J Biosci Vol. 42, No. 4, December 2017, pp. 657–664
DOI: 10.1007/s12038-017-9718-1

In vitro leishmanicidal, antibacterial and antitumour potential of anhydrocochlioquinone A obtained from the fungus *Cochliobolus* sp.

Fernanda F Campos[1,2]*, Jonas P Ramos[3], Djalma M De Oliveira[4], Tânia M A Alves[2], Elaine M De Souza-Fagundes[3], Carlos L Zani[2], Fábio C Sampaio[5], Attilio Converti[6] and Betania B Cota[2]

[1]*Faculty of Medicine, Federal University of Vales do Jequitinhonha e Mucuri, Diamantina, Brazil*

[2]*Laboratory of Chemistry of Bioactive Natural Products, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil*

[3]*Department of Physiology and Biofisics, Federal University of Minas Gerais, Belo Horizonte, Brazil*

[4]*Department of Chemistry, Southwest Bahia State University, Jequié, Bahia, Brazil*

[5]*Department of Pharmacy, Federal University of Vales do Jequitinhonha e Mucuri, Diamantina, Brazil*

[6]*Department of Civil, Chemical and Environmental Engineering, University of Genova, Genova, Italy*

**Corresponding author (Email, ffcmicro@gmail.com)*

MS received 3 June 2017; accepted 28 September 2017; published online 7 November 2017

The bioassay-guided fractionation of the ethyl acetate extract of the fungus *Cochliobolus* sp. highlighted leishmanicidal activity and allowed for anhydrocochlioquinone A (ANDC-A) isolation. MS, 1D and 2D NMR spectra of this compound were in agreement with those published in the literature. ANDC-A exhibited leishmanicidal activity with EC_{50} value of 22.4 μg/mL (44 μM) and, when submitted to the microdilution assay against Gram-positive and Gram-negative bacteria, showed a minimal inhibitory concentration against *Staphylococcus aureus* ATCC 25295 of 128 μg/mL (248.7 μM). It was also active against five human cancer cell lines, showing IC_{50} values from 5.4 to 20.3 μM. ANDC-A demonstrated a differential selectivity for HL-60 (SI 5.5) and THP-1 (SI 4.3) cell lines in comparison with Vero cells and was more selective than cisplatin and doxorubicin against MCF-7 cell line in comparison with human peripheral blood mononuclear cells. ANDC-A was able to eradicate clonogenic tumour cells at concentrations of 20 and 50 μM and induced apoptosis in all tumour cell lines at 20 μM. These results suggest that ANDC-A might be used as a biochemical tool in the study of tumour cells biochemistry as well as an anticancer agent with durable effects on tumours.

Keywords. Anhydrocochlioquinone; *Cochliobolus* sp.; leishmanicidal; antimicrobial; tumour cells

1. Introduction

Leishmaniases are diseases caused by protozoan parasites belonging to more than 20 *Leishmania* species that are transmitted to humans by the bites of infected female phlebotomine sandflies (WHO 2017). In the Americas, a total of 51,098 cases of cutaneous leishmaniasis and mucosal form were reported in 2014, with an incidence rate of 19.76 cases per 100,000 inhabitants, being that Brazil (20,418 cases), Colombia (11,586) and Peru (6231) held 75% of all cases (PAHO 2016). The main forms of the disease, i.e. cutaneous, visceral and mucocutaneous, affect poor and vulnerable groups in the world, hence remaining a leading cause of morbidity and mortality in most of the endemic and poor countries (WHO 2010; Savoia 2015); furthermore, most of antileishmanial drugs were introduced many decades ago (Ehrenberg and Ault 2005; WHO 2010).

Antibiotics have increased the life span and guaranteed the success of some advanced medical practices (Gould and Bal 2013). However, the emergence of resistant bacteria is occurring worldwide, jeopardizing the efficacy of antibiotics, mainly due to (Barlett *et al.* 2013; Ventola 2015): (i) overuse, (ii) inappropriate prescribing, (iii) addressing antibiotic abuse in farm animal and (iv) drop in development

of newer antibiotics. In relation to the last item, Ventola (2015) said that the number of new antibiotics developed and approved has decreased steadily over the past three decades (although four new drugs were approved in 2014), leaving fewer options to treat resistant bacteria.

The population growth and ageing combined with lifestyle risk factors have increased the number of cases of cancer. Currently, cancer is a leading cause of death and disability in low-income and middle-income countries, and the treatments of some cancer types are palliative and remain neglected compared with other diseases (Farmer *et al.* 2010). On the other hand, uniformly the cancers are fatal, often as a result of development of resistance to traditional drugs (Housman *et al.* 2014). Thus, to overcome the chemoresistance to current therapies and improve patient outcome, novel treatment agents are highly needed.

In turn, microorganisms have been studied as source of many chemotherapeutics, and amongst them, fungi can be promising sources. In the present work, the bioassay-guided fractionation of the ethyl acetate extract from the endophytic fungus *Cochliobolus* sp. resulted in the isolation of anhydrocochlioquinone A (ANDC-A), which was then tested for its leishmanicidal and antimicrobial activities. In addition, its cytotoxic effects on five human tumour cells and differential toxicity in comparison with Vero cells and PBMC were assessed. In previous study, Campos *et al.* (2008) demonstrated that the chromatographic fractionation of the extract of *Cochliobolus* sp. was validated as a drug target for *Trypanosoma cruzi*. These authors identified Cochlioquinone A and isocochlioquinone A as enzyme inhibitors that showed activity in the assay with *Leishmania amazonensis*. These results stimulated us to search and isolate new bioactive compounds from this fungus with leishmanicidal, antibacterial and antitumour potential.

2. Materials and methods

2.1 *Microorganism maintenance and extraction of secondary metabolites*

The endophytic fungus *Cochliobolus* sp. was grown and maintained in potato dextrose agar (PDA) as previously described by Campos *et al.* (2008). After the inoculum, dishes were stored at 4 °C and subcultured every two weeks. The fungus was grown on PDA in 1000 Petri dishes and extracted with ethyl acetate at room temperature for 48 h. The organic phase was filtered and the solvent evaporated under vacuum in a rotary-evaporator at 45°C (model 4000, Laborota Heidolph, Germany). Residual solvent was eliminated after vacuum centrifugation at 40°C using a Spress SpeedVac® vacuum centrifuge (Thermo-Savant SPD SC250 Express, Holbrook, NY, USA). Sample for leishmanicidal (10 mg mL^{-1}), antimicrobial (according to MIC determination described in section 2.5) and cytotoxicity (10 mg mL^{-1}) testing were prepared in 0.5% aqueous dimethyl sulfoxide.

2.2 *Chromatographic fractionation of the extract*

An aliquot of the organic extract (3.0 g) was subjected to preparative purification by medium pressure liquid chromatography (MPLC) with methanol:water (10:90→100:0) as a gradient elution system. A total of 148 fractions of 50 mL each were collected. They were pooled into 24 groups based on their similarity, as assessed by thin-layer chromatography after elution with a 95:5 dichloromethane:methanol mixture. Group 22 (121 mg) was active in *in vitro* leishmanicidal screening, and its purity by HPLC analysis was 99%.

The MPLC was carried out using a preparative C18 (LiChroprep RP-18, 40–63 μM, Merck, Darmstadt, Germany) reverse-phase column (250×20 mm) and a LC8-AD pump (Shimadzu, Kyoto, Japan) at 20 mL/min flow rate. Thin-layer chromatographic analyses were conducted on pre-coated commercial silica gel G-60/F254 (Merck) plates. The spots were visualized after spraying the plate with a 1:1 mixture of 1% (w/v) vanillin and 10% (v/v) H_2SO_4 ethanolic solutions followed by heating at 100°C for 5–10 min. Analytical RP-HPLC analyses were developed in a HPLC system (Shimadzu) equipped with a LC6-AD pump and a SPD M-10A VP Diode Array.

2.3 *Structure elucidation of the isolated bioactive compound*

The bioactive compound isolated from group 22 was dissolved in perdeuterated solvent containing 0.1% tetramethylsilane as the internal chemical shift standard, and spectral data were obtained according to Campos *et al.* (2008). The mass spectra (MS) were recorded by a mass spectrometer, model LCQ Advantage (Thermo Finnigan, San Jose, CA, USA), equipped with an electrospray ion (ESI) source, and the NMR ones by a spectrometer, model DRX 400 (Bruker, Rheinstetten, Germany).

The isolated bioactive compound is a red wine amorphous solid with UV_{max} absorption in methanol of 199, 273 485 nm. The electrospray ionization mass spectra (ESI-MS) analysis exhibited quasi-molecular ion peaks at *m/z* 537.3 $[M+Na]^+$ and 477.1 $[M+Na\text{-}Ac]^+$). ^1H NMR spectra data ($CDCl_3$, 400 MHz) were: δ_H 0.84 (3H, m, H-1), 0.86 (3H, d, *J* 7.0 Hz, H-28), 1.08 (1H, dd, *J* 7.0, 4.5 Hz, H-2a), 1.13 (3H, br. s, H-25), 1.13 (3H, d, *J* 6.5 Hz, H-27),, 1.19 (3H, br. s, H-23), 1.20 (3 H, br. s, H-24), 1.42-1.46 (1H, m, H-2b), 1.47-1.55, (1H, m, H-3), 1.49-1.53 (1H, m, H-19α), 1.54-1.62 (1H, m, H-20*β*), 1.55 (3H, br. s, H-26), 1.67-1.72 (1H,

m, H-16β), 1.70-1.75 (1H, m, H-20α), 1.78-1.85 (1H, m, H-16α), 2.02 (1H, m, H-15α), 2.05 (3H, br s., H-30), 2.11 (1H, ddd, *J* 12.0, 12.0, 5.0 Hz, H-19β), 2.31 (1H, dt, *J* 13.5, 3.5, 3.5 Hz H-15β), 3.19 (1H, br. dd, *J* 12.0, 4.0, Hz, H-21), 3.20 (1H, dd, *J* 11.5, 4.0 Hz, H-17), 3.40 (1H, br. dddd, *J* 6.5, 6.5, 6.5 and 6.5 Hz), 4.93 (1H, dd, *J* 6.5, 5.5 Hz, H-4), 6.33 (1H, s, H-12) and 6.63 (1H, s, H-11). On the other hand, ^{13}C NMR (CD_3OD, 100 MHz) spectra showed: δ_C 11.09 (C-1), 15.46 (C-28), 18.26 (C-27), 20.19 (C-25), 20.85 (C-30), 21.65 (C-20), 23.85(C-23), 23.95 (C-2), 24.51 (C-16), 26.07 (C-24), 27.05 (C-26), 32.64 (C-5), 34.83 (C-19), 36.43 (C-3), 37.85 (C-15), 38.64 (C-18), 71.83 (C-22), 80.03 (C-4), 81.09 (C-17), 81.51 (C-14), 84.62 (C-21), 110.55 (C-12), 117.17 (C-9), 132.88 (C-11), 147.56 (C-13), 148.95 (C-6), 148.95 (C-8), 170.61 (C-29), 181.16 (C-7), 184.98 (C-10).

2.4 *Leishmanicidal activity*

Leishmanicidal activity was determined using promastigotes of *Leishmania amazonensis* IFLA/BR/196/PH-8 obtained from lesions of infected hamsters (Callahan *et al.* 1997). Parasites were differentiated into amastigotes by rising temperature from 26 to 32°C and lowering the pH of the Schneider's medium from 7.2 to 6.0. After 7 days, amastigote density was adjusted to 1×10^8 parasites per mL, and 90 µL of this suspension were added to each well of 96-well plates. Ten microliters of test samples and controls solutions were added to obtain the desired concentrations. Plates were incubated at 32°C for 72 h, and then cells viability was determined by the methyl thiazolyl tetrazolium (MTT) assay (Teixeira *et al.* 2002). Results were expressed as percent inhibition in relation to the controls without drug according to Campos *et al.* (2008). Amphotericin B at 0.2 µg/mL was used as a positive drug control. The effective concentration to kill 50% of the parasites (EC_{50}) was obtained from dose-response curves fit of two or more independent experimental datasets to a four-parameter logistic dose-response (Campos *et al.* 2008) using the software GraphPad Prism (version 4.03, CA, USA).

2.5 *Antimicrobial activity*

Antimicrobial activity either of the *Cochliobolus* sp. extract or the ANDC-A-rich fraction obtained by MPLC was tested against six target bacterial strains, namely *Staphylococcus aureus* ATCC 25295, *Escherichia coli* ATCC 18804, *Salmonella typhimurium* ATCC14028, *Pseudomonas aeruginosa* ATCC27853, *Klebsiella oxytoca* ATCC 49131 and *Listeria monocytogenes* ATCC 19115. Bacterial strains were maintained on Brain Heart Infusion agar according Campos *et al.* (2008). Mueller Hinton broth was prepared in accordance with the CLSI document M7-A6 and used for Minimal Inhibitory Concentration (MIC) bacterial assays according to NCCLS (NCCLS 2003) with modifications (Campos *et al.* 2008).

2.6 *Cell line assays*

2.6.1 *Human tumour and Vero cell lines:* The *in vitro* cytotoxicity activity of the ANDC-A-rich fraction obtained by MPLC was tested against five different human tumour cell lines, namely human promyelocytic leukemia cell line (HL-60), human immortalized line of T lymphocyte cells (Jurkat), acute monocytic leukemia cell line (THP-1), human breast cancer cell line (MCF-7), and human colorectal carcinoma cell line (HCT-116). In addition, normal cells of African green monkey kidney line (Vero) and human peripheral blood mononuclear cells (PBMC) were used as models. The cells (except PBMC) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium or Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin. Leukemia cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum enriched with 2 mM L-glutamine.

Cell lines were inoculated at 1×10^4 cells (MCF-7, HCT and Vero), 5×10^4 cells (HL-60) and 1×10^5 cells (Jurkat and THP-1) per well. Plates were pre-incubated for 24 h at 37 °C before the addition of the test compounds. The half maximal inhibitory concentration (IC_{50}) was determined using non-serial eight dilutions (from 100 to 1.5 µg/mL). All cell cultures were incubated in a 5% CO_2/95% air-humidified atmosphere at 37 °C for 48 h. Dimethyl sulfoxide at concentration of 0.5% (v/v) was used as negative control. Cell viability was estimated using MTT (Monks *et al.* 1991). Etoposide, doxorubicin and cisplatin were used as positive controls. The selectivity indexes (SI) were determined as the ratios of the IC_{50} values for tumour cell lines to those for normal (PBMC and Vero) cells (Badisa *et al.* 2009).

2.6.2 *Human peripheral blood mononuclear cells:* Human peripheral blood mononuclear cells (PBMC) were obtained from healthy adult volunteers of both sexes (HEMOMINAS, protocol n. 105/2004) by centrifugation of heparinized venous blood over Ficoll cushion (Pinto *et al.* 2011). PBMC were collected from the interphase after Ficoll separation and washed thrice in RPMI 1640 medium before further processing. Cells were cultured in complete RPMI 1640 medium, supplemented with 5% (v/v) heat-inactivated, pooled human serum type AB and 2.0 mM L-glutamine, containing 1,000 U/mL penicillin, 1,000 µg/mL streptomycin and 25 µg/mL fungisone.

Briefly 1×10^6 cells/mL (1.5 x 10^5 cells per well) were seed in flat-bottomed microtiter plates. Cultures were

stimulated with 2.5 μg/mL phytohemagglutinin (PHA) and incubated for 72 h at 37°C in a 5% CO_2/95% air-humidified atmosphere (Gazzinelli *et al.* 1983). Cell proliferation was determined using a MTT-based colorimetric assay (Jiang and Xu, 2003). The half maximal inhibitory concentration (IC_{50}) was determined using non-serial eight dilutions (from 100 to 1.5 μg/mL).

2.6.3 *Determination of subdiploid DNA content:* Quantification of subdiploid DNA-content as indicative of DNA fragmentation by apoptosis was performed by propidium iodide (PI) staining and flow cytometry analysis (Nicoletti *et al.* 1991). All tumour cells were treated with ANDC-A at final concentration of 20 μM and incubated in a 5% CO_2/95% air-humidified atmosphere at 37°C for 24 h. Afterwards, cells were centrifuged, resuspended in a hypotonic fluorochrome solution (50 μg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100) and incubated at 4°C for 4 h. The PI fluorescence of 10.000 individual nuclei was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Data were analysed by the FlowJo software (TreeStar Inc, Ashland, OR, USA).

2.6.4 *Clonogenic cell survival assay:* MCF-7 and HCT-116 cells were inoculated in D-MEM up to a concentration of 200 cells per mL. Then, 2.0 mL of the resulting suspensions were seeded into each well of six-well plates. After 4 h, they were treated with vehicle control (0.5% v/v DMSO per well) or with 20 or 50 μM ANDC-A. After 24 h, the medium was replaced by 4.0 mL of fresh medium to stop the treatment. After 14 days, cells were stained with crystal violet, and colonies with up to 50 cells were counted. Data were expressed as survival fraction (Mordant *et al.* 2010).

2.7 *Statistical analysis*

All measurements were performed in two independent experiments carried out in triplicate. The significance of some variations was presented in the form of probability values (*$p<0.05$, **$p<0.01$, ***$p<0.001$ or $^{ns}p>0.05$, where *ns* means 'not significant') using the Student's t-test (Software GraphPad Prism, version 4.03, CA, USA).

3. Results

The ethyl acetate extract of *Cochliobolus* sp. was fractionated by reverse-phase MPLC, and the fraction 22 that showed a single major compound was active against axenically grown *Leishmania amazonensis* amastigotes, exhibiting 78% inhibition at 20 μg/mL (test screening). This compond showed MS, 1D and 2D NMR spectra in full agreement with those reported for anhydrocochlioquinone A (ANDC-A) (figure 1) (Phuwapraisirisan *et al.* 2007) and an EC_{50} value of 22 μg/mL (42.7 μM) (table 1) in the *in-vitro* leishmanicidal assay. This value is almost 100 times higher than that obtained for amphotericin and approximately 4 times lower than that obtained for the *Cochliobolus* sp. extract.

ANDC-A was also evaluated for its activity against several opportunistic bacterial strains (table 1) according to the susceptibility test. The results showed that ANDC-A was only partially active against *Staphylococcus aureus* ATCC 25295, with a MIC value of 128 μg/mL (248.7 μM), while for the other bacteria the MIC was higher than 256 μg/mL (497.4 μM). In turn, the extract of *Cochliobolus* sp. showed no activity against the tested bacteria (MIC>256 μg/mL).

When ANDC-A was tested against a panel of five lines of human cancer cells, it was able to reduce the viability of all them and showed IC_{50} (effective concentration to kill 50% of the parasites) values ranging from 5.5 to 20.3 μM (table 2). In the same assay, etoposide, drugs used in clinic to treat leukemia, showed $IC_{50}<3$ μM for HL-60 and THP-1 cells lines and >100 μM for the other cells lines. Doxorubicin, a drug used in breast cancer, showed IC_{50} of 3.61±2.6 μM and 10.93±2.35 μM for MCF-7 and HCT-116 cell lines, respectively (table 2), while cisplatin, an adjuvant chemotherapic to treat solid tumours, showed $IC_{50}>100$ and <40 μM for MCF-7 and HCT-116 cancer cells, respectively.

ANDC-A and control drugs were also tested for their cytotoxicity against non-tumour cells, exhibiting IC_{50} values of 29.9 and 19.3 μM for Vero cells and normal lymphocytes (PBMC), respectively (table 2), as well as a differential selectivity for HL-60 and THP-1 cell lines in comparison to Vero cells (SI_M of 5.5 and 4.3, respectively) and to human cells (SI_H of 3.5 and 2.8, respectively). Whereas these SI values were one order of magnitude lower than those exhibited by etoposide (55.5 and 47.6 for THP-1 and HL-60, respectively), for Jukart cells those of both ANDC-A and etoposide as control were <2.0. For both MCF-7 and HCT-116 cells, the ANDC-A SI_M values (>2.0) were higher than those of cisplatin (≤1.9), but lower than those of doxorubicin (≥5.7), while the corresponding SI_H values, including those of doxorubicin and cisplatin as controls, were all <2.0.

As illustrated in figure 2, after 24 h-treatment at concentration of 20 μM, ANDC-A was also able to significantly induce a statistically significant increase in subdiploid DNA content associated with DNA fragmentation in all cell lines compared with 0.5% DMSO used as a control ($p<0.0001$). The same significant difference was observed when comparing the etoposide (HL-50, Jukart and THP-1) and doxorubicin (MCF-7) controls with DMSO. Except for HL-60 cells, the percentage of subdiploid DNA was higher in the presence of ANDC-A when compared to controls. The highest values of DNA fragmentation (>70%) induced by ANDC-A were observed in Jurkat and THP-1 and MCF-7 cells lines.

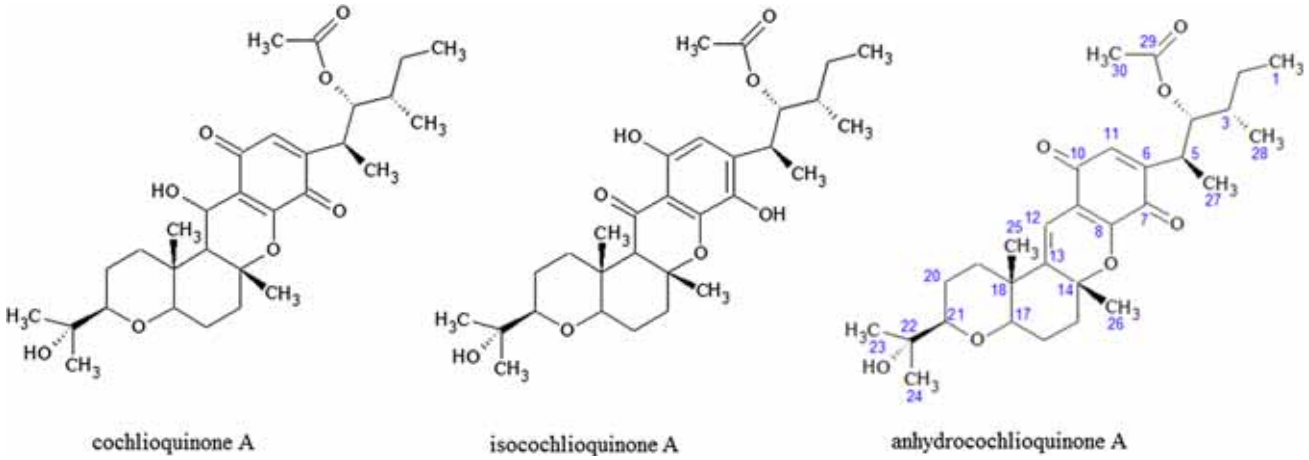

Figure 1. Chemical structure of ANDC-A isolated from *Cochliobolus* sp.

Table 1. *In vitro* leishmanicidal and antimicrobial activities of *Cochliobolus* sp. extract and anhydrocochlioquinone A compared to those of chloramphenicol and amphotericin B selected as controls

Sample	EC_{50} (μg/mL)[a]	MIC (μg/mL)[b]					
	L.A[c]	*S.A*[d]	*E.C*[e]	*S.T*[f]	*P.A*[g]	*K.O*[h]	*L.M*[i]
Cochliobolus extract	87 ± 3***	> 256	> 256	> 256	> 256	> 256	> 256
ANDC-A[j]	22 ± 2**	128	> 256	> 256	> 256	> 256	> 256
Chloramphenicol	NA[k]	16	8	16	8	8	8
Amphotericin B	0.024±0.004	NA	NA	NA	NA	NA	NA

[a] EC_{50}, half effective concentration; [b]MIC, minimal inhibitory concentration; [c]L.A, *Leishmania amazonensis* IFLA/BR/196/PH-8; [d]S.A, *Staphylococcus aureus* ATCC 25295; [e]E.C, *Escherichia coli* ATCC 18804; [f]S.T, *Salmonella typhimurium* ATCC14028; [g]P.A, *Pseudomonas aeruginosa* ATCC27853; [h]K.O, *Klebsiella oxytoca* ATCC 49131; [i]L.M, *Listeria monocytogenes* ATCC 19115; [j]ANDC-A, anhydrocochlioquinone A; [k]NA, not applicable; $^{**}p<0.01$ or $^{***}p<0.001$, statistically significant EC_{50} values when compared with Amphotericin B as control by the Student's *t*-test

Finally, the treatment of MCF-7 and HCT-116 cells with ANDC-A at concentrations of 20 and 50 μM for 24 h (figure 3) resulted in a strong decrease of survival fraction of viable cells, eradicating clonogenic cells with self-renewal capacity.

4. Discussion

Although ANDC-A has been isolated in large quantity from the extract in the present study, the compounds closely related to it, i.e. cochlioquinone A and isocochlioquinone A (figure 1), were detected neither by TLC nor by HPLC. This finding may be due to any biochemical alteration of the fungal isolate as a result of storage and/or of several mycelial transfers at different times under different conditions (Kale *et al.* 1994).

ANDC-A was less active than cochlioquinone A and isocochlioquinone A, which exhibited EC_{50} values of 1.7 μM and 4.1 μM, respectively, in the same assay with axenically grown *L. amazonensis* amastigotes (Campos *et al.* 2008).

According to the literature, cochlioquinone A has antimicrobial potential against *Staphylococcus aureus* CCT 4295 (MIC>15 ppm), *Bacillus subtilis* CCT 0089 (MIC>15 ppm) and *Apergillus niger* CCT 1435 (MIC>250 ppm) (Campos *et al.* 2008). On the other hand, when tested according to the microdilution assay against either Gram-positive or Gram-negative bacteria, ANDC-A exhibited weak activity against *S. aureus* (Bicalho *et al.* 2013). Accordingly, in the present work ANDC-A showed a partial activity against *S. aureus*. As shown in figure 1, compared with cochlioquinone A, ANDC-A has a double bond in positions 12 and 13 as a result of the loss of one water molecule, which may have been responsible for such difference in biological activity (Pinto *et al.* 2014).

To the best of our knowledge, ANDC-A was shown to inhibit the growth of HeLa cells (IC_{50}=5.9 μg/mL, corresponding to 11.48 μM) (Phuwapraisirisan *et al.* 2007). When ANDC-A was tested in the present work against five human cancer cell lines, it was active in the same concentration range or below than 25 μM, thus confirming the potential of this substance for antitumour therapies.

Table 2. *In vitro* cytotoxic activity of anhydrocochlioquinone A and control drugs against five tumour cell lines expressed as half maximal inhibitory concentration (IC_{50}, μM/mL) and selectivity indexes (SI, dimensionless) using IC_{50} values for normal monkey (Vero, SI_M)[a] or human (PBMC, SI_H)[b] cell models

	ANDC-A[c]			Etoposide[d]			Doxorubicin[e]			Cisplatin[f]		
Tumour cells	IC_{50}	SI_M	SI_H	IC_{50}	SI_M	SI_H	IC_{50}	SI_M	SI_H	IC_{50}	SI_M	SI_H
HL-60[g]	5.48 ± 2.54	5.5	3.5	1.79 ± 0.72[ns]	55.5	55.5	ND[l]	ND	ND	ND	ND	ND
Jukat[h]	20.34 ± 2.21	1.5	1.0	>100.00***	<1.0	<1.0	ND	ND	ND	ND	ND	ND
THP-1[i]	6.91 ± 0.29	4.3	2.8	2.10 ± 1.18*	47.6	47.6	ND	ND	ND	ND	ND	ND
MCF-7[j]	13.31 ± 3.07	2.2	1.4	>100.00***	I[m]	I	10.93 ± 2.35[ns]	5.7	0.6	>100.00***	0.7	0.03
HCT-116[k]	11.00 ± 4.18	2.7	1.8	>100.00***	I	I	3.61 ± 2.60[ns]	15.8	1.8	38.05 ± 5.27*	1.9	0.09
Vero[a]	29.93 ± 5.83	NA[n]	NA	>100.00***	NA	NA	57.24 ± 6.65*	NA	NA	76.69 ± 16.48*	NA	NA
PBMC[b]	19.29 ± 2.31	NA	NA	>100.00***	NA	NA	6.4 ± 0.3*	NA	NA	3.43 ± 1.18*	NA	NA

[a] Vero, African green monkey kidney line; [b]PBMC, peripheral blood mononuclear cells; [c]ANDC-A, anhydrocochlioquinone A; [d]Control, drug used in clinic to treat leukemia; [e]Control, drug used in clinic to treat breast cancer; [f]Control, adjuvant chemotherapic to treat solid tumours; [g]HL-60, human promyelocytic leukemia; [h]Jurkat, human immortalized line of T lymphocyte; [i]THP-1, acute monocytic line; [j]MCF-7, human breast cancer line; [k]HCT-116, human colorectal carcinoma line; [l]ND, not determined; [m]I, inactive; [n]NA, not applicable; *p<0.05 or ***p<0.001, statistically significant IC_{50} values when compared with the ANDC-A treatment for each cell line by the Student-t test; ns, not statistically significant difference between average values (p>0.05) using the Student's t-test

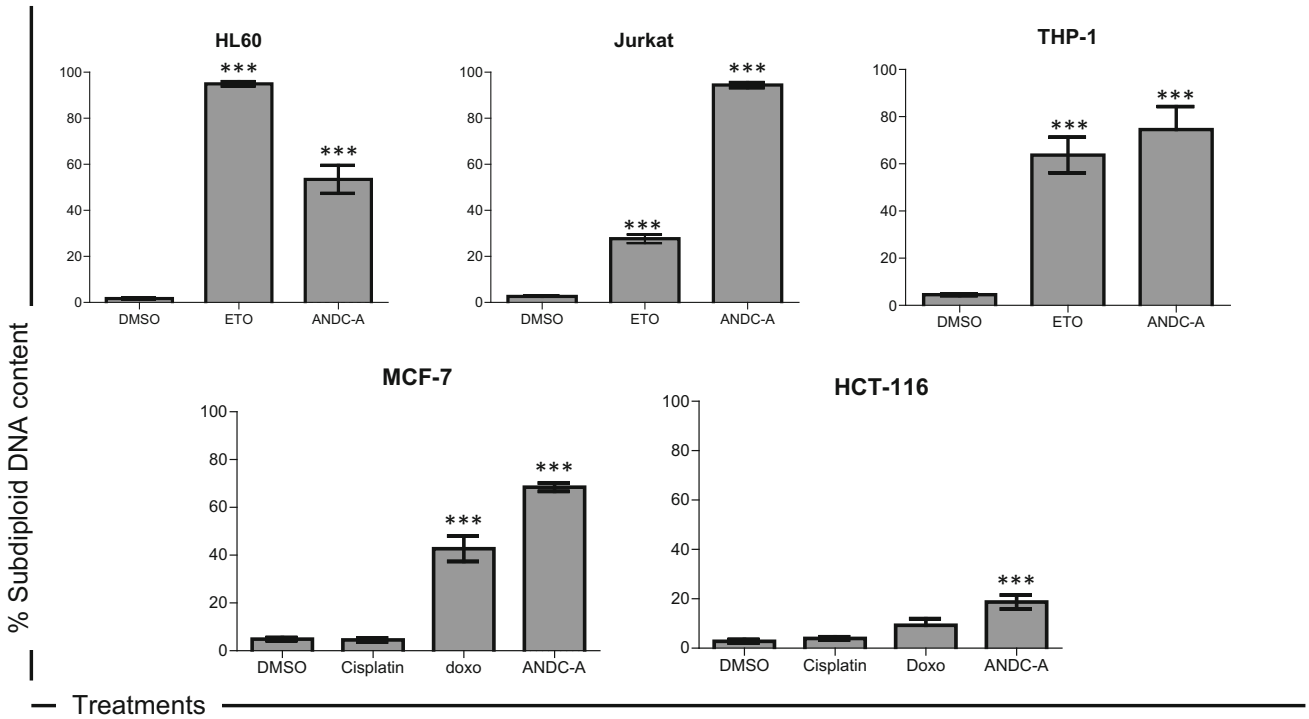

Figure 2. Increase of subdiploid DNA content in human tumour cells induced by ANDC-A. ANDC-A was incubated with the different cell lines for 24 h at 37°C and 5% CO_2. The subdiploid DNA content was used as indicative of DNA fragmentation after labeling with propidine iodide and flow cytometry analysis. The mean data (grey bar) and error bar are representative data of two independent experiments carried out in triplicate. Either ANDC-A or control drugs of clinical use, namely etoposide (ETO), cisplatin (CIS) and doxorubicin (DOXO), were used at concentration of 20 μM. ***Statistically significant values (p<0.0001).

As is well known, the selectivity index (SI) of a compound is an important parameter that provides a measure of its potential *in vivo* toxicity, in that compounds with SI>2 are considered to be selective against tumour cells (Badisa *et al.* 2009). ANDC-A showed SI>2 only for HL-60 and THP-1 cell lines compared with monkey (Vero) and human

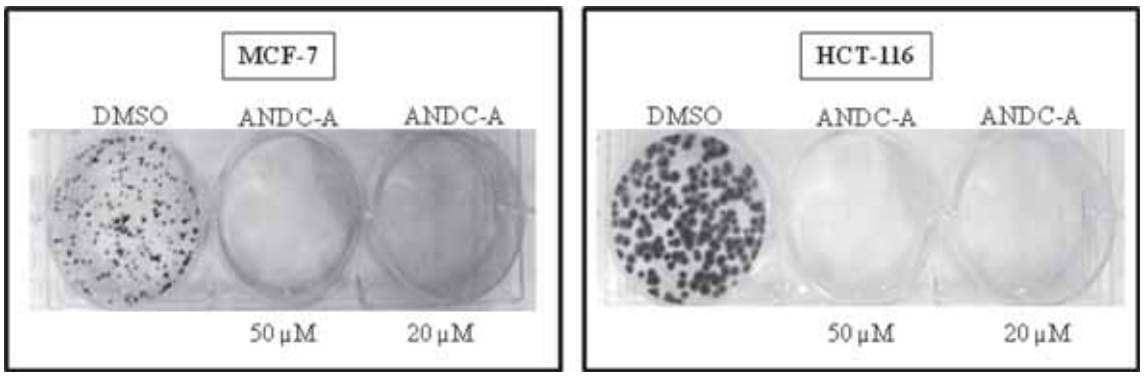

Figure 3. Effects of ANDC-A at concentrations of 20 and 50 µM on survival of clonogenic MCF-7 and HCT-116 cells. After cells incubation with ANDC-A or with control treated with vehicle (0.05% v/v DMSO) for 24 h, the medium was replaced by a drug-free one, and the incubation was continued for 14 days. Cell colonies were stained with crystal violet and counted. At least two independent experiments were performed in duplicate.

cells (PBMC) as models. To provide a comparison basis, 4-hydroxy tamoxifen, an antiestrogen used in the treatment of human breast cancer, exhibited SI=1.29 against MCF-7 cells in comparison with CRL-1439 normal liver cell line (Badisa *et al.* 2009). Moreover, although ANDC-A showed SI<2 for Jurkat, MCF-7 and HCT-116 cell lines in comparison with PBMC, it was more selective than doxorubicin and cisplatin, two drugs in clinical use.

Recently, ANDC-A isolated together with other three cochlioquinone derivatives from the ethyl acetate extract of the phytopathogenic fungus *Bipolaris luttrellii* was the only compound able to induce apoptosis and caspase activity in a dose-dependent manner (10–30 µM) in HCT116 cells, as the likely result of Bcl-2 downregulation and cytochrome c release from mitochondria (Qi *et al.* 2014). It has been demonstrated that resistance to chemotherapy mainly involves apoptosis, which is a potential therapeutic target for cancer treatment (Nicholson 2000; Fesik 2005). Also, in our tests the treatment with 20 µM ANDC-A was able to induce DNA fragmentation both in leukemic and solid lines, with the strongest effect on Jurkat cell line.

Even after post-treatment with drugs, senescent cancer cells can maintain their membrane intact and remain metabolically active for a long time. These cells may then be considered as "survivors" in short-term assays of cell viability loss such as MTT. Clonogenic assay is suitable to determine all modes of cell death and includes both early and late responses such as delayed growth arrest. For these reasons, it is considered the "gold standard" assay for the assessment of cytotoxicity (Mirzayans *et al.* 2007). ANDC-A was able to eradicate all MCF-7 and HCT-116 clonogenic cells at concentration of 20 µM. This activity is quite important to achieve durable results in anticancer therapy, because it requires effective elimination of subpopulations of cancer cells that otherwise could remain unaffected.

In this study we showed that anhydrocochlioquinone A markedly reduces cell viability of tumour cells from different cancer models (leukemia and solid tumour) and is able to induce apoptosis in all cell lines. Moreover, it was shown to eradicate cells with self-renewal capacity, suggesting good effectiveness and antitumour potential.

Acknowledgements

The authors acknowledge Caryne Margotto Bertollo and Daniela Nabak Bueno for technical assistance. This work was supported by the Fundação Oswaldo Cruz (RPT10A), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (CBB - APQ-00145-12-296841) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (472175/2012-2).

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Corresponding editor: Seyed E Hasnain