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α-Mangostin Synergizes the Antineoplastic Effects of 5-Fluorouracil Allowing a Significant Dose Reduction in Breast Cancer Cells

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Abstract: Breast cancer is the most common neoplasm and the leading cause of cancer death in women worldwide. Although 5-fluorouracil is a conventional chemotherapeutic agent for breast cancer treatment, its use may result in severe side effects. Thus, there is widespread interest in lowering 5-fluorouracil drawbacks, without affecting its therapeutic efficacy by the concomitant use with natural products. Herein, we aimed at evaluating whether α-mangostin, a natural antineoplastic compound, could increase the anticancer effect of 5-fluorouracil in different breast cancer cell lines, allowing for dose reduction. Cell proliferation was evaluated by sulforhodamine-B assays, inhibitory concentrations and potency were calculated by dose-response curves, followed by analysis of their pharmacological interaction by the combination-index method and dose-reduction index. Cell cycle distribution was evaluated by flow cytometry. Each compound inhibited cell proliferation in a dose-dependent manner, the triple negative breast cancer cells being the most sensitive. When 5-fluorouracil and α-mangostin were used concomitantly, synergistic antiproliferative effect was observed. The calculated dose-reduction index suggested that this combination exhibits therapeutic potential for reducing 5-fluorouracil dosage in breast cancer. Mechanistically, the cotreatment induced cell death in a greater extent than each drug alone. Therefore, α-mangostin could be used as a potent co-adjuvant for 5-fluorouracil in breast cancer.

Keywords: α-mangostin; breast cancer; combination index; dose-reduction index; 5-fluorouracil

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1. Introduction

Breast cancer is the most frequently diagnosed tumor and is the leading cause of oncological-related mortality among women worldwide [1]. Considering the molecular expression profile, breast cancer is generally classified into four different subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER-2) enriched and triple negative breast cancer (TNBC) [2–5]. Luminal tumors are estrogen receptor positive (ER+) [2–5], thus benefiting from hormonal therapy such as tamoxifen, fulvestrant and aromatase inhibitors [2,6], while HER-2 enriched breast tumors are mainly treated with monoclonal antibodies or tyrosine kinase inhibitors [7]. However, the TNBC are not candidates for

targeted therapy, and there are few existing therapeutic alternatives, including chemotherapeutic agents such as 5-fluorouracil (5-FU), a false nucleotide [6,8–10]. However, the well-known side effects of 5-FU limit its use [10–12]. A promising way for overcoming these drawbacks is to reduce 5-FU dose by its combination with less toxic agents with anti-neoplastic activity, such as natural compounds. In this regard, α-mangostin (AM), a xanthone obtained from the pericarp of mangosteen (*Garcinia Mangostana* Linn), exhibits a broad spectrum of anticancer effects including apoptosis, inhibition of cell proliferation and metastasis [13–15]. The antineoplastic mechanisms of AM have been widely studied in leukemia [16,17], prostate [18–20], pancreatic [21], colon [22–24], and breast cancer [25–30]. In the last, different outcomes have been reported regarding AM potency in non-hormone and hormone-dependent breast cancer [25,28,30]. On one hand, Li and colleagues showed that AM induces apoptosis with similar potency regardless of ER expression by inhibiting fatty acid synthase, which is required for cell proliferation [28]. On the other hand, Won and colleagues reported that only ER+ breast cancer cells are highly sensitive to AM [30]. Similarly, Balunas and colleagues showed that, in this phenotype, AM inhibits aromatase activity, blocking the main survival pathway of these cells [25]. Of note, the antineoplastic effect of AM has been evaluated in conjunction with chemotherapeutic agents in colon [31,32], melanoma [33], pancreatic [34], cervical [35] and gallbladder cancer cells [36], where the effect was greater than mono-treatments. The above suggests that the xanthone could act as an adjuvant agent of conventional chemotherapy. Regarding breast cancer, there are only two reports studying the effects of the ethanolic extract of mangosteen or AM combined with doxorubicin [37,38]. However, to our knowledge, there are no studies looking specifically at the combination of AM in combination with 5-FU in breast cancer cells with different phenotypes.

Therefore, in this study we investigated the effects of AM alone or combined with 5-FU on proliferation of breast cancer cell lines with a different phenotype. Additionally, we evaluated the nature of the pharmacological interaction between the chemotherapeutic agent and the natural compound, as well as the effect on cell cycle distribution in order to gain insight into the mechanisms involved.

2. Materials and Methods

2.1. Cell Culture

In this study, we used breast cancer cell lines with different phenotypes (Table 1). The established human breast cancer cell lines SUM-229PE (Asterand, San Francisco, CA, USA), HCC-1806 and T-47D (ATCC, Manassas, VA, USA) were maintained following indications from suppliers. The M serial breast cancer ductal F primary cell culture (MBCDF) and MBCDF-D5 were kindly provided by Dr. María de Jesús Ibarra-Sánchez and Dr. José Esparza-López (Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, México). All experimental procedures were performed in DMEM-F12 medium supplemented with 100 units/mL penicillin plus 100 µg/mL streptomycin and 5% charcoal-stripped-heat-inactivated fetal bovine serum (Gibco, Dublin, Ireland) under standard cell culture conditions.

Table 1. Breast cancer cells phenotype.

Cell Line	Phenotype	References
SUM-229PE	TNBC	[39]
MBCDF-D5	TNBC	[40,41]
HCC-1806	TNBC	[39]
MBCDF	HER-2 enriched	[41]
T-47D	ER+	[42]

The SUM-229PE, MBCDF-D5 and HCC-1806 cells represent a triple negative breast cancer (TNBC) phenotype. MBCDF is human epidermal growth factor receptor 2 (HER-2) enriched, and T-47D cells are estrogen receptor positive (ER+).

2.2. Proliferation Studies

Cells were seeded in 96-well plates (1000 cells/well) and after 24 h they were incubated in the presence of different concentrations of 5-FU (0.01–6.0 μM, Sigma-Aldrich, St Louis, MO, USA), AM (0.1–8.0 μM, Sigma-Aldrich) or their vehicle (0.1% v/v DMSO) for 6 days. Afterward, cell proliferation was evaluated by the sulforhodamine B (SRB) colorimetric assay [43]. Briefly, cells were fixed in ice-cold trichloroacetic acid at 4 °C for 1 h and air-dried, then SRB (dissolved in acetic acid at 0.057%) was added to each well and incubated at room temperature for 1 h. The unbound dye was removed with acetic acid (1% v/v) and the protein-bound dye was extracted from viable cells with an alkaline solution (10 mM Tris base, pH 10.5) and shaking. The absorbance was read at 492 nm in a microplate reader (Synergy HT Multi Mode Microplate Reader, BioTek, VT, USA). The concentration values that caused 20% (IC_{20}) and 50% (IC_{50}) cell growth inhibition were calculated by the dose-response fitting function, using the scientific graphing software Origin 9.0 (OriginLab Corporation, Northampton, MA, USA).

2.3. Combination Index and Dose Reduction Index Determination

The IC_{20} and IC_{50} values of each compound were used to determine the pharmacological interaction between 5-FU and AM, by calculating the combination index and dose reduction index (DRI), as previously reported [44,45]. Combination index values < 1, =1 or >1 depict synergism, additive effect or antagonism, respectively, while DRI values < 1, =1 or >1 indicate not favorable dose-reduction, no dose-reduction or favorable dose reduction, respectively [44,45].

2.4. Cell Cycle Distribution

Cells were seeded in 6-well plates (30,000–70,000 cells per well, depending on the cell line). After 24 h, the cells were incubated with vehicle (DMSO 0.1%) or 5-FU (IC_{20} or IC_{50}) in the presence or absence of AM (IC_{50}) during 72 h. After treatment, cells were collected, washed in PBS, fixed in ethanol (70% v/v) and kept at −20 °C. For cell cycle analyses, samples were washed twice with PBS pH 7.2 and incubated in a solution containing RNase (10 μg/mL), Triton X-100 (0.1% v/v) and 7-amino actinomycin D (7AAD, 1 μg/mL) in the dark at room temperature for 20 min. The DNA content was determined using the FACSCanto II flow cytometer (Becton Dickinson, CA, USA) and results were analyzed by FlowJo V10 software (Becton Dickinson, Ashland, OR, USA).

2.5. Statistical Analysis

Statistical differences were established by one-way ANOVA followed by appropriate post hoc tests for multiple comparisons. The comparisons between two treatments were analyzed by Student´s *t*-test using a specialized software package (SigmaStat 3.5, Jandel Scientific, CA, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. 5-FU and AM Alone Inhibited Breast Cancer Cell Proliferation in a Dose-Dependent Manner

The antiproliferative effects of 5-FU and AM were evaluated in a panel of breast cancer cell lines. Briefly, the cells were treated with different concentrations of 5-FU or AM for 6 days followed by the analysis of proliferation by the SRB assay. After analysis, both compounds significantly inhibited breast cancer cell proliferation in a dose-dependent manner (Figure 1). According to the dose-response curves, the greatest growth inhibitory effect of the compounds was observed in the TNBC cells. When using drug concentrations higher than 2 μM and 5 μM for 5-FU and AM, respectively, a complete inhibition of cell proliferation was observed.

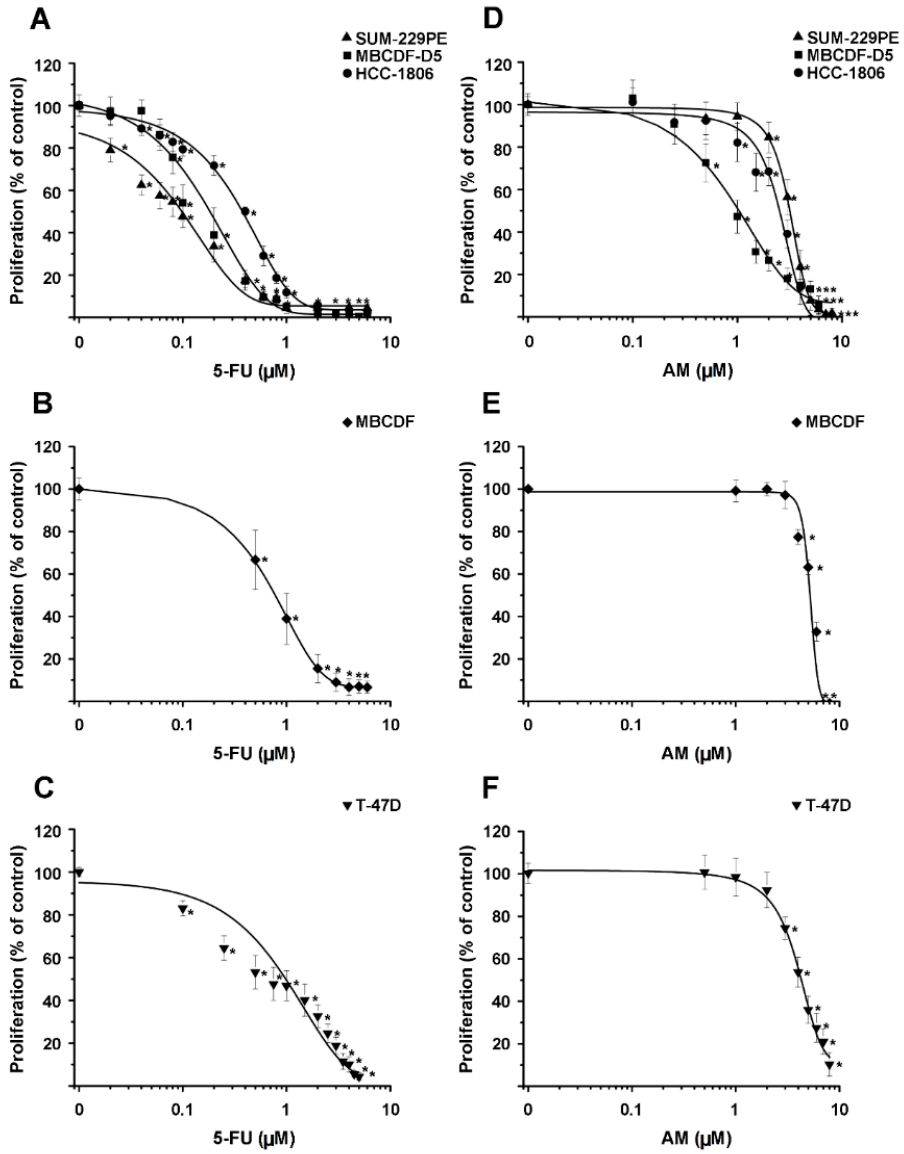

Figure 1. Antiproliferative effects of 5-fluorouracil and α-mangostin in a panel of breast cancer cells. The chemotherapeutic agent 5-fluorouracil (5-FU) and the xanthone α-mangostin (AM) dose-dependently inhibited cell proliferation with different potency, depending on the cell phenotype: triple negative (**A**,**D**), HER-2 enriched (**B**,**E**) and ER+ (**C**,**F**). The results are depicted as the mean ± SEM of six replicates in at least three independent experiments. Data from vehicle-treated cells were normalized to 100%. * $P < 0.001$ vs. control.

Based on the dose-response curves, the IC_{20} and IC_{50} values were calculated for both drugs (Table 2). These concentrations were taken into account for the combined treatments. Considering IC_{50} values, the following scale of sensitivity was obtained among the cells studied, for 5-FU: SUM-229PE > MBCDF-D5 > HCC-1806 > T-47D > MBCDF, and for AM: MBCDF-D5 > HCC-1806 > SUM-229PE > T-47D > MBCDF. According to these results, TNBC cells were the most sensitive for 5-FU and AM, while the HER-2 enriched were the less sensitive cells. The mean values of IC_{20} and IC_{50} of 5-FU in TNBC cells are 0.105 ± 0.11 μM and 0.213 ± 0.192 μM, respectively; while mean values for AM are 1.47 ± 1.16 μM and 2.16 ± 1.236 μM, respectively.

Table 2. IC_{20} and IC_{50} values of 5-fluorouracil and α-mangostin.

Cell line	5-FU (μM)		AM (μM)	
	IC_{20}	IC_{50}	IC_{20}	IC_{50}
SUM-229PE	0.016 ± 0.01	0.061 ± 0.01	2.39 ± 0.12	3.13 ± 0.09
MBCDF-D5	0.07 ± 0.01	0.15 ± 0.01	0.16 ± 0.11	0.77 ± 0.22
HCC-1806	0.23 ± 0.02	0.43 ± 0.02	1.87 ± 0.20	2.59 ± 0.17
MBCDF	0.36 ± 0.03	0.70 ± 0.03	4.67 ± 0.24	5.23 ± 0.19
T-47D	0.18 ± 0.07	0.54 ± 0.13	2.70 ± 0.11	4.36 ± 0.17

Inhibitory concentrations at 20% (IC_{20}) and 50% (IC_{50}) were calculated based on the dose-response curves of 5-fluorouracil (5-FU) and α-mangostin (AM). Results are depicted as the mean ± SEM.

3.2. The Antiproliferative Activity of 5-FU Was Significanlty Enhanced by AM in Cultured Breast Cancer Cells

To evaluate the antiproliferative effects of 5-FU combined with AM, the following combination schemes (5-FU/AM) were considered: IC_{20}/IC_{20}, IC_{20}/IC_{50}, IC_{50}/IC_{20} and IC_{50}/IC_{50}. As shown in Figure 2, the combination of both drugs significantly reduced breast cancer cells proliferation in a greater extent than each compound alone. The IC_{50}/IC_{50} combination inhibited cell growth up to ∼90% in SUM-229PE, 86% in HCC1806 and 80% in MBCDF-D5, MBCDF and T-47D (Figure 2). Although in the cell lines MBCDF-D5 (Figure 2B) and HCC-1806 cells (Figure 2C) all the combination schemes resulted in a similar growth inhibitory potency, in SUM-229PE, MBCDF and T-47D (Figure 2A, D and E, respectively), the scheme using higher doses of both compounds (IC_{50}/IC_{50}) was more potent as compared to the IC_{20}/IC_{20} combination.

3.3. The Combination of 5-FU with AM Acted Synergistically to Inhibit Cell Growth in Most Cell Lines Tested, Allowing for a Significant 5-FU Dose-Reduction While Preserving Its Potency

To discern the pharmacological interaction of 5-FU with AM, the combination index and DRI were calculated taking into account the combination schemes. In SUM-229PE, MBCDF-D5 and T-47D cells, all the co-treatment schemes showed combination index values < 1, reflecting synergism (Figure 3). A similar result was obtained in HCC-1806 cells with IC_{20}/IC_{20} (5-FU/AM), while an additive effect was observed with IC_{50}/IC_{20} and IC_{50}/IC_{50}. Regarding MBCDF cells, the drug combinations, although inhibitory, did not reflected synergistic effect (Figure 3). Of note, combination index values closer to zero reflect greater synergism than the values closer to one, while fraction affected values closer to one indicate greater inhibitory growth effect than the values closer to zero. In a general way and comparing the combination schemes per cell line, the greatest synergistic effect was observed with IC_{20}/IC_{20} (Figure 3, circles) while the greatest fraction affected was observed with IC_{50}/IC_{50} (Figure 3, diamonds).

With the aim to determine how many folds the dose of each drug in combination may be reduced, we calculated the DRI values on the combination schemes showing synergistic and additive antiproliferative effects (Table 3). Remarkably, in all cell lines tested, incubations in the presence of AM allowed to reduce 5-FU doses while maintaining the same efficacy as the drug alone. The cell lines in which 5-FU and AM doses could be significantly reduced were those where the compounds alone showed the highest antiproliferative effect (Table 2), as is the case of SUM-229PE and MBCDF-D5 (Table 3), where 5-FU and AM doses could be reduced up to 7- and 15-folds, respectively.

3.4. Breast Cancer Cell Death Was Promoted by AM, 5-FU and Their Combination

To get mechanistic insights, we studied the cell cycle distribution effects of both compounds alone and combined in one cell line *per* breast tumor phenotype. Representative histograms depicting the cell cycle distribution of SUM-229PE cells treated with 5-FU, AM or their combinations are shown in Figure 4A. As seen, only 5-FU significantly induced cell death (Sub G1) in SUM-229PE cells, an effect that was further enhanced by its combination with AM (Figure 4A,B). Such effect was accompanied with a reduction in the G1 population

(Figure 4B). The opposite was observed in MBCDF and T-47D cells, where AM per se significantly promoted cell death, while 5-FU did not (Figure 4C,D). In these two cell lines, the drug combination did not further modify the effect of AM.

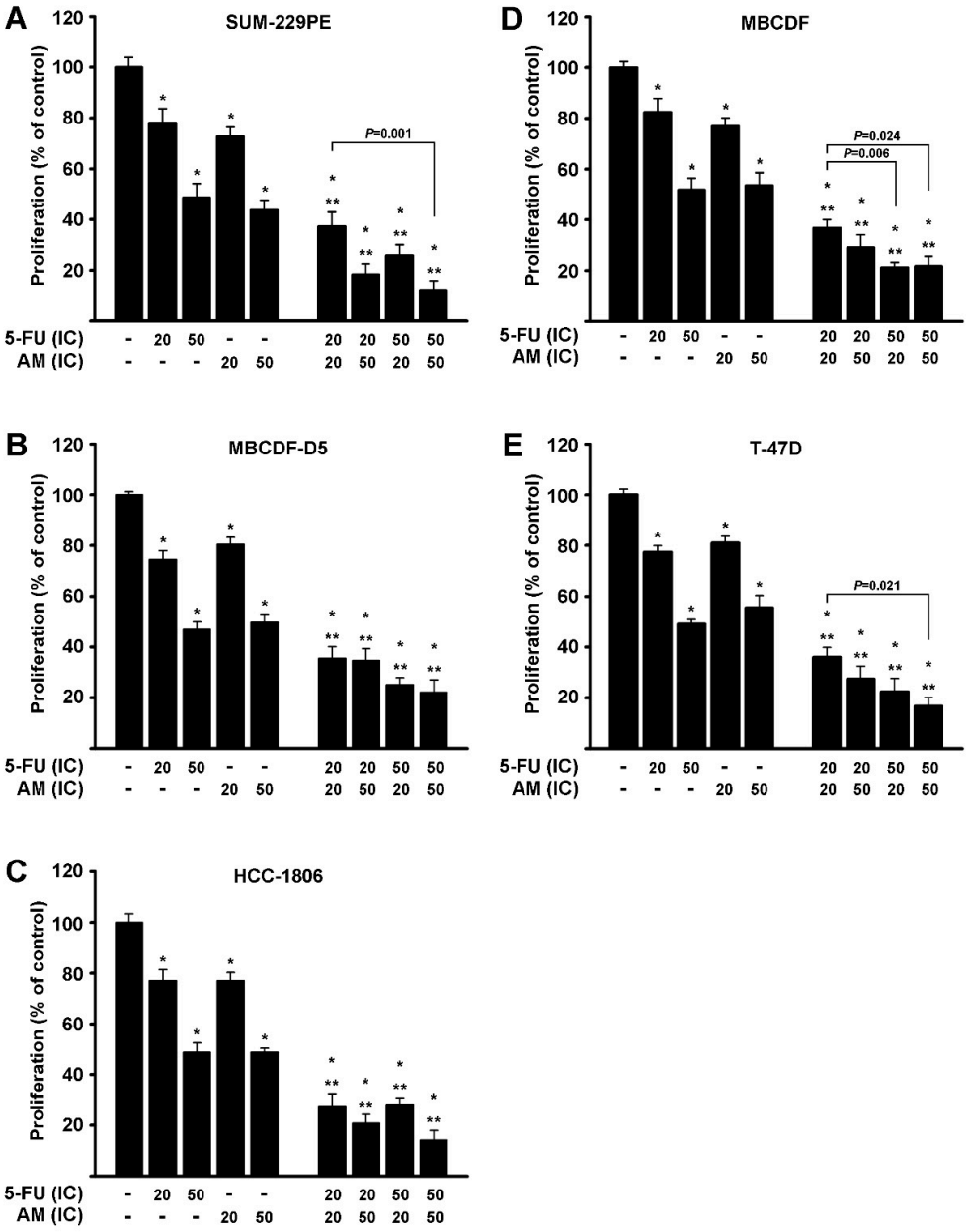

Figure 2. The combined treatment inhibited cell proliferation in a greater extent than each compound alone. The antiproliferative effect of the respective inhibitory concentrations (IC) at 20% and 50% of 5-fluorouracil (5-FU) and α-mangostin (AM) per se or in combination was evaluated in a panel of breast cancer cell lines. Results are depicted as the mean ± SEM of six replicates in at least three independent experiments. Data from vehicle-treated cells were normalized to 100%. * $P < 0.001$ vs. control, ** $P < 0.001$ vs. monotreatment (5-FU or AM alone).

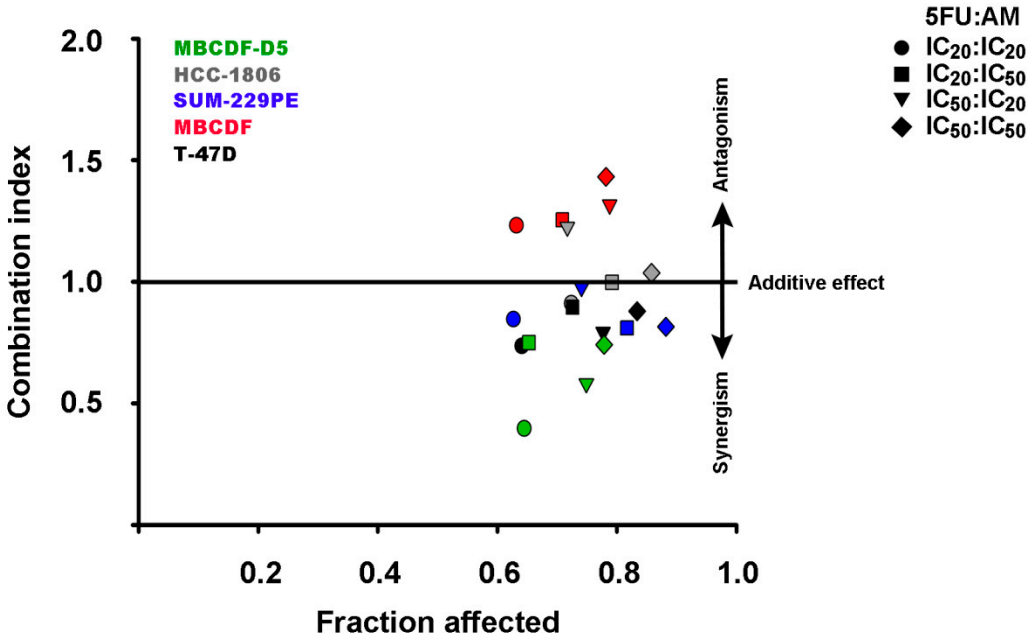

Figure 3. Combination index versus fraction affected in cells exposed to different combinations of 5-fluorouracil with α-mangostin. The combination index and the fraction affected were determined by co-incubating the cells in the presence of the inhibitory concentrations at 20% (IC_{20}) and/or 50% (IC_{50}) of 5-fluorouracil (5-FU) and α-mangostin (AM) in the breast cancer cell lines MBCDF-D5, HCC-1806, SUM-229PE, MBCDF and T-47D. The combination schemes evaluated (5-FU/AM) were IC_{20}/IC_{20}, IC_{20}/IC_{50}, IC_{50}/IC_{20} and IC_{50}/IC_{50}. The symbols under, on or over the horizontal line depict synergism, addition or antagonism, respectively. Fraction affected values closer to 1 indicate greater inhibitory growth effect than values closer to 0. N ≥ 3 independent experiments with six replicates each.

Table 3. Dose reduction index of 5-fluorouracil and α-mangostin.

Cell Line	Combination Schemes	DRI (Folds)	
	5FU/AM	5-FU	AM
MBCDF-D5	IC_{20}/IC_{20}	3.32	**10.38**
	IC_{20}/IC_{50}	**3.40**	2.20
	IC_{50}/IC_{20}	1.94	**15.45**
	IC_{50}/IC_{50}	**2.11**	1.24
SUM-229PE	IC_{20}/IC_{20}	**6.43**	1.45
	IC_{20}/IC_{50}	**6.44**	1.34
	IC_{50}/IC_{20}	**1.87**	1.61
	IC_{50}/IC_{50}	**7.25**	1.48
T-47D	IC_{20}/IC_{20}	**4.73**	1.91
	IC_{20}/IC_{50}	**6.48**	1.35
	IC_{50}/IC_{20}	**2.68**	2.39
	IC_{50}/IC_{50}	**3.58**	1.67
HCC-1806	IC_{20}/IC_{20}	**3.00**	1.73
	IC_{20}/IC_{50}	**3.76**	1.37
	IC_{50}/IC_{50}	**2.63**	1.52

The dose reduction index (DRI) calculation was based on the synergic or additive effect between 5-fluorouracil (5-FU) and α-mangostin (AM) using their inhibitory concentrations at 20% (IC_{20}) and 50% (IC_{50}). DRI values higher than one indicate a favorable dose reduction. DRI values highlighted in bold indicate the higher DRI value for each combination scheme.

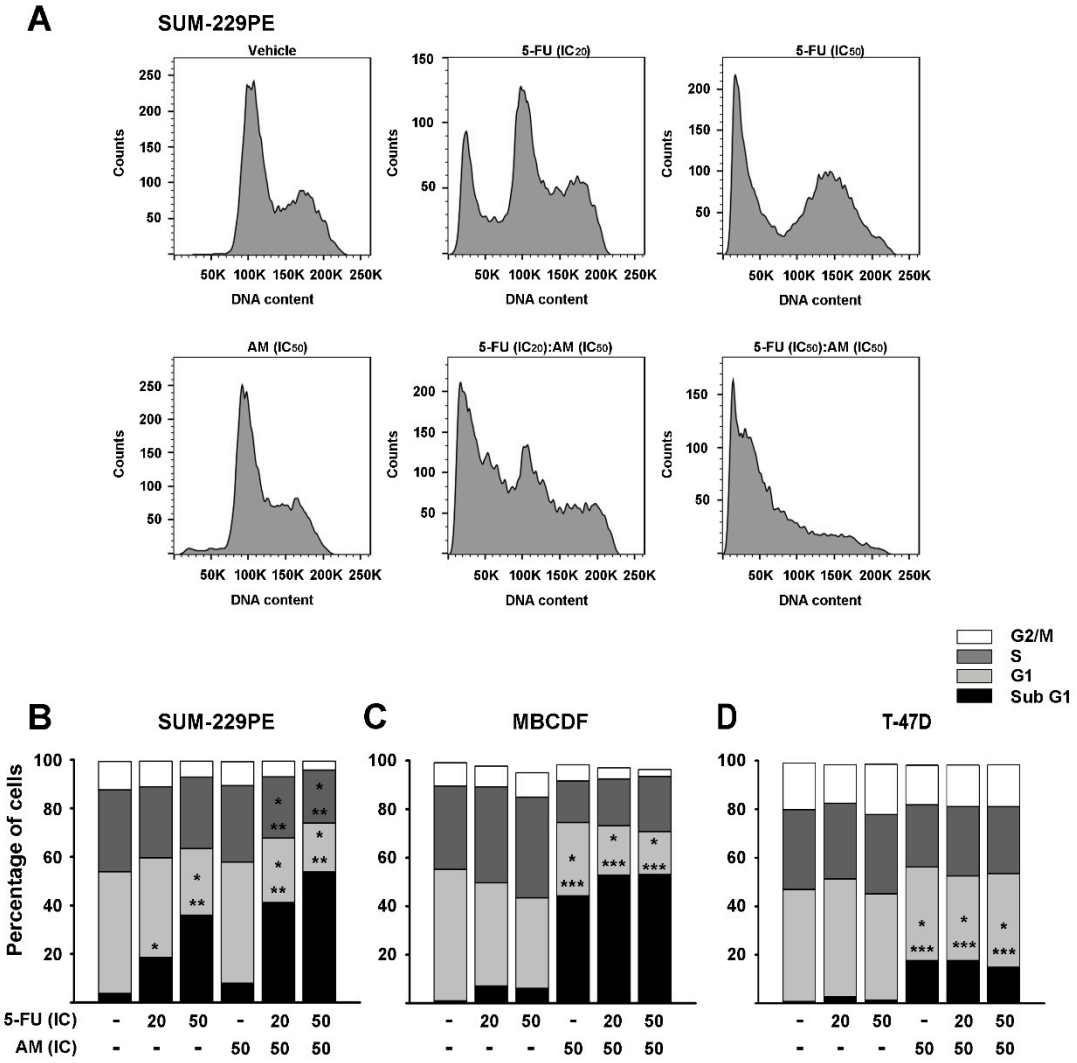

Figure 4. Effects of 5-fluorouracil, α-mangostin or their combination on the cell cycle distribution in neoplastic cells. The effects of the inhibitory concentrations at 20% (IC_{20}) and 50% (IC_{50}) of 5-fluorouracil (5-FU) and IC_{50} of α-mangostin (AM) and/or 5-FU were evaluated in the cell cycle distribution of SUM-229PE (**A**,**B**), MBCDF (**C**), and T-47D (**D**) breast cancer cells. Results are depicted as the percentage of cells in each phase of the cell cycle from at least three independent experiments. * $P < 0.05$ vs. control, ** $P < 0.05$ vs. AM, *** $P < 0.05$ vs. 5-FU.

4. Discussion

One of the motivations of this study was to determine AM antiproliferative activity and potency in breast cancer cells with different phenotypes, given the discrepancy prevailing in the literature in this topic [25,28,30]. In addition, as chemotherapy is the main option for TNBC, we thought of importance to study if AM allowed for 5-FU dose reduction while maintaining a similar antiproliferative activity. Our results showed that AM consistently and significantly reduced proliferation in all breast cancer phenotypes, with TNBC being the most sensitive (IC_{50} = 2.16 ± 1.24 μM), followed by ER+ cells (IC_{50} = 4.36 ± 0.17 μM) and HER-2 enriched cells (IC_{50} = 5.23 ± 0.19). These results support the idea that AM induces its antineoplastic effects regardless of ER expression. As expected, 5-FU also reduced proliferation in all breast cancer cells and the TNBC was the most sensitive. These results were consistent with other studies showing increased sensitivity to 5-FU in ER negative tumors [46,47]. A possible explanation for the increased effects of both compounds in TNBC cells may reside in their increased sensitivity to oxidative stress due to defects in the DNA damage response pathways, which can lead to mitotic catastrophe [48,49]. Regarding

this, both AM [18,32] and 5-FU [11] induce reactive oxygen species (ROS) accumulation, promoting oxidative stress and cell death.

Our results regarding the combination index strongly suggest that AM may function as an excellent adjuvant of 5-FU, given the synergic pharmacological activity observed in several breast cancer cell lines. The observed synergism may be the result of mutually exclusive antineoplastic mechanisms of both compounds [44]. While 5-FU targets cancer cells by blocking the synthesis of nucleic acids [8–10], AM exerts its antineoplastic effects by inhibiting cyclin-dependent kinases (CDK) involved in cell cycle progression [50,51], the expression and activity of fatty acid synthase [28], aromatase activity [25] and (4) the expression of ER in breast cancer [30].

Regarding the mechanisms of action of AM involved in the synergic effect observed herein, further studies are warranted; however, previous studies in other neoplasms such as leukemia and colon cancer have shown that AM induced apoptosis via the caspase-3 pathway or through the rupture of the mitochondrial membrane and the subsequent release of endonuclease-G [17,24].

One major aim for achieving synergy in drug combination studies is the dose-reduction of the cytotoxic agent while preserving the therapeutic effect [44]. Our results showed favorable dose reduction (DRI > 1), supporting the possibility to scale down the dose of 5-FU in breast cancer patients by combining it with AM. The combination of 5-FU and AM has been previously tested in colon cancer cells resistant to the chemotherapeutic agent, showing the sensitization of these cells to 5-FU–dependent apoptosis [32]. Similarly, other studies have shown an enhanced effect of chemotherapeutic agents when combined with AM in different tumors [31–36]. Particularly in breast cancer MCF-7 cells, the combination of doxorubicin with mangosteen derivatives has resulted in improved antineoplastic effects [38], including the reduction of stemness and the induction of cell death [37]. The above, together with our results, strongly support the concept that AM could act as an adjuvant of conventional chemotherapy. The current chemotherapeutic regimen to treat breast cancer includes 5-FU, commonly combined with other cytotoxic agents such as doxorubicin, epirubicin, cyclophosphamide and/or methotrexate [6]. The advantage to combine 5-FU with AM instead of these drugs would be the induction of synergism without increasing systemic toxicity. In line with this, in vivo studies have shown AM cardioprotective [52] and neuroprotective [53] properties, suggesting that the xanthone could counteract the adverse effects of 5-FU.

To our knowledge, this is the first report showing the combined effects of 5-FU with AM in breast cancer cells with different phenotypes. Interestingly, the synergic interaction between 5-FU and AM was not observed in MBCDF cells [54], which can satisfactorily be explained by the high cellular heterogeneity prevailing in this cell line [41,55].

Several studies have been carried out in order to elucidate AM anticancer mechanism. In TNBC, AM has been shown to promote apoptosis, cell cycle arrest [27], and spheroids-cell adhesion reduction [56]. Similarly, in ER+ cells, the xanthone induces apoptosis [26] and reduces metastasis [57]. The present study demonstrates that the combination of AM with 5-FU promoted greater accumulation of cells in Sub G1 phase, suggesting that death is part of the mechanisms involved. A limitation of this investigation is the lack of in vivo experiments, which remain the subject of future studies.

In summary, herein we describe the effects of combining 5-FU with AM on cell growth in various established and primary breast cancer cell lines. We determined the pharmacological interaction of the combined effect and the possibility to scale down the doses of both compounds, contributing to new knowledge in the field. Overall, our results provide scientific basis to test 5-FU with AM as a therapeutic option for patients whose only alternative is chemotherapy, specifically those with TNBC tumors.

5. Conclusions

The xanthone AM inhibited breast cancer cells proliferation independently of their phenotype, with greater potency in TNBC cells. AM allowed to significantly reduce 5-FU

dose while maintaining its efficacy to inhibit cell growth. Given the synergic interaction between 5-FU and AM, the use of this natural compound as co-adjuvant might result in less chemotherapy-derived adverse effects. We believe that our results may help to conceptualize further preclinical and clinical studies to provide TNBC-patients with an affordable and effective new therapeutic approach with reduced undesirable side effects.

Author Contributions: G.L.-S. performed the experiments and analysis of data. L.D. and J.G.-Q. performed analysis/interpretation of data and wrote the manuscript. R.G.-B. designed the figures. L.D., F.L., R.G.-B. and E.A. made substantive intellectual contributions to the study and helped to draft the manuscript. H.P.-G. carried out experimental flow cytometry analysis and interpretation of data, reviewed and edited the manuscript. M.d.J.I.-S. and J.E.-L. contributed with critical revision of the manuscript. G.M.-G. performed reviewed and edited the manuscript. J.G.-Q. conceived, designed and coordinated the study. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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