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Folic acid enhances the apoptotic and genotoxic activity of carboplatin in HeLa cell line

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ABSTRACT

In human tumor cells, experimental and clinical evidence indicates that some factors involved in signal transduction and cell growth can also modulate the response to chemotherapeutic treatment. The aim of the present study was to investigate the role of folic acid (FA) as a modulator of carboplatin (CBDCA) activity.
Genotoxicity and cytotoxicity induced by CBDCA alone and in combination with FA were assessed in cultured HeLa cells. We used comet assay, mitotic index analysis, MTT and NR assays, cytokinesis-block micronucleus cytome assay and annexin V-IP as different cytotoxicity and genotoxicity approaches for human cervical carcinoma cell line studies.
The results showed that addition of 900 nM FA together with 40.4 mM CBDCA enhanced the activity of the platinum compound, increasing its effect on cell death by nearly 20%, as evidenced by the MTT and NR assays. Moreover, not only higher levels of DNA and chromosomal damage were reached but also the number of necrotic and apoptotic cells were significantly increased when cell cultures were treated with the combined procedure.
This situation opens the possibility to explore the use of FA in platinum-based chemotherapy protocols to reduce the platinum doses for patient treatment and decrease the chance of developing the known side effects without losing biological activity.

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1. Introduction

Platinum-based compounds are known since the 19th century. In fact, cisplatin was first synthesized in 1845, but the discovery of its antitumor properties was not made until 1969 (Rosenberg and Vancamp, 1969). Today, platinating compounds are still front-line clinical therapies and constitute part of the treatment regimen for patients with many types of cancers, including head and neck, testicular, ovarian, cervical, lung, colorectal and relapsed lymphoma. Despite this success, nephrotoxicity, neurotoxicity and the emetic properties of cisplatin have limited its use (Basu and Krishnamurthy, 2010). Also, the reduced water solubility, the relatively narrow range of treatable cancers and the ability of carcinomas to develop chemotherapy resistance have pressed researchers to develop a new generation of platinum-based molecules. In this sense, carboplatin (CBDCA) is a second generation drug introduced and approved by the FDA in the late 20th century. Since that time, it has gained popularity in clinical treatment due to its reduced side-effects, when compared with its related compound cisplatin. Particularly, although CBDCA has low nephrotoxicity effects, it shows undesirables myelosuppressive characteristics (Wagstaff et al., 1989).

Even though platinum-based compounds have the ability to bind to a wide spectrum of biomolecules, such as RNA, proteins or phospholipids, DNA is their main biological target (Pascoe and Roberts, 1974; Knox et al., 1986). Cisplatin and CBDCA can enter cells via passive diffusion through a process known as aquation (Gately and Howell, 1993), or via endocytosis using the copper transporter CTR1, which is a key influx transporter and plays an important role in mediating the uptake of platinum compounds (Shen et al., 2012). Once into the cells, platinum compounds bind to the nitrogens of purine bases in DNA, inducing intrastrand crosslinks. The presence of these adducts distorts the double helix, blocking DNA replication and transcription (Jamieson and Lippard, 1999). Specifically, CBDCA mostly forms the intrastrand crosslink cis-Pt(NH3)2d(pGpG)(Pt-GG), requiring >200-fold CBDCA than cisplatin to obtain equal levels of platination. On the other hand, >20 times CBDCA is needed to obtain equal levels of cytotoxicity in CHO cells, after 1 h of exposure (Blommaert et al., 1995). To treat locally advanced cervical cancer, platinum-containing agents are used alone or in combination. Some studies have shown that the use of cisplatin combined with paclitaxel requires a prolonged infusion and is less convenient and more toxic than the combination of CBDCA and paclitaxel, thus leading to a more widespread use of CBDCA (Moore et al., 2007).

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Folate, or folic acid (FA), is a water-soluble B-vitamin also known as vitamin B9 or folacin. In nature, folates exist in a wide range of chemical forms and they can be found in food as well as in metabolically active forms in the human body. FA is the major synthetic form found in fortified foods and vitamin supplements; however, it has no biological activity unless converted into folate (National Academy Press, 1998). In human physiology, the only function of folate coenzymes appears to be linked to the transfer of one-carbon units, playing a major role in *S*-adenosylmethionine formation, the universal methyl donor, as well as in the construction of purine and thymidine in a variety of reactions critical to the metabolism of nucleic acids and amino acids (Bailey and Gregory, 1999; Choi and Mason, 2000).

Epidemiologic data have revealed that a reduced folate status is associated with cervical, colorectum, lung, esophagus, brain, pancreas, and breast cancer. Intervention trials using supraphysiologic doses of folate, mainly with individuals at increased risk of cervical and colorectum cancer, showed inconsistent results (Choi and Mason, 2000). Nevertheless, folates appear to be essential for cell growth and cell cycle, key processes in cell transformation. On the other hand, folate deficiency induces lower proliferation rates by increased apoptosis and cell cycle arrest (Courtemanche, 2003). It has been widely demonstrated that folate receptor is overexpressed in several human epithelial cancer cells, but is absent or negligibly expressed in most normal cells, highlighting the need for folates by transformed cells. In general, aggressive cancer types express the highest amounts of folate receptor alfa or FRα (Hansen et al., 2015).

In human tumor cells, experimental and clinical evidence indicates that some factors involved in signal transduction and cell growth can also modulate the response to chemotherapeutic treatment (Toffoli et al., 1998). For example, the cellular transformation by different oncogenes can stimulate the proliferation of ovarian cancer cells and confer cellular resistance to cisplatin (Basu and Cline, 1995). In this way, an increased folate internalization may have a role in the constitutive control of cell proliferation (Sun et al., 1995), thereby modifying the response to chemotherapeutic agents.

Human cervical carcinoma HeLa cells strongly express the folate receptor, as demonstrated in several works (Zhang et al., 2006). In this context, the use of folate as a cell response modulator against a chemotherapeutic agent is fully grounded, thus allowing the use of these cells as a model for studying the cellular response to a chemotherapeutic agent modulated by FA.

In this paper, we investigate the genotoxicity and cytotoxicity induced by CBDCA alone and in combination with FA. We used alkaline single cell gel electrophoresis assay (SCGE), mitotic index analysis (MI), 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cytokinesis-block micronucleus cytome (CBMN Cyt) assay as different cytotoxicity and genotoxicity approaches for human cervical carcinoma cell line studies.

2. Materials and methods

2.1. Cells

HeLa cells were obtained from the American Type Culture Collection (Manassas, VA, USA). They were cultured in Falcon T-25 flasks (Nunc, Denmark) using MEM medium (Gibco BRL, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (Notocor Laboratories, Cordoba, Argentina), 50 IU penicillin and 50 μg/ml streptomycin (Bagó Laboratories, Buenos Aires, Argentina) in a humidified atmosphere with 5% CO_2. In our laboratory, all cell lines are routinely tested for contamination by mycoplasma. Hela cells were shown to be mycoplasma-free.

2.2. Experimental design

Preliminary experiments were conducted to define the adequate concentration of CBDCA and FA to perform all the analyses. With this aim, MTT assays were done with cells cultured in duplicate in 96-well plates for 24 h.

FA (Sigma, St. Louis, MO, USA) was tested at concentrations of 300, 600, 750 and 900 nM. FA concentration selection was based on previous studies conducted by Beetstra et al. (2005). Also, cell cultures were treated with increasing CBDCA concentration: 40.4, 80.8, 121.2, 161.6, 242.4 and 323.2 mM (Carboxtie® 150 mg, Bio Profarma, Buenos Aires, Argentina). Finally, combined CBDCA-FA treatments were used to determine changes in CBDCA activity modulated by FA, measuring changes in the levels of cellular viability. Combined treatments, expressed as mM CBDCA and nM FA, were as follows: CBDCA40.4/FA150; CBDCA40.4/FA300; CBDCA40.4/FA600; CBDCA40.4/FA750; CBDCA40.4/FA900; CBDCA80.8/FA300; CBDCA80.8/FA600; CBDCA80.8/FA900.

The final methodological design for all tests was as follows: cells were cultured in monolayers for 24 h and then treated for another 24 h with CBDCA (40.4 mM), FA (900 nM), or the combined treatment (40.4 mM CBDCA/900 nM FA). Analytical tests were performed at the end of the treatments. Mannitol (part of Carboxtie® formulation) was considered as a control of excipients, Bleomycin (1 μg/ml) was used as a positive control for SCGE, MI and CBMN Cyt assays and cells grown in MEM without any other component were used as negative control. Ethanol 10% was used as a positive control for MTT assay. Bleomycin (Gador Laboratories Nippon Kayaku Co., Japan) was dissolved in distilled water before treatment.

All the experiments were performed in triplicate to allow an accurate estimate of the inter-experimental variation.

2.3. Comet assay

SCGE was performed using the alkaline version described by Singh et al. (1988) with some modifications (Tice and Strauss, 1995). This version of the technique detects DNA migration caused by strand breaks, alkaline labile sites and transient repair sites. Briefly, slides were covered with a first layer of 180 μl of 0.5% normal agarose (Carlsbad, Ca, USA). An amount of 75 μl of 0.5% low melting point agarose (Carlsbad, Ca, USA) was mixed with approximately 15,000 cells suspended in 15 μl and layered onto the slides, which were immediately covered with coverslips. After agarose solidification at 4 °C for 10 min, coverslips were removed and slides were immersed overnight at 4 °C in fresh lysis solution. The slides were equilibrated in alkaline solution (pH 13) for 20 min. Electrophoresis was carried out for 30 min at 25 V and 300 mA (1.25 V/cm). Afterwards, slides were neutralized by washing them three times with Tris buffer (pH 7.5) every 5 min and subsequently washed in distilled water. Slides were stained with 1/1000 SYBR Green I (Molecular Probes, Eugene, Oregon, USA) solution (Olive, 1999). Two hundred randomly selected comet images were analyzed per treatment.

Scoring was performed at 400× magnification using a fluorescent microscope (Olympus BX40 equipped with a 515–560 nm excitation filter) connected through a Sony 3 CCD-IRIS Color Video Camera. Based on the extent of strand breakage, cells were classified according to their tail length in five categories, ranging from 0 (no visible tail) to 4 (still a detectable head of the comet but most of the DNA in the tail) (Olive, 1999; Collins, 2004). With this data an Index Damage (ID) was obtained according to Collins (2004). The total score for the sample gel was between 0 and 400 "arbitrary units". Visual scoring (arbitrary units) is rapid as well as simple and there is very close agreement between this method and computer image analysis (percentage of DNA in the tail) (Collins, 2004).

2.4. MTT and neutral red (NR) assay

HeLa cells (2×10^5/ml) were cultured in MEM complete culture medium on 96-well microplates. The procedure of the MTT assay (Thiazolyl Blue Tetrazolium Bromide) was performed following the techniques described by Kosmider et al. (2004). After exposure with

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tested compounds, the culture medium was removed and 20 μl MTT (5 mg/ml) was added for an additional 3 h period. Then the formazan crystals were dissolved in 100 μl DMSO. The protocol described by Borenfreund and Puerner (1985) was employed for the NR assay. Following exposure with test compounds, cells were incubated for an additional 3 h period in the presence of 100 μg/ml neutral red dye dissolved in PBS. Then the cells were washed with PBS and the dye was extracted in each well. Absorbance at 550 nm was measured with a microplate spectrophotometer (Biotek Instruments Inc., Bedfordshire, United Kingdom). Results were expressed as the mean percentage of mitochondrial activity from three independent experiments performed in parallel.

2.5. Apoptosis detection by annexin V affinity assay

HeLa cells were incubated at 37 °C in a 5% CO2 atmosphere in a final media volume of 1 ml and treated with test compounds during 24 h. Negative and positive controls were run simultaneously with treated cultures. At the end of the treatment period, cells were washed in PBS, resuspended in 250 μl of 1× binding buffer (10 mm HEPES, 140 mm NaCl, and 2.5 mm $CaCl_2$) and exposed to annexin V-FITC (1 μl, 15 min at room temperature). Then, cells were washed with binding buffer, and 3 μl of propidium iodide (PI; stock solution, 10 μg/ml) was added to each sample. Samples were analyzed under a fluorescent microscope (Olympus BX40) equipped with an appropriate filter combination. HeLa cells were visually classified following the examination criteria reported by Pläsier et al. (1999), and the proportions of alive (annexin V negative/PI negative), early apoptotic (annexin V positive/PI negative), late apoptotic (annexin V positive/PI positive), and necrotic cells (annexin V negative/PI positive) were determined. At least 400 cells were scored per experimental point, and the results are expressed as the mean percentages of alive, total apoptotic and necrotic cells among all cells from three independent experiments performed in parallel.

2.6. Mitotic index (MI)

MI was determined by counting the number of mitotic cells (all stages) in 1000 cells analyzed per treatment. Two hours before fixation, colchicine (Merck, Darmstadt, Germany) at 1 mg/ml final concentration was added to the cultures. Cells were then removed from each flask by trypsinization and agitation. The cell suspension was centrifuged and the pellet resuspended in 5 ml hypotonic solution (KCl 0.075 M) for 20–22 min at 37 °C. One milliliter of fixative (methanol:acetic acid 3:1) was added to the suspension before the cells were pelleted and resuspended in fixative. Cells were dropped onto clean slides and stained with 4% Giemsa for 10 min. At least 1000 metaphases per experimental point were scored under light microscopy.

2.7. Cytokinesis-block micronucleus cytome (CBMN cyt) assay

Cytostatic effects, cytotoxicity and chromosomal damage were assessed by the CBMN cyt assay according to the method of Fenech (2007) with slight modifications. Briefly, after 2 d in culture, cells were resuspended in fresh culture medium in the presence of Cytochalasin-B (3 μg/ml final concentration) (Sigma, St. Louis, MO, USA) for 26 h. Cells were harvested on day 3 and removed by trypsinization and agitation. The cell suspension was centrifuged and the pellet was resuspended in 5 ml of fixative 1 (sodium chloride:methanol:acetic acid 6:5:1). The cells were washed two times with fresh fixative 2 (methanol:acetic acid 5:1); they were later resuspended, dropped onto clean slides and finally stained with 5% Giemsa for 10 min. The chromosome damage biomarkers scored were micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBuds). One thousand binucleated cells (BN) were analyzed per experimental point. Cytostatic effects were analyzed through the nuclear division index (NDI), estimated by the ratio of mono-, bi-, and multinucleated cells. Five hundred viable cells were scored per experimental point to determine the frequency of cells with 1, 2, 3 or 4 nuclei and calculate the NDI using the formula $(M_1 + 2\,M_2 + 3\,M_3 + 4\,M_4) / N$, where M_{1-4} represent the number of cells with 1–4 nuclei and N is the total number of viable cells scored (Fenech, 2007). Cytotoxicity events were assessed by the frequency of necrotic and apoptotic cells scoring in 500 cells. Fenech's scoring criteria for MNi, NPBs, NBuds, necrotic and apoptotic cell determinations were used (Fenech, 2007).

2.8. Statistical analysis

Data were statistically analyzed using simple ANOVA and Multiple Ranges test for MI analysis, SCGE and CBMN cyt assays. Student's *t*-test was used for MTT and NR assays. Chi square was used for annexin V-FITC. Statgraphics® 5.1 software (Manugistics Inc., Rockville, MD) was used for all the analyses. Data were expressed as mean ± standard error and $p < 0.05$ was considered statistically significant.

3. Results

As shown in Fig. 1A, CBDCA alone displayed a concentration-dependent reduction in cell viability, measured as the percentage of mitochondrial activity in the MTT assay. Such decrease stabilized over the 161.6 mM CBDCA, where the drug induced a marked reduction, at the same level of 10% ethanol which was used as a positive control for cell survival assays ($p < 0.001$). With these results, we adjusted the best CBDCA concentration to 40.4 mM. At this level, CBDCA reduces HeLa survival and viability by approximately 20%, leaving enough viable cells to proceed with all of the cellular and genomic analyses.

Previous work studying the radioprotective effects of FA supplementation on low doses of ionizing radiation showed that 300 nM FA had radioprotective effects and did not induce genomic damage. Following these results, the levels of FA concentrations were increased up to 900 nM in order to determine the safety of this compound in cell culture. MTT analysis showed that increasing FA concentrations in the range of 300 to 900 nM did not induce a significant reduction in cell viability or genomic damage (Fig. 1B).

Once the cell response to the different compounds was established separately, a set of CBDCA-FA combinations was tested to determine the role of FA on CBDCA activity in HeLa cells. In this sense, increasing concentrations of FA were added to the 40.4 mM CBDCA. MTT analysis demonstrated that FA had a little or directly no effect below 750 nM (Fig. 1C). Using these data as a baseline for the experimental design, the CBDCA-FA combination was finally adjusted to 900 nM FA and 40.4 mM CBDCA, and this concentration was used for all the genomic and cellular analyses. Mannitol, a sugar compound used as an excipient in carboplatinum formulations, was also evaluated in order to set another control. As observed in Fig. 1D, exposure of cell cultures to 150 μg/ml mannitol alone or in combination with FA, did not affect cell viability in the MTT test. Fig. 1D also shows the results obtained with MTT assay when HeLa cells were treated with 40.4 mM CBDCA and 900 nM FA. The effect of CBDCA was enhanced, as demonstrated by the significant drop in cell viability, as compared with that obtained when CBDCA was used alone ($p < 0.001$). Fig. 2 shows the results for cell viability using the NR assay, another test for cytotoxicity evaluation. As seen in this figure, FA did not affect cell viability at all, meanwhile the combination of CBDCA-FA produced a significant drop in lysosomal activity respected to the negative control and CBDCA alone.

The same trend was seen for the MI analysis. Table 1 shows that the number of observed mitosis for the combined treatment was reduced by half, when compared with the treatment with CBDCA alone. Although this trend was clear, the reduction in MI was not significant, due to the small number of mitosis observed in the assay after treatment.

The levels of platinum-induced DNA damage were also evaluated using the alkaline SCGE. Following treatment of cells with 40.4 mM CBDCA, an increment in the level of DNA damage was observed ($p < 0.001$), indicating that the method has the sensitivity and accuracy

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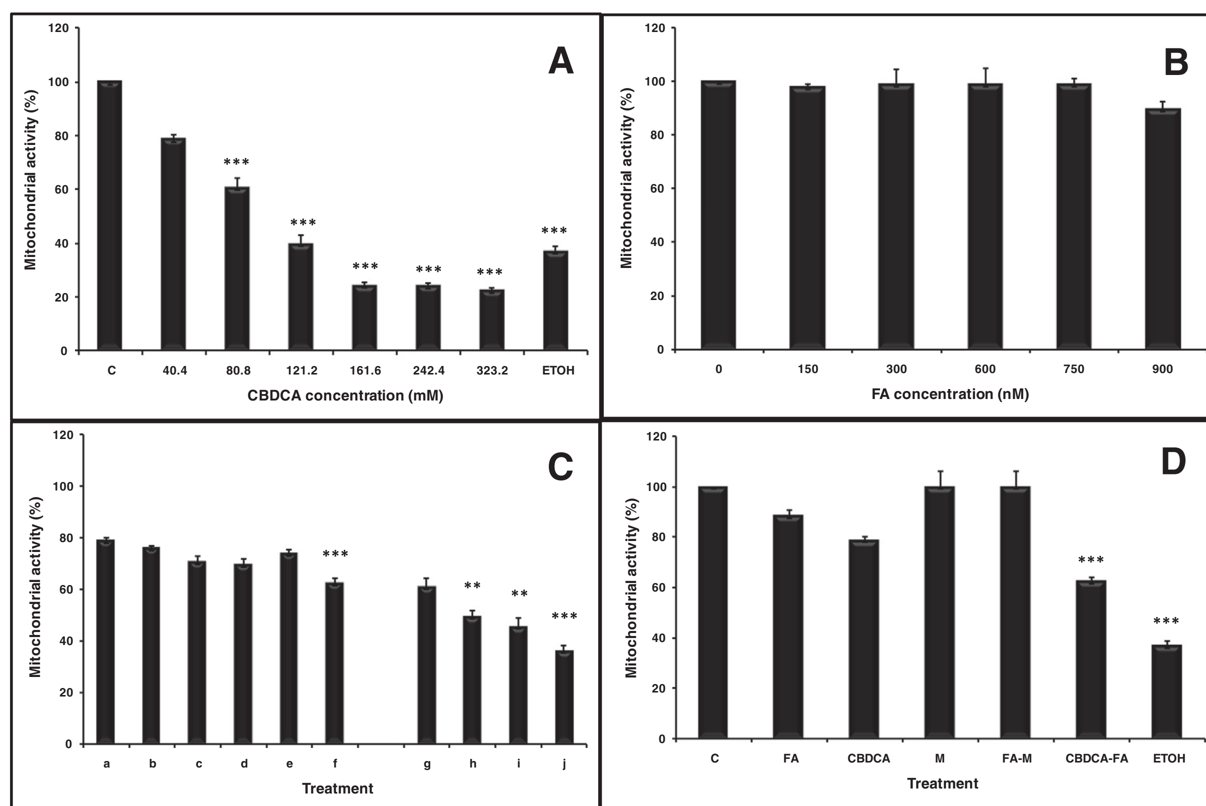

Fig. 1. Cellular metabolism inhibition evaluated by MTT assay. Results are expressed as the mean percentage of cell growth inhibition from three independent experiment. Ethanol (ETOH) 10% was used as positive control. HeLa cells treated with (A) 40.4–323.2 mM carboplatin (CBDCA); (B) 150–900 nM folic acid (FA); (C) different combinations of CBDCA–FA (a = CBDCA40.4, b = CBDCA40.4FA150, c = CBDCA40.4FA300, d = CBDCA40.4FA600, e = CBDCA40.4FA750, f = CBDCA40.4FA900, g = CBDCA80.8, h = CBDCA80.8FA300, i = CBDCA80.8FA600, j = CBDCA80.8FA900); (D) CBDCA40.4 –FA900 and respective controls, mannitol (M). **, $p < 0.01$; ***, $p < 0.001$.

to detect and quantify platinum-induced DNA damage (Table 2). The levels of DNA damage analyzed with SCGE in cells treated with FA alone and with the combined treatment (40.4 mM CBDCA with 900 nM FA) are also depicted in Table 2. Taking into account that grade 3 and 4 damaged cells were negligibly found, statistical data analysis was restricted to grade 1 and grade 2 comets, considering these two grades as one category named “slightly damaged cells”. Multiple range test showed statistically significant differences between the combined CBDCA-FA treatment with respect to CBDCA alone, FA alone and the negative control. As shown in Fig. 2, the combination of 900 nM FA with 40.4 mM CBDCA gave the highest levels of DNA damage. Results of chromosomal damage and cytostatic and cytotoxic effects (CBMN cyt assay) for cells treated with CBDCA alone, FA alone and CBDCA-FA combination are shown in Table 3. A significant increment in the frequency of MNi was found when cells were treated with CBDCA alone and in the combination of 900 nM FA and 40.4 mM CBDCA. Again, the combined treatment gave the highest frequency of chromosomal damage, showing significant differences as compared with the CBDCA treatment. There were no significant differences in the frequencies of NPBs and NBUDs (data not shown) for cells treated with CBDCA and the CBDCA-FA combination. As seen in Table 3, the number of necrotic and apoptotic cells increased with CBDCA treatment, but the highest number of these cells appeared when cultures were treated with the CBDCA-FA combination ($p < 0.001$). Finally, Table 3 also shows a trend in the reduction of NDI for CBDCA alone and the combination of CBDCA-FA, but these differences were no significant.

Annexin V-FITC assay was performed in order to confirm the data obtained for cell death using CBMN. As seen in Table 4, the number of necrotic and apoptotic cells increased significantly when cell cultures were treated with CBDCA and CBDCA-FA. The increment in apoptotic and necrotic cells when using CBDCA-FA respected CBDCA alone were 1.27 and 2.45 respectively. Moreover, the results obtained with the annexin-FITC assay demonstrated again a synergistic effect between CBDCA and FA.

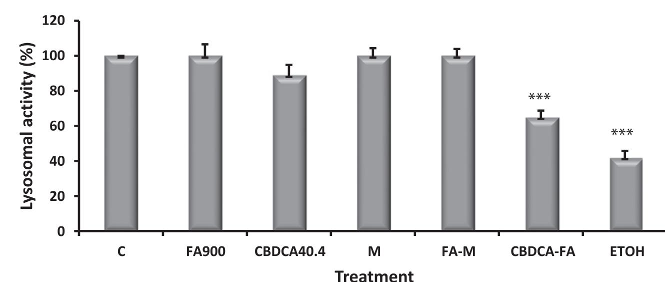

Fig. 2. Lysosomal activity evaluated by NR assay. Results are expressed as the mean percentage of cell growth inhibition from three independent experiment. Ethanol (ETOH) 10% was used as positive control. HeLa cells were treated with 40.4 mM µg/ml carboplatin (CBDCA); 900 nM folic acid (FA); combination CBDCA-FA; mannitol (M) and FA-M. ***, $p < 0.001$.

Table 1
Mitotic index in HeLa cells treated with FA and CBDCA.

Treatment	MI
Negative control	18 ± 1.2
FA (900 nM)	16 ± 0.9
CBDCA (40.4 mM)	6 ± 0.4
CBDCA-FA combination	2 ± 0.2
Bleomycin 1 µg/ml	3 ± 0.2

Values are expressed as standard error of the mean.

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Table 2
DNA damage measured with the comet assay in HeLa cells treated with FA and CBDCA.

Treatment	0	1	2	3	4	ID
Negative control	87 ± 0.2	12 ± 0.0	1 ± 0.0	0	0	14 ± 0.8[a]
FA 900 nM	93 ± 0.2	7 ± 0.0	0	0	0	7 ± 0.3[b]
CBDCA 40.4 mM	16 ± 0.2	16 ± 0.0	7 ± 0.0	6 ± 0.0	2 ± 0.0	56 ± 2.5[c]
CBDCA-FA	47 ± 0.1	37 ± 0.1	11 ± 0.3	4 ± 0.0	1 ± 0.0	75 ± 0.7[d]
Bleomycin 1 μg/ml	31 ± 0.2	25 ± 0.0	19 ± 0.2	14 ± 0.0	1 ± 0.0	129 ± 2.2[e]

0: grade 0 images (%); 1: grade 1 images (%); 2: grade 2 images (%); 3: grade 3 images (%); 4: grade 4 images (%).
[a, b, c, d, e]Homogeneous groups.
Values are expressed as standard error of the mean.

4. Discussion

Carboplatin is mainly used for the treatment of ovarian cancer but also for other types of malignancies, including lung, head and neck, esophageal, bladder, breast, endometrial and cervical, central nervous system or germ cell tumors. Moreover, CBDCA is also used as preparation for stem cell or bone marrow transplantation. Kidney injury and the myelosuppressive characteristics are common pathological effects of CBDCA usage in a variety of solid tumor therapies. Considering that these side effects are dose dependent, strategies to minimize CBDCA toxicity through mechanisms that lower the doses of this compound are of great clinical interest.

The aim of the present study was to investigate the role of FA as a modulator of CBDCA activity in cultured HeLa cells. In order to examine the drug sensitivity of HeLa cells to carboplatinum chemotherapy, alone or in combination with folic acid, changes in cellular and genomic responses regarding cell survival, DNA and chromosomal damage, mitotic activity and cytostatic and cytotoxic effect were analyzed.

The obtained results demonstrated that a concentration of 40.4 mM CBDCA in HeLa cell cultures inhibited cell growth and survival, induced DNA double-strand breaks, and promoted chromosomal damage and apoptosis. But more interestingly, the results also showed that addition of 900 nM FA together with 40.4 mM CBDCA enhanced the activity of the platinum compound, increasing its effect on cell death by nearly 20%, as evidenced by the colorimetric assays. Moreover, not only higher levels of DNA and chromosomal damage were reached but also the number of necrotic and apoptotic cells was significantly increased when cell cultures were treated with the combined CBDCA-FA protocol, according to CBMN and annexin V-FITC results. The results observed after CBDCA-FA treatment, and according to the definition of Warne (2003), showed the synergistic effect of CBDCA-FA combination respected to both compounds alone. This situation opens the possibility to explore the use of FA in platinum-based chemotherapy protocols to reduce the platinum doses for patient treatment and decrease the chance of developing the known side effects without losing biological activity.

One-carbon metabolism is composed of a biochemical network that provides methyl groups for a variety of essential biological processes. FA is a water-soluble type of the B vitamin family, and its deficiency has been extensively studied, mainly in relation to blood cells formation and fetus development. Folate and other B vitamins are essential nutrients which play important roles in DNA synthesis, repair, methylation and regulation of gene expression. These functions also indicate the potential association of this group of vitamins with cancer development (Ma et al., 2009), suggesting an inverse association between folate intake and the risk for several types of cancer (Cancarini et al., 2015; Lin et al., 2015; Liu et al., 2015). On the other hand, FA has displayed radioprotective effects against low and high radiation doses (Pote et al., 2006; Batra and Devasagayam, 2009; Jelveh et al., 2013; Padula et al., 2016) and protective effects against oxidative damage (Acharyya et al., 2015; Rathor et al., 2015; Vanzin et al., 2015).

More recently, FA has been used as a targeted delivery system in tumors overexpressing the folate receptor, in a wide variety of cancer types (Liang et al., 2011; Liu et al., 2011; Sadat et al., 2014). This scheme of cancer treatment could be a potential delivery system with excellent therapeutic efficacy for targeting the drugs directly to cancer cells. Increasing experience in the application of this technology has opened a promising future in cancer treatment, with high efficacy and few side effects.

In this sense, this is the first report using FA not as a transporter but certainly as a modulator of CBDCA activity in vitro, being not quite different from the targeted delivery systems. The enhanced activity of the combined FA-CBDCA treatment to induce apoptosis, genomic and chromosomal damage in HeLa cells could be explained through the spontaneous formation of FA-CBDCA complexes when they are applied together, and entering to the cells via the overexpressed folate receptors. Further studies will be necessary to confirm the hypothesis and to elucidate the mechanisms involved in the cellular response to CBDCA-mediated FA modulation.

Table 3
Cytostatic effects, cytotoxicity and chromosomal damage assessed by the CBMN cyt assay in HeLa cells treated with FA and CBDCA.

Treatment	NDI	Necrotic	Apoptotic	MNi ‰	NPB ‰
Negative control	1.45	3 ± 0.4	2 ± 0.7	31 ± 2.6[a]	4 ± 0.9
FA 900 nM	1.50	1 ± 0.4	0	24 ± 2.1[a]	0
CBDCA 40 mM	1.33	11 ± 1.2	6 ± 0.9	43 ± 1.9[b]	5 ± 0.9
CBDCA-FA	1.22	20 ± 2.3	16 ± 1.5	71 ± 2.7[c]	2 ± 0.4
Bleomycin 1 μg/ml	1.17	NA	NA	70 ± 1.5[c]	5 ± 0.9

Values are expressed as standard error of the mean.
NDI: nuclear division index; Necrotic: necrotic cells in 500 counted; Apoptotic: apoptotic cells in 500 counted; MNi: micronuclei in 1000 cells counted; NPB: nucleoplasmic bridges in 1000 cells counted.
[a, b, c]Homogeneous groups.
NA not analyzed data.

Table 4
Analysis of apoptosis as measured by annexin V-FITC in HeLa cells exposed 24 h to carboplatin (CBDCA) alone, and in combination with folic acid (FA).

Compound	Percentage of cells (%)[a]		
	Alive	Total apoptotic[f]	Necrotic
Control	93.8 ± 0.8	5.8 ± 0.9	0.3 ± 0.2
M[b]	93.7 ± 0.4	6.3 ± 0.4	0.0 ± 0.0
FA[c]	88.5 ± 1.2	11.0 ± 1.2	0.0 ± 0.0
FA-M	91.4 ± 0.4*	8.6 ± 0.4	0.0 ± 0.0
CBDCA[d]	42.5 ± 1.4***	56.0 ± 1.7***	1.5 ± 0.7**
CBDCA-FA	25.0 ± 0.3***	71.3 ± 1.0***	3.7 ± 1.2***
ETOH[e]	49.2 ± 2.4***	38.7 ± 2.9***	12.2 ± 1.2***

[a] Results are presented as mean values of pooled data from three independent experiments ± SE of the mean.
[b] Mannitol (M) was used as excipient control for CBDCA.
[c] FA: 900 nM.
[d] CBDCA: 40.4 mM.
[e] Ethanol (ETOH, 5%) was used as positive control.
[f] Total apoptotic = (Early apoptotic + Late apoptotic).
* $p < 0.05$; values in regards to control values.
** $p < 0.01$; values in regards to control values.
*** $p < 0.001$; values in regards to control values.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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References

Acharyya, N., Deb, B., Chattopadhyay, S., Maiti, S., 2015. Arsenic-induced antioxidant depletion, oxidative DNA breakage, and tissue damages are prevented by the combined action of folate and vitamin B12. Biol. Trace Elem. Res. 168, 122–132.

Bailey, L.B., Gregory, J.F., 1999. Folate metabolism and requirements. J. Nutr. 129, 779–782.

Basu, A., Cline, J.S., 1995. Oncogenic transformation alters cisplatin-induced apoptosis in rat embryo fibroblasts. Int. J. Cancer 63, 597–603.

Basu, A., Krishnamurthy, S., 2010. Cellular responses to cisplatin-induced DNA damage. Journal of Nucleic Acids 2010, 1–16.

Batra, V., Devasagayam, T.P.A., 2009. Interaction between cytotoxic effects of γ-radiation and folate deficiency in relation to choline reserves. Toxicology 255, 91–99.

Beetstra, S., Thomas, P., Salisbury, C., Turner, J., Fenech, M., 2005. Folic acid deficiency increases chromosomal instability, chromosome 21 aneuploidy and sensitivity to radiation-induced micronuclei. Mutat. Res. 578, 317–326.

Blommaert, F.A., van Dijk-Knijnenburg, H.C., Dijt, F.J., den Engelse, L., Baan, R.A., Berends, F., Fichtinger-Schepman, A.M., 1995. Formation of DNA adducts by the anticancer drug carboplatin: different nucleotide sequence preferences in vitro and in cells. Biochemistry 34, 8474–8480.

Borenfreund, E., Puerner, J.A., 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 24, 119–124.

Cancarini, I., Krogh, V., Agnoli, C., Grioni, S., Matullo, G., Pala, V., Pedraglio, S., Contiero, P., Riva, C., Muti, P., Sieri, S., 2015. Micronutrients involved in one-carbon metabolism and risk of breast cancer subtypes. PLoS One 10. http://dx.doi.org/10.1371/journal.pone.0138318.

Choi, S.W., Mason, J.B., 2000. Folate and carcinogenesis: an integrated scheme. J. Nutr. 130, 129–132.

Collins, A.R., 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol. Biotechnol. 26, 249–261.

Courtemanche, C., 2003. Folate deficiency and ionizing radiation cause DNA breaks in primary human lymphocytes: a comparison. FASEB J. 18 (1), 209–211.

Fenech, M., 2007. Cytokinesis-block micronucleus cytome assay. Nat. Protoc. 2, 1084–1104.

Gately, D.P., Howell, S.B., 1993. Cellular accumulation of the anticancer agent cisplatin: a review. Br. J. Cancer 67, 1171–1176.

Hansen, M.F., Greibe, E., Skovbjerg, S., Rohde, S., Kristensen, A.C.M., Jensen, T.R., Stentoft, C., Kjær, K.H., Kronborg, C.S., Martensen, P.M., 2015. Folic acid mediates activation of the pro-oncogene STAT3 via the Folate Receptor alpha. Cell. Signal. 27, 1356–1368.

Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline, 1998t. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. The National Academies Collection: Reports Funded by National Institutes of Health. National Academies Press (US), Washington (DC).

Jamieson, E.R., Lippard, S.J., 1999. Structure, recognition, and processing of Cisplatin-DNA adducts. Chem. Rev. 99, 2467–2498.

Jelveh, S., Kaspler, P., Bhogal, N., Mahmood, J., Lindsay, P.E., Okunieff, P., Doctrow, S.R., Bristow, R.G., Hill, R.P., 2013. Investigations of antioxidant-mediated protection and mitigation of radiation-induced DNA damage and lipid peroxidation in murine skin. Int. J. Radiat. Biol. 89, 618–627.

Knox, R.J., Friedlos, F., Lydall, D.A., Roberts, J.J., 1986. Mechanism of cytotoxicity of anticancer platinum drugs: evidence that cis-diamminedichloroplatinum (II) and cis-diammine-(1, 1-cyclobutanedicarboxylato) platinum (II) differ only in the kinetics of their interaction with DNA. Cancer Res. 46, 1972–1979.

Kosmider, B., Zyner, E., Osiecka, R., Ochocki, J., 2004. Induction of apoptosis and necrosis in A549 cells by the cis-Pt(II) complex of 3-aminoflavone in comparison with cis-DDP. Mutat. Res. 563, 61–70.

Liang, C., Yang, Y., Ling, Y., Huang, Y., Li, T., Li, X., 2011. Improved therapeutic effect of folate-decorated PLGA-PEG nanoparticles for endometrial carcinoma. Bioorg. Med. Chem. 19, 4057–4066.

Lin, P.-H., Aronson, W., Freedland, S.J., 2015. Nutrition, dietary interventions and prostate cancer: the latest evidence. BMC Medicine 13, 3.

Liu, Y., Zhang, B., Yan, B., 2011. Enabling anticancer therapeutics by nanoparticle carriers: the delivery of paclitaxel. Int. J. Mol. Sci. 12, 4395–4413.

Liu, Y., Yu, Q., Zhu, Z., Zhang, J., Chen, M., Tang, P., Li, K., 2015. Vitamin and multiple-vitamin supplement intake and incidence of colorectal cancer: a meta-analysis of cohort studies. Med. Oncol. 32, 434.

Ma, E., Iwasaki, M., Junko, I., Hamada, G.S., Nishimoto, I.N., Carvalho, S.M.T., Motola, J., Laginha, F.M., Tsugane, S., 2009. Dietary intake of folate, vitamin B6, and vitamin B12, genetic polymorphism of related enzymes, and risk of breast cancer: a case–control study in Brazilian women. BMC Cancer 9, 122.

Moore, K.N., Herzog, T.J., Lewin, S., Giuntoli, R.L., Armstrong, D.K., Rocconi, R.P., Spannuth, W.A., Gold, M.A., 2007. A comparison of cisplatin/paclitaxel and carboplatin/paclitaxel in stage IVB, recurrent or persistent cervical cancer. Gynecol. Oncol. 105, 299–303.

Olive, P.L., 1999. DNA damage and repair in individual cells: applications of the comet assay in radiobiology. Int. J. Radiat. Biol. 75, 395–405.

Padula, G., Ponzinibbio, M.V., Seoane, A., 2016. Possible radio-protective effect of folic acid supplementation on low dose ionizing radiation-induced genomic instability in vitro. Indian J. Exp. Biol. 54, 537–543.

Pascoe, J.M., Roberts, J.J., 1974. Interactions between mammalian cell DNA and inorganic platinum compounds. I. DNA interstrand cross-linking and cytotoxic properties of platinum(II) compounds. Biochem. Pharmacol. 23, 1359–1365.

Pläsier, B., Lloyd, D.R., Paul, G.C., Thomas, C.R., Al-Rubeai, M., 1999. Automatic image analysis for quantification of apoptosis in animal cell culture by annexin-V affinity assay. J. Immunol. Methods 229, 81–95.

Pote, M.S., Gandhi, N.M., Mishra, K.P., 2006. Antiatherogenic and radioprotective role of folic acid in whole body gamma-irradiated mice. Mol. Cell. Biochem. 292, 19–25.

Rathor, L., Akhoon, B.A., Pandey, S., Srivastava, S., Pandey, R., 2015. Folic acid supplementation at lower doses increases oxidative stress resistance and longevity in *Caenorhabditis elegans*. Age (Dordr.) 37, 113.

Rosenberg, B., Vancamp, L., 1969. Platinum compounds: a new class of potent antitumour agents. Nature 222, 385–386.

Sadat, T.M.F., Nejati-Koshki, K., Akbarzadeh, A., Yamchi, M.R., Milani, M., Zarghami, N., Zeighamian, V., Rahimzadeh, A., Alimohammadi, S., Hanifehpour, Y., Joo, S.W., 2014. PLGA-based nanoparticles as cancer drug delivery systems. Asian Pac. J. Cancer Prev. 15, 517–535.

Shen, D.-W., Pouliot, L.M., Hall, M.D., Gottesman, M.M., 2012. Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes. Pharmacol. Rev. 64, 706–721.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

Sun, X.-L., Murphy, B.R., Li, Q.-J., Gullapalli, S., Mackins, J., Jayaram, H.N., Srivastava, A., Antony, A.C., 1995. Transduction of folate receptor cDNA into cervical carcinoma cells using recombinant adeno-associated virions delays cell proliferation in vitro and in vivo. J. Clin. Invest. 96, 1535.

Tice, R.R., Strauss, G.H., 1995. The single cell gel electrophoresis/comet assay: a potential tool for detecting radiation-induced DNA damage in humans. Stem Cells 13 (Suppl 1), 207–214.

Toffoli, G., Russo, A., Gallo, A., Cernigoi, C., Miotti, S., Sorio, R., Tumolo, S., Boiocchi, M., 1998. Expression of folate binding protein as a prognostic factor for response to platinum-containing chemotherapy and survival in human ovarian cancer. Int. J. Cancer 79, 121–126.

Vanzin, C.S., Mescka, C.P., Donida, B., Hammerschimidt, T.G., Ribas, G.S., Kolling, J., Scherer, E.B., Vilarinho, L., Nogueira, C., Coitinho, A.S., Wajner, M., Wyse, A.T.S., Vargas, C.R., 2015. Lipid, oxidative and inflammatory profile and alterations in the enzymes paraoxonase and butyrylcholinesterase in plasma of patients with homocystinuria due CBS deficiency: the vitamin B12 and folic acid importance. Cell. Mol. Neurobiol. 35, 899–911.

Wagstaff, A.J., Ward, A., Benfield, P., Heel, R.C., 1989. Carboplatin. A preliminary review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in the treatment of cancer. Drugs 37, 162–190.

Warne, M.S.J., 2003. A review of the ecotoxicity of mixtures, approaches to, and recommendations for, their management. In: Langley, A., Gilbey, M., Kennedy, B. (Eds.), Proceedings of the Fifth National Workshop on the Assessment of Site Contamination. NEPC Service Corporation, Level 5, 81 Flinders Street, Adelaide SA 5000, pp. 253–276.

Zhang, Q., Xiang, G., Zhang, Y., Yang, K., Fan, W., Lin, J., Zeng, F., Wu, J., 2006. Increase of doxorubicin sensitivity for folate receptor positive cells when given as the prodrug N-(phenylacetyl) doxorubicin in combination with folate-conjugated PGA. J. Pharm. Sci. 95, 2266–2275.

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