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ORIGINAL ARTICLE

Effects of folic acid on the antiproliferative efficiency of doxorubicin, camptothecin and methyl methanesulfonate in MCF-7 cells by mRNA endpoints



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Abstract There is a lack of consensus on whether the role of folate in cancer cells is protective or harmful. The use of folates in combination with cancer-targeting therapeutic regimens requires detailed information to ensure their safe and proper use. Therefore, we evaluated the effects of folic acid (FA) in combination with the chemotherapeutic compounds doxorubicin (DXR), camptothecin (CPT) and methyl methanesulfonate (MMS) on the growth of MCF-7 cells. The data generated from the RTCA assays demonstrated that FA did not affect proliferation in MCF-7 cells treated with DXR and CPT; however, FA reduced the efficacy of MMS treatment. RTCA data also confirmed that DXR and CPT exert their cytotoxic effects in a time-dependent manner and that CPT induced a significantly greater decrease in MCF-7 cell proliferation compared with DXR. The MTT assay failed to detect a reduction in cell proliferation in cells treated with MMS. We quantified the mRNA expression levels of genes associated with cellular stress response, cell cycle and apoptosis pathways using RT-qPCR. The addition of FA to DXR or CPT promoted a similar shift in the gene expression profile of MCF-7 cells compared with cells treated with DXR or CPT without FA; however, this shift did not alter the bioactivity of these drugs. Rather, it indicated that these drugs promoted cell death by alternative mechanisms. In contrast, the addition of FA to MMS reduced the efficacy of the drug without changing the gene expression profile. None of the genes encoding folate receptors that were analyzed were differentially expressed in cells treated with

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or without FA. In conclusion, supplementation with 450 μM FA was not cytotoxic to MCF-7 cells. However, the addition of FA to anti-cancer drugs must be performed cautiously as the properties of FA might lead to a reduction in drug efficacy.

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1. Introduction

Folate (folic acid) is one of the key components of one-carbon metabolism. It plays an important role as a source of fuel to drive the cellular functions required for growth, proliferation, and survival, such as DNA replication and cell division. Folate is also critical to the synthesis, repair and methylation of DNA. The disruption of these activities can promote DNA instability, which potentially increases the risk of carcinogenic processes and promotes the genetic diversity underlying the development of other hallmarks of cancer (Kim, 1999; Negrini et al., 2010; Duthie, 2011; Hanahan and Weinberg, 2011; Crider et al., 2012; Gonen and Assaraf, 2012).

Despite these observations, the results of systematic reviews and meta-analyses evaluating the precise role of folate deficiency or supplementation in cancer development and progression and the potential role of folate in cancer prevention remain controversial. There has been a debate as to whether the use of folates is beneficial in primary cancer prevention, and there are speculations that folate exerts potentially harmful effects in the early stages of cancer. There is a fine balance between preventing and promoting carcinogenesis in different contexts depending on folate levels. For example, there are potential tissue- and cell-specific differences in the effects of folic acid on cellular functions (Ulrich and Potter, 2007; Maruti et al., 2009; Wien et al., 2012; Vollset et al., 2013).

Epidemiologic studies suggest that there is an inverse correlation between the risk of developing breast cancer and folate levels. Reviews of many published case-control studies investigating the potential association between dietary folate intake and breast cancer risk have strengthened the validity of these findings. Alternatively, the increased risk may be attributable to a combination of chance, measurement error or other confounding factors. The reviews have reported both a direct and inverse relationship that was not statistically significant, became non-significant after adjustment, or could not be distinguished from other risk factors (Kim, 2006; Lewis et al., 2006; Vollset et al., 2013).

It has been known for decades that the covalent attachment of folic acid to macromolecules generates a conjugate that can be internalized by folate receptor-bearing cells by a mechanism similar to that employed by free folic acid. This knowledge has been exploited for therapeutic drug delivery purposes. In addition to several other interesting features, the observation that the folate receptor is a highly selective tumor marker overexpressed on the cell surface of various tumor types suggests that folic acid might be part of a promising treatment approach for targeted personalized cancer therapy (Leamon and Reddy, 2004; Lu and Low, 2012; Assaraf et al., 2014).

As there is no consensus on the role of folate in cancer cells and considering that folate is used in some targeted approaches to treating cancer, information regarding the interaction of folate and anti-cancer therapies is imperative to

ensure the safe and proper use of this approach. Many questions regarding the effect of folate in cancer remain unanswered. Does folate have a protective action against cell damage or does it further promote it? If folate has a protective or synergistic effect, can it interfere with chemotherapy efficacy? To address these issues, our study aimed to evaluate the effects of folic acid when used in combination with the chemotherapeutic agents doxorubicin, camptothecin and methyl methanesulfonate in a human breast cancer cell line (MCF-7). We investigated the potential mechanisms by which folate interacts with these drugs with respect to cytotoxicity, cell proliferation and gene expression.

2. Materials and methods

2.1. Chemicals

Folic acid (FA) was purchased from Acros Organics® (96–100%; CAS n° 66-27-3) and used at a final concentration of 450 μM . This concentration represents a high dose of folic acid and corresponds to a dose 50-fold greater than the folic acid concentration in the culture medium.

To evaluate the effects of FA, the cells were treated with doxorubicin (DXR) (CAS n°23214-92-8, Adriblastina, Pharmacia, Milan, Italy), camptothecin (CPT) (CAS n°7689-03-4) and methyl methanesulfonate; (MMS) (CAS n°66-27-3) at final concentrations of 2 μM , 3 μM and 450 μM , respectively. These concentrations were based on previous tests performed in our laboratory.

2.2. Cell line and culture conditions

MCF-7 cells were acquired from the Rio de Janeiro Cell Bank and cultured in 25 cm² bottles containing 10 mL of Dulbecco's Modified Eagle's medium (DMEM – Gibco®, Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS – Gibco®) and maintained in an atmosphere of 5% CO₂ at 37 °C.

2.3. Cytotoxicity analysis – MTT assay

The MTT assay was performed as previously described (Mosmann, 1983) with some modifications. The cell number was determined using the Countess® Automated Cell Counter (Life Technologies, Grand Island, NY) and 6×10^3 cells/well in a volume of 200 μL were seeded into a 96-well plate. The plates were maintained at 37 °C in a humidified atmosphere with 5% CO₂ for stabilization. After removing the media, positive control cells were treated with each chemical (2 μM DXR, 3 μM CPT and 450 μM MMS) diluted in 200 μL of culture medium supplemented with 10% FBS. Test cells were treated with each chemical, as described above, in combination with

450 μM of FA. A negative control (DMEM plus 10% FBS) and a folate control (cells treated with 450 μM of FA) were also used. After each treatment period (24 h and 48 h), the culture medium was completely removed and 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (CAS 298931; Sigma Aldrich, St. Louis, MO) (MTT – 0.5 mg/mL) was added. After 4 h, the MTT solution was removed and the formazan crystals generated were solubilized using 100 μL of DMSO. Three independent biological replicates were used in the 8 replicate experiments of each condition. The absorbance of the cells was measured using a spectrophotometer at 570 nm. The mean absorbance of each reaction was converted to cell viability (%) using the following equation: (mean absorbance treatment/mean absorbance control) \times 100.

2.4. Real Time Cell Analysis (RTCA)

Roche's xCELLigence RTCA SP System (Mannheim, Germany) was used to investigate the effect of FA on MCF-7 cell proliferation using the same experimental design as described for the MTT assay. The assay was performed according to the manufacturer's protocol. Briefly, a resistance test of the equipment specific plate (a 96-well plate containing a gold base with microelectrodes that assess the impedance of each well in the presence of an electric field) was performed by adding 50 μL of DMEM supplemented with 10% FBS. After this test, 6×10^3 cells/well were seeded into the wells at a final volume of 100 μL /well. Initial attachment and growth were continuously monitored for approximately 24 h at 37 $^{\circ}\text{C}$ and 5% CO_2 for stabilization. Then, each chemical agent and FA were added to the wells (final volume of 200 μL /well) and the effects were continuously recorded every 30 min for a period of 72 h. The plate remained in the RTCA Station for 96 h, and cell proliferation was monitored in real time and plotted on a graph using the RTCA software. Three biological replicates were evaluated in each experiment.

Changes in electrical impedance, which occur as cells attach or detach from the surface electrodes, were measured, evaluated by complex mathematical algorithms and plotted as cell index (CI) values. The impedance readings can vary based on the quality of the cell interactions and adherence properties between cells and the electrodes. There is a direct correlation between the number of cells attached to the electrode and the number of cells attached to the electrodes with the CI readout on the machine (Urcan et al., 2010; Pan et al., 2013; Moniri et al., 2015). A normalized CI (NCI) value was calculated and used to generate baseline values. The xCELLigence software permits normalization to any time point, and results can be directly viewed in the software window. We conducted the normalization at one time point before the treatment.

2.5. Gene expression analysis using RT-qPCR

Eight flasks were seeded with 4×10^4 cells/flasks in 25 cm^2 flasks at a final volume of 5 mL for 24 h to allow for cell stabilization. Then, each bottle received 5 mL of the indicated treatments: flask 1 – control (complete culture medium); flask 2–450 μM FA; flask 3–2 μM doxorubicin; flask 4–2 μM doxorubicin + 450 μM FA; flask 5–3 μM camptothecin; flask 6–3 μM camptothecin + 450 μM FA; flask 7–450 μM MMS;

and flask 8–450 μM methyl methanesulfonate + 450 μM FA. After a 24 h incubation, the culture medium was removed and the total RNA was extracted using TRIzol (Invitrogen® Life Technology, Grand Island, NY) and the RNeasy® Mini Kit (Qiagen, Valencia, CA–Cat. n°74106) according to the supplier's instructions. The experiments were performed in triplicate. The purity and concentration of the isolated RNA were determined using a spectrophotometer, and RNA integrity was evaluated using denaturing agarose gel electrophoresis as previously described (Aranda et al., 2012).

cDNA synthesis reactions were performed in triplicate for each sample in a T100 Thermal Cycler (BIO-RAD, Singapore) using 500 ng of total RNA diluted in a final volume of 16 μL , 8 μL of DEPC-treated water, 4 μL of oligo dT (20 pmol/ μL) and 4 μL of dNTPs (2.5 mM). The first reaction mix was incubated for 10 min at 65 $^{\circ}\text{C}$. Each reaction was subjected to thermal shock in ice. Then, 4 μL of a second reaction mix was added [1.3 μL of DEPC-treated H_2O , 2 μL of 10 \times buffer, 0.6 μL of MgCl_2 (50 mM), 0.05 μL of RNase Out (Invitrogen) and 0.05 μL of M-MLV enzyme (Invitrogen)]. The final solution was incubated at 37 $^{\circ}\text{C}$ for 50 min to allow cDNA synthesis to proceed, and the enzymatic reaction was terminated at 70 $^{\circ}\text{C}$ for 15 min.

The qPCR reactions were also performed in triplicate in a CFX96™ Real-Time System (BIO-RAD, Singapore) using 5 μL of Platinum SYBR Green qPCR Supermix-UDG (Invitrogen), 1 μL of each oligonucleotide primer (10 pmol/ μL) and 5 μL of cDNA (50 ng/ μL) (1:10 dilution of input RNA). The reaction conditions were as follows: pre-incubation at 50 $^{\circ}\text{C}$ for 2 min (UDG incubation), initial denaturation at 95 $^{\circ}\text{C}$ for 5 min; and 40 cycles of 95 $^{\circ}\text{C}$ for 20 s, 60 $^{\circ}\text{C}$ for 30 s and 72 $^{\circ}\text{C}$ for 20 s. A melting curve analysis ranging from 55 $^{\circ}\text{C}$ to 95 $^{\circ}\text{C}$ was generated at the end of the reaction, with 5 s readings at every 0.5 $^{\circ}\text{C}$. The software CFX Manager 3.1 (BIO-RAD) was used to collect the data and the efficiency of the reactions was calculated using LinRegPCR software (Amsterdam, the Netherlands), as previously described (Ruijter et al., 2009).

Pre-designed KiCqStart SYBR® Green primers (Sigma–Aldrich) were used for the qPCR reactions and *GAPDH* was used as the reference gene. The following target genes were evaluated: *FOLR1*, *SLC19A1* and *SLC46A1* (encoding the folate receptors $\text{FR}\alpha$, RFC and PCFT, respectively); *GADD45A*, *DDIT3*, *BIRC5*, *CCNA2* and *TP53* (genes associated with the DNA damage response and cell cycle regulation); and *BAK*, *BAX*, *BCL-2*, *BCL-XL*, *CASP7*, *CASP8* and *CASP9* (genes associated with apoptosis).

2.6. Statistical analysis

The absorbance values obtained from the MTT assay and RTCA cell index were compared using ANOVA and Tukey's test ($p < 0.05$) using GraphPad Prism® 5 software (San Diego, CA). Statistical evaluation of reference gene and test gene expression levels were performed using the stand-alone software REST® 2009 (Relative Expression Software Tool, M. Pfaffl, Munich, Germany and QIAGEN, Hilden, Germany) as previously described (Pfaffl et al., 2002) with efficiency correction. A statistically significant difference was defined as a 2-fold change in expression and a p -value < 0.05 compared with the corresponding control.

3. Results

The absorbance values obtained from the MTT assay after 24 and 48 h of treatment were converted to percentages of cell viability (Fig. 1). No significant effect on viability was observed in cells treated with only folic acid (FA) compared with the control cells at both time points evaluated. The viability of MCF-7 cells after 24 h of treatment with DXR, CPT and MMS was 67.12%, 39.31% and 38.93%, respectively. After 48 h, the viability continued to decline significantly in cells treated with DXR (37.66%) and CPT (15.48%) but remained unchanged in cells treated with MMS (35.80%).

At both time points analyzed, CPT induced a significantly greater decrease in MCF-7 cell viability compared with DXR. At 24 h, the reduction in cell viability induced by MMS treatment was similar to the reduction observed with CPT, and the reduction in cell viability induced by DXR was significantly compared with MMS and CPT. However, the effects of MMS in MCF-7 cells stabilized after 48 h, and the reduction in cell viability was surpassed by CPT and was equivalent to DXR, which continued to decrease cell viability after 24 h. DXR and CPT, but not MMS, exerted their cytotoxic effects in a time-dependent manner. The cytotoxic profiles of all three drugs were unaffected by the addition of FA. The viability of cells treated with FA in combination with DXR, CPT and MMS was 69.46%, 44.45% and 40.17%, respectively, at 24 h, and 37.95%, 17.67% and 33.27%, respectively, at 48 h.

To ensure that the cytotoxicity observed in the MTT assay was associated with the reduction in the proliferation of MCF-7 cells, promoting a cytostatic effect and/or death, we conducted a Real Time Cell analysis (RTCA). The RTCA monitoring of MCF-7 cells proliferation was performed for 72 h after the addition of treatment. The results are plotted in Fig. 2. There was no significant effect on the proliferation of cells treated with FA only compared with the control. The RTCA data from cells treated with DXR and CPT were consistent with the results of the MTT assay, which demonstrated no significant differences in the anti-proliferative effect of DXR, CPT and MMS when used alone or in combination with FA. Similar to the MTT assay, the RTCA demonstrated that DXR and CPT decrease cell viability in a time-dependent

manner and that CPT induces a significantly greater decrease in MCF-7 cell proliferation compared with DXR. The cellular index (CI) of cells treated with CPT obtained at 24 h (0.0782) was approximately 50% of the CI value obtained for cells treated with DXR (0.1573).

The RTCA data revealed that CPT and DXR exhibited similar proliferation profiles in the first 10 h following treatment. After 10 h, CPT induced a greater CI reduction compared with DXR. After 15 h of treatment, we detected a 2:1 ratio between the CI of DXR- and CPT-treated cells. This correlation was observed at all other points analyzed until the curves began to decline, demonstrating the significantly faster action of CPT compared with DXR in MCF-7 cells. The MTT assay cannot provide this type of information. The RTCA also revealed that both drugs killed nearly the entire cell population after approximately 24 h of treatment. In contrast, the MTT assay indicated a continuous reduction in cell viability until 48 h.

Additionally, in contrast to the MTT assay outcomes, RTCA data revealed that MMS treatment did not provide a greater reduction in MCF-7 cell viability compared with DXR after 24 h. The differences between the effects of the drugs were more evident at 48 h. The MTT assay revealed that cell viability was similar in cells treated with DXR and MMS. However, the RTCA data revealed that cell viability was significantly lower in cells treated with DXR compared with CPT at 48 h. In fact, at both the 24 and 48 h time points, cell viability was significantly lower in cells treated with DXR and CPT compared with MMS. The proliferation curves associated with DXR and CPT exhibited a steeper decline compared with MMS, which exhibited a partial reduction followed by a recovery of proliferation.

In contrast to DXR and CPT, the anti-proliferative effect of MMS was influenced by FA. FA significantly diminished the anti-proliferative effect of MMS as early as 10 h after treatment. At this time point, the CI values of the control, MMS and MMS + FA groups were 1.5415, 1.0370 and 1.1158, respectively. The reduction in MMS activity induced by FA significantly increased over the incubation time. After 24 h, the CI values of the control, MMS and MMS + FA groups were 2.7632, 0.6489 and 0.8202, respectively.

In addition, proliferation significantly recovered after approximately 30 h in MCF-7 cells treated with MMS and

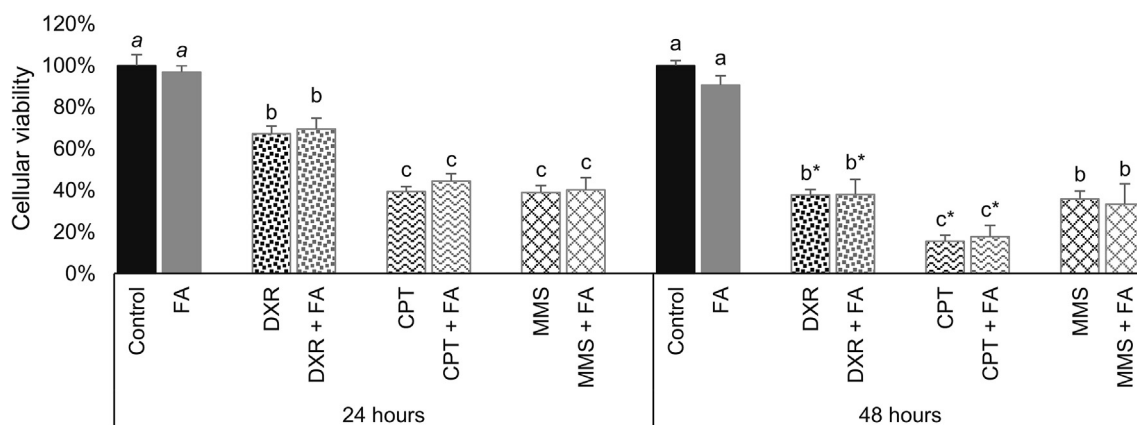


Figure 1 MCF-7 cells treated with doxorubicin (DXR), camptothecin (CPT) or methyl methanesulfonate (MMS) for 24 h and 48 h with or without the addition of folic acid (FA) were evaluated using the MTT assay. The values in the column represent the mean \pm S.D. Letters represent statistically significant differences between treatments (24 or 48 h). (*) represents the statistically significant difference between the 24 h and 48 h treatments.

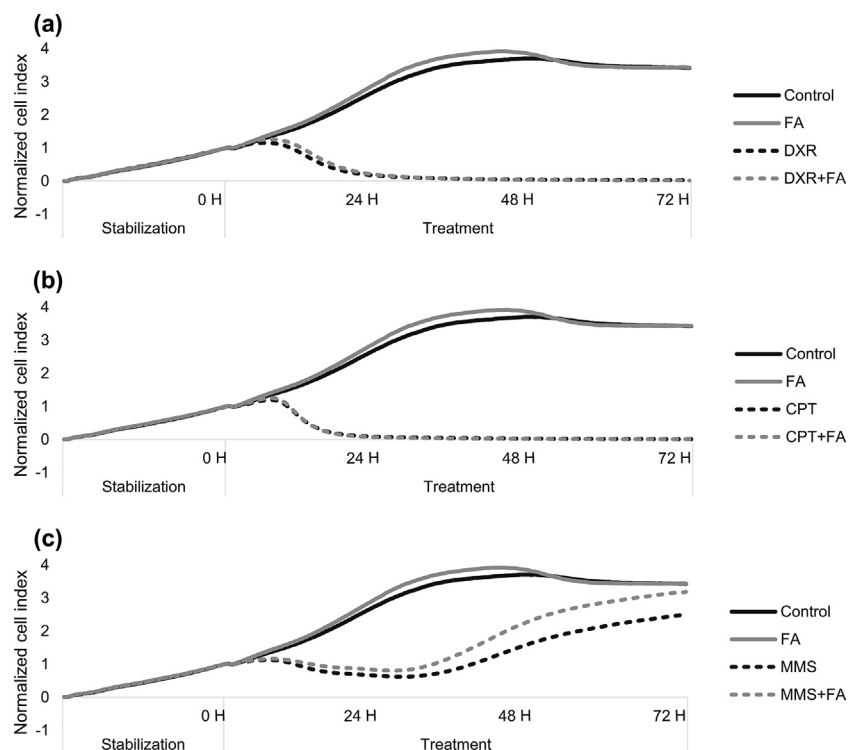


Figure 2 Real Time Cell Analysis (RTCA) of MCF-7 cells treated with (a) doxorubicin (DXR), (b) camptothecin (CPT) or (c) methylmethanesulfonate (MMS) with or without the addition of folic acid (FA). Cells were analyzed 72 h after the addition of treatment.

MMS + FA, and the significant difference in the viability of these 2 groups was maintained. At 48 h, the CI values of MMS- and MMS + FA-treated cells were 1.6320 and 2.3180, respectively. As was previously observed, the MTT assay did not detect the restoration of MCF-7 cell growth at 48 h or the significant difference in the viability of MMS- and MMS + FA-treated cells. At 72 h, RTCA revealed that the CI values of the control, MMS and MMS + FA groups changed to 3.4196, 2.4951 and 3.1767, respectively. In summary, both proliferation curves (MMS and MMS + FA) are significantly different from the control, and the addition of FA significantly impaired the efficacy of MMS treatment. The MTT assay was unable to detect these differences.

Gene expression data were analyzed using REST 2009, and the results are plotted in Fig. 3. The reference gene *GAPDH* was validated and proved that there is no statistically significant difference between the analyzed conditions by the same software. The expression ratio is the median value obtained from all the experiments performed in each condition. We analyzed the gene expression levels of the folate receptors *FOLR1*, *SLC19A1* and *SLC46A1* in response to DXR, CPT and MMS treatments with or without FA. No significant differences in gene expression were observed between the 2 conditions (data not shown).

We analyzed a set of genes involved in stress signaling, cell cycle and apoptosis pathways (*GADD45A*, *DDIT3*, *BIRC5*, *CCNA2*, *TP53*, *BAX*, *BAK*, *BCL-XL*, *BCL-2*, *CASP7*, *CASP8* and *CASP9*). None of the genes evaluated were differentially expressed in cells treated with FA only compared with the control, demonstrating that folate treatment alone does not alter the normal expression levels of these genes in MCF-7 cells

under the conditions evaluated in this study. Both DXR and CPT significantly reduced the expression of all the target genes evaluated. There was a decline in the expression of *BAK* and *CASP7* in viable cells treated with DXR; however, the reduction was not significantly compared with the control.

The addition of FA to DXR and CPT significantly altered gene expression profiles. In cells treated with DXR or CPT in combination with FA, the gene expression levels of *GADD45A* and *DDIT3* increased compared with the control, and the expression levels of *BAX*, *BAK*, *BCL-XL*, *BCL-2*, *CASP7*, *CASP8* and *CASP9* were restored to normal levels (equivalent to the control). However, as demonstrated by the MTT assay and RTCA, the shift in the gene expression profiles did not influence the cytotoxic effects of DXR and CPT.

In summary, the addition of FA to DXR and CPT treatments significantly increased the expression of genes whose expression levels decreased in cells treated with DXR or CPT only (in the absence of FA), with the exception of *TP53*, *BIRC5* and *CCNA2*, which were expressed at similar levels in the presence or absence of FA. These results demonstrate that DXR and CPT induce similar changes in gene expression profiles generated when administered without FA and induce a similar response to the addition of FA in MCF-7 cells.

In contrast to DXR and CPT, the addition of FA to MMS did not induce significant changes in gene expression. DXR and CPT significantly increased the expression of *GADD45A* and *DDIT3* and decreased the expression of *TP53*, *BIRC5* and *CCNA2*. We observed a trend in which pro-apoptotic genes (*BAX* and *BAK*) were up-regulated and anti-apoptotic genes (*BCL-XL* and *BCL-2*) were down-regulated in cells treated with MMS with or without the addition of FA.

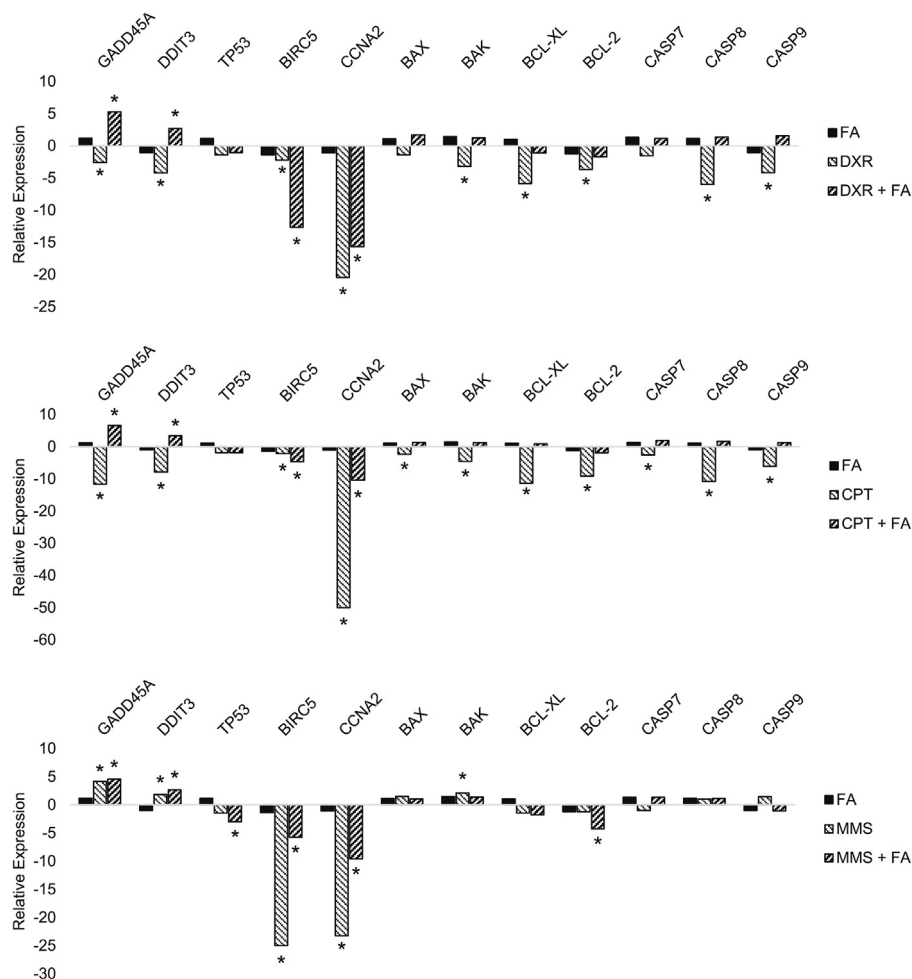


Figure 3 Relative gene expression levels in MCF-7 cells treated for 24 h with doxorubicin (DXR), camptothecin (CPT) or methyl methanesulfonate (MMS) with or without folic acid (FA) were evaluated using RT-qPCR. (*) represents a statistically significant difference between the treatments with and without FA compared with the control.

4. Discussion

In recent years, the free radical scavenging properties and antioxidant activity of folic acid have been demonstrated (Joshi et al., 2001; Gliszczynska-Swigło, 2007; Sarna et al., 2012). As the addition of FA was the only difference between the conditions evaluated in this study, we postulate that the antioxidant effects of FA modulate gene expression in MCF-7 cells treated with DXR and CPT.

Several studies have scrutinized the sensitivity and accuracy of cytotoxicity end-point assays. These studies compared the strengths and weaknesses of the most commonly used assays, especially the MTT assay (Sieuwert et al., 1995; Lobner, 2000; Putnam et al., 2002; Weyermann et al., 2005; Fotakis and Timbrell, 2006; Scherlie, 2011). They emphasize the relevance of using the appropriate assay to avoid overestimating or underestimating the toxicity of a substance and to reduce the risk of false-positive or false-negative results. These studies also indicate that more than one assay should be used to evaluate cytotoxicity *in vitro*. It is not possible to rely solely on the results of 1 method. The results of different assays can widely vary as the methods are based on different mechanisms. The

results can also vary as a result of the particular mechanism of action of the substance analyzed or other technical factors. In addition, to increase the reliability of the results, incubations with various concentrations of a substance at many time points and microscopic analysis of cell cultures before and after performing an assay are recommended.

FA does not affect the cytotoxicity and anti-proliferative effects promoted by DRX and CPT in MCF-7 cells; however, FA reduces the efficacy of MMS. Accordingly, the failure of the MTT assay to detect the actual influence of folic acid on MMS activity in our study is not surprising. Other studies have also described discrepancies between metabolic-based MTT and impedance-based viability assays. These reports suggest that data generated using RTCA reflects cellular viability more accurately and is not influenced by metabolic status, indicating that it is a more sensitive and suitable method for evaluating antineoplastic agents (Gumulec et al., 2014a,b). Therefore, the results observed in our study confirmed the advantages of RTCA that have been previously described. In addition, our findings indicate that RTCA overcomes the limitations associated with the MTT assay and can more accurately assess the effects of FA, such as the reduction in efficacy of MMS in

MCF-7 cells, when used in combination with chemotherapeutics. In summary, the RTCA system has proven to be a more reliable and informative technique to evaluate cytotoxicity *in vitro* and demonstrates that FA does not affect the cytotoxicity and antiproliferative effect of DRX and CPT, but it does reduce the efficacy of MMS in MCF-7 cells.

Our results also suggest that folic acid supplementation to the chemotherapeutics evaluated in this study does not influence the expression of genes encoding folate receptors. The expression levels of the folate receptors *FOLR1*, *SLC19A1* and *SLC46A1* were not significantly different in cells treated with DXR, CPT and MMS compared with cells treated with DXR, CPT and MMS in combination with FA.

DXR and CPT have similar (but not equal) mechanisms of action that promote similar cellular responses (Li et al., 2006; Thorn et al., 2011; Tacar et al., 2013; Dezhenkova et al., 2014). DXR binds to multiple DNA-associated enzymes, including topoisomerases I and II, to induce a range of cytotoxic effects. The generation of free radicals and the damaging effects of free radicals on cellular membranes, DNA and proteins are additional cytotoxic mechanisms employed by DXR and CPT. Both drugs can induce antiproliferative effects and DNA damage, thereby triggering apoptotic cell death pathways when attempts to repair DNA breaks fail and cell cycle progression arrests at the G1 and G2 phases (Thorn et al., 2011; Tacar et al., 2013; Dezhenkova et al., 2014). CPT binds to the topoisomerase I-DNA complex, which results in the accumulation of DNA double stranded breaks during replication. CPT also exerts a range of cytotoxic effects that ultimately cause cell death. CPT-induced cytotoxicity is especially pronounced during the S-phase of the cell cycle as it is more toxic to cells undergoing DNA synthesis. ROS production is also involved in CPT-induced apoptosis (Li et al., 2006; Dezhenkova et al., 2014). Our results confirm that CPT and DXR exert similar cytotoxic effects in MCF-7 cells as the cytotoxic profile and the proliferation curves, obtained by MTT and RTCA assays, respectively, were similar for the 2 drugs. The primary difference between DXR and CPT was the intensity of their effects.

Our results showed that, despite the FA-induced changes in gene expression, the capacity of DXR and CPT to promote cell death was unaffected by FA. Therefore, the antioxidant properties of FA might protect MCF-7 cells from damage induced by ROS formation when administered in combination with DXR and CPT. We hypothesize that the similar changes in gene expression induced by DXR and CPT result from the ROS formation associated with the mechanism of cell death exhibited by these drugs. When DXR and CPT are administered in combination with FA, FA's free radical scavenging and antioxidant properties might neutralize the effects of ROS and trigger a shift in gene expression consistent with a response to a mechanism of cell death independent of ROS.

There are two partially interconnected apoptotic mechanisms, caspase-dependent classical apoptosis and caspase-independent programmed cell death. The two forms of cell death might be interconnected and potentially lead to the activation of non-caspase proteases (Ola et al., 2011; Chaabane et al., 2013). As reviewed by Sinha et al. (2013), an alternative pathway modulated by oxidative stress can also promote apoptosis independent of *CASP7*, *CASP8* and *CASP9* by activating TNF α . In this alternative pathway, the receptor-interacting protein (RIP) binds to TNFR1-associated death domain (TRADD) and ultimately activates caspase-2. This activation

was reported to potentially be a response to severe stress and to promote rapid cell death.

In our study, DXR and CPT treatment of MCF-7 cells resulted in the down-regulation of the *CASP7*, *CASP8* and *CASP9* genes. Moreover, the release of AIF from the mitochondria can be prevented by the overexpression of Bcl-2 (Yu et al., 2003; Ola et al., 2011); however, our data revealed that the *BCL-2* gene was down-regulated in response to DXR and CPT treatments.

Our results also demonstrated that the *TP53* gene is down-regulated in MCF-7 cells treated with DXR and CPT. However, *TP53* overexpression is normally observed in the classical apoptotic pathways. In fact, all classical pro-apoptotic (*BAX* and *BAK*) and anti-apoptotic (*BCL-2* and *BCL-XL*) genes are down-regulated by both treatments, as previously described.

GADD45A and *DDIT3* play significant roles in the response to cellular genotoxic and non-genotoxic stress, whereby they function as stress sensors and tumor suppressors. The expression of *GADD45A* and *DDIT3* are rapidly induced by DNA damage and they promote cell cycle arrest and/or apoptosis or actively participate in DNA repair mechanisms (Liebermann and Hoffman, 2008; Tamura et al., 2012). Our RT-qPCR results demonstrated that the *GADD45A* and *DDIT3* genes were down-regulated in MCF-7 cells treated with DXR and CPT, indicating that the induction of these genes was not a result of DNA damage. These results suggest that these agents primarily promote cell death by an alternative mechanism. Some authors corroborated that apoptosis may be attributable to off-target effects independent of DNA damage (Havelka et al., 2007).

Methyl methanesulfonate (MMS) is an alkylating agent capable of randomly inducing DNA lesions that lead to single and double strand breaks in genomic DNA. In response to this damage, the cell cycle arrests and repair pathways are activated. Once the DNA repair process is complete, cell cycle progression can resume. Alternatively, if the damage cannot be repaired, cell death mechanisms are initiated (Schwartz and Kmiec, 2005; Kondo et al., 2010).

Our RTCA results demonstrated that proliferation was restored in MCF-7 cells after 30 h of treatment with either MMS + FA or MMS. Therefore, it is possible that the cytotoxicity observed in the MTT assay reflects a decrease in metabolically active cells due to cell cycle arrest rather than a decrease in cellular viability due to cell death. This type of effect has been previously reported by other groups (Schwartz and Kmiec, 2005).

Our gene expression results also support this hypothesis. We showed that MMS and MMS + FA treatments increased the expression of *GADD45A* and *DDIT3*, genes activated by DNA damage and significantly reduced the expression of the *TP53*, *BIRC5* and *CCNA2* genes, indicating that cell cycle arrest had occurred. However, the expression levels of pro-apoptotic and anti-apoptotic genes did not change. These findings, coupled with the observation that proliferation recovers in cells treated with MMS or MMS + FA indicate that most MCF-7 cells did not undergo cell death but underwent DNA damage repair in response to the cytotoxic effects of MMS with or without FA. Together, our results demonstrate that folic acid promoted a similar shift in the gene expression profiles of MCF-7 cells treated with DXR and CPT but did not alter the expression profile of MCF-7 cells treated with MMS.

5. Conclusion

Our study emphasizes the need for increased awareness about the choice of tests for analyzing cell viability *in vitro*. Our study revealed that the RTCA system is more accurate, sensitive and reliable compared with the MTT assay. We demonstrated that 450 μM of FA supplementation is not cytotoxic to MCF-7 cells and does not alter the expression of folate receptor genes even in combination with the chemotherapeutic drugs evaluated in this study. However, the addition of FA to chemotherapy drugs must be performed cautiously, as the antioxidant properties of FA might not always be beneficial and might interfere with the mechanisms underlying drug efficacy.

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References

- Aranda, P.S., LaJoie, D.M., Jorcyk, C.L., 2012. Bleach gel: a simple agarose gel for analyzing RNA quality. *Electrophoresis* 33, 366–369.
- Assaraf, Y.G., Leamon, C.P., Reddy, J.A., 2014. The folate receptor as a rational therapeutic target for personalized cancer treatment. *Drug Resist. Updat.* 17, 89–95.
- Chaabane, W., User, S.D., El-Gazzah, M., Jaksik, R., Sajjadi, E., Rzeszowska-Wolny, J., Łos, M.J., 2013. Autophagy, apoptosis, mitoptosis and necrosis: interdependence between those pathways and effects on cancer. *Arch. Immunol. Ther. Exp.* 61, 43–58.
- Crider, K.S., Yang, T.P., Berry, R.J., Bailey, L.B., 2012. Folate and DNA methylation: a review of molecular mechanisms and the evidence for folate's role. *Adv. Nutr.* 3, 21–38.
- Dezhenkova, L.G., Tsvetkov, V.B., Shtil, A.A., 2014. Topoisomerase I and II inhibitors: chemical structure, mechanisms of action and role in cancer chemotherapy. *Russ. Chem. Rev.* 83, 82–94.
- Duthie, S.J., 2011. Folate and cancer: how DNA damage, repair and methylation impact on colon carcinogenesis. *J. Inherit. Metab. Dis.* 34, 101–109.
- Fotakis, G., Timbrell, J.A., 2006. *In vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol. Lett.* 160, 171–177.
- Gliszczynska-Swigło, A., 2007. Foliates as antioxidants. *Food Chem.* 101, 1480–1483.
- Gonen, N., Assaraf, Y.G., 2012. Antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. *Drug Resist. Updat.* 15, 183–210.
- Gumulec, J., Balvan, J., Sztalmachova, M., Raudenska, M., Dvorakova, V., Knopfova, L., Polanska, H., Hudcova, K., Ruttkay-nedecky, B., Babula, P., Adam, V., Kizek, R., Stiborova, M., Masarik, M., 2014a. Cisplatin-resistant prostate cancer model: differences in antioxidant system, apoptosis and cell cycle. *Int. J. Oncol.* 44, 923–933.
- Gumulec, J., Fojtu, M., Raudenska, M., Sztalmachova, M., Skotakova, A., Vlachova, J., Skalickova, S., Nejdil, L., Kopel, P., Knopfova, L., Adam, V., Kizek, R., Stiborova, M., Babula, P., Masarik, M., 2014b. Modulation of induced cytotoxicity of doxorubicin by using apoferritin and liposomal cages. *Int. J. Mol. Sci.* 15, 22960–22977.
- Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *Cell* 144, 646–674.
- Havelka, A.M., Berndtsson, M., Olofsson, M.H., Shoshan, M.C., Linder, V., 2007. Mechanisms of action of DNA-damaging anticancer drugs in treatment of carcinomas: is acute apoptosis an “Off-Target” effect? *Mini-Rev. Med. Chem.* 7, 1035–1039.
- Joshi, R., Adhikari, S., Patro, B.S., Chattopadhyay, S., Mukherjee, T., 2001. Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. *Free Radic. Biol. Med.* 30, 1390–1399.
- Kim, Y.I., 1999. Folate and carcinogenesis: evidence, mechanisms, and implications. *J. Nutr. Biochem.* 10, 66–88.
- Kim, Y.I., 2006. Does a high folate intake increase the risk of breast cancer? *Nutr. Rev.* 64, 468–475.
- Kondo, K., Obitsu, S., Ohta, S., Matsunami, K., Otsuka, H., Teshima, R., 2010. Poly(ADP-ribose) polymerase (PARP)-1-independent apoptosis-inducing factor (AIF) release and cell death are induced by eleostearic acid and blocked by α -tocopherol and MEK inhibition. *J. Biol. Chem.* 285, 13079–13091.
- Leamon, C.P., Reddy, J.A., 2004. Folate-targeted chemotherapy. *Adv. Drug Deliv. Rev.* 56, 1127–1141.
- Lewis, S.J., Harbord, R.M., Harris, R., Smith, G.D., 2006. Meta-analyses of observational and genetic association studies of folate intakes or levels and breast cancer risk. *J. Natl. Cancer Inst.* 98, 1607–1622.
- Li, Q.Y., Zu, Y.G., Shi, R.Z., Yao, L.P., 2006. Review camptothecin: current perspectives. *Curr. Med. Chem.* 13, 2021–2039.
- Liebermann, D.A., Hoffman, B., 2008. Gadd45 in stress signaling. *J. Mol. Signal* 3, 15.
- Lobner, D., 2000. Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis. *J. Neurosci. Methods* 96, 147–152.
- Lu, Y., Low, P.S., 2012. Folate-mediated delivery of macromolecular anticancer therapeutic agents. *Adv. Drug Deliv. Rev.* 64, 342–352.
- Maruti, S.S., Ulrich, C.M., White, E., 2009. Folate and one-carbon metabolism nutrients from supplements and diet in relation to breast cancer risk. *Am. J. Clin. Nutr.* 89, 624–633.
- Moniri, M.R., Young, A., Reinheimer, K., Rayat, J., Dai, L.J., Warnock, G.L., 2015. Dynamic assessment of cell viability, proliferation and migration using real time cell analyzer system (RTCA). *Cytotechnology* 67, 379–386.
- Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival; application to proliferation cytotoxic assays. *J. Immunol. Methods* 65, 55–63.
- Negrini, S., Gorgoulis, V.G., Halazonetis, T.D., 2010. Genomic instability – an evolving hallmark of cancer. *Nat. Rev. Mol. Cell Biol.* 11, 220–228.
- Ola, M.S., Nawaz, M., Ahsan, H., 2011. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. *Mol. Cell Biochem.* 351, 41–58.
- Pan, T., Khare, S., Ackah, F., Huang, B., Zhang, W., Gabos, S., Jin, C., Stampf, M., 2013. *In vitro* cytotoxicity assessment based on KC50 with real-time cell analyzer (RTCA) assay. *Comput. Biol. Chem.* 47, 113–120.
- Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real time PCR. *Nucleic Acids Res.* 30, e36.
- Putnam, K.P., Bombick, D.W., Doolittle, D.J., 2002. Evaluation of eight *in vitro* assays for assessing the cytotoxicity of cigarette smoke condensate. *Toxicol. In Vitro* 16, 599–607.
- Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., van den Hoff, M.J.B., Moorman, A.F.M., 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 37, e45.
- Sarna, L.K., Wu, N., Wang, P., Hwang, S.Y., Siow, Y.L., Karmin, O., 2012. Folic acid supplementation attenuates high fat diet induced hepatic oxidative stress via regulation of NADPH oxidase. *Can. J. Physiol. Pharmacol.* 90, 155–165.
- Scherlie, R., 2011. The MTT assay as tool to evaluate and compare excipient toxicity *in vitro* on respiratory epithelial cells. *Int. J. Pharm.* 411, 98–105.

- Schwartz, T.R., Kmiec, E.B., 2005. Using methyl methanesulfonate (MMS) to stimulate targeted gene repair activity in mammalian cells. *Gene Ther. Mol. Biol.* 9, 193–202.
- Sieuwert, A.M., Klijn, J.G.M., Peters, H.A., Foekens, J.A., 1995. The MTT tetrazolium salt assay scrutinized: how to use this assay reliably to measure metabolic activity of cell cultures in vitro for the assessment of growth characteristics, IC₅₀-values and cell survival. *Clin. Chem. Lab. Med.* 33, 813–824.
- Sinha, K., Das, J., Pal, P.B., Sil, P.C., 2013. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Arch. Toxicol.* 87, 1157–1180.
- Tacar, O., Sriamornsak, P., Dass, C.R., 2013. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. *J. Pharm. Pharmacol.* 65, 157–170.
- Tamura, R.E., Vasconcellos, J.F., Sarkar, D., Libermann, T. A., Fisher, P.B., Zerbini, L.F., 2012. GADD45 proteins: central players in tumorigenesis. *Curr. Mol. Med.* 12, 634–651.
- Thorn, C.F., Oshiro, C., Marsh, S., Hernandez-Boussard, T., McLeod, H., Klein, T.E., Altman, R.B., 2011. Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenet. Genomics* 21, 440–446.
- Ulrich, C.M., Potter, J.D., 2007. Folate and cancer-timing is everything. *JAMA* 297, 2408–2409.
- Urcan, E., Haertel, U., Styllou, M., Hickel, R., Scherthan, H., Reichl, F.X., 2010. Real-time xCELLigence impedance analysis of the cytotoxicity of dental composite components on human gingival fibroblasts. *Dent. Mater.* 26, 51–58.
- Vollset, S.E., Clarke, R., Lewington, S., Ebbing, M., Halsey, J., Lonn, E., Armitage, J., Manson, J.E., Hankey, G.J., Spence, J.D., Galan, P., Bønaa, K.H., Jamison, R., Gaziano, J.M., Guarino, P., Baron, J.A., Logan, R.F.A., Giovannucci, E.L., den Heijer, M., Ueland, P. M., Bennett, D., Collins, R., Peto, R., 2013. Effects of folic acid supplementation on overall and site-specific cancer incidence during the randomised trials: meta-analyses of data on 50,000 individuals. *Lancet* 381, 1029–1036.
- Weyermann, J., Lochmann, D., Zimmer, A., 2005. A practical note on the use of cytotoxicity assays. *Int. J. Pharm.* 288, 369–376.
- Wien, T.N., Pike, E., Wisløff, T., Staff, A., Smeland, S., Klemp, M., 2012. Cancer risk with folic acid supplements: a systematic review and meta-analysis. *BMJ Open*, e000653.
- Yu, S.W., Wang, H., Dawson, T.M., Dawson, V.L., 2003. Poly(ADP-ribose) polymerase-1 and apoptosis inducing factor in neurotoxicity. *Neurobiol. Dis.* 14, 303–317.