-7 human breast cancer and Hep2 laryngeal carcinoma cells with AgNPs induced classic cytotoxic effects, including oxidative stress, loss of mitochondrial membrane potential and apoptosis (Jeyaraj et al. 2013; Rosarin et al. 2013). In SKBR3 breast cancer cells, AgNPs caused endoplasmic reticulum stress, oxidative stress and mitochondrial disorder leading to cell death mainly through autophagy (Buttacavoli et al., 2018). Sriram et al. (2010) showed that the intraperitoneal injection of AgNPs increased the survival of mice with Dalton's lymphoma subjected to transplant, likely due to the induction of apoptosis in lymphoma cells.

AgNPs have been tested against GBM, with relative success (AshaRani et al. 2009; Urbańska et al. 2015). Chemically-synthesized AgNPs (at 40 μg mL^{-1}) had antiproliferative effect against the commercial GBM cell line U87, leading to a decrease of 35% in the growth rate, when compared to untreated or placebo-treated cells (Urbańska et al. 2015). When conjugated with other cytotoxic agents, AgNPs were also effective against GBM (AshaRani et al. 2009). AgNPs impregnated with an alginate-chitosan blend (produced by a green method) had a potent antiproliferative effect against the GBM cell line U87MG (IC_{50} = 2.4 μg mL^{-1}), leading to extensive DNA damage, oxidative stress, mitochondrial dysfunction (with loss of mitochondrial membrane potential) and apoptosis (Sharma et al. 2014). Lipophilic AgNPs (entrapped in PEG-based polymeric nanoparticles) conjugated with the peptide chlorotoxin also had a significant cytotoxic effect against U87MG cells in vitro (IC_{50} = 45 μM) (Locatelli et al. 2012). Importantly, polymeric nanocomposites of AgNPs and the cytotoxic drug alisertib reduced GBM tumor growth in vivo (Locatelli et al. 2014).

Silver/silver chloride nanoparticles (Ag/AgCl-NPs), which have similar physicochemical properties to those of AgNPs, were initially developed in an attempt to produce AgNPs of higher efficiency (Vigneshwaran et al. 2007). Since then, the synthesis of Ag/AgCl-NPs by microorganisms and by plant materials have been the focus of many studies investigating eco-friendly alternatives to produce

nanoparticles that may be used to treat clinically important diseases (Jain and Aggarwal 2012; Huang et al. 2007; Durán et al. 2016b). Nevertheless, compared with AgNPs, Ag/AgCl-NPs have received little attention for scientific research and industrial applications. Nevertheless, Ag/AgCl-NPs share important properties with AgNPs, including the release of Ag^+ ions (Villanueva-Ibáñez et al. 2015). Recently, our group showed that biogenic Ag/AgCl-NPs produced using yeast cultures have strong antiproliferative effect against the pathogenic bacteria *Staphylococcus aureus* (gram-positive) and *Klebsiella pneumoniae* (gram-negative) (Eugenio et al. 2016), and Durán et al. (2016b) showed that biogenic Ag/AgCl-NPs from a different source have microbicidal effect towards both bacteria and fungi. However, the antitumor potential of Ag/AgCl-NPs has not been examined to date.

In this work we investigated further the therapeutic potential of Ag/AgCl nanoparticles, by testing the antitumor activity of these particles against GBM cells. Ag/AgCl-NPs were tested in vitro against a human glioblastoma cell line established previously in our laboratory (GBM02) and against non-tumoral astrocytes, alone or in combination with TMZ. The effect of treatment on cell proliferation was evaluated by automated high-content analysis (HCA), a highly efficient tool for automated image acquisition and analysis, and which has been widely applied to phenotypic screening, providing the identification of complex morphological changes in individual cells in response to treatment (Usaj et al. 2016). HCA has been used successfully in drug discovery (Brayden et al. 2015) and toxicology (Ramery and O'Brien 2014)—for the rapid (and impartial) analysis of multiple conditions within the same experiment—but has rarely been applied to the evaluation of cellular responses to metallic nanoparticles (Stojiljković et al. 2016).

Experimental section

Biosynthesis of Ag/AgCl-NPs

The synthesis of the Ag/AgCl nanoparticles was performed according to Eugenio et al. (2016). Briefly, the yeast *Candida lusitaniae* was cultivated in rich medium (4% glucose, 1% bacteriological peptone and 1% yeast extract, pH 6.5), for 24 h at 30 °C (with an initial inoculum of 10^7 cells/mL). Then, an aqueous solution of silver nitrate ($AgNO_3$) was added to the culture medium for a final concentration of 3.5 mM, and cultures were kept under agitation (150 rpm) for 5 days at 30 °C, and in the dark.

To confirm the production of Ag/AgCl-NPs, culture samples were collected after 5 days of incubation in the presence of $AgNO_3$, added to sterile 96-well plates (Biofil®, Guangzhou, China), and the absorbance was measured at increasing wavelengths (from 200 to 700 nm, with 1-nm increments), in an UV–Visible Spectra Max 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Purification of Ag/AgCl-NPs

Culture supernatants containing nanoparticles were separated from cell pellets by centrifugation at $2728\times g$ for 15 min, at room temperature, and Ag/AgCl-NPs were purified from these supernatants by centrifugation at $38{,}361\times g$ for 20 min, at room temperature. The pellet containing Ag/AgCl-NPs was resuspended in 1% sodium citrate in distilled water (pH 8.0) and was kept at room temperature before the experimental procedures.

Cell lines and cell culture

The human tumor cell line GBM02 was described previously (Faria et al. 2006). The use of patient's surgical specimens for the establishment of cell lines intended for research had the written informed consent from patients and was approved by the Brazilian Ministry of Health Ethics Committee, under Institutional Review Board (IRB, Research Ethics Committee of the Clementino Fraga Filho University Hospital, Rio de Janeiro, Brazil; consent CEP-HUCFF No. 002/01).

GBM02 cells were cultivated in DMEM-F12 medium (Invitrogen—Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, São Paulo, Brazil) and kept at 37 °C (5% CO_2) until reaching 80% of confluence. Cells were then detached from the flask using a solution of 0.05% trypsin (Sigma-Aldrich, São Paulo, Brazil)/0.02% ethylenediamine tetraacetic acid (EDTA) (Sigma-Aldrich, São Paulo, Brazil) for

5 min, at 37 °C, and seeded in 96-well plates at a density of 10^3 cells/well, for HCA assays.

Primary human astrocytes were isolated from surgically resected anterior temporal lobe tissue, from adult patients selected for surgical treatment of temporal-lobe epilepsy associated with hippocampus sclerosis. All patients gave written consent for the study and the procedures were in agreement with the Brazilian Ministry of Health Ethics Committee (CONEP 2340). Cultures of astrocytes were established from healthy cortical brain tissue only, as described previously (Diniz et al. 2012). Briefly, tissue samples were washed in DMEM-F12, mechanically dissociated, cut into small pieces with a sterile scalpel, and incubated in 10 mL of 0.25% trypsin (in water) at 37 °C for 10 min. After centrifugation, the cell pellet was resuspended in DMEM-F12 supplemented with 10% FBS, and cells were plated into tissue culture flasks and allowed to adhere at 37 °C (5% CO_2) for 2 h. Non-adherent astrocytes were then transferred to fresh culture flasks previously coated with 0.1% poly-L-lysine (Sigma-Aldrich, São Paulo, Brazil). When cultures reached 80% confluence, astrocytes were detached with 0.05% trypsin/0.02% EDTA and then seeded onto 96-well plates, at a density of 10^3 cells/well, for HCA assays (see below).

Nanoparticles and drug treatment

GBM02 cells and astrocytes were seeded at 10^3 cells/wells into flat-bottom 96-well plates (Corning Incorporated Costar®, Corning, NY, USA), in 300 µL/well of DMEM-F12 supplemented with 10% FBS and containing 0.5 µg mL^{-1} Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA). After 24 h, cells were incubated in the presence of different concentrations of Ag/AgCl-NPs (0.1, 0.5, 1.0, 2.5 and 5.0 µg mL^{-1}), TMZ (Sigma-Aldrich, São Paulo, Brazil) (9.7, 19.4, 29.1, 38.5 and 48.5 µg mL^{-1}) or a combination of Ag/AgCl-NPs and TMZ (0.1 + 9.7, 0.5 + 19.4, 1.0 + 29.1, 2.5 + 38.5, 5.0 + 48.5 µg mL^{-1}) for 24, 48 and 72 h at 37 °C (5% CO_2). The untreated control was performed by omitting either the nanoparticles or TMZ.

High-content analysis (HCA) of cell proliferation

Digital images of labeled cells left untreated or treated with Ag/AgCl-NPs and/or TMZ as described above were acquired in an In Cell Analyzer 2000 (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). Fluorescence images were taken of six random fields per sample (totalizing 24 images per treatment, for 4 time-points), in the emission wavelength range of 410–480 nm, and using a 10x objective lens. The nucleus (labeled with Hoechst 33258) of individual cells was segmented using the software In Cell Investigation (GE HealthCare Life Sciences), and the total number of nuclei per field was counted. The cell proliferation after 24, 48 and 72 h of treatment was calculated as a percentage of the number of cells (nuclei) counted in control (untreated) conditions.

The growth rate was estimated according to Sherley et al. (1995) using the formula:

$$N_t = N_0 2^{ft}$$

where N_t, total number of cells; N_0, initial number of cells; f, growth rate; and t, treatment time. The growth rate values were then used to calculate the population doubling time, using the formula, according Sherley et al. (1995):

$$PDT = \frac{\ln(2)}{f}$$

where PDT, population doubling time; f, growth rate and ln = natural logarithm.

Statistical analysis

All experiments were performed in biological triplicates. Cell proliferation data were expressed as mean ± standard deviation of three independent experiments. IC_{50} (concentration that inhibits 50% of cell proliferation) of the treated GBM02 and human astrocytes was determined by using Graphpad Prism 5 software. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test, using Graphpad Prism 5. Statistical significance was established as $p < 0.05$.

Results

Ag/AgCl-NPs produced by yeasts inhibit glioblastoma cell proliferation more effectively than TMZ, and within the therapeutic window of silver nanoparticles

Ag/AgCl-NPs have potent microbicidal effect, but their effect against tumor cell growth had not been

examined. Here, we used an automated, image-based high-content analysis (HCA)—with fast and accurate counting of individual cell nuclei labeled with Hoechst—to study the effect of different concentrations of Ag/AgCl-NPs, alone or in combination with the anticancer drug TMZ, on the proliferation of a glioblastoma cell line (GBM02). As a control, we also tested Ag/AgCl-NPs against human astrocytes isolated from healthy cortical brain tissue (removed from patients subjected to surgery for epilepsy), to evaluate the selectivity of treatments against tumor cells. We chose TMZ concentrations ranging from 9.7 μg mL^{-1} to a maximum of 48.5 μg mL^{-1}, which has a similar impact on cell viability to the dose recommended by Clinical Practice Guidelines in Oncology (NCCN Guidelines) for the treatment of central nervous system tumors (350 μM, or 67.9 μg mL^{-1}) (Balça-Silva et al. 2015).

Representative images of Hoechst 33258-stained nuclei (Fig. S1, S2 and S3) show the response of both GBM02 cells and astrocytes to each treatment condition. The proliferation of GBM02 cells was inhibited by Ag/AgCl-NPs and TMZ in a time- and dose-dependent manner, and cell proliferation inhibition was considerably less pronounced in astrocytes (Fig. 1, Supplementary Table 1). The inhibition of GBM02 proliferation started at a concentration of 0.1 μg mL^{-1} Ag/AgCl-NPs, after 24 h of treatment (5 ± 4% inhibition), while for astrocytes proliferation inhibition was only noticeable from 1.0 μg mL^{-1} Ag/AgCl-NPs (Fig. 1, Supplementary Table 1). At this concentration inhibition reached 2 (± 2) % in astrocytes. We did not observe growth inhibition when astrocytes were incubated in the presence of Ag/AgCl-NPs ranging from 0.1 to 1.0 μg mL^{-1} for 48 h or 72 h.

At 24 h, the lower concentrations of TMZ used here (9.7 and 19.4 μg mL^{-1}) inhibited the proliferation of GBM02 by 13 (± 5) and 10 (± 2) %, respectively (Fig. 1a; Supplementary Table 1). Although GBM02 cell proliferation inhibition was limited (5 ± 4%) at the lower concentrations of Ag/AgCl-NPs used here (0.1 and 0.5 μg mL^{-1}), these concentrations had only a minor effect on astrocyte proliferation (2 ± 2%), while the lower concentrations of TMZ used here decreased astrocyte proliferation by 5 (± 2) to 8 (± 6) % (Fig. 1d; Supplementary Table 1). The same pattern was observed at 48 h and 72 h: TMZ was more effective than Ag/AgCl-NPs on the inhibition of tumor cell proliferation, but had stronger effects on astrocyte proliferation than the nanoparticles (Fig. 1b, c, e, f).

At the highest concentrations, the maximum inhibition of GBM02 cell proliferation obtained with monotherapy were 82 (± 1) and 62 (± 2) %, following 72 h of exposure to 5.0 and 48.5 μg mL^{-1} of Ag/AgCl-NPs and TMZ, respectively (Fig. 1c; Supplementary Table 1). At the same concentration of nanoparticles astrocyte growth inhibition was 27 (± 7) % (3-fold less than that for tumor cells), while the highest concentration of TMZ inhibited astrocyte proliferation by 37 (± 5) %.

The combined treatment with Ag/AgCl-NPs and TMZ led to a time- and dose-dependent reduction in the cell proliferation of GBM and astrocytes (Fig. 1; Supplementary Table 1), as occurred with both Ag/AgCl-NPs and TMZ-treated cells. Interestingly, the antiproliferative effect of lower doses of Ag/AgCl-NPs and TMZ on GBM02 cells was similar to that of TMZ alone, whereas at higher doses the combination had a similar effect to that of Ag/AgCl-NPs alone (Fig. 1a–c; Supplementary Table 1). On astrocytes, treatment with Ag/AgCl-NPs and TMZ inhibited cell proliferation at similar levels to TMZ, irrespective of the concentration; thus, the combination was relatively more toxic to astrocytes than Ag/AgCl-NPs alone (1.2–6 fold higher proliferation inhibition) (Fig. 1d–f; Supplementary Table 1).

The IC_{50} (i.e., concentration that inhibits 50% of cell proliferation) data clearly indicate that treatment with Ag/AgCl-NPs was in general more effective against GMB02 (i.e., had lower IC_{50} values) than against astrocytes (Table 1). At 24 and 48 h of treatment, the IC_{50} for the treatment of astrocytes with Ag/AgCl-NPs was ∼ 3-fold higher than that for GBM02, and at 72 h this difference increased to ∼ 4-fold. Surprisingly, at 24 and 48 h, TMZ treatment was more aggressive towards astrocytes than towards GBM02 cells, leading to ∼ 23 and ∼ 34% higher inhibition of astrocyte growth, respectively. On the other hand, at 72 h, TMZ treatment was more aggressive towards tumor cells, with growth inhibition values towards these cells ∼ 85% lower than those for astrocytes (Table 1).

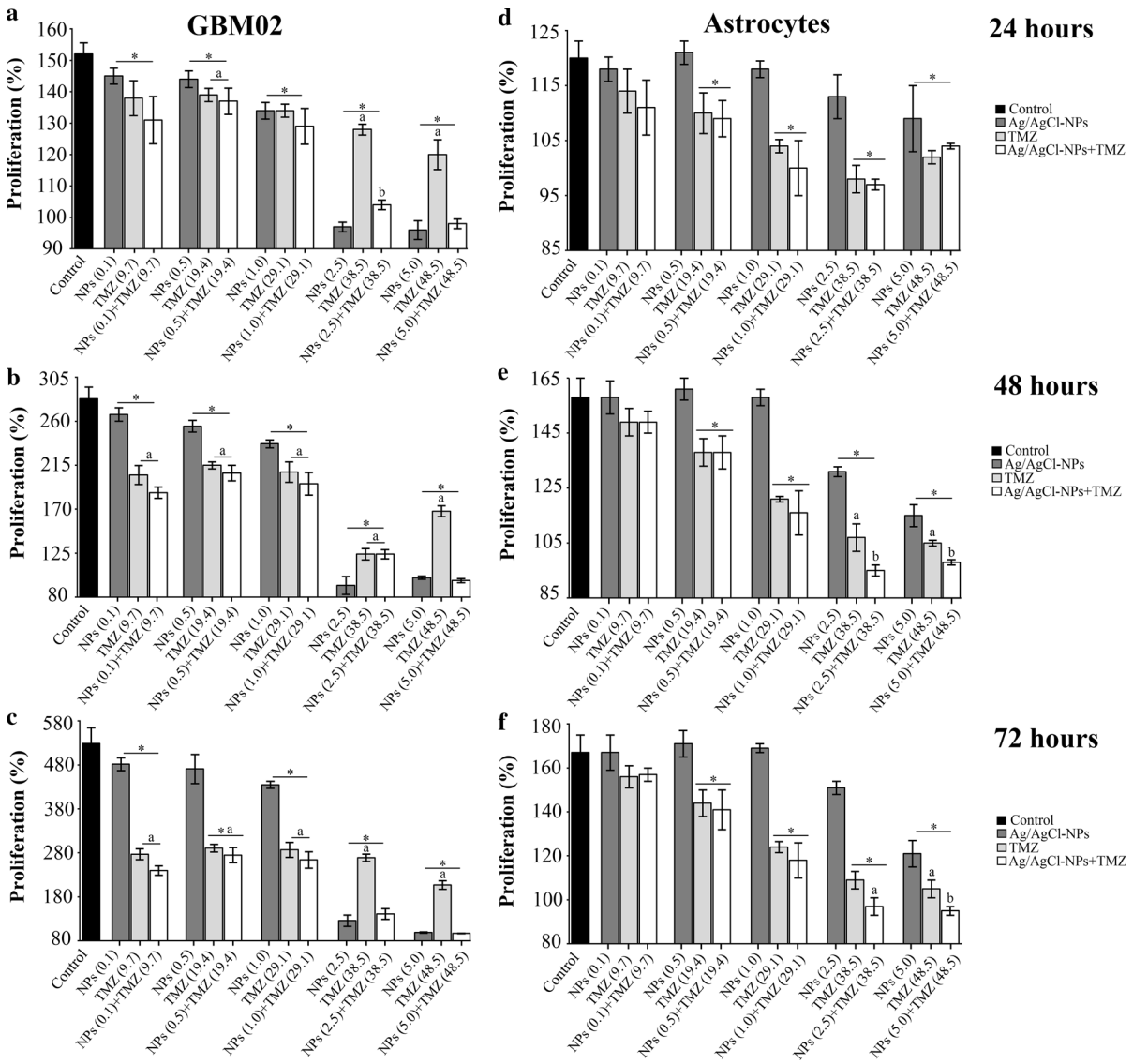

Fig. 1 Effect of Ag/AgCl-NPs and TMZ on the proliferation of GBM02 cells and astrocytes. Cells labeled with Hoechst 33258 were treated with Ag/AgCl-NPs and TMZ, alone or in combination, for 24 (**a**, **b**), 48 (**c**, **d**) and 72 h (**e**, **f**), and then subjected to high-content analysis (HCA) of cell proliferation. Numbers in parenthesis represent concentration values, in μg mL^{-1}. Cell proliferation was calculated as a percentage of the number of cells (nuclei) counted in control (untreated) conditions. Data represent mean ± standard deviation values of three independent experiments. The groups labeled with the same letter, within the same treatment concentration, are not statistically different (considering $p \leq 0.05$, by one-way ANOVA followed by Tukey's test). The statistical differences between the treated groups and the untreated control are represented by $^*p < 0.05$

Table 1 IC_{50} (μg mL^{-1}) for the treatment of GBM02 and astrocytes with Ag/AgCl-NPs or with temozolomide (TMZ)

Treatment	Time (h)	IC_{50} GBM02	IC_{50} astrocytes
Ag/AgCl-NPs	24	6.3 (± 0.3)	18.5 (± 0.7)
Ag/AgCl-NPs	48	3.0 (± 0.3)	8.4 (± 0.7)
Ag/AgCl-NPs	72	2.3 (± 0.3)	9.1 (± 1.4)
TMZ	24	198.1 (± 5.1)	152.2 (± 6.1)
TMZ	48	98.0 (± 4.9)	65.8 (± 2.6)
TMZ	72	8.8 (0.3)	60.3 (± 2.1)

Population growth parameters indicate that Ag/AgCl-NPs alone are more effective against glioblastoma cells than TMZ, or the combination of Ag/AgCl-NPs and TMZ

We further estimated the growth rate (Fig. 2) and population doubling time (PDT, Table 2) for the treatment of GBM02 and astrocytes with Ag/AgCl-NPs, TMZ and Ag/AgCl-NPs + TMZ for 72 h, to improve our understanding of the treatment effects, as described previously (Assanga 2013; Gavish et al. 2016). Exposure to lower concentrations (0.1 to 1.0 μg mL^{-1}) of Ag/AgCl-NPs for 72 h led to a limited decrease (∼ 12%) in the growth rate of GBM02 (compared with untreated cells), while the growth rate of these cells decreased sharply, by 80 and 98%, after treatment with 2.5 and 5.0 μg mL^{-1} Ag/AgCl-NPs, respectively (Fig. 2a). In contrast, lower concentrations of TMZ led to a considerable reduction in the growth rate of GBM02 cells, but TMZ at higher doses was less effective than higher doses of Ag/AgCl-NPs at reducing the growth rate of glioblastoma cells (Fig. 2).

Treatment with TMZ led to a dose-dependent decrease in the growth rate of astrocytes, reaching a growth rate reduction of 90%, at the highest TMZ concentration (Fig. 2e). In contrast, concentrations of Ag/AgCl-NPs up to 1.0 μg mL^{-1} did not alter the growth rate of astrocytes, while 2.5 μg mL^{-1} Ag/AgCl-NPs had a modest effect (18% decrease) on the growth rate of these cells (Fig. 2d); however, this growth rate was still ∼ 1.2-fold higher than that of GBM02 cells treated with the same concentration of Ag/AgCl-NPs (Fig. 2a), showing that higher concentrations of Ag/AgCl-NPs were less toxic to astrocytes than to glioblastoma cells. Treatment with the highest concentration of Ag/AgCl-NPs (5.0 μg mL^{-1}) decreased the astrocyte growth rate considerably (62%, Fig. 2d), which was still ∼ 3.7-fold higher than the growth rate of astrocytes treated with the highest concentration of TMZ (Fig. 2e). The highest TMZ concentrations used here, which are closer to the range used in the clinic, (Balça-Silva et al. 2015)

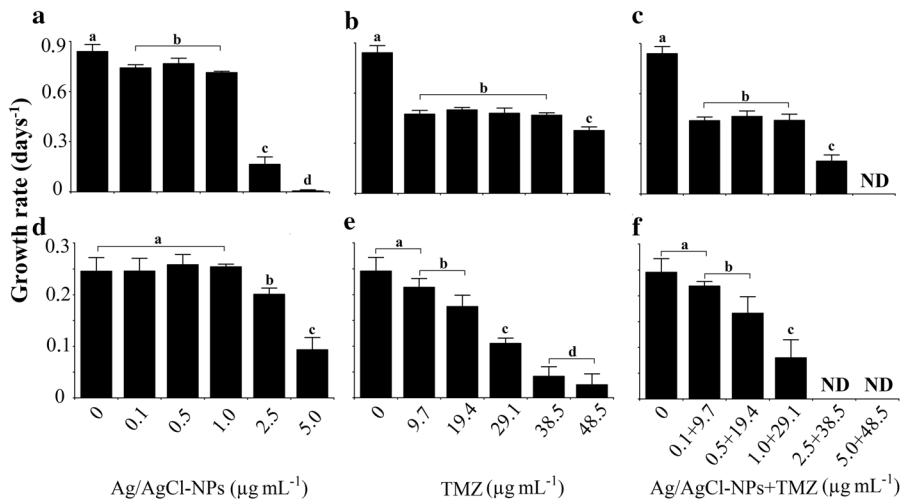

Fig. 2 Population growth rate of GBM02 cells and astrocytes treated for 72 h with Ag/AgCl-NPs and TMZ, alone or in combination. GBM02 cells treated with Ag/AgCl-NPs (**a**), TMZ (**b**) or Ag/AgCl-NPs and TMZ (**c**). Astrocytes treated with Ag/AgCl-NPs (**d**), TMZ (**e**) or Ag/AgCl-NPs and TMZ (**f**). Data represent mean ± standard deviation values of three independent experiments. The groups labeled with the same letter are not statistically different (considering $p \leq 0.05$, by one-way ANOVA followed by Tukey's test)

Table 2 Effect of treatment with Ag/AgCl-NPs, alone or combined with temozolomide (TMZ), on the population doubling time (PDT, in hours) of GBM02 cells and astrocytes

Treatment	Concentration (μg mL^{-1})	PDT (h) GBM02	Astrocytes
Control	0	20 (± 1)	68 (± 7)
Ag/AgCl-NPs	0.1	22 (± 0.5)	68 (± 6)
Ag/AgCl-NPs	0.5	22 (± 1)	64 (± 4)
Ag/AgCl-NPs	1.0	23 (± 0.2)	65 (± 1)
Ag/AgCl-NPs	2.5	107 (± 34)	83 (± 5)
Ag/AgCl-NPs	5.0	2514 (± 1673)	188 (± 56)
TMZ	9.7	35 (± 2)	78 (± 6)
TMZ	19.4	33 (± 1)	95 (± 12)
TMZ	29.1	35 (± 2)	159 (± 15)
TMZ	38.5	36 (± 1)	483 (± 282)
TMZ	48.5	44 (± 3)	1120 (± 938)
Ag/AgCl-NPs + TMZ	0.1 + 9.7	38 (± 2)	76 (± 3)
Ag/AgCl-NPs + TMZ	0.5 + 19.4	36 (± 3)	102 (± 21)
Ag/AgCl-NPs + TMZ	1.0 + 29.1	38 (± 3)	245 (± 130)
Ag/AgCl-NPs + TMZ	2.5 + 38.5	86 (± 15)	ND
Ag/AgCl-NPs + TMZ	5.0 + 48.5	ND	ND

ND (not determined)—the PDT of some treatments could not be determined because the final number of cells counted (after 72 h) was ≤ the starting cell number (at 0 h)

were ∼ 4-fold more cytotoxic to astrocytes than to glioblastoma cells (Fig. 2b, e).

Treatment with a combination of Ag/AgCl-NPs and TMZ decreased significantly the growth rate of GBM02 cells (Fig. 2c), when compared with untreated cells. However, the combination also decreased considerably the growth rate of astrocytes (Fig. 2f). We could not estimate the growth rate of GBM02 and astrocytes after treatment with the highest dose of the Ag/AgCl-NPs + TMZ combination, because no growth was detected at 72 h, compared with the untreated control, evidencing the deleterious of these treatment conditions.

The PDT of GBM02 treated with up to 1.0 μg mL^{-1} Ag/AgCl-NPs was slightly higher (by up to 15%) than that of untreated cells, while treatment with 2.5 and 5.0 μg mL^{-1} Ag/AgCl-NPs abruptly increased (by ∼ 5 and ∼ 130-fold, respectively) the PDT of GBM02 cells (Table 2), clearly indicating that Ag/AgCl-NP treatment at these concentrations dramatically affects glioblastoma cell proliferation. The high value found to 5.0 μg mL^{-1} treated GBM cells (2514 (± 1673)) should be indicative of cell death, instead of cell cycle arrest. For astrocytes, treatment with Ag/AgCl-NPs decreased by ∼ 5% the PDT at concentrations up to 1.0 μg mL^{-1}, and at 2.5 μg mL^{-1} the PDT was increased by ∼ 20% only (compared with an increase of ∼ 5-fold for GBM02). At the highest concentration of Ag/AgCl-NPs (5.0 μg mL^{-1}), the PDT of astrocytes increased ∼ 2.7 fold compared with the untreated control; however, it was approximately 6-fold lower than that of cells treated with TMZ alone, at 48.5 μg mL^{-1}.

After treatment with the combination of 1.0 μg mL^{-1} Ag/AgCl-NPs and 29.1 μg mL^{-1} TMZ, we observed an increase of ∼ 3.6-fold in the PDT of astrocytes. We could not calculate the PDT for the highest doses of the combined treatment, due to the drastic effect of these conditions on population growth. These results confirm that Ag/AgCl-NPs were less toxic to astrocytes than either TMZ or Ag/AgCl-NPs + TMZ. TMZ treatment was only able to increase the PDT of GBM02 by ∼ 2.2-fold, representing the least effective treatment. Combining 2.5 μg mL^{-1} Ag/AgCl-NPs + 38.5 μg mL^{-1} TMZ treatment increased significantly the PDT of glioblastoma cells (by ∼ 4.4-fold compared to the untreated cells), but this effect was still less pronounced than that of Ag/AgCl-NPs alone at the same concentration used in the combination (5.4-fold compared with untreated cells).

Discussion

Ag/AgCl-NPs share several physicochemical properties with AgNPs and, thus, are likely to have comparable biomedical/catalytic applications to those of

silver nanoparticles (Eugenio et al. 2016; Devi et al. 2016). However, few studies have evaluated the biomedical potential of Ag/AgCl-NPs, and these have been limited to their antibacterial and antifungal effect (Durán et al. 2016b; Eugenio et al. 2016).

Given the wide range of biomedical applications of AgNPs (Allahverdiyev et al. 2011; Durán et al. 2016a), we examined whether an important AgNPs property—their activity against cancer cells in vitro—was shared by Ag/AgCl-NPs. We investigated the effect of increasing concentrations (0.1–5.0 μg mL^{-1}) of biogenic Ag/AgCl-NPs (with a size range from 2 to 22 nm) (Eugenio et al. 2016) on the proliferation of human glioblastoma cells in vitro, and compared these results with the administration of TMZ, an antitumor drug currently used in the treatment of GBMs (Stupp et al. 2009). Also, we combined Ag/AgCl-NPs and TMZ, to assess a possible cooperative effect on the proliferation of glioblastoma cells. To produce a fast and accurate analysis of the effect of Ag/AgCl-NPs on glioblastoma cells, while minimizing operator bias (Zanella et al. 2010), we quantified cell proliferation directly, using an HCA-based method described in previous works (Chan et al. 2013; Stengl et al. 2017). HCA has frequently been used in drug discovery (Usaj et al. 2016) and to evaluate nanoparticle applications (Brayden et al. 2015) and we have now extended the use of HCA to the study nanoparticle activity in preclinical cancer research.

GBM is the most malignant type of brain tumor, and patient prognosis did not improve despite the efforts of researchers from widely different fields (Louis et al. 2007; Goldlust et al. 2008; Stupp et al. 2009). In the last two decades, GBM treatment using chemotherapy underwent changes such as the replacement of alkylating substances like carmustine, nimustine and lomustine with temozolomide (TMZ). The low efficacy of TMZ clearly illustrates the need for further changes in the treatment of this disease (Beier et al. 2011). It is recognized that GBM may contain cancer stem cells with stem cell-like properties. Through signals from the microenvironment, they can generate highly resistant tumor cells, which are the main cause of recurrence of the disease. It is extremely difficult to combat this type of GBM derived from cancer stem cells, since the current treatments have proven to be inefficient. This aspect emphasizes the need for therapeutic alternatives (Glaser et al. 2017).

The use of silver-based nanoparticles as antitumor agents has attracted attention in the last few years (Rosarin et al. 2013; Sriram et al. 2010). AgNPs, alone or in combination with other agents, have antiproliferative activity against GBM in vitro (AshaRani et al. 2009; Urbańska et al. 2015), but the combination of metallic silver nanoparticles and silver chloride nanoparticles (Ag/AgCl-NPs) for the treatment of GBM had not been reported in the literature, to our knowledge.

Our results using a concentration range considerably lower than that used in other studies (from 25 to 222 μg mL^{-1}) (AshaRani et al. 2009; Sharma et al. 2014; Urbańska et al. 2015) showed that Ag/AgCl-NPs elicit highly deleterious effects on the growth rate of GMB cells (83% inhibition at 5.0 μg mL^{-1}). We also showed that the nanoparticles synthetized by our group were more effective at inhibiting tumor cell proliferation than TMZ, the standard chemotherapeutical drug of choice for glioblastoma treatment, while having little impact on the proliferation of astrocytes. AshaRani et al. (2009) showed that starch-coated AgNPs generated cytotoxic effects—including mitochondrial and DNA damage, increased ROS production, and cell cycle arrest—on human glioblastoma cells (U251). However, these cytotoxic effects were observed in the presence of AgNPs at concentrations much higher (25–400 μg mL^{-1}) than the Ag/AgCl-NP concentrations used here. Although the authors noticed similar effects in normal human lung fibroblasts (IMR-90), these cells recovered efficiently from AgNP treatment, whereas the cancer cells ceased to proliferate. These data support the results observed in our study, in which non-tumor cells (astrocytes) were less affected by nanoparticles than tumor cells.

Based on data from previous studies on the use of AgNPs we believe that the antiproliferative effect of the Ag/AgCl-NPs on GBM02 may be attributed to the physical–chemical interaction of silver with functional groups in intracellular proteins, as well as with nitrogenous bases and phosphate groups in DNA (Rutberg et al. 2008). Ag/AgCl-NPs may also accumulate in the nucleus, causing chromosomal instability, and disrupting mitosis. It is also possible that Ag/AgCl-NPs interact with the actin cytoskeleton, causing structural and functional damage to cytoskeletal elements (Urbańska et al. 2015). In addition, the active surface of AgNPs can directly induce the generation of free radicals and the dissolution of AgNPs into Ag

ions, which is a characteristic shared by silver-based nanoparticles, including Ag/AgCl-NPs (Kang et al. 2016). Ag ions damage the DNA and disrupt of the mitochondrial membrane potential, leading to the release of cytochrome c and to mitochondrial-dependent apoptosis (Hsin et al. 2008; Piao et al. 2011). These are interesting hypotheses that should be investigated in future studies on the mechanism of Ag/AgCl-NPs inhibition of glioblastoma cell proliferation.

MacEwan et al. (2010) reported that nanoparticles could respond to the low extracellular pH of the tumor microenvironment. From this finding, Urbańska et al. (2015) hypothesized that the release of silver ions from AgNPs would be higher in the tumor compared to normal cells. That would explain the differential antiproliferative effect observed for tumor versus healthy cells (astrocytes), as described by others (AshaRani et al. 2009). On the other hand, our data show that TMZ was more aggressive for astrocytes than for GBM cells. The reason for this remains to be determined, since the action of TMZ involves DNA damage through methylation of the O6-position of guanines, blocking DNA replication and inducing either cell death or cell cycle arrest, in a process that is similar in tumor and non-tumor cells (Glaser et al. 2017).

The advantages of combining Ag/AgCl-NPs and TMZ would be a possible increase in treatment efficacy and a decrease in the drug dose, as previously demonstrated (Naqvi et al. 2013; Silva et al. 2015). Liang et al. (2017) showed that chemically-generated AgNPs combined with TMZ increased the sensitivity of human glioma cells (U251) to TMZ, with dose-dependent cytotoxicity. In contrast, we showed here that the treatment of GBM02 cells with a combination of Ag/AgCl-NPs and TMZ was clearly no more effective than treatment with Ag/AgCl-NPs only. Also, our data showed that both monotherapy with TMZ and the combined treatment with Ag/AgCl-NPs and TMZ were significantly more aggressive towards astrocytes than Ag/AgCl-NPs alone.

Based on the data reported here, the treatment with Ag/AgCl-NPs at the concentration of 2.5 μg mL^{-1} is the most effective in this model, due to the high proliferation inhibition in GBM02 coupled with the low antiproliferative effect on astrocytes. Taken together, our results indicate that Ag/AgCl-NPs deserve more attention as a therapeutic option against glioblastoma.

Conclusion

Here, we showed that 'green' (i.e., biogenically produced) Ag/AgCl-NPs have antiproliferative activity against cells from human glioblastoma, the most aggressive brain tumor type, with extremely poor patient prognosis. Importantly, Ag/AgCl-NPs had a considerably lower antiproliferative effect towards healthy human astrocytes, and our data indicate that the concentration of 2.5 μg mL^{-1} Ag/AgCl-NPs was the safest dose tested here, since it had a strong antiproliferative effect against GBM02 cells—and was more potent than TMZ—with low antiproliferative effect on healthy astrocytes. These results suggest that Ag/AgCl-NPs have potential application in the treatment of patients with GBM. Our data also show that HCA can be used to investigate the antiproliferative effect of metallic nanoparticles against human cells, by high-throughput image analyses of cell proliferation, based on single-nuclei counting.

Acknowledgements This work was supported by the National Council for Scientific and Technological Development (CNPq), the Carlos Chagas Filho Foundation for Research Support of the State of Rio de Janeiro (FAPERJ) and the Coordination for the Improvement of Higher Education Personnel (CAPES).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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