DNA damage and reticular stress in cytotoxicity and oncotic cell death of MCF-7 cells treated with fluopsin C

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**ABSTRACT**

Fluopsin C is an antibiotic compound derived from secondary metabolism of different microorganisms, which possesses antitumor, antibiotic, and antifungal activity. Related to fluopsin C antiproliferative activity, the aim of this study was to examine the following parameters: cytotoxicity, genotoxicity, cell cycle arrest, cell death induction (apoptosis), mitochondrial membrane potential (MMP), colony formation, and mRNA expression of genes involved in adaptive stress responses and cellular death utilizing a monolayer. In addition, a three-dimensional cell culture was used to evaluate the effects on growth of tumor spheroids. Fluopsin C was cytotoxic (1) producing cell division arrest in the G1 phase, (2) elevating expression of mRNA of the CDKN1A gene and (3) decrease in expression of mRNA H2AFX gene. Further, fluopsin C enhanced DNA damage as evidenced by increased expression of mRNA of GADD45A and GPX1 genes, indicating that reactive oxygen species (ROS) may be involved in the observed genotoxic response. Reticulum stress was also detected as noted from activation of the ribonuclease inositol-requiring protein 1 (IRE1) pathway, since a rise in mRNA expression of the ERN1 and TRAF2 genes was observed. During the cell death process, an increase in mRNA expression of the BBC3 gene was noted, indicating participation of this antibiotic in oncotic (ischemic) cell death. Data thus demonstrated for the first time that fluopsin C interferes with the volume of tumor spheroids, in order to attenuate their growth. Our findings show that fluopsin C modulates essential molecular processes in response to stress and cell death.

**KEYWORDS**

Antitumor; cell stress; programmed necrosis; spheroid; antibiotic

**Introduction**

Breast cancer is a disease that accounts for numerous deaths annually in women and in 2020 alone 2.3 million new cases and 684,996 deaths were estimated worldwide (Sung et al. 2021). Currently, the available treatments include surgery to remove the tumor or breast (McLaughlin 2013), radiotherapy (EBCTCG et al., 2011), and systemic therapies such as chemotherapy and hormonal treatment (EBCTCG et al., 2012).

In addition to antibiotic drug usage against microorganisms, antibiotic compounds are one of the main classes of natural products used clinically as chemotherapeutic agents including doxorubicin, daunorubicin, epirubicin, mitoxantrone, pirarubicin, mitomycin, bleomycin, pingyangmycin, actinomycin, and mithramycin (Blanchard 2015; Cragg and Newman 2001; Demain and Vaishnav 2011; Eskandari et al. 2021; Khasraw, Bell, and Dang 2012; Liu et al. 2018; Marquez et al. 2020; Saednia 2015; Zhao et al. 2017). However, the search for new antitumor drugs is still necessary due to side effects and resistance to chemotherapy (Abushaheen et al. 2020; Cagan and Meyer 2017; Nezhad et al. 2021; Wencewicz 2019).

Fluopsin C is an antibiotic molecule that has been gaining notoriety given its antibacterial (Egawa et al. 1970; Ito et al. 1971; Miyamura et al. 1972; Navarro et al. 2019; Patteson et al. 2021), antifungal (Egawa et al. 1970; Miyamura et al. 1972; Patteson et al. 2021), and antitumor potential.
(Ito et al. 1971; Ma et al. 2013; Miyamura et al. 1972). Fluopsin C is considered a strong candidate to counteract multi-resistant microorganisms (Navarro et al. 2019; Sharma 2020), and for inducing programmed oncosis (ischemic)-like necrosis in tumor cells (Ma et al. 2013; Yu et al. 2020).

Fluopsin C is a metalloantibiotic compound obtained from secondary metabolism of *Pseudomonas* spp. (Egawa et al. 1970), *Streptomyces* sp. (Miyamura et al. 1972), and *Pseudomonas aeruginosa* strain LV (Navarro et al. 2019). The molecular composition of fluopsin C consists of two thioformin molecules associated with a central copper atom, forming a low molecular weight polydentate copper complex (Egawa et al. 1971). In relation to breast cancer, antitumor activity was noted by Ma et al. (2013) using adenocarcinoma breast cancer MCF-7 cells as an *in vitro* model. Ma et al. (2013) demonstrated that fluopsin C induced the following consequences including oncotic cell death, loss of mitochondrial membrane potential (MMP) integrity, release of cytoplasmic content, elevated production of reactive oxygen species (ROS), depletion of cellular ATP, and destruction of the cytoskeleton.

Oncosis is a type of death characterized by (1) the presence of cellular and organelles swelling, (2) formation of protrusions in the plasma membrane (blebbing), (3) increased membrane permeability, (4) failure of the plasma membrane ion pumps, (5) ATP depletion, and (6) endoplasmic reticulum dilation (Escobar et al. 2011; Majno and Joris 1995; Weerasinghe and M 2012). Oncosis is associated with pulmonary diseases, liver failure, and, especially, myocardial infarction (Fricker et al. 2018; Loh, Wang, and Liao 2018; Weerasinghe et al. 2013).

Due to the scarcity of information regarding the antiproliferative activity and mechanisms of actions of fluopsin C, the aim of this study was to investigate cytotoxicity, genotoxicity, cell cycle arrest, formation of new colonies, cell death, and expression of poly(A)-mRNA of genes related to molecular responses attributed to cellular stress using this antibiotic in a monolayer system. In addition, the effects of fluopsin C was examined upon growth of tumor spheroids of adenocarcinoma breast cancer MCF-7 cells.

**Material and methods**

**Cell lineage, culture conditions, and chemical agents**

The mycoplasma-free mammary carcinoma cell line of molecular subtype A (MCF-7) was obtained from the Cell Bank of Rio de Janeiro. Cells were grown in Modified Eagle’s Medium – low glucose culture medium (Gibco, catalog no. 12800–017) supplemented with 10% fetal bovine serum (FBS) (GIBCO, cod: 12657–029) and 1% antibiotic and antimitotic (GIBCO, cod: 15240–062) and kept in a humid incubator with a controlled atmosphere containing 5% CO₂ at 37°C. For all experiments, cell viability was previously determined with trypan blue in cell counter (LifeTechnology) with only viability above 85% being accepted. In addition, for each assay, three experiments were performed with cells treated only after a 24-hr period of cell stabilization.

To carry out this investigation, the compound fluopsin C (Figure 1a) was used (C₉H₈CuN₂O₂S₂), provided by the Laboratory of Microbial Ecology of the State University of Londrina (Bedoya et al. 2019), dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Cat. No: 472301) and diluted in culture medium at the time of treatment. For experimental assays, camptothecin (CPT, Sigma, cat. No. C9911), doxorubicin (DXR, Doxolem, Zodiac), and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were employed as positive controls. Control treatment was prepared with 0.0045% DMSO; this value corresponds to the concentration of DMSO present in the highest concentration of fluopsin C tested.

**Cytotoxicity assay (resazurin)**

The cytotoxic effect of fluopsin C was evaluated using the dye resazurin (7-hydroxy-3 H-phenoxazine-3-one-10-oxide) in 1.5 × 10⁴ cells/well in a 96-well plate. Cells were treated with fluopsin C at concentrations of 0.25, 0.5, 0.75, 1, or 1.5 µM, control, or DXR (2 µM) for 24 hr and incubated with resazurin (60 µM) for a further 2 hr. Fluorescence was measured using a Glomax® spectrophotometer (520 nm excitation and 580–640 nm emission). Cell viability was determined using the formula: percentage viability = ([A<sub>treatment</sub> − A<sub>white</sub>]/[A<sub>control</sub> − A<sub>white</sub>]) × 100, where A represents the fluorescence value. The IC<sub>50</sub>, the cytotoxic concentration necessary to
reduce cell viability by 50%, and the correlation coefficient were calculated utilizing GraphPad Prism 7.0 (GraphPad Software, USA).

**Apoptosis and cell cycle assay by flow cytometry**

To investigate whether fluopsin C induces cell death by apoptosis, the GUAVA NEXIN apoptosis kit (Merck/Millipore 4500–0450) was employed, evaluating the externalization profile of phosphatidylserine through interaction with annexin, thus exhibiting % viable cells [Annexin V (-) and 7-AAD (-)], initial apoptosis [Annexin V (+) and 7-AAD (-)], late apoptosis [Annexin V (+) and 7-AAD (+)], and dead cells [Annexin V (-) and 7-AAD (+)]. To measure DNA content and infer the relative % profile of cell cycle phases (G₁, S, and G₂/M) cells were permeabilized with 100 µl citrate/triton solution (0.1% sodium citrate/ 0.1% Triton X-100) and DNA stained with 5 µl propidium iodide (50 µg/ml). In a 6-well plate, 10⁶ cells/well were seeded with culture medium without FBS for 24 hr, to ensure a pre-synchronization of the cell cycle. After this stabilization period, cells were treated with fluopsin C (0.25, 0.5 or 1 µM), control, or CPT (3 µM) for 24 hr, washed with PBS, trypsinized (0.025%), and centrifuged at 900 g for 5 min. The supernatant was removed, and cells resuspended in 300 µl PBS. For both assays, the fluorescence intensity of each marker was quantified utilizing a MilliporeSigma Muse® flow cytometer, analyzing 5,000 events/sample.

**Mitochondrial membrane potential assay (TMRE)**

To observe whether fluopsin C produced alterations in mitochondrial membrane potential (MMP) (Concato et al. 2020), the tetramethylrhodamine ethyl ester (TMRE) marker was used, which is a cationic fluorescent dye that is incorporated into metabolically active mitochondria. Cells (1.5x10⁴) were seeded in a black 96-well plate and treated with fluopsin C (0.25, 0.5 or 1 µM), control, or CCCP (100 µM), as a positive control for 24 hr. Subsequently, cells were washed with PBS and incubated with TMRE (25 nM) for 30 min at 37°C and analyzed at excitation wavelength at 480 nm and emission at 580 nm in a fluorescence microplate reader (Victor X3, PerkinElmer, Finland).

**Genotoxicity assay (comet)**

To assess whether fluopsin C induced DNA damage, the comet assay was employed (Collins et al. 2008). A cell concentration of 1 x 10⁶ cells/well was seeded in culture tubes polypropylene and cells treated with...
fluopsin C (0.25, 0.5 or 1 µM), control, and DXR (2 µM) for 3 hr. The cells were centrifuged at 900 g for 5 min and pellet was resuspended in (0.8%) low melting point agarose and distributed on slides pre-gelatinized with normal melting point agarose (1.5%). After solidification, the slides were subjected to a chemical lysis treatment (2.25 M NaCl, 89 mM EDTA, 8.9 mM Tris-HCL, 10% control, and 1% Triton X-100, pH 10 adjusted with NaOH) for one hr at 4°C, then to a treatment with alkaline solution (1 mM EDTA-Titriplex and 0.3 N NaOH) for 20 min. Slide electrophoresis was performed in alkaline buffer (pH>13), 25 V and 300 mA for 20 min, followed by 2x neutralization (0.4 N Tris at pH 7.5), fixation in absolute ethanol, and storage at 4°C. For analysis, slides were stained with ethidium bromide (2 µg/ml) and a total of 100 cells/treatment were evaluated. Photomicrographs were obtained using an EVOS® FL Auto Cell Imaging System (Thermo Fisher) microscope at 100X magnification. For each image, the size of the comet tail and tail moment (multiplication of the length of the tail by % DNA in the tail) were evaluated using CometScore® software.

**Clonogenic assay**

To investigate whether fluopsin C interferes with the ability to form new colonies in MCF-7 cells, the protocol described by Franken et al. (2006) was used. For this, 1.5 × 10⁶ cells/well were seeded in a 6-well plate and treated with fluopsin C (0.25, 0.5 or 1 µM), control, or DXR (2 µM) for 24 hr. Subsequently, the supernatant was removed and 500 cells/well were seeded in a 24-well plate and maintained with culture medium without treatment for 14 days and with medium renewal every 3 days. Subsequently, the total colonies/treatment were incubated with resazurin (60 µM) for 2 hr, and fluorescence intensity was quantified in a Glomax® spectrophotometer (520 nm excitation and 580 nm emission). Finally, the colonies were fixed in a solution of methanol + acetic acid (3:1) for 5 min and stained with crystal violet (0.5%) for 15 min.

**3D Cell culture – spheroids**

For the preparation of a non-adherent surface, in a 96-well plate, 50 µl/well were added of sterile solution containing low melting point agarose (1.5%) dissolved in culture medium without FBS, according to the methodology described by Friedrich et al. (2009). After solidification, 3 × 10³ cells/well were seeded in a final volume of 200 µl culture medium supplemented with FBS, and centrifuged at 900 g for 5 min. After the spheroidization period (3 days) in an incubator, the required time for cells to aggregate and form spheroids, these spheroids were treated with fluopsin C (0.25 0.5 or and 1 µM), control, or DXR (2 µM).

Qualitative and quantitative parameters were evaluated from 6 spheroids/treatments at times 0, 24, 48, and 72 hr post spheroidization. The volumes of the spheroids and the rendering of 3D graphics were obtained using AnaSP (Piccinini 2015) and ReViSP software (Piccinini et al. 2015) and expressed as mean mm³. In addition, spheroids were labeled with fluorescent dyes Rhodamine123 and Hoechst33342, to observe mitochondrial activity and peripheral cell DNA, respectively. After 72 hr incubation spheroids were transferred to a 48-well plate and 300 µl/well of untreated culture medium were added to observe recovery of cell proliferation for 24 hr on the adherent surface. Photomicrographs were captured using the EVOS™ FL Auto Imaging System microscope (ThermoFisher Scientific, USA) and EVOS® XL Core Cell Imaging System (Thermo Fisher).

**Real-time relative gene expression (RT-qPCR)**

The real-time gene expression assay (RT-qPCR) was used to measure relative changes in mRNA expression of genes related to molecular pathways of cell death (BBC3, BIRC5, CASP3, CASP8, CASP9, PARP1, BCL2, and TNF), oxidative stress (GPX1), reticulum stress (ERN1 and EIF2AK3), DNA damage (GADD45A and H2AFX), cell cycle regulation (CDKN1A, M-TOR, NF-KB, TRAF-2, C-MYC, and TP53), autophagy (BECN1), and invasion (MMP9 and MMP1) in MCF-7 cells treated with 1 µM fluopsin C (Primer list – Table S1). GAPDH and ACTB served as reference genes.

For this, 1 × 10⁶ cells/well were seeded in 6-well plates and treated with fluopsin C (1 µM) or control for 6 hr. Subsequently total RNA was extracted with the MiniKit (Qiagen) RNeasy®, following manufacturer's instructions. The purity and quantification of total RNA were noted using a BioDrop µLite
spectrophotometer, based upon the absorbance ratio (A260/280 mm) and only samples with a ratio between 1.9 and 2 were considered valid. The verification of the integrity of the material was carried out on 1% agarose gel. The cDNA synthesis was performed in a T100TM Thermal Cycler BIO-RAD thermocycler, with three reactions/treatment/repli ca, each containing 1000 ng total RNA diluted in 12.4 µl DEPC water, 1 µl oligo dT (20 pm/ml, Invitrogen, catalog No. 18418012), and 2 µl dNTPs (10 mM, Invitrogen, catalog No. 10297018), maintained at 60°C for 15 min. Next, 0.1 µl RNase OUT (4 units, Invitrogen, catalog No. 10777019), 4 µl 5x first-strand buffer (Invitrogen, catalog No. Y02321), and 0.5 µl M-MLV-RT (Invitrogen, catalog No. 28025-013) were added to the reaction mixtures with samples kept at 37°C for 2 min, 50°C for 45 min, and 70°C for 15 min.

For real-time PCR, a thermocycler LightCycler® Nano (Roche) was employed at a setting of 95°C for 5 min and 35 cycles of 95°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec. Each RT-qPCR reaction contained 11 µl final volume, containing 5 µl cDNA (1:10), 0.5 µl each (initiator) primer (forward and reverse) and 5 µl QuantiNova SYBR Green PCR Mix (Qiagen cat. No. 208052). The melting curve was analyzed at the end of the reaction from 50°C to 90°C, every 0.5°C/20 sec.

**Statistical analysis**

Numerical data were submitted to the Shapiro-Wilk’s test to verify normality. Parametric data from cytotoxicity, apoptosis, cell cycle, genotoxicity, mitochondrial, and clonogenic membrane potential assays were submitted to analysis by variance (one-way ANOVA) and Dunnett’s post-hoc test. For the 3D culture assay, data were submitted to Analysis of Variance (two-way ANOVA) and post-hoc Tukey’s test of multiple comparisons. GraphPad Prism® 7.0 software (GraphPad Software, Inc.) was used and results expressed as mean ± standard deviation. The criterion for significance was set at p < .05. For the analysis of relative gene expression, the *Pairwise Fixed Reallocation Randomization Test* method was used, inserted in the Rest 2009 software (Pfaffl, Horgan, and Dempfle 2002) and expressions with fold-change ≥ 2 and ≤ 0.5 were considered as significant.

**Results**

**Fluopsin C cytotoxic and morphological alterations in MCF-7 cells**

After 24 hr incubation, fluopsin C was cytotoxic to MCF-7 cell line (Figure 1b) at concentrations of 0.25 µM (86.23 ± 1.14), 0.5 µM (64.23 ± 4.97), 0.75 µM (55.86 ± 3.88), 1 µM (51.62 ± 4.13), and 1.5 µM (41.19 ± 3.58). Cell viability was concentration-dependently reduced, since this quantity exhibited a significant coefficient of −0.941 between cell viability and fluopsin C concentration. The IC50 calculated was 1 µM. In addition, alterations in cell morphology were noted after treatment with fluopsin C in relation to control (Figure 1c). This antibiotic was found to disturb cell adhesion, increase the volume of cytosol resulting in of cellular swelling (Figure 1d), and induced formation of blebs in the plasma membrane (Figure 1e).

**Fluopsin C effect on cell cycle arrest in G1 phase**

After 24 hr incubation, fluopsin C significantly elevated the relative % cells in the G1 phases at concentrations of 0.25 µM (61.53 ± 0.45), 0.5 µM (63.07 ± 0.45), and 1 µM (70.87 ± 0.6), compared to control (60.47 ± 0.21). In the DNA synthesis step, concentrations of 0.5 µM (11.17 ± 0.61) and 1 µM (11.47 ± 0.41) decreased the relative % compared to control (12.57 ± 0.15). Further, an attenuation was detected in the G2/M phases at levels of 0.5 µM (25.67 ± 0.6) and 1 µM (17.67 ± 0.25) compared to control (26.87 ± 0.32). Therefore, due to the rise in G1 and reduction in relative % in the S and G2/M phases, evidence suggested that fluopsin C significantly induced cell cycle arrest in the G1 phase (Figure 2a).

**Influence of fluopsin c on apoptosis**

Since apoptotic cells present externalization of phosphatidylserine and the annexin dye interacts with this phospholipid component, it is conceivable that fluopsin C (1 µM) produced a different type of cell death in particular apoptosis. This is attributed to a 5-fold increase in the mean relative value of dead/necrotic cells (30.06 ± 5.2) compared to the control (5.92 ± 1.41), while the relative % of cells in apoptosis did not exhibit significant differences between treatments (Figure 2b).
Figure 2. Cell cycle and apoptosis analysis by flow cytometry. (A) Relative percentage mean of cells in different phases of the cell cycle after 24 hr treatment with 0.25–1 µM of fluopsin C (FC). DNA content was analyzed using propidium iodide in flow cytometry. (B) Relative percentage of living, apoptotic, and dead cells after 24 hr incubation with 0.25–1 µM of fluopsin C. Cells were labeled with Annexin V and 7-AAD and analyzed in flow cytometry. (C) FC induces loss in mitochondrial membrane potential. (*) represents p-value ≤ 0.05 in relation to the control, by the ANOVA test followed by the Dunnett test. Values expressed as mean ± SD.
Figure 3. Fluopsin C was genotoxic to MCF-7 and decreased colony formation. (A) After three hours of exposure, fluopsin C (1.0 µM) produced DNA damage in MCF-7 cells. (A1) Mean size of the comet’s tail. (A2) Mean tail moment (multiplication of the length of the tail by the percentage of DNA in the tail). One hundred cells/treatment were evaluated (B) – Clonogenic analysis for MCF-7 treated with fluopsin C for 24 hr; (B1) Control colonies and colonies after treatment with 0.25, 0.5 µM, or 1 µM fluopsin C. (*) represents p-value ≤ 0.05 in relation to the control, by the ANOVA test followed by the Dunnett’s test. Values expressed as mean ± standard deviation.

**Influence of fluopsin c on Mitochondrial Membrane Potential (MMP)**

After 24 hr exposure, fluopsin C decreased the MMP by 47.8% at a concentration of 1 µM (1632 ± 86.42), compared to control (3126 ± 530.4) (Figure 2c), thus demonstrating that at 1 µM fluopsin C inhibited mitochondrial metabolic activity, which is yet another mode of action of fluopsin C in MCF-7 cells.

**Effect of fluopsin c is genotoxicity and colony formation**

Fluopsin C was found to be genotoxic at a concentration of 1 µM, inducing DNA strand breaks after 3 hr exposure (Figure 3a). For the parameter mean size of the comet’s tail (Figure 3A1), at the concentration of 1 µM (10.63 ± 1.72) a threefold rise was observed in relation to control (3.36 ± 0.23). In the case of mean tail moment (Figure 3A2) a concentration of 1 µM (2.33 ± 0.53) elevated this parameter 6-fold, in relation to control (0.37 ± 0.05). In both parameters, the concentrations 0.25 µM and 0.5 µM did not display significant differences.

Regarding colony formation (Figure 3b), after 14 days of removal of fluopsin C treatment, a significant decrease in resazurin metabolism by colonies was noted (Figure 3b1) by 43% for 0.5 µM (2600 ± 562.7) and by 69% for 1 µM (1427 ± 74.68), compared to the control (4588 ± 526). The concentration of 0.25 µM produced no significant effect.

**Effect of fluopsin c on MCF-7 cell spheroids behavior**

After 24 hr incubation fluopsin C produced cell death on proliferative layer of homotypic spheroids of MCF-7 cells (Figure 4a), which was accentuated at 48 and 72 hr. The most severe alteration was found with 1 µM of fluopsin C (Figure 4b). In addition, labeling with Hoechst 33342 or Rhodamine 123 reinforced the presence of dead cells around the spheroids, once only DNA content was labeled, unlike that detected in control, where the peripheral cells of spheroid were found to incorporate rhodamine 123.

For each treatment, alterations in the mean spheroidal volume (mm³) for 3 days were determined (Figure 5). For control, increases in volume
Figure 4. Fluopsin C alters tumor spheroid morphology of MCF-7 cells. (A) Control spheroids and those treated with fluopsin C, at concentrations of 0.25 µM, 0.5 µM, and 1 µM for 24, 48, and 72 hr. Fluopsin C at 1 µM accentuates cell death in the peripheral layer of the spheroids compared to the control (magnification 200×). (B) Control spheroids and spheroids treated with fluopsin C at 1 µM for 72 hr showing mitochondrial activity, labeled with Rhodamine123 (red) and DNA content, labeled with Hoechst33342 (blue). After 72 hr, due the cytotoxicity of fluopsin C, a reduced in mitochondrial activity was observed in the peripheral layer compared to the control spheroids (magnification 100×).
Figure 5. Fluopsin C influences the mean spheroidal volume (A). Fluopsin C interferes with the volume of tumor spheroids, in order to attenuate their growth, mainly at 1 µM. Letters represent the differences (p-value ≤ 0.05) between the spheroid means at different times and treatments as a result of the analysis of variance (two-way ANOVA) and post-hoc Tukey's multiple comparison test. Values expressed as mean ± standard deviation. (B) Growth curve in mean spheroid volume at times 0, 24, 48, and 72 hr after treatment with fluopsin C. (C) After 72 hr treatment with fluopsin C the spheroids were transferred to a plate with an adherent surface, in a condition without exposure to fluopsin C for 24 hr, and then it was observed that fluopsin C interferes with the recovery of the proliferative profile (highlighted in red) of the spheroids treated compared to the control spheroids.

Further, for the concentration of 1 µM, an elevation of 23.6% (0.04 ± 0.01) in volume was found at 72 hr in relation to time 0 (0.03 ± 0.01).

In relation to differences between treatments (Figure 5ab), at time 0, there were no significant differences. Within 24 hr, the volume of spheroids at concentrations of 0.25 µM (0.03 ± 0.01), 0.5 µM (0.03 ± 0.01), and 1 µM (0.03 ± 0.01) of fluopsin C were statistically equal to each other, and markedly less than control, indicating that fluopsin C attenuated the rise in spheroid volume after 1 day of treatment. In 48 hr at concentrations of 0.25, 0.5 or 1 µM, the volume of the spheroids was equal to the volume of control at 24 hr suggesting a delay of 1 day spheroid volume elevation. In addition, at 72 hr, the volume in treatments with 0.25 and 0.5 µM were equal to control at 48 hr, while in the treatment with 1 µM (0.04 ± 0.01) the volume was equal to value at 24 hr in control.

Twenty-four hr after the transfer of spheroids to a plate with an adherent surface and without treatment, with only complete culture medium, distinct proliferative patterns were detected between
treatments (Figure 5c). Control spheroids exhibited a more accentuated cell proliferation, while for the fluopsin C concentrations tested, there was a smaller area of cell occupation.

**Fluopsin C effects on gene expression**

Regarding relative gene expression (Figure 6), an increase in mRNA expression of genes CDKN1A (4.1x), TRAF-2 (2.2x), BBC3 (3.2x), ERN1 (2.7x), GADD45A (2x), and GPX1 (4.2x) was observed accompanied by a decrease in H2AFX (2.7x) after 6 hr incubation with 1 µM fluopsin C.

**Discussion**

Antibiotic molecules present versatility in their bioactivity acting against microorganisms and tumor cells (Gao et al. 2020; Vasconcelos et al. 2020; Viana et al. 2022). In the current study, fluopsin C was cytotoxic to MCF-7 cells, interfering with cell viability in a concentration-dependent manner, presenting an IC₅₀ of 1 µM after 24 hr treatment. Our findings corroborate the work of Ma et al. (2013) where an IC₅₀ of 0.9 µM and 1.03 µM was observed for MCF-7 and MD-MBA-231 mammary tumor lines, respectively. In addition, Ma et al. (2013) demonstrated that fluopsin C was more cytotoxic to tumor than normal breast epithelial cells and immortalized human mammary epithelial cells (HMLE) lineage (IC₅₀ 2.4 µM). In a murine model, a lack of nephrotoxicity, moderate hepatotoxicity and an LD₅₀ of 4 mg/kg were noted by Navarro et al. (2019).

As it is cytotoxic to MCF-7 cells, data demonstrated that fluopsin C exerted different modes of action that induced cell death, including (1) disruption of cell metabolism, (2) DNA damage, (3) cell cycle arrest in G₁, (4) adverse MMP alterations, and (5) attenuation of the formation of new clones. Thus, fluopsin C displayed antitumor activity similar to other antibiotics, currently used in the clinic, such as anthracycline and non-anthracycline antibiotics (Bolzán and Bianchi 2018; Gao et al. 2020; Lüpert et al. 2010; Slingerland, Guchelaar, and Gelderblom 2012). For example, DXR as its mode of action enhances production of ROS resulting in DNA damage and arresting the cell cycle (Lüpert et al. 2010), which is used in hematologic cancers and solid tumors (Slingerland, Guchelaar, and Gelderblom 2012), as well as bleomycin, used in cancers of the digestive tract, cervix lung, and esophagus (Gao et al. 2020), where the predominant main mode of action is a genotoxic effect (Bolzán and Bianchi 2018).

When eukaryotic cells are exposed to a toxic agent, these cells activate evolutionarily conserved molecular mechanisms to attenuate stressful events and ensure cell homeostasis (Galluzzi, Yamazaki, and Kroemer 2018). However, when the stress adaptation threshold is reached, molecular mechanisms of regulated cell death (RCD) are activated (Galluzzi, Yamazaki, and Kroemer 2018). Considering the cytotoxic effect of fluopsin C, our findings showed that the MCF-7 lineage activates molecular responses indicative of cellular stress in vitro, such as (1) DNA damage response, (2) unfolded protein response, (3) mitochondrial stress signaling, and (4) cell death, when the adaptation threshold is reached (Galluzzi, Yamazaki, and Kroemer 2018), regulating gene expression of GADD45A, GPX1, CDKN1A, TRAF-2, ERN1, H2AFX, and BBC3, sensor molecules involved in this cellular response. Regarding the arrest of cell division progression, an increase in CDKN1A (4.1x) gene expression was observed and attenuation of the gene H2AFX (2.7x) 6 hr following fluopsin C incubation (1 µM). The enhanced expression of the CDKN1A gene may be involved in the arrest of cell division progression, as its role in the arrest of the cell cycle in G₁ phase was previously reported (Wang et al. 2017; Zhang et al. 2019). CDKN1A encodes the protein p21WAF1/CIP1 which possesses cyclin-dependent kinase inhibitory activity and participates in cell cycle regulation (Kreis, Louwen, and Yuan 2019), as well as, in response to DNA damage in a p53-dependent or independent manner (Deng et al. 2018). It is also of interest that a decrease in the expression of the gene H2AFX was observed. This attenuation might be related to the arrest of the cell cycle in G₁, since the presence of the poly(A) mRNA of this gene is fundamental for progression of the cell cycle. Griesbach et al. (2021) found that depletion of this poly(A) mRNA in HeLa cells reduced the expression of histone H2A. X SL and H4 SL mRNA, which are specifically polymerized in the S phase, indicating arrest of cell
division progression in the G1 phase. In this context in addition to the rise in CDKN1A expression, data suggest that fluopsin C induces G1 phase arrest by diminishing expression of H2AFX poly(A) mRNA in MCF-7 cells.

As demonstrated by Ma et al. (2013), in terms of cytotoxicity, the concentration of 1 μM fluopsin C did not produce significant cytotoxicity in MCF-7 cells after 6 hr. For this reason, the comet assay was performed after 3 hr exposure to fluopsin C (1 μM), ensuring that the positive response in this assay was not associated with significant cytotoxicity. Our findings demonstrated a genotoxic response after 3 hr incubation with fluopsin C (1 μM), and an elevation in mRNA expression of GADD45A gene (twofold) after 6 hr treatment. The GADD45A gene encodes the GADD45α protein, which participates in DNA damage signaling, the DNA repair system, and cell growth arrest, with an increase in transcripts in response to stress conditions, such as DNA damage produced by agents harmful to this biomolecule (Cretu et al. 2009; Pietrasik et al. 2020), mainly in the presence of oxidative stress (Tong et al. 2020; Zhang et al. 2006). Based upon these observations evidenced suggested that the genotoxic activity of fluopsin C is mediated by oxidative stress, since fluopsin C elevated the concentration of ROS in MCF-7 cells (Ma et al. 2013), and by enhanced expression of mRNA of GADD45A. Since fluopsin C initiated DNA damage and elevated ROS levels, regulation of mRNA expression of the GPX1 gene was investigated. This gene encoding the glutathione peroxidase enzyme, which in a situation of oxidative stress displays enhanced expression of its transcripts (Wagner et al. 2009). Data demonstrated that the MCF-7 cells positively regulated expression of mRNA of the GPX1 gene when exposed to fluopsin C, indicating a requirement of cellular expression of this gene, due to the presence of oxidative stress.

Another event that was observed, and which may be involved in cellular stress is the rise in mRNA expression of the ERN1 gene, the gene encoding the ribonuclease inositol-requiring protein 1 (IRE1) receptor protein that participates in the unfolded protein response (UPRER) signaling pathway in response to endoplasmic reticulum stress, due to accumulation of nonfunctional proteins (Hetz and Papa 2018; Wang and Kaufman 2016). Upon prolonged activation, IRE1 interacts with the tumor necrosis factor receptor-associated factor 2 (TRAF-2) molecule and initiates cell death via the ASK-1/JNK/MAPK pathway (Almanza et al. 2019). Our findings showed that fluopsin C induced endoplasmic reticulum stress through up-regulation of mRNA expression of the ERN1 and TRAF-2 genes. Since fluopsin C enhanced reticulum stress, it is conceivable that cellular processes such as protein maturation, post-translational modifications, fatty acid and sterol biosynthesis, xenobiotic detoxification, and intracellular calcium accumulation are compromised after treatment with this antibiotic (Anelli and Sitia 2008).

In addition to these mechanisms, alterations in cell morphology were noted that are characteristic of oncosis-type cell death process, such as formation of protrusions in the plasma membrane and increase in cytoplasmic volume, in agreement with the findings of Ma et al. (2013). This process of volume rise results from influx of water into the intracellular environment, attributed to an osmotic imbalance initiated by disruption of the ion pumps, arising from depletion of ATP (Weerasinghe and M 2012). Data demonstrated that fluopsin C depleted ATP by disrupting the MMP. Another aspect that reinforces the evidence of death from oncosis and not apoptosis is the labeling with the annexin dye, as evidenced by (1) an absence of externalization of phosphatidylserine, characteristic of the apoptotic process, and (2) obtaining only dead cells due to necrosis using flow cytometry after 24 hr treatment with fluopsin C (1 μM). Further, Ma et al. (2013) reported that fluopsin C induced cell death through a process independent of caspases evidence of the absence of death by apoptosis. Regarding the expression of genes involved in cell death, it was observed that fluopsin C markedly enhanced mRNA expression of the BBC3 gene 3-fold an indication of the requirement of this gene product during the process of death by oncosis. This gene encodes a protein of the BCL-2 family, present in mitochondria, acting to control mitochondrial permeability, response to reticulum stress, and plays a role in the apoptotic process of cell death (Li, Lee, and Lee 2006; Reimertz...
et al. 2003). Other natural compounds that induce oncrosis in tumor cells are sanguinarine, artesunate, and dihydroabietic acid derivatives (QC2 and QC4) (Gonzalez et al. 2010; Hallock et al. 2007; Yu et al. 2020; Zhou et al. 2013).

In the current study for the first-time fluopsin C was found to attenuate the rise in tumor spheroid volume of MCF-7 cells, reducing the volume of spheroids at the three concentrations tested. Specifically considering the IC_{50} content, fluopsin C interfered with cell proliferation, maintaining the spheroidal volume at 72 hr equal to the volume of control at 24 hr. Interestingly, peripheral cells exhibited lower mitochondrial activity, indicating possible mitochondria dysfunction as noted in monolayer culture. This behavior might be justified due to the cytotoxic effect on peripheral cells of the tumoral spheroid. Peripheral cell damage was also reported by Baek et al. (2016) in spheroids of the HeLa, A549, 293 T, SH-SY5Y, and U2OS cell lines treated with DXR, initiating ATP depletion through mitochondrial dysfunction. Regarding the recovery profile of cell proliferation on an adherent surface, the 1 μM concentration showed a low proliferative rate, indicating interference of cell division progression in peripheral cells of the treated spheroids. Further a decrease in the proliferative rate was also observed in the clonogenic assay, demonstrating that after 24 hr treatment, the proliferation/formation of new colonies was influenced by fluopsin C. This influence may be attributed to enhanced expression of GADD45A, since the elevation in GADD45α reduces cell growth, as well as formation of new colonies in T24 and 5637 cells (Han et al. 2019).

Considering the modes and mechanisms of action of fluopsin C exerted on MCF-7 cells, data suggest that this biomolecule presents different targets for antitumor strategies. Among the tumor characteristics described as therapeutic targets by Hanahan and Weinberg (2016), fluopsin was found to (1) prevent cell proliferation, (2) produce genetic instability, (3) induce cell death, and (4) deregulate energy metabolism, thus suggesting fluopsin C be considered a strong candidate for a new class of chemotherapeutic agent for breast cancer therapy, mainly due to the presence of programmed necrotic induction mediated by a natural antibiotic compound (Yu et al. 2020).

Conclusions

Data demonstrated that fluopsin C was concentration-dependent cytotoxic and exhibited antiproliferative characteristics in MCF-7 cells. Fluopsin C induced arrest of cell division progression, DNA damage, and decreased colony formation associated with its mechanism of action by involving alterations of mRNA expression of genes associated with adaptive responses to cell stress and cell death (Figure 7). Further, data showed that fluopsin C interfered with the volume of tumor spheroids in

Figure 7. Graphical abstract. (A) Different targets for antitumor strategies mediated by fluopsin C, such as antiproliferative activity, genetic instability, alteration of gene expression, induction of cell death, and deregulation of energy metabolism. (B) Fluopsin C results in morphological changes in MCF-7 cells, causing the formation of blebs in the cytoplasmic membrane (bubbles) and cellular swelling. In addition, fluopsin C induces the arrest of cell division progression, DNA damage, decreased colony formation, mitochondrial dysfunction, and its mechanism of action by altering the mRNA expression of genes involved in adaptive responses to cell stress and cell death.
order to attenuate their growth. Thus, the observed antiproliferative properties of fluopsin C suggest a potential use as an anticancer agent.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

All data generated or analyzed in the current study were included in the published article.

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