Evaluation of the Antitumor Potential of the Resorcinolic Lipid 3-Heptyl-3,4,6-trimethoxy-3H-isobenzofuran-1-one in Breast Cancer Cells

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Abstract. *Background/Aim:* In recent years, the search for new anticancer experimental agents derived from natural products or synthetic analogues, such as resorcinolic lipids, has received increased attention. The present study aimed to evaluate the antitumor potential, describe the cell death mechanism and the effects of 3-Heptyl-3,4,6-trimethoxy-3H-isobenzofuran-1-one (AMS35AA) in combination with different chemotherapeutic agents in the MCF-7 cell line. *Materials and Methods:* Analysis of cytotoxic, genotoxic, membrane integrity, cell death and gene expression induced by the compound was performed. *Results:* The AMS35AA

and its association with 5-FU demonstrated reduction of cell viability; increase of cell death; enhancement of genomic damage and accumulation of cells in G₂/M phase. *Conclusion:* AMS35AA has potential for breast cancer treatment since it is capable of exerting cytotoxic and cytostatic effects in a breast cell line and also could be an adjuvant in cancer therapy when combined with 5-FU.

Non-isoprenenic resorcinolic lipids or alkylresorcinols (ARs) agents are widely distributed and are being produced by bacteria, protozoa, algae, plants and animals (1). They are amphiphilic in nature due to the non-isoprenoid side chains attached to the hydroxybenzene ring and are believed to be also derived from the polyketide (acetate) pathway. In general, these resorcinolic lipids have a long carbon chain, varying between C-5 and C-27 being able to be saturated or unsaturated, *e.g.* mono-, di-, tri-, tetra-, penta-, hexane linked to a resorcinol ring, with one or both hydroxyls methylated. The aromatic ring may contain a methyl (methylcardol) group and the hydroxyls may be mono or dimethylated and diacetylated. Some exhibit a carboxyl group, esterified or free, between the hydroxyls (2).

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Among the resorcinolic lipids those that stressed relevance are the 3-Heptyl-3,4,6-trimethoxy-3H-isobenzofuran-1-one (AMS35AA) (3), the 3-Heptyl-4,6-dihydroxy-3H-isobenzofuran-1-one (AMS49) (4) and the 3-hexyl-5,7-dimethoxy-isochromen-1-one (isocoumarin) (5). These compounds share the ability to increase the frequency of apoptosis in the kidneys and liver of treated animals. In addition, AMS35AA and AMS49 did not show any toxicogenicity, considering that they do not induce micronuclei and they further potentiate the genotoxic effects of cyclophosphamide (3, 4). On the other hand, isocoumarin has genotoxic action (increases comet frequency and micronuclei) and at the same time has antigenotoxic action against damage induced by cyclophosphamide and cisplatin (5). Thus, it is perceived that AMS35AA and AMS49 are candidates as new chemotherapeutics and adjuvants, which was not occurred in the isocoumarin experiment because it reduced the commercial drug effects.

We further emphasize that AMS35AA was effective in causing immunomodulation and consequently in increasing the number of lymphocytes. In general, the reduction of lymphocytes in patients treated with chemotherapy is an undesirable adverse effect that prevents the treatment of patients with cancer. Thus, we chose to use AMS35AA in combination with commercial chemotherapeutics. The present study evaluated the effects of AMS35AA in combination with the chemotherapeutic agents: tamoxifen, doxorubicin, cisplatin, 5-fluorouracil and irinotecan with respect to ability to induce cytotoxicity and cell death in MCF-7 mammary adenocarcinoma cells.

Materials and Methods

Chemical agents. The following chemotherapeutics agents were used: tamoxifen (Sanofi Aventis; Paris, France); doxorubicin (Bergamo; Bergamo, Italy); cisplatin (Gunther, São Paulo, Brazil); 5-fluorouracil (Sigma Chemical Co., St. Louis, MO, USA) and irinotecan (Janssen Beerse, Belgium®). The resorcinol lipid AMS35AA (3-Heptyl-3,4,6-trimethoxy-3H-isobenzofuran-1-one), the test compound, was obtained as previously described by Navarro, *et al.* (2014) (3).

Cell line and culture conditions. MCF-7 cells, human breast adenocarcinoma cell line was used in this study. The cells were grown at 37°C in a humidified atmosphere containing 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) (Gibco® Life Technologies, Grand Island, NY, USA), supplemented with 10% foetal bovine serum (FBS) (Gibco® Life Technologies, CA, USA), 0.1% penicillin/streptomycin (10,000 U, 30110-01, LGC® (Gibco® Life Technologies).

Cell viability assay. The cytotoxic potential of AMS35AA was evaluated by the MTT colorimetric test performed as described by Pesarini *et al.* (2017) and Schweich *et al.* (2017), with modifications (6, 7). Cells were seeded at a density of 3×10³ in 96 well culture plates in a supplemented DMEM medium and

incubated in 5% CO₂ atmosphere at 37°C for 24 h for adherence and stabilization. Thereafter, the treatments were performed for 72 h in supplemented DMEM culture medium: A) for the determination of IC₅₀ (half-maximal inhibitory concentration) of the chemotherapeutic agents; a.1) tamoxifen (2.5; 5; 7.5; 10; 20 µM); a.2) doxorubicin (0.1; 0.2; 0.3; 0.4; 0.5 µM); a.3) cisplatin (2.5; 5.0; 10; 25; 50 µM); a.4) 5-fluorouracil (5-FU) (0.1; 1.0; 2.5; 5; 10 µM); and a.5) irinotecan (5; 10; 20; 40; 60 µM). b) different concentrations of the AMS35AA (14, 28, 56, 84, 112, 140, 210 and 280 µM); B: The AMS35AA IC₅₀ (54 µM) associated with the chemotherapeutic agents IC₅₀: tamoxifen (7 µM), doxorubicin (0.35 µM), cisplatin (2 µM), 5-FU (2 µM) and irinotecan (20 µM) established according to experiment A. At the end of treatment, the cells were incubated with 100 µl of MTT (Invitrogen® Life Technologies, 0.005 g MTT, 5 ml PBS, 10 ml HDMEM) for 4 h under the same conditions. Plates were dried and 100 µl of DMSO were added. The absorbance was measured at 540nm in ELISA Plate Analyzer ROBONIK® spectrophotometer. Three independent replicates were performed in quintuplicate. The combination Index (CI) was calculated from the values of the affected cell fractions (FA): CI<1 indicates synergism; CI=1 indicates additive effect and CI>1 indicates antagonistic effect using CompuSyn software (www.combosyn.com) (8). The percentage of damage reduction (DR%, positive values) (9) as well as the percentage of damage increase (DI%, negative values) were also calculated as described by Navarro, 2014 and Oliveira, 2015 with adaptations (3, 4), according to the formula presented below:

$$DR \text{ or } DI\% = \frac{\text{Drug viability mean} - \text{association viability mean (Drug + AMS35AA)} \times 100}{\text{Drug viability mean} - \text{control mean}}$$

Genotoxicity test. The Comet assay alkaline version was performed as described by Singh *et al.* (1988), with modifications. All samples were processed in triplicate. 1×10⁵ cells were seeded in a DMEM medium supplemented with 10% foetal bovine serum and incubated in 5% CO₂ atmosphere at 37°C for 24 h. The cells were exposed to the treatment for 4 h and the concentrations were: 14, 28, 56 and 112 µM for AMS35AA; it was also submitted to the treatment the AMS35AA IC₅₀ (54 µM) in combination with 5-FU IC₅₀ (2.5 µM); Positive Control (5-FU; 2.5 µM) and Negative Control. The cells were trypsinized and centrifuged at 1200 rpm for 5 min. Microscope slides were covered with a thin layer of Low Melting Point Agarose. 20 µl of cell suspension was added to 120 µl of low melting point at 37°C. The cells were lysed in a cold high salt and detergent containing solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0-10.5, with freshly added 1% Triton X-100 and 10% dimethyl sulfoxide pH=10) for 1 h in fridge. The slides were then carefully placed in a horizontal electrophoresis chamber unit containing freshly-made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 20 min for DNA denaturation. The slides were electrophoresed for 20 min at 25 volts and 300 mA, and then the buffer was neutralized with 0.4 M Tris (pH 7.5). The material was stained (100 µl ethidium bromide, 2×10⁴ mg/ml) and analyzed under 400x magnification with an epifluorescence microscope (Bioval®, Model L 2000A, Valencia, Spain) equipped with a 420-490 nm excitation filter and 520 nm barrier filter. The comets were classified according to Kobayashi *et al.* (1995) (10). The %DR and/or %DI were calculated as described in the cell viability assay. All steps were conducted in the dark.

Table I. Oligonucleotides sequences

Gene	Forward	Reverse	Size (pb)
<i>ATR</i>	CCTTCAGATTTCCCTTGAATAC	GCAGTTCATGTTTTGATGAG	137
<i>p53</i>	TACCACCATCCACTACAAC	GACAGGCACAAACACGCAC	145
<i>GADD45</i>	TCAGCGCACGATCACTGTC	CCAGCAGGCACAACACCAC	82
<i>p21</i>	TAGCAGCGGAACAAGGAG	AAACGGGAACCAGGACAC	249
<i>p38</i>	ATGTCTCAGGAGAGGCCACGTTCT	AATGGTCTGGAGAGCTTCTTCA	242
<i>JNK</i>	CGACCTTCTATGACGATGC	GTGGTGGTGATGTGCC	253
<i>BAK</i>	CTGTTTGAGAGTGGCATC	ATGCTGGTAGACGTGTAG	84
<i>BAX</i>	CCTTCTTTGAGTTCGGTG	TTCAGGTACTCAGTCATCCAC	100
<i>CASP-6</i>	CCAGACAGACAAGCTGGACA	TGTACCAGGAGCCATTCAACA	235
<i>CASP-7</i>	TCACCATGCGATCCATCAAGACCA	TTTGTCTGTTCCGTTTCGAACGCC	148
<i>CASP-9</i>	CTCTACTTTCCAGGTTT	TTTACCAGAAACAGCATT	168
<i>BCL-2</i>	GGACGAACTGGACAGTAAC	GCAAAGTAGAAAAGGCGCAAC	127

Cell death. To evaluate the apoptotic or necrotic cells, a morphological assay was performed according to the protocol for cell death of Oliveira *et al.* (2007), with modifications (9). In a 12-well plate, 2×10^5 cells were seeded in a DMEM medium and incubated in 5% CO₂ atmosphere at 37°C for 24 h. The cells were exposed to the treatment for 4 h and the concentrations were: 14, 28, 56 and 112 µM for AMS35AA; IC₅₀ of the compound (54 µM) in combination with 5-FU IC₅₀ (2.5 µM); Positive Control (5-FU) and Negative Control. A second experiment was realized in the same conditions, after 4 h followed by 16 h (to verify repair) in a drug free medium. The cells were trypsinized and centrifuged at 1200 rpm for 5 min. For the preparation of the slides, 20 µl of cell suspension and 2 µl of ethidium bromide dye (100 µg/ml) and 2 µl of acridine orange (100 µg/ml) were used, following the protocol of Oliveira *et al.* (2007) (9). The assay was performed in three independent replicates. 100 cells per slide were analyzed under a fluorescence microscope at a magnification of 400×.

Cell-cycle analysis. In brief, MCF-7 cells were seeded at a density of 2×10^5 cells per well in a supplemented DMEM medium and incubated in 5% CO₂ atmosphere at 37°C for 24 h. Then, the cells were exposed to the treatment for 24 h, as following: a) AMS35AA (IC₅₀ 54 µM); b) AMS35AA (IC₅₀ 54 µM) in combination with 5-FU (IC₅₀ 2.5 µM); Positive Control (5-FU; 2.5 µM) and Negative Control. After 24 h of treatment, the cells were trypsinized, centrifuged at 1,200 rpm for 5 min and resuspended in 100 µl of PBS. Next, 5 µl of RNase was added, and the cells were incubated at 37°C for 30 min. Lysis of DNA was performed using 100 µl of lysis solution (50 µg/l PI, 0.1% sodium citrate, 0.1% Triton X-100), and incubated on ice in the dark for 30 min. Fluorescence intensity (from 10,000 cells) was immediately analyzed by flow cytometry (BD Accuri™ C6, Becton Dickinson, Franklin Lakes, EUA) with 488-nm laser excitation (7).

Membrane Integrity. The cells were prepared as described above. After that, 25 µl of PI 50 µg/ml was added to resuspended cells for 5 min at room temperature in the dark. Fluorescence intensity (from 10,000 cells) was immediately analyzed by flow cytometry (BD Accuri™ C6) with 488-nm laser excitation (7).

Gene expression analysis. RT-qPCR analysis has been applied in an attempt to determine changes in gene expression profiles. Table I contains the oligonucleotides sequences used to amplify genes involved in the DNA damage (*ATR*, *p53*, *GADD45* and *p21*) and apoptosis (*BAK*, *BAX*, *CASP6*, *CASP7*, *CASP9* and *BCL-2*). In a 6 well plates a total of 2×10^5 cells/well were seeded in a supplemented DMEM medium and incubated in 5% CO₂ atmosphere at 37°C for 24 h. Then, the cells were incubated for 24 h with: a) different concentrations of AMS35AA (14, 28, 56 and 112 µM); b) IC₅₀ of the compound in combination with IC₅₀ of 5 FU (2.5 µM), c) Negative Control and d) Positive Control. Briefly, for RNA extraction, the cells were treated with guanidine isothiocyanate buffer (GuSCN 5 mol/l, Tris HCL 11.2 g/l pH6.4, EDTA NaOH 7.43 g/l, 7.8 ml Triton X-100) and 50 µl of magnetic beads (bioMérieux® Marcy-l'Étoile, France) was added to each sample (200 µl). Right after successive washes in GuSCN/citrate and ethanol were performed. The nucleic acids are eluted in 30 µl of RNase free water. The samples were quantified using a spectrophotometer. The ratio of absorbance at 260 nm and 280 nm (NanoVue™ Plus spectrophotometer - Life Sciences®) is used to assess the purity of RNA, and only samples with a ratio between 1.8 and 2.1 were used in further experiments. The cDNA was obtained by reverse transcription and amplified by PCR (T100™ - Thermal Cycler, Bio-Rad™, Hercules, California, EUA) using the GoScript Reverse transcription (RT) System (Promega®, Madison, EUA) following the instructions from the manufacturer. Real-time PCR was carried out in triplicate by a Rotor Gene® (Qiagen, Hilden, Alemanha) instrument. Reactions were prepared in a total volume of 20 µl, containing 10 µl of GoTaq® Master Mix (Promega Madison, EUA), 2 pmol of each oligonucleotide, 500 ng of cDNA and ddH₂O. The cycling parameters were as follows: 95°C for 5 min (initial denaturation), 40 cycles at 95°C for 2 sec (denaturation), and 60°C for 30 sec (annealing and extension). At the end, the melting curve was generated to analyze the specificity. The GAPDH (beta-actin) was used as a reference gene. The results were analyzed in the Rotor Gene® v2.3.1 (Qiagen) software.

Statistical analysis. The results were expressed as means±standard error of the mean (SEM) and analyzed by ANOVA/Tukey test using the GraphPad Prism software (version 3.02; San Diego, CA, USA). The level of significance adopted was $p \leq 0.05$.

Figure 1. Determination of IC_{50} values. MCF-7 cells were treated for 72 h with various concentrations of tamoxifen, doxorubicin, cisplatin, 5-FU and Irinotecan as mentioned in Materials and Methods section. MTT assay was then used to determine the cell viability (%). For each experiment, three independent replicates were performed in quintuplicates. The mean and standard error of the mean (SEM) – (bar), are shown in the plot.

Figure 2. Determination of range and IC_{50} value of AMS35AA. (A) The range determination of AMS35AA in MCF-7 cells. (B) Determination of IC_{50} value of AMS35AA. The cells were treated for 72 h with different concentrations (14, 28, 56 and 112 μ M). For each experiment, three independent replicates were performed in quintuplicates. The mean and (SEM) – (bar) are shown in the plot.

Results

IC_{50} determination of the commercial chemotherapeutic agents. The IC_{50} of the commercial chemotherapeutic agents was established by pilot run using CompusSyn software. The IC_{50} of tamoxifen (Figure 1A), doxorubicin (Figure 1B), cisplatin (Figure 1C), 5-FU (Figure 1D), irinotecan (Figure 1E), was, respectively, 7 μ M, 0.35 μ M, 2 μ M, 2.5 μ M and 20 μ M.

IC_{50} determination of the AMS35AA and cell viability study. The first MTT assay demonstrated that concentrations higher than 14 μ M were capable of reducing significantly cell viability of MCF-7 (Figure 2A). After that, a smaller range was tested to establish the IC_{50} . In this second run, concentrations of 14, 28, 56 and 112 μ M of AMS35AA reduced the cell viability to ($p < 0.05$) 88.50 ± 3.5 , 64.82 ± 4.65 , 51.36 ± 5.62 and 27.37 ± 0.76 , respectively and the IC_{50} was 54 μ M (Figure 2B).

Cell viability: effects of AMS35AA associated with chemotherapeutic agents. The cytotoxic effect of tamoxifen, doxorubicin, cisplatin and irinotecan was reduced by AMS35AA and the percentages of damage reduction were 4%, 6%, 2% and 143.3%, respectively. According to the combination index AMS35AA has antagonistic action on tamoxifen, doxorubicin, cisplatin and irinotecan. When AMS35AA was combined with 5-FU, a 30% increase in damage was observed and, therefore, the combination index indicated an additive effect (Figure 3).

AMS35AA causes genomic damage and potentiates the effects of 5-FU. AMS35AA is genotoxic and increases the frequency of DNA lesions by 2.61 $\times$, 2.79 $\times$, 3.60 $\times$ and 2.82 $\times$ at 14, 28,

56 and 112 μ M and the score by 2.53 $\times$, 2.93 $\times$, 3.84 $\times$ and 2.88 $\times$, for the same concentrations, respectively (Table II). When AMS35AA was combined with 5-FU at their IC_{50} values there was a 21.03% increase in the frequency of lesioned cells and a 63.19% increase in the score (Table II). When the same test was performed after 16 h of recovery, the frequency of lesioned cells was increased by 1.88 $\times$, 2 $\times$, 2.97 $\times$ and 2.03 $\times$ and the score at 1.89 $\times$, 2.02 $\times$, 3.32 $\times$ and 1.97 $\times$, respectively. It was also observed that the increase in damages was in the order of 27.6% for the frequency of lesioned cells and 178.72% for the score, respectively (Table III).

AMS35AA induces cell death. A cytological analysis of cell death (differential apoptosis and necrosis) 4h after treatment demonstrated that all concentrations caused a significant increase in the frequency of cell death. The increases were 2.6 $\times$, 2.76 $\times$, 3.4 $\times$ and 2.86 $\times$, relative to the control, for the concentrations of 14, 28, 56 and 112 μ M, respectively. In the evaluation after 16 h, a significant increase was observed only at concentrations above 28 μ M. There was an increase by 1.54 $\times$; 2.77 $\times$; 2.45 $\times$ and 2.12 $\times$, relative to the control, for the concentrations of 14 (non-significant), 28, 56 and 112 μ M, respectively (Figure 4). At the concentration of 56 μ M, there was more apoptosis in the experiment after 16 h. The combination of AMS35AA with 5-FU at their IC_{50} values demonstrated potentiation ($p < 0.05$) of apoptotic effects of 5-FU by 7.4 $\times$ and 5.96 $\times$ for the experiment evaluated with and without recovery (Figure 4). According to this cytological assay cell death occurred by apoptosis and no necrotic cells were observed.

AMS35AA does not cause rupture of plasma membrane. The MCF-7 cells were negative for PI test by flow cytometry (Figure 5), confirming the membrane integrity.

Figure 3. Continued

Figure 3. Effects of AMS35AA when associated with chemotherapeutic agents on cell viability in the MCF-7 breast cancer tumor line using the MTT assay. Chemotherapeutics alone (black line); chemotherapeutics combined with AMS35AA (gray line). (A) Tamoxifen IC₅₀ and tamoxifen IC₅₀ in combination with AMS35AA IC₅₀; (B) Combination Index between tamoxifen and AMS35AA; (C) Doxorubicin IC₅₀ and doxorubicin IC₅₀ in combination with AMS35AA IC₅₀; (D) Combination Index between doxorubicin and AMS35AA; (E) Cisplatin IC₅₀ and cisplatin IC₅₀ in combination with AMS35AA IC₅₀; (F) Combination Index between cisplatin and AMS35AA (G) 5-FU IC₅₀ and 5-FU IC₅₀ in combination with AMS35AA IC₅₀; (H) Combination Index between 5-FU and AMS35AA; (I) Irinotecan IC₅₀ and irinotecan IC₅₀ in combination with AMS35AA IC₅₀. The combination index of irinotecan was not calculated since the affected fraction cannot be negative to generate CI. For each experiment, three independent replicates were performed in quintuplicates. The mean and (SEM) – (bar) are shown in the graphs (A), (C), (E), (G) and (I). The combination index values were calculated according to the Chou and Talalay method using the CompuSyn software. (J) Percentual of damage reduction (DR%, positive values) and Percentual of damage increase (DI%, negative values).

Combination of AMS35AA with 5-FU induces inhibition of cell cycle progression (sub-G₁ and G₂/M arrest). AMS35AA and 5-FU caused a statistically significant reduction of cells at the S phase and increase of cells in the G₂/M phase. In addition, the combination of these two compounds was still able to reduce the frequency of cells in G₁ and S phases and increase the frequency in the G₂/M phase. This increase in G₂/M was 1.48x comparing to 5-FU and AMS35AA + 5-FU (Figure 6).

Gene expression. AMS35AA caused a significant increase in the expression of p53 levels (Figure 7A) and a significant reduction in BAX expression (Figure 7B). 5-FU increased ($p < 0.05$) expression of ATR, p53, p21, GADD45 (Figure 7A) and BAK (Figure 7B) and reduced ($p < 0.05$) BCL-2 expression (Figure 7B). The combination of these two compounds caused a significant increase in the expression of ATR, p53, p21, GADD45 (Figure 7A), BAK, CASP7 and CASP9 (Figure 7B), and significant reduction of BCL-2 (Figure 7B).

Discussion

AMS35AA is a resorcinol lipid that was firstly synthesized and tested by our research group. A therapeutic potential for cancer therapy was demonstrated (3). Since then, we have challenged breast cancer cells (MCF-7) with this compound

and our results are promising. This study verified that AMS35AA has a cytotoxic and cytostatic effect on MCF-7 cells since a reduction of cell viability and cell cycle arrest were found.

According to Navarro *et al.* (2014) AMS35AA is neither genotoxic nor mutagenic in Swiss mice (3). However, the present search indicates that this resorcinolic lipid is genotoxic to MCF-7 cells. These results may suggest a selective action on tumor cells, a fact also suggested by Navarro *et al.* (2014) (3). This ability to cause genomic damage was confirmed by the comet assay and also corroborates for this finding: (i) the reduction of cell viability observed in the MTT assay; (ii) the increase of cell death verified in the cytological assay (differential apoptosis/necrosis); and (iii) the increase of p53 levels.

Most likely the three methoxy groups of AMS35AA can be demethylated on position 4 and 6 and maybe also in position 3. This benzofuran can become very reactive, especially when the demethylation occurs at the 3-position. The furan side can be opened, which leads to a reactive molecule that may explain the genotoxicity towards MCF-7 cells. The hydroxyl groups on position 4 and 6 are less reactive. Catechol setting, *i.e.* 4 and 5 or 5 and 6 hydroxyl-groups, are chemically more reactive (11).

It is believed that these DNA damages lead the cells to apoptosis since AMS35AA did not cause damage to

Table II. Frequency of cells with total DNA damage, class of damage and score of the Comet assay on MCF-7 breast cancer line after 4 h of treatment with AMS35AA.

Experimental groups	DNA damage	Class of damage				Score
		0	1	2	3	
Control	18.04±3.46 ^a	81.96±2.19	10.04±3.79	4.67±1.20	3.33±1.20	29.37±4.26 ^a
5-Fluorouracil	76.67±4.46 ^c	23.33±3.15	42.67±4.63	13.00±1.53	21.00±1.15	131.67±4.35 ^c
14 µM AMS35AA	47.01±1.67 ^b	53±1.26	28.33±10.5	10.01±1.15	8.67±2.60	74.36±0.88 ^b
28 µM AMS35AA	50.33±1.85 ^b	49.67±1.84	27.00±2.00	11.00±2.08	12.33±1.86	85.99±3.38 ^b
56 µM AMS35AA	65±4.04 ^c	34.97±3.46	31.66±3.71	19.00±2.00	14.33±2.73	112.65±1.86 ^c
112 µM AMS35AA	51±5.52 ^b	49.02±4.15	28.00±3.51	12.33±1.45	10.67±1.76	84.66±8.17 ^b
ASSOCIATION	89±10.00 ^d	10.1±2.55	19±3.78	32.66±3.18	37.33±2.08	196.31±5.33 ^d

Association (AMS35AA IC₅₀ + 5 – FU IC₅₀). Mean±SEM of number of total cells/300 cells (100 cells/treatment/repetition). Different letters indicate statistically significant differences. ANOVA/Tukey-Kramer ($p \leq 0.05$).

Table III. Frequency of cells with total DNA damage, class of damage and score of the Comet assay on the MCF-7 breast cancer line after 4 h of treatment with AMS35AA followed by 16 h free of treatment.

Experimental groups	DNA damage	Class of damage				Score
		0	1	2	3	
Control	24.66±3.48 ^a	75.34±6.02	12.36±1.28	7.66±2.08	4.66±3.78	41.66±15.53 ^a
5-fluorouracil	79.65 ±4.16 ^c	20.35±8.62	46.66±8.62	12.33±3.05	20.66±3.05	95±4.04 ^c
14 µM AMS35AA	46.33±4.09 ^{a,b}	53.66±7.37	26.00±6.55	8.33±2.51	12.00±7.00	78.66±22.48 ^{a,b}
28 µM AMS35AA	49.33±2.18 ^b	50.66±3.78	25.33±2.51	13.00±3.60	11.00±1.00	84.33±9.01 ^b
56 µM AMS35AA	73.33±3.93 ^c	26.67±6.80	30.00±4.35	21.66±4.61	21.67±2.08	138.33±10.40 ^c
112 µM AMS35AA	50±2.51 ^b	50.02±4.35	26.66±3.21	14.66±4.04	8.66±2.08	82.00±9.16 ^b
ASSOCIATION	94.83±0.88 ^d	5.17±6.55	46.66±7.76	22.84±5.25	25.33±2.30	190.33±15.04 ^d

Association (AMS35AA IC₅₀ + 5 – FU IC₅₀). Mean±SEM of number of total cells/300 cells (100 cells/treatment/repetition). Different letters indicate statistically significant differences. ANOVA/Tukey-Kramer ($p < 0.05$).

membrane integrity and there was already indication of cell death by apoptosis in the morphological assay. In addition, accumulation of cells in G₂/M phase was observed when the cells were treated with the resorcinol lipid. This cell cycle arrest in G₂/M phase may occur in response to DNA damage (p53 increase). It was also supposed to happen a late apoptosis after treatment with AMS35AA. This late apoptosis could be suggested since comet and apoptosis assays demonstrated that even in the absence of treatment, the frequency of DNA damage and cell death were maintained at similar statistical levels. The reduction of BAX, a pro-apoptotic protein, may be related to the occurrence of late apoptosis (12).

Another fact that may justify the reduction of BAX is that BAX protein levels declined in the cytosol at 3 and 8 h respectively after treatment, which was subsequent to cytochrome *c* release. It is, therefore, possible that in these cells, sufficient BAX resides at the mitochondrial membrane to induce cytochrome *c* release after death signal, and further

apoptotic insult leads to increasing amounts of BAX translocating to the mitochondria (13).

These data indicate that AMS35AA may be cytotoxic and cytostatic in MCF-7 cells, suggesting two possible mechanisms of action for its anticancer effect. Therefore, the present study indicates an alternative cell death pathway (DNA damage-mediated apoptosis) for the literature proposed for resorcinolic lipids until now (14, 15).

A Chinese research group in 2008 found evidence, by molecular biology studies, that cytosporone B, also a resorcinolic lipid, showed to be cytotoxic on many strains of tumors behaves as a natural ligand to the orphan nuclear receptor Nur77 of eukaryote cells. (14, 15). Its expression is induced in response to cellular stress, serum withdraw or treatment with apoptotic agents or mitogenic factors. The amoitone B, another resorcinolic lipids, is one of the most active analogues of cytosporone B. It has similar response with respect to cellular viability assays and apoptosis. It also

Figure 4. Graph showing the percentage of apoptotic cells treated with different concentrations of AMS35AA (14, 28, 56 and 112 μ M); IC₅₀ (5-FU) and combination of AMS35AA and 5-FU at their IC₅₀ values in MCF-7 cells for 4 h (gray bars) and 4 h followed by 16 h (black bars) in drug free medium. 100 cells were analyzed per slide under a fluorescence microscope at a magnification of 400 $\times$. The cells were classified by morphological appearance and differential staining by ethidium bromide and orange acridine. The graphs represent the mean and SEM of three independent replicates. Necrotic cells were not found. Statistical differences were analyzed by ANOVA/Tukey. Different letters indicate statistical differences ($p \leq 0.05$).

Figure 5. Cytoplasmic membrane integrity MCF-7 cells assessed by flow cytometry shows a percentage increase for the viable cell population of Control, AMS35AA and AMS35AA + 5-FU. Data are presented as mean $\pm$ SEM of three separate experiments. Statistical differences were analyzed by ANOVA/Tukey. Different letters indicate statistical differences ($p \leq 0.05$).

Figure 6. Results of the cell-cycle analysis by flow cytometry. The cell-cycle distribution was quantified and is shown in bar graphs including sub G₁, G₀/G₁, S and G₂/M phases. AMS35AA in combination with 5-FU induces inhibition of cell cycle progression (sub-G₁ arrest and G₂/M). Data are presented as mean $\pm$ SEM of three separate experiments. Statistical differences were analyzed by ANOVA/Tukey. Different letters indicate statistical differences ($p \leq 0.05$).

demonstrates affinity for Nur77 and antitumor activity in vivo (16). In addition, some alkylresorcinols exhibit activity in colon neoplastic cells (SW620), inhibiting proliferation by more than 70%, in lung neoplastic cells (H1299) and in

hepatome cells (HepG2) by more than 40%. In hepatocytes (HL-7702), human hepatoblasts (ME-Hep4) and fibroblasts (NIH3T3), the effect was smaller, suggesting that their activity by Nur77 is cell type dependent (14, 16).

The chemotherapeutics drugs cisplatin, tamoxifen, doxorubicin, 5-FU and irinotecan used in the treatment of breast cancer were analyzed in a cell viability test to verify the ability of AMS35AA to potentiate their effects. 5-FU was the only drug that demonstrated agonism when combined with AMS35AA. All the others presented antagonism. On that account, the combination of AMS35AA with cisplatin, tamoxifen, doxorubicin and irinotecan are not indicated in view of their reduction in anticancer action. Equivalent finding was presented by Mauro *et al.*, 2017 in a similar study (16).

5-FU was the only chemotherapeutic agent that potentiated the effect of AMS35AA. For this reason, the other agents were not used in the upcoming association tests. The combination index value between the resorcinolic lipid and 5-FU were less than 1 indicating strong additivism additive effect in MCF-7 cells (8).

Experimental agents derived from natural or synthetic products offer opportunities to evaluate not only totally new chemical classes of anticancer agents, but also relevant mechanisms of action. In some studies on anticancer properties of natural or synthetic products, it was shown that resorcinolic lipids are able to inhibit both DNA and RNA synthesis and to possess the ability for DNA strand scission (17). Regarding the 5-FU mechanism of action, it was found that this pyrimidine antagonist is converted to the active element 5-FdUMP (5-fluorodeoxyuridine-monophosphate) (18). This metabolite interacts with the enzyme thymidylate synthase causing the suppression of thymidine triphosphate (TTP) and, thus, preventing DNA synthesis. In addition, 5-FU inhibits RNA processing and is incorporated into DNA at the site where TTP is suppressed. 5-FU is also capable of causing single strand breaks and double strands of DNA (17, 19).

These combined mechanisms of action may be responsible for the additive effect (increase of 30% of the affected fraction) observed for AMS35AA and 5-FU according to the combination index proposed by Chou and Talalay (1983) (8). The data below corroborate for this: potentiation of the anticancer effects and the increase of DNA damage evaluated by the comet assay, increase in the expression of ATR, p53, p21 and GADD45 (genes involved in DNA damage), which occurred in both 5-FU treated cells and in those treated with the combination of 5-FU + AMS35AA; and also, cell-cycle arrest in G₂/M phase observed more prominently in the combined treatment. In addition, there was an increase in the frequency of apoptosis, evaluated by the cytological method, which is associated with absence of cell membrane damage (evaluated by the flow cytometry test) and increased expression of the BAK, CASP7, CASP 9 and reduction of BCL-2 expression (genes involved in apoptosis) in the combined treatment (5-FU + AMS35AA). Many studies report the presence of proteins that may be linked to this accumulation in sub G₁ phase, as is the case of p21 increase expression (20).

Figure 7. Relative expression of genes involved in (A) DNA-damage ATR, P53, P21, GADD45 and (B) Apoptosis BAK, BAX, CASP6, CASP7, CASP9 and BCL-2 obtained by Real Time PCR.

Gamper, 2017, has reported similar results in a study of alkylresorcinol in human hepatocarcinoma cell, demonstrating: (1) morphological alterations of apoptosis; (2) DNA fragmentation, detected by laddering and appearance of a sub-G₁ phase population; and (3) condensed and fragmented nuclei by acridine orange– PI (20).

MCF-7 breast cancer cells do not express caspase 3, thought by some to be a critical component of the apoptosis cascade. Despite this, MCF-7 cells undergo morphological apoptosis after treatment with a variety of agents and conditions (21). In agreement with this finding, the present work demonstrates that MCF-7 cells expressed damaged and apoptosis genes after treatment with AMS35AA. The underlying molecular mechanisms of toxicity of various antineoplastic drugs are only partially known, in spite of their extended clinical use. There is compelling evidence that one

of their main effectors is the p53, known as an important mediator in the action of many drugs (22). DNA damage signal through ATM/ATR or *via* CHK1/CHK2 can activate p53 in a direct or indirect manner, respectively (23). The results of this study indicate that P21 may function as a primary response gene. In addition, it appears to be involved in G₂/M phase cell accumulation, explaining its high expression in the Real Time PCR test (24). This research indicates that AMS35AA treatment down-regulates Bcl-2 (anti-apoptotic) expression. It could be inducing a conformational change of Bcl-2, with activation of caspase-7 and -9 in MCF-7 cells which favor spontaneous apoptotic cell death (25). The expression of BAX (pro-apoptotic) was also down regulated. Therefore, this finding suggests that the pathway that leads to apoptosis is BAX-dependent. The compound inhibited ATR, the upstream activator of CHK1, avoiding the DNA repair. But when combined with 5-FU there was an increase in ATR, however, not enough to repair the damage.

Our results suggest that AMS35AA has potential for the treatment of breast cancer since it is capable of exerting cytotoxic and cytostatic action in MCF-7 tumoral lineage. In addition, it is possible to affirm that cell death is mediated by DNA damage that leads cells to apoptosis by caspases activation. Finally, our results further suggest that AMS35AA is able to potentiate the effects of the chemotherapeutic agent 5-FU, for this reason it is a candidate to be an adjuvant in breast cancer therapy.

Conflicts of Interest

The Authors declare that they have no conflicts of interest regarding this study.

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