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Antitumoral effect of novel synthetic 8-hydroxy-2-((4-nitrophenyl)thio) naphthalene-1,4-dione (CNN16) *via* ROS-mediated DNA damage, apoptosis and anti-migratory effect in colon cancer cell line

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ABSTRACT

Colorectal cancer (CRC) is estimated as the third most incident cancer and second in mortality worldwide. Moreover, CRC metastasis reduces patients' survival rates. Thus, the study and identification of new compounds with anticancer activity selectively to tumor cells are encouraged in the CRC treatment. Naphtoquinones are compounds with several pharmacologic activities, including antitumoral properties. Therefore, this study aimed to investigate the anticancer mechanism of synthetic 8-Hydroxy-2-(P-Nitrothiophenol)-1,4-Naphthoquinone (CNN16) in colon cancer cell line HCT-116. CNN16 showed an IC_{50} of 5.32 μM in HCT-116, and 9.36, 10.77, and 24.57 μM in the non-cancerous cells MRC-5, MNP-01, and PMBC, respectively, evaluated by the MTT assay. CNN16 showed an anticlonogenic effect in HCT-116 and induced cell fragmentation identified by flow cytometry analysis. Furthermore, we observed that CNN16 presented genotoxicity and induces reactive oxygen species (ROS) after 3 h of treatment visualized by alkaline comet assay and DCFH-DA dye fluorescence, respectively. Furthermore, CNN16 caused cellular membrane disruption, reduction in the mitochondrial membrane polarization, and the presence of apoptotic bodies and chromatin condensation was visualized by differential stained (HO/FD/PI) in fluorescent microscopy along with *PARP1*, *TP53*, *BCL-2,* and *BAX* analyzed by RT-qPCR. Results also evidenced inhibition in the migratory process analyzed by wound healing assay. Therefore, CNN16 can be considered as a potential new leader molecule for CRC treatment, although further studies are still necessary to comprehend the effects of CNN16 in *in vivo* models to evaluate the anti-migratory effect, and toxicology and assure compound safety and selectively.

1. Introduction

Colorectal cancer (CRC) was estimated as the third most incident cancer (10%) and second in mortality (9.4%) in the world, with more fatalities in countries with low human development index (HDI) (Sung et al., 2021). CRC cases are related to environmental factors like poor diet, high alcohol, and tobacco consumption. Genetic mutations also have a substantial role in the development of CRC as well, due to mutations in key genes leading to uncontrolled replication and thus cellular hyperproliferation and enhancing cell metastatic profile (Arnold et al., 2017; Sawicki et al., 2021; Siegel et al., 2022).

In CRC, metastasis are frequently diagnosed in more than half of the

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patients, which reduces the median survival period to <8 months and around 11% of cases present a 5-year survival rate as the best prognosis when the metastasis remains local (Recio-Boiles and Cagir, 2022; Valderrama-Treviño et al., 2017). CRC has a high metastatic profile with most common sites in the liver, lung, and peritoneum, but cell infiltrations have also been described in bone, spleen, brain, and distant lymph nodes (Chow and Chok, 2019; Moro et al., 2018; Valderrama-Treviño et al., 2017; Yang et al., 2018). CRC incidence rate has increased in patients aged <50 years with the incidence rising by 1% to 4% per year in countries such as the United States, Canada, Australia, and others with high HDI levels. Furthermore, by 2030, it is estimated that the incidence rates of colon and rectal cancers in patients aged 20 to 34 years will increase by 90% and 124.2%, respectively (Bailey et al., 2015; Siegel et al., 2019; Vuik et al., 2019).

In metastatic CRC, chemotherapy regimens are constituted by the administration of oxaliplatin, 5-fluorouracil, and/or irinotecan in combination with targeted agents including bevacizumab or aflibercept (antiangiogenic) and, according to the RAS/BRAF status, cetuximab or panitumumab (anti-epidermal growth factor receptor (EGFR) drugs. However, mutations in drive genes lead to the acquisition of intrinsic resistance to the usual chemotherapy and reduce the overall survival rates in the clinic (Armaghany et al., 2012; Carethers and Jung, 2015; Lièvre et al., 2008). Studies have focused on identifying new compounds with anticancer properties as a crucial step for controlling this disease. However, results are often limited due to the high toxicity observed in the chemotherapy regimen (Aoullay et al., 2020; Gobran et al., 2020; Nishijima et al., 2018).

Quinones are organic compounds widely distributed in nature, occurring in animals, plants, and microorganisms. These molecules present essential biological attributes and are related to metabolic energy production, being a target for new pharmacological studies (López et al., 2015; Pereyra et al., 2019). Among quinones, naphthoquinones are compounds that have presented several pharmacological properties, such as antitumoral, anti-inflammatory, antimicrobial, anthelmintic, antifungal, and antiviral potential (Checker et al., 2009; Dias et al., 2018; Futuro et al., 2018; Gonzaga et al., 2019; Lee et al., 2019; Mata-Santos et al., 2015; Portilho et al., 2022, 2021; Qiu et al., 2018).

Cancer therapy aims to trigger cell death mechanisms, that can be induced by direct or indirect DNA damage resulting in deregulation of the tumor replication machinery by genotoxic agents (Li et al., 2021). Endogenous agents, such as Reactive Oxygen Species (ROS), and/or exogenous agents such as UV light, ionizing radiation (IR), and chemotherapy agents can trigger DNA damage, initiating the apoptotic process pathway in cells (Cavalcante et al., 2019). In this sense, the p53 and poly [ADP-ribose] polymerase 1 (PARP-1) proteins are involved in several mechanisms in response to cellular stress, such as DNA repair, cell-cycle arrest, senesce, apoptosis, and along with anti-apoptotic mediators are important pharmaceutic targets in colon cancer treatment (Abraha and Ketema, 2016; Reilly et al., 2019).

Therefore, this study aimed to evaluate the anticancer properties of novel synthetic naphthoquinones *in vitro*. Besides, understand the mechanism of action of 8-hydroxy-2-((4-nitrophenyl)thio)naphthalene-1,4-dione (CNN16) (Fig. 1) compound in the colon cancer cell line HCT-116. As such, the role of CNN16 in proliferation, DNA damage, redox potential, cell death mechanism, migration, and modulation of apoptotic pathway-related genes to attest its potential as possible new chemotherapy for CRC therapy.

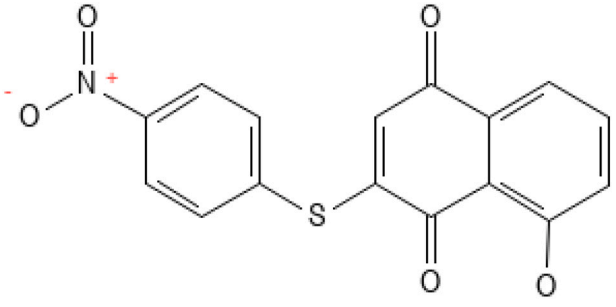

Fig. 1. Chemical structure of 8-hydroxy-2-((4-nitrophenyl)thio)naphthalene-1,4-dione (CNN16).

2. Methods

2.1. Cell culture

Human cell lines used in this study were established from the colon (HCT-116), gastric intestinal adenocarcinoma (AGP-01), gastric diffuse adenocarcinoma (ACP-02), melanoma (SKMEL-19), normal pulmonar fibroblast (MRC-5), and non-malignant gastric epithelium (MNP-01). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco®) supplemented with 10% fetal bovine serum (FBS, Gibco®), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco®), at 37 °C and 5% CO_2.

2.2. Isolation and in vitro culture of peripheral mononuclear blood cells

Peripheral mononuclear blood cells (PMBC) were isolated as described by Bausinger and Speit (Bausinger and Speit, 2016). Healthy volunteers were selected, and blood samples (20 mL) of three nonsmoker 18 to 30-year-old men with no history of exposure to mutagens were obtained by venipuncture. Research participants gave written informed consent and the study was approved by the Research Ethics Committee of the Federal University of Ceará (CAAE: 52352121.0.0000.5054). After isolation, PBMC cells were seeded in RPMI 1640 medium, supplemented with 10% FBS, 4% phytohemagglutinin A (Gibco-Invitrogen, Carlsberg, CA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco®) and cultured at 37 °C and 5% CO_2.

2.3. Chemicals

The 8-hydroxy-2-((4-nitrophenyl)thio)naphthalene-1,4-dione (CNN16) was synthesized as described previously by Portilho et al. (2021). The CNN16 was generously donated by Professor David Rodrigues da Rocha from the Federal Fluminense University, Brazil. Doxorubicin was purchased from Sigma® and used as a positive control. Compounds were dissolved in DMSO to a concentration of 1 mM and stored at −20 °C as a master stock solution.

2.4. Cytotoxicity assay

At first, CNN16 was evaluated at a concentration of 10 μM against a panel of cell lines. HCT-116, AGP-01, ACP-02, SKMEL-19, and MRC-5 cell lines were plated in a concentration of (3×10^3 cells/well), after cell attachment, compounds were added for 72 h and then viability was measured by MTT salt (3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide) (0,5 mg/mL) and absorbance was determined by spectrometry (570 nm) (Berridge et al., 2005). Then, cell lines with 80% of cell death at 10 μM, were selected to be evaluated in a concentration-response curve (CNN16: 0.325 μM - 10 μM). To assess the selectivity, the compound was also tested against MNP-01 (0.312 μM – 20 μM), and PMBC (0.781 μM - 50 μM) non-tumoral cell lines. Inhibition concentration (IC_{50}) was determined by absorbance at 570 nm. For subsequent assays, CNN16 was used in three different concentrations (2.5, 5, and 10 μM) in triplicate based on its IC_{50} after 72 h in the HCT-116 cell line.

2.5. Clonogenic assay

Cells were seeded in a 6-well-plate at 3×10^2 cells/well respecting an equal number of cells in every group, and then treated with CNN16 for

24 h. After that, the supernatant was removed and wells were washed three times with PBS 1×. Culture medium was added and clonogenic assay was followed for 7 days. Afterwards, cells were washed three times with PBS 1×, fixed in a solution with methanol, acetic acid, and destilated water (1:1:8) for 30 min, then colonies were stained with Giemsa solution (1:20) for 30 min and photographed to further analysis (Franken et al., 2006; Plumb, 2004; Rafehi et al., 2011). Doxorubicin (0.5 μM) was used as the positive control.

2.6. Cell cycle analyses

To investigate CNN16 action in cell cycle progression, cells were seeded at a concentration of 1×10^5 cells/ well in a 12-well-plate. After 24 h, cells were treated with CNN16 for 72 h. Next, the supernatant was collected and cells were dissociated. All cellular content was centrifuged, then the pellet was resuspended and fixed in 80% ethanol solution at 4 °C for 30 min in order to allow cellular permeabilization. Following, cells were centrifuged, pellet resuspended, and incubated with propidium iodide (50 μg/mL) for 25 min. Thereafter, cells were resuspended in PBS 1× (free serum) and were evaluated using flow cytometry (BD FACSVerse™). A total of 10.000 events were analyzed using FlowJo® software (Dobashi, 2005; Dobashi et al., 2003).

2.7. Quantification of Reactive Oxygen Species (ROS)

In order to analyze modifications in ROS levels under CNN16 exposure, the HCT-116 cell line was seeded at a concentration of 3×10^5 cells/well in a 12-well-plate, after cell attachment, cells were treated for 1.5 and 3 h with different concentrations of CNN16, and the H_2O_2 was used as positive control (2 mM). Next, DCFH-DA dye was added (10 μM) for 30 min, then fluorescence was measured (Ex/Em: 488/525 nm) in BioTek Synergy HTX microplate reader, and ROS levels were estimated (Bilski et al., 2002).

2.8. Alkaline comet assay

To analyze the genotoxicity potential of CNN16 in the colon cancer cell line, we performed an alkaline comet assay as previously described with modifications (Singh et al., 1988). HCT-116 were plated in a 6-well-plate at a density of 2.5×10^5 and after cell attachment, CNN16 and Doxorubicin (1 μM) were added for 3 h. Next, cells were removed and the pellet was resuspended in low melting agarose (0.8%) and pippeted on microscope slides previously covered with normal melt agarose (1.5%) and chilled at 4 °C for 5 min.

After agarose polymerization, slides were incubated in alkaline lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10) at 4 °C overnight. Then, slides were submitted to horizontal electrophoresis at 34 V and 300 mA for 20 min in an alkaline buffer solution (300 mM NaOH and 1 mM EDTA, pH ≥ 13.0), slides were washed twice with Mili Q water at 4 °C and fixed with ethanol 100% for 3 min and stained with ethidium bromide (20 μg/mL) for analyses.

A total of 100 nuclei (comets) were counted for samples in fluorescence microscopy (Olympus® BX41). Thus, the damage index (DI) was calculated based on tail size (DNA fragmentation), and cells were further classified into five scores depending on the damage level (0–4) (Burlinson et al., 2007).

2.9. Evaluation of cellular membrane integrity

Propidium Iodide (PI) dye was used to assess cellular membrane integrity by flow cytometry (Portilho et al., 2022). Thereby, the HCT-116 cell line was seeded in a 24-well-plate in a concentration of $1{,}5 \times 10^4$ cells/well, after 24 h cells were treated with CNN16 (2.5, 5, and 10 μM) for 72 h, Doxorubicin was used as the positive control (0.5 μM). At the end of treatment time, cells were harvested and all content was centrifugated and resuspended in IP (5 μg/mL). After 15 min, cells were centrifuged again, resuspended in PBS 1×, and analyzed in the flow cytometry (BD FACSVerseTM), at the end a total of 10.000 events were evaluated using FlowJo® software.

2.10. Analysis of mitochondrial membrane potential

To investigate the potential of CNN16 to alter the mitochondrial transmembrane potential (ΔΨM) (Aranha et al., 2021; Green and Reed, 1998) the HCT-116 cell line was seeded in a 24-well-plate in a concentration of $1{,}5 \times 10^4$ cells/well, after cell attachment, HCH-116 was treated for 72 h with different concentrations of CNN16 (2.5, 5, and 10 μM), and Doxorubicin was used as the positive control (0.5 μM). Then, cells were collected and stained with Rhodamine 123 (5 μg/mL) dye for 30 min. Lastly, cells were resuspended in PBS 1× and analyzed by flow cytometry (BD FACSVerseTM), a sum of 10.000 events were analyzed using FlowJo® software.

2.11. Morphologic analyses of cell death by fluorescence microscopy

HCT-116 was plated in a 12-well-plate in a concentration of 1×10^4 cells/well, after 24 h, cells were treated with CNN16. Doxorubicin was used as the positive control (0.5 μM) for 72 h. Thus, cells were detached and the pellet was resuspended in a solution with fluorescent stains Hoechst 44432 (HO), fluorescein diacetate (FD), and propidium iodide (PI) for 5 min at 37 °C. 150 cells per sample were analyzed in a fluorescence microscope (Olympus® BX41). Differential stains provide visualization of viable, apoptotic, and necrotic cells according to changes in cell morphology (Chan et al., 2015). Viable cells present intact nucleus and plasmatic membrane with no iodide propidium stained, and cells in apoptosis were identified with chromatin condensation, fragmentation, and apoptotic body formation. Cells with necrotic profiles presented a loss in membrane integrity and propidium iodide incorporation (Mesquita et al., 2018).

2.12. Wound healing assay

Analysis of the anti-migratory potential of CNN16 was performed by wound healing assay as described previously (Liang et al., 2007). HCT-116 was seeded at a concentration of 3×10^6 cells/well into a 12-well-plate. After 24 h, scratches were performed with a sterile 10 μL tip, then wells were washed twice with PBS and fresh medium was added. Cells were treated with CNN16 and pictures were taken at different times (0, 7, 14 and 21 h). The migration was followed until the doubling time (21h) of HCT-116 (data not shown), assuring no interference of cell proliferation in the migratory profile. Migration of the cells was observed in an optical microscope (Evos® XL) and the scratch area was measured using ImageJ Software ® (Suarez-Arnedo et al., 2020).

2.13. Total mRNA extraction and qRT-PCR analysis

For analysis of gene expression, HCT-116 cell line were plated in the concentration of 6×10^4 cells/well in a 12-well plate, after adherence cells were treated with CNN16 (5 μM) and positive control Doxorrubicin (0.5 μM) in a non-cytotoxic time of 24 h (Supplementary Fig. 1). After exposure, cells were trypsinized and collected and the total mRNA extraction was performed by TRIzol® Reagent (Life Technologies®, USA) according to the manufacturer's protocol. Then, the RNA concentration was determined using NanoDrop (Thermo Scientific), and then reverse transcription was performed using the High-Capacity cDNA kit (Life Technologies, USA).

Quantitative real-time PCR (RT-qPCR) was performed by the Fast SyberGreen kit (Applied Biosystems, USA). Expression levels of *PARP1* (NM_001618.3), *TP53* (*NM*_205264.1), *BCL-2* (NM_000633.3), and *BAX* (NM_138764.5) relative expression levels were normalized and determined using *ACTB* (NM_001101.5) gene as an endogenous control. Primer efficiency was determined for all genes described.

All requirements proposed in Minimum Information for Publication of Quantitative Real-Time PCR Experiments - MIQE Guidelines were followed (Bustin et al., 2009). The expression level was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008), considering non-treated cells (negative control) as a calibrator of the experiments.

2.14. Statistics analyses

Assays were executed in three independent experiments in triplicate. Results were shown as a mean ± standard deviation (SD). Negative control was compared to treated samples by Analysis of Variance (ANOVA) followed by Bonferroni's post-test. Significant differences were considered with an interval of confidence of 95% ($p < 0.05$). GraphPad Prism 5.01 software was used for data analysis and graph design.

3. Results

3.1. CNN16 displays cytotoxicity preferentially to colon cancer cell line

First, the cytotoxic effect of CNN16 was evaluated in a single dose of 10 μM against malignant and non-malignant cell lines. Our results showed that CNN16 only presented a cytotoxic effect below 10 μM against colon cancer cell HCT-116 (IC_{50}: 5.32 μM). Then, CNN16 was evaluated in a concentration-response curve showing minor cytotoxic against MRC-5 (IC_{50}: 9.36 μM). and non-malignant gastric cell line MNP-01 (IC_{50}: 10.77 μM), demonstrating almost 2-fold selectivity. Then, CNN16 was also evaluated against peripheral mononuclear blood cells (PMBC), and the selectivity was approximately 5-fold (IC_{50}: 24.57 μM), ensuring that CNN16 is a safe compound with low toxicity to normal cells when compared to clinical use, Doxorubicin (Table 1).

3.2. CNN16 induces a reduction in the number of colonies and enhances cell fragmentation in colon cancer cell line

To confirm the MTT assay, the cytotoxic effect of CNN16 in HCT-116 colony formation was evaluated by clonogenic assay. There was a reduction in the number of colonies at around 40%, 70%, and 98% in concentrations of 2.5, 5, and 10 μM, respectively (Fig. 2A). All concentrations caused a significant reduction in the surviving fraction of cells ($p < 0.0001$) when compared to untreated cells (Fig. 2B).

To further understand the *in vitro* mechanism of synthetic naphthoquinone CNN16 in cell cycle progression of colon cancer cell line HCT-116, we performed a cell cycle analyses by flow cytometry. In our study, as shown in Fig. 2C, was observed that CNN16 did not cause cell cycle arrest in any concentration. However, it was evidenced cell fragmentation in all concentrations tested ($p < 0.001$), characterized by the increase of cells in the Sub-G1 phase (Fig. 2D).

3.3. CNN16 leads to enhanced ROS levels followed by DNA damage in the HCT-116 cell line

We sought to investigate the effect of CNN16 on reactive oxygen species (ROS) production and its genotoxic effect in the HCT-116 cell line. The DCFH-DA dye was used to measure *in vitro* ROS levels after 1.5 and 3 h of treatment with CNN16 in the HCT-116 colon cancer cell line. In 1.5 h was not observed difference in ROS production when compared to non-treated cells (CN), otherwise, CNN16 significantly enhanced ROS levels by around 83%, 94%, and 96% in 2.5, 5, and 10 μM, respectively, after 3 h of exposure. (Fig. 3A). Next, the comet alkaline assay was performed to evaluate the genotoxic effect of CNN16, in Fig. 3B we can visualize the increase in the tail size (DNA fragmentation) of the comet along with CNN16 concentration enhancement, the tail size was used to classify the DNA damage into five scores (Fig. 3C), proving that CNN16 caused genotoxic damage after 3 h of treatment in all concentrations tested.

3.4. CNN16 causes alteration in cellular membrane integrity and alters mitochondrial transmembrane potential (ΔΨm)

To investigate cellular alterations related to cell death mechanism, we evaluate the cellular membrane integrity and mitochondrial transmembrane potential (ΔΨM) using Propidium iodide (PI) and Rhodamine 123 (Rho 123) fluorophores, respectively, by flow cytometry (Fig. 4). Results showed that CNN16 leads to enhance in cellular membrane disruption defined by cells PI + (Fig. 4A). A increase in cells negative for Rho 123 staining (cells Rho 123 -) (Fig. 4 C) was also visualized after 72 h of treatment with CNN16, indicating a reduction in the mitochondrial transmembrane potential (ΔΨM). Altogether, as visualized in Figs. 4B and C, the data indicate that CNN16 causes cellular alterations typical of the apoptotic mechanism in a concentration-dependent manner (2.5 μM $p < 0.001$, 5 and 10 μM $p < 0.0001$). Thus, the cytotoxic mechanism of CNN16 may be intrinsically related to the triggering of the apoptotic mechanism in the HCT-116 cell line.

3.5. CNN16 leads to cell death by apoptosis in colon cancer cell line

To best characterized the apoptotic mechanism, fluorescent microscopy was used to visualize morphologic alterations typical of the apoptotic process. In agreement with ours previous results, the synthetic naphthoquinone CNN16 was able to induce cell death in a concentration-dependent manner. As shown in Fig. 5, HCT-16 cells showed morphological changes typical of the apoptotic mechanism, such as chromatin condensation and the formation of cell membrane blebbing and apoptotic bodies (red arrows). Cells without treatment remain with intact chromatin and no changes in the plasmatic membrane. Moreover, cells were classified according to the morphological changes into viable, apoptotic, and necrotic. CNN16 increased the number of cells with apoptotic features in the concentration of 2.5 (15%), 5 (43%), and 10 μM (67%) after 72 h of treatment. The compound did not show a significant modification in the percentage of necrotic cells.

3.6. CNN16 inhibits cell migration in vitro

The anti-migratory effect of CNN16 in HCT-116 cells was evaluated by wound healing assay. It is worth citing that the experiments were performed before cell doubling time (21 h) to avoid the effect of cell

Table 1
Screening of synthetic naphthoquinone CNN16 against malignant and non-malignant cell lines. Cells were treated with CNN16 for 72 h and cytotoxicity was evaluated by MTT assay. CI [95%]: 95% confidence interval, IC_{50}: half maximal inhibitory concentration, PMBC: peripheral mononuclear blood cells, ND: not determined.

IC_{50} (μM) CI [95%]							
Compounds	HCT-116	AGP-01	ACP-02	SKMEL-19	MRC-5	MNP-01	PMBC
CNN16	5.32 (3.9–7.26)	ND	ND	ND	9.36 (8.28–10.58)	10.77 (8.69–13.35)	24.57 (16.82–35.87)
Doxorubicin	0.45 (0.3–0.5)	0.25 (0.19–0.33)	0.82 (0.63–1.07)	0.45 (0.27–0.78)	0.2 (0.16–0.25)	0.6 (0.27–1.3)	ND

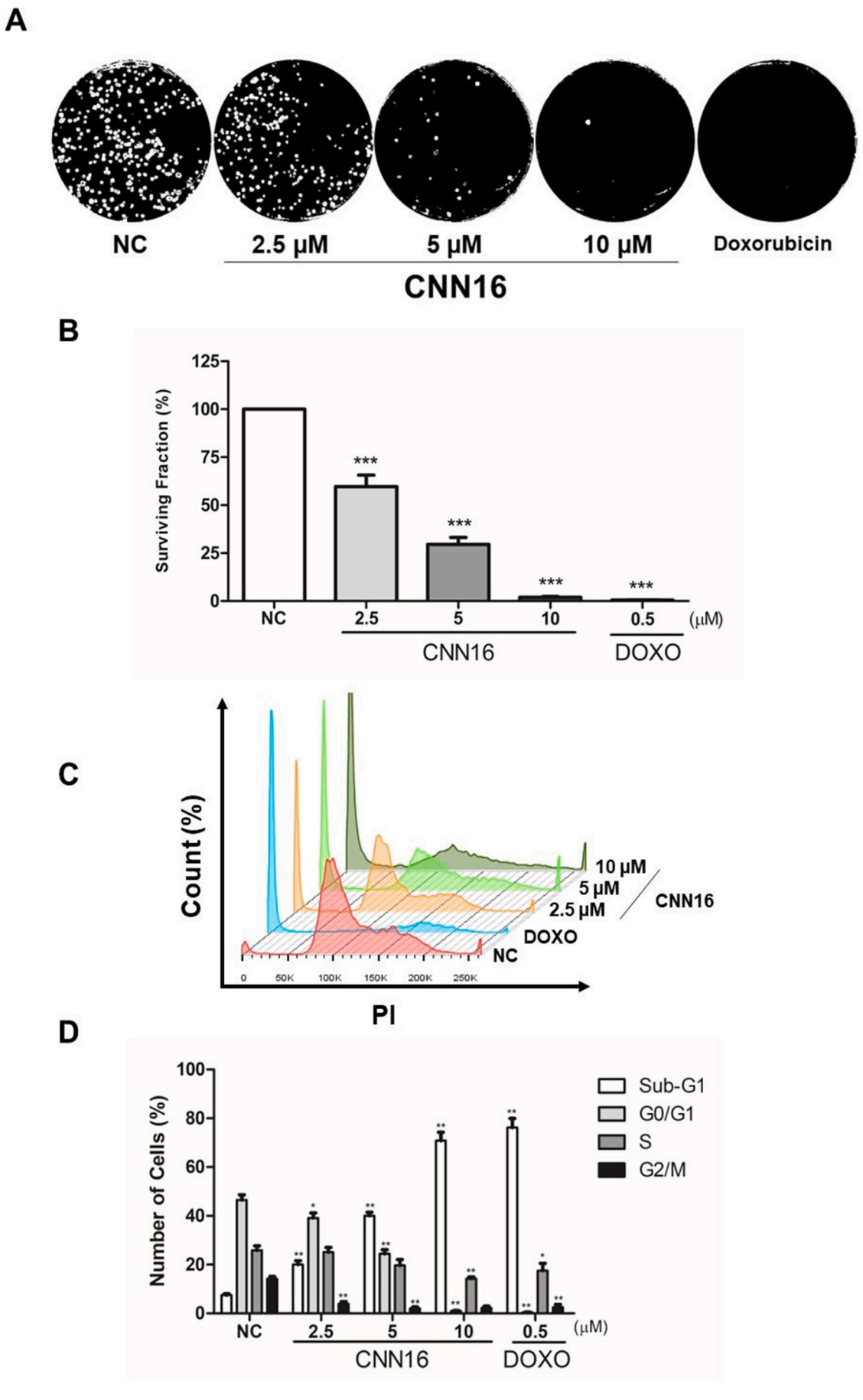

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Fig. 2. CNN16 shows an anticlonogenic effect with highly cell fragmentation in the HCT-116 cancer cell line. (A) Pictures of cell colonies after 7 days of culture. (B) The surviving fraction of colonies after treatment. (C) Cells were exposed to CNN16 (2.5, 5, and 10 μM) for 72 h and analyzed in flow cytometry for DNA content with Propidium Iodide (PI). The number of cells in Sub-G1, G0/G1, S, and G2/M phases was calculated using FlowJo® software. The histogram is presented in all conditions evaluated showing the number of cells intercalated with IP. (D) Data are presented as the mean ± SD of three independent experiments. Doxorubicin (0.5 μM) was used as the positive control in both assays. Statistical analysis was performed with ANOVA followed by Bonferroni's posttest. Significant differences: $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$. DOXO: Doxorubicin. NC: Negative Control. PI: Propidium Iodite.

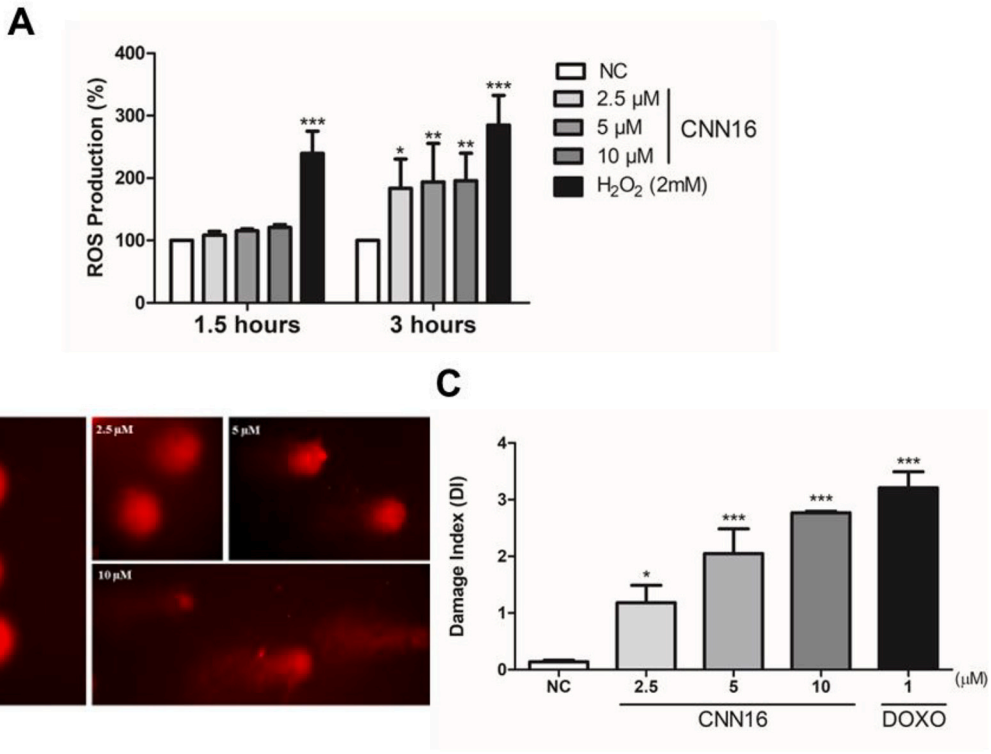

Fig. 3. Synthetic naphthoquinone CNN16 alters ROS levels and presents a genotoxic effect in the HCT-116 cell line. (A) The HCT-116 cell line was treated for 1.5 and 3 h with different concentrations of CNN16 to determine the ROS levels with DCFH-DA dye. (B) In the comet assay cells were visualized in fluorescent microscopy (40×) and classified into five scores depending on the damage level (0–4). (C) Data indicated the increase in the damage index (DI) along with the increase in the concentration of CNN16 after 3 h of treatment. The H_2O_2 (1 mM) and Doxorubicin (1 μM) were used as positive control for ROS evaluation and DNA damage, respectively. Data are presented as the mean ± SD of three independent experiments. Statistical analysis was performed with ANOVA followed by Bonferroni's posttest. Significant differences: $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$. Scale bar: 100 μm. DOXO: Doxorubicin. NC: Negative Control.

proliferation (data not shown). As shown in Fig. 6, we observed that the synthetic compound prevented cell migration in all concentrations evaluated from 7 h of exposure when compared to the negative control (untreated).

3.7. CNN16 alters the expression of genes related to DNA repair and apoptotic pathway

Finally, we evaluated the expression of the genes associated with DNA repair and mediators of the apoptotic pathway. Genes *PARP1* and *BCL-2* associated with DNA repair and apoptosis prevention in cancer, respectively, presented a reduction in transcript levels when treated with CNN16 ($p < 0.0001$). On the other hand, the suppressor tumoral gene *TP53* ($p < 0.0001$) and the pro-apoptotic transcript *BAX* ($p < 0.001$) displayed an increase in gene expression in response to CNN16 treatment (Fig. 7).

4. Discussion

Colorectal cancer (CRC) is one of the most incident neoplasms in the world, and have been noticed an increase in the number of diagnosed cases (Bray et al., 2018; Sung et al., 2021). Chemotherapeutic compounds such as doxorubicin, 5-fluorouracil, oxaliplatin, and irinotecan are commonly used for the CRC treatment, however, adverse reactions and chemoresistance have been described in the literature (De Rosa et al., 2015; Liang et al., 2010; Xiong and Xiao, 2018). Hence, the study of new compounds with antitumoral activity and selective properties is still necessary to reduce adverse reactions and improve treatment responses in patients with CRC.

Synthetic compound 8-Hydroxy-2-(P-Nitrothiophenol)-1,4-Naphthoquinone (CNN16) showed cytotoxicity in colon cancer cell line HCT-116, with low toxicity in normal cells. Results reported that CNN16 leads to reduce in the colony number and an increase in cellular cell fragmentation. After 3 h of treatment were observed enhancement in ROS levels linked to CNN16 genotoxic effect *in vitro*. Furthermore, CNN16 triggered a loss of cellular membrane integrity and a reduction in the mitochondrial membrane potential along with morphologic alterations typical of the apoptotic mechanism. The synthetic naphthoquinone also modulated the migratory profile of the colon cancer cell line and altered the mRNA expression of genes related to DNA repair and apoptotic pathway which may be associated with its highly antitumoral potential against the HCT-116 cell line.

Naphtoquines are compounds with wide pharmacological applicability. The synthesis of naphthoquinone derivatives have been shown to enhance its pharmacological potential and reduce the toxicity effect *in vitro* (Cardoso et al., 2017; Lara et al., 2021; Moreira et al., 2021;

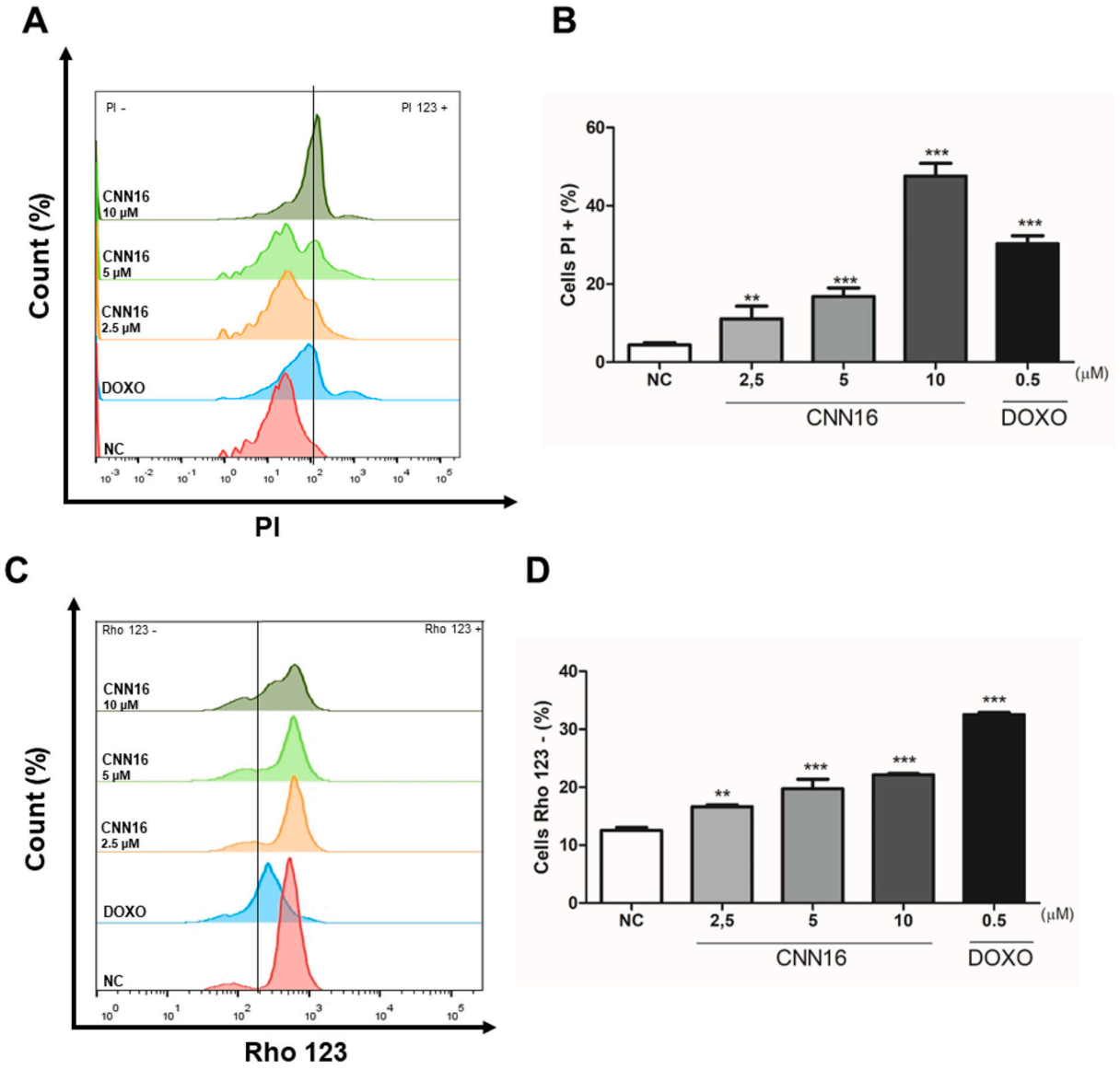

Fig. 4. CNN16 induces enhance in cell membrane fragmentation and loss in mitochondrial transmembrane potential. The HCT-116 cell line was treated with CNN16 for 72 h and then stained with Propidium Iodide (PI) and Rhodamine 123 (Rho 123) to analyze cellular modifications by flow cytometry. (A) Representative histogram of PI staining (10,000 events). (B) The average of PI positive cells (PI +). (C) Representative histogram of Rho 123 staining (10,000 events). (D) The average of Rho 123 negative cells (Rho 123 -). Data were analyzed by FlowJo software and are presented in bars as the mean ± SD of three independent experiments. Doxorubicin (0.5 μM) was used as the positive control in both assays. Statistical analysis was performed with ANOVA followed by Bonferroni's posttest. Significant differences: $^{**}p < 0.001$, $^{***}p < 0.0001$. DOXO: Doxorubicin. NC: Negative Control. PI: Propidium Iodite. Rho 123: Rhodamine 123.

Moreira et al., 2017; Portilho et al., 2022; Portilho et al., 2021). The synthetic naphthoquinone CNN16 counts with the addition of a 4-NO_2Ph radical (Portilho et al., 2021), studies describe nitro compounds (NO_2) as active substances that have been associated with mutagenicity and genotoxicity potential. The nitro group possesses a strong electron-attracting capability which generates sites with electrons deficits within the molecules, thus leading to direct interaction with biological nucleophiles present in living systems, such as proteins, amino acids, nucleic acids, and enzymes, this interaction is directly related to its pharmacological activity (Jezuita et al., 2021; Nepali et al., 2018).

Studies have sought to better understand the *in vitro* mechanism of naphthoquinones in cancer treatment and its selectivity. In this study, we showed that CNN16 presented antitumoral activity against colon cancer cell line HCT-116 below 10 μM being at least 2-fold more selective to tumoral cell lines than to normal cell lines. In fact, it was visualized a selectivity of 5-fold to colon cancer cells when compared to PMBC cells with other compounds derived from naphthoquinones (Iijima et al., 2019, p.; Komarnicka et al., 2021). Similar to our study, Zhang et al. (Zhang et al., 2018) evaluated eight synthetic compounds derivated from 1,4-naphthoquinone *in vitro*, and among them, one compound presented high toxicity against breast, liver, and ovarian cancer cell lines, and low toxicity against normal cell line HSF, showing that synthetic naphthoquinones seem to present less toxicity to the normal cell than to tumoral one, which may take to low adverse effects in clinical practice (Iijima et al., 2019; Komarnicka et al., 2021).

The antiproliferative effect of CNN16 in colon cancer cells was confirmed by clonogenic assay, in which HCT-116 presented a reduction in colony number in all concentrations. Liang et al. (Liang et al., 2017) also attested the anticlonogenic activity of another natural naphthoquinone Shikonin in CRC, as well as in our study, in two different colon cancer cell lines (HCT-116 and SW480) in a concentration-dependent manner. Therefore, we attested the cytotoxic and cytostatic effect of CNN16 in the CRC cell line, indicating that its intracellular mechanism can be related to cell cycle regulation and thus in the

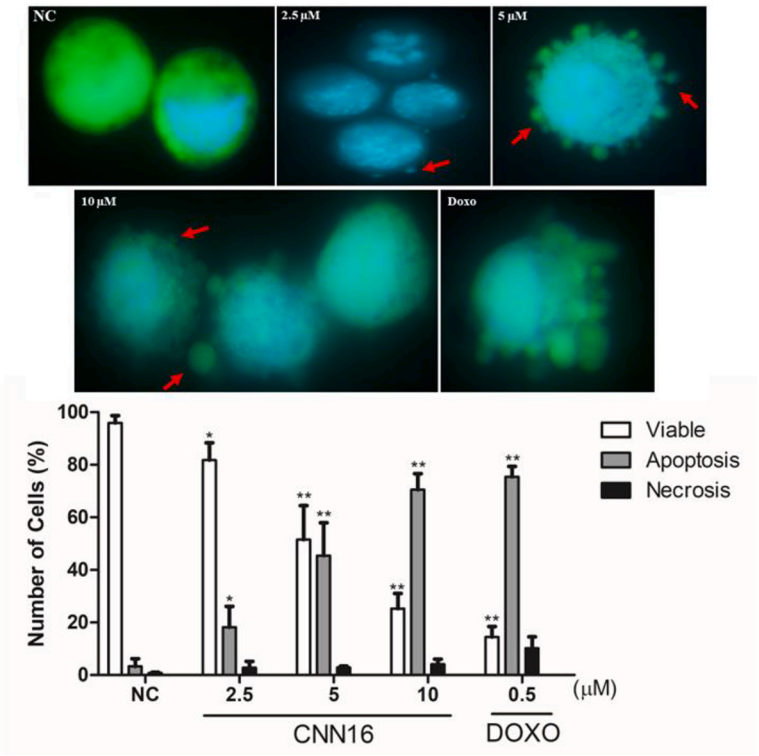

Fig. 5. Synthetic naphthoquinone CNN16 induces modifications in colon cancer cell line morphology typical of apoptotic cell death. Doxorubicin (0.5 μM) was used as the positive control for cell death. Cells with a viable profile present cytoplasm stained by fluorescein diacetate (FD) and nucleus by Hoechst 3342 (HO), apoptotic cells show cytoplasmatic modifications as cell membrane blebbing/apoptotic bodies formation (red arrows) and chromatin condensation stained by HO visualized in fluorescent microscopy (100 x). Data are presented as the mean ± SD of three independent experiments. Statistical analysis was performed with ANOVA followed by Bonferroni's posttest. Significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar: 40 μm. DOXO: Doxorubicin. NC: Negative Control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

replication process.

Cancer cells have a high proliferative rate when compared to most normal cells, as a consequence, the target cell division process can reduce its effects on normal cells and the adverse effects of chemotherapy (Alimbetov et al., 2018). For example, the tumor suppressor gene *TP53* is highly downregulated in cancer, and around 43% of CRC tumors harbor p53 mutations reducing its activity and contributing to tumor development once antitumoral responses to DNA repair, cell cycle arrest, cell death, and differentiation are affected (Liebl and Hofmann, 2021). Another gene, such as *PARP1* gene has also an important role in response to DNA damage, once it acts in DNA damage repair. The inhibition of *PARP1* expression can enhance cancer cells' sensitivity to DNA-damaging agents, favoring selectively cellular death (Abu-Sanad et al., 2015; Alhusaini et al., 2021). We visualized that CNN16 caused a decrease in the number of cells in the S phase in the higher concentration tested and also reduced the *PARP1* gene expression, upregulated nearly 35-fold the expression of *TP53* transcripts, as well as, showed a genotoxic effect *in vitro*. The HCT-116 cells express wildtype p53 but do not express the *MLH1* gene, a tumor suppressor gene involved in DNA mismatch repair (MMR), and are considered MMR-defective, being considered a good *in vitro* CRC cell line to evaluate the response to DNA damage (Koi et al., 1994; Stubbert et al., 2010). Thus, our data attests to the potential of CNN16 to trigger cell cycle arrest, and sensitive colon cancer cell lines to DNA damage (Jarolim et al., 2018; Liang et al., 2017; Wang et al., 2019; Wellington, 2015).

Several studies have shown the cytotoxic and genotoxic activity of naphthoquinones describing increasing in the ROS production due to the redox potential of these compounds, high levels of ROS can lead to DNA break and so trigger mechanisms of cell death (Wellington, 2015). Apoptosis is a promising target for cellular death due to its no inflammatory effect like necrosis. During carcinogenesis, to support uncontrolled growth, cancer cells lose sensitivity to apoptosis. Thus, drugs with the potential to reactivate this process are excellent candidates to become new chemotherapeutic agents (Fouad and Aanei, 2017; Pfeffer and Singh, 2018).

Studies in leukemia, liver, and gastric cancer have also shown enhancement in ROS levels linked to apoptotic cell death mechanisms (Li et al., 2018; Liu et al., 2018; Wang et al., 2019). Increased intracellular ROS levels lead to up-regulation of anti-apoptotic genes such as *BCL-2* and *BCL-XL*, and pro-apoptotic genes such as *BAX*. Such action alters membrane mitochondrial potential, increasing mitochondrial outer membrane permeabilization (MOMP) and the release of death-promoting proteins such as cytochrome *c*, with consequent activation of caspases-9 and -3 (Chipuk et al., 2006; Liang et al., 2017). As shown, enhancement in ROS levels can lead to DNA damage and apoptosis by the intrinsic pathway. Interestingly, CNN16 displayed genotoxic activity with enhancing ROS levels, cell fragmentation, mitochondrial depolarization, apoptotic cell death, reduction in *BCL-2* transcript levels, and improved expression of pro-apoptotic gene *BAX*. Thus, the regulation of pro and anti-apoptotic genes along with the triggering of the intrinsic apoptotic pathway may be related to the cytotoxic effect of CNN16 against colon cancer cells.

Studies have identified CRC metastasis in different organs, reducing the survival rate of patients (Yang et al., 2018). In our study, we showed the anti-migratory potential of CNN16 in colon cancer cells. Kee et al. (Kee et al., 2017) showed the antimetastatic effect of a natural naphthoquinone, β-Lapachone, in colon cancer cell line (CT26), and in *in vivo* experimental model, the compound was able to inhibit the migration and invasion potential of CTC26 cells *in vitro*. Altogether, these results show that naphthoquinones display antimetastatic mechanisms which are essential for the treatment of patients with CRC metastasis. Therefore, we evidenced the antitumoral role of CNN16 *in vitro* against HCT-116 cell line, indicating the synthetic compound as possible brand-new chemotherapy with a selective response to the colon cancer cells.

5. Conclusions

Thus, our findings indicate that novel synthetic 8-Hydroxy-2-(P-Nitrothiophenol)-1,4-Naphthoquinone (CNN16) is a novel and promising anticancer agent with selective activity against colon cancer cell line (HCT-116). Results suggested that CNN16 causes DNA damage by ROS production, regulation of pro and anti-apoptotic gene expression,

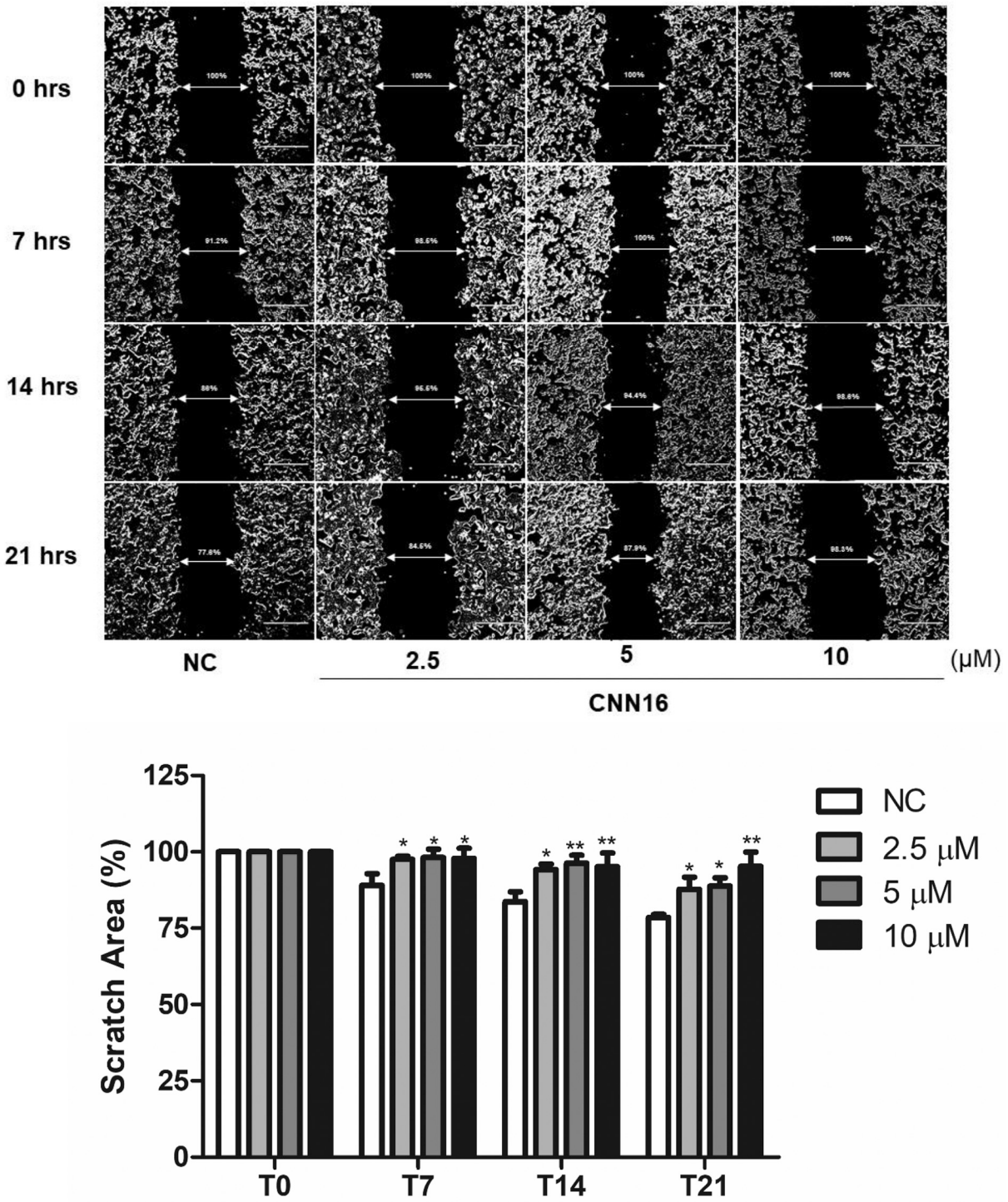

Fig. 6. Synthetic compound CNN16 inhibits cell migration *in vitro*. The migratory profile of HCT-116 was observed in an optical microscope. Pictures showed that the compound tested prevented scratch area closure when compared to wells with no previous treatment. Scratch area in time 0 h (T0) was set as 100%. Data are presented as the mean ± SD of three independent experiments. Statistical analysis was performed with ANOVA followed by Bonferroni's posttest. Significant differences: $*p < 0.01$, $**p < 0.001$. Scale bar: 400 μm. NC: Negative Control.

and triggering mitochondrial polarization, which can be involved in activation of the intrinsic apoptosis pathway, all these data together can help us to better understand cytotoxicity mechanisms of this compound *in vitro*. We also evidenced the anti-migratory effect of the compound *in vitro*, being a good pharmacological option for the treatment of metastatic CRC. Still, further studies are necessary to comprehend the effects of CNN16 in other colon cancer models and affirm its therapeutic efficacy.

CRediT authorship contribution statement

Emerson Lucena da Silva: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Felipe Pantoja Mesquita:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Ingryd Nayara de Farias Ramos:** Conceptualization, Methodology,

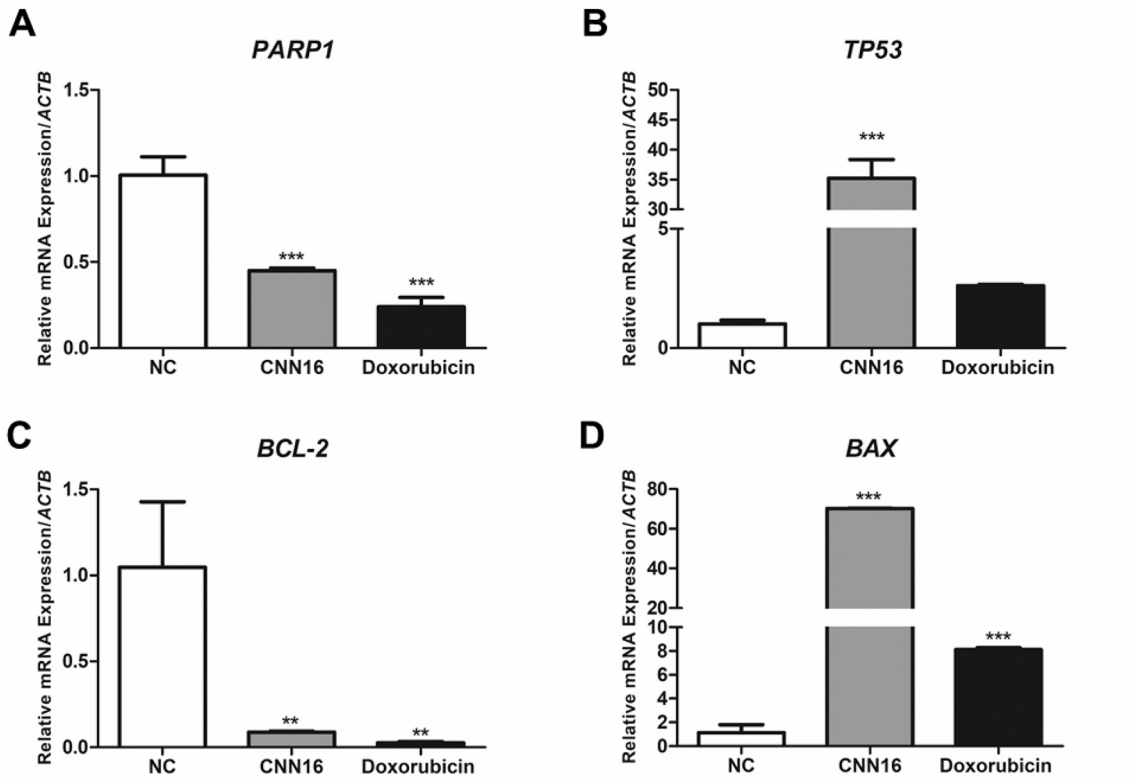

Fig. 7. CNN16 modifies expression of genes related to the DNA repair and intrinsic apoptotic pathway in the HCT-116 cell line. After 24 h of exposure to CNN16 (5 μM) mRNA levels of (A) *PARP1*, (B) *TP53*, (C) *BCL-2,* and (C) *BAX* were analyzed by qPCR. Gene expression was normalized by endogenous gene *ACTB* and the NC group was used as a calibrator of the experiment. Data are presented as the mean ± SD of three independent experiments. Cells without previous treatment (NC) were compared to cells treated for 24 h with CNN16 (5 μM) and with the positive control Doxorubicin (0.5 μM) with ANOVA followed by Bonferroni's posttest. Significant differences: $^{**}p < 0.001$, $^{***}p < 0.0001$. NC: Negative Control.

Formal analysis, Investigation, Visualization. **Carinne Borges de Souza Moraes Rego Gomes:** Methodology, Formal analysis. **Caroline dos Santos Moreira:** Methodology, Formal analysis. **Vítor Francisco Ferreira:** Conceptualization, Resources, Supervision, Funding acquisition. **David Rodrigues da Rocha:** Conceptualization, Resources, Supervision, Funding acquisition. **Marcelo de Oliveira Bahia:** Resources, Supervision. **Caroline Aquino Moreira-Nunes:** Conceptualization, Writing – original draft, Supervision. **Carolina Rosal Teixeira de Souza:** Conceptualization, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Rommel Mario Rodrigues Burbano:** Resources, Supervision, Funding acquisition. **Raquel Carvalho Montenegro:** Conceptualization, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Raquel Carvalho Montenegro reports financial support was provided by National Council for Scientific and Technological Development. Raquel Carvalho Montenegro has patent #BR1020180073850 pending to INPI - National Institute of Industrial Property.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2022.116256.

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