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Amygdalin folic acid nanoparticles inhibits the proliferation of breast cancer and enhances the effect of radiotherapy through modulation of tumor promoting factors/immunosuppressive modulators in vitro

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ABSTRACT

Purpose: Breast Cancer (BC) cells often develop multiple mechanisms of chemo and radio-resistance during tumor progression, which is the major reason for the failure of breast cancer therapy. Targeted nano-medicines have tremendous therapeutic potential in BC treatment over their free drug counterparts. Searching for chemo-and radio sensitizers to overcome such resistance is therefore urgently required. The goal of this study is to evaluate and compare the radio-sensitizers efficacy of Amygdalin-Folic acid-nanoparticles (Amy-F) on MCF-7 and MDA-MB-231 cells.

Methods: The effects of Amy-F on MCF-7 and MDA-MB-231 cells proliferation and IC50 were assessed using MTT assay. The expression of proteins involved in several mechanisms induced by Amy-F in MCF-7 and MDA-MB-231 cells, including growth inhibition, apoptosis, tumor growth regulators, immuno-modulators and radio sensitizing activities were evaluated via flow cytometry and ELISA assay.

Results: Nanoparticles demonstrated sustained Amy-F release properties and apparent selectivity towards BC cells. Cell-based assays revealed that Amy-F markedly suppresses cancer cell growth and improves Radiotherapy (RT) through inducing cell cycle arrest (G_1 and sub G_1), and increases apoptosis as well as reduces the proliferation of BC by down-regulating Mitogen-Activated Protein Kinases (MAPK/P38), Iron level (Fe), Nitric Oxide (NO), and up-regulating the Reactive Oxygen Species level (ROS). Amy-F has also shown to suppress the expression of the Cluster of Differentiations (CD4 and CD80), and interfering with the Transforming growth factor beta (TGF- β)/Interferon Gamma (INF-g)/ Interleukin-2 (IL-2)/Interleukin-6 (IL-6)/Vascular Endothelial Growth Factor (VEGF) induced suppression in its signaling hub, while up-regulating Natural Killer Group 2D receptor (NKG2D) and CD8 expression.

Conclusions: Collectively, the novel Amy-F either alone or in combination with RT abrogated BC proliferation.

Key words: Amygdalin; Anticancer; Immunosuppressive modulators; Nanoparticles; Radio-sensitization; Tumor promoting factors

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INTRODUCTION

Breast Cancer (BC) is one of the major threats challenging women's health all over the world. This threat represents the shared type of malignant tumor in females, with the highest mortality rate [1]. Several regimens and protocols are approved as treatment for this lethal disease, including chemotherapy, radiotherapy, and surgery in addition to different protocols of combination therapy. Chemotherapy remains the first choice for breast cancer treatment. Nevertheless, the deadly side effects resulting from these cytotoxic chemotherapeutics have posed hindrances along the way [2]. Cancer cells generate an immunosuppressive microenvironment called Tumor Microenvironment (TME) to regulate tumor growth, promote tumor immune escape, and as a source of Tumor Promoting Factors (TPFs) [3]. TPFs include growth factors, cytokines, extracellular matrix proteins, and hypoxia inducible factors, among others, which promote tumor growth, survival, and metastasis [4]. The TME is the networks of cells (such as immune cells, immune cell receptors (CD4, CD8, CD80, and NKG2D), and cancer-associated fibroblasts), promoting factors (*i.e.* tumor promotors (MAPK and P38), cytokines (IL-2, IL-6, and INF-γ), Growth Factors (TGF-β and VEGF), hormones, and signal stimulators (Fe, NO, and ROS), associated with the extracellular matrix and surrounding vasculature that surrounds cancer cells. The formation of TME relies essentially on tumor metabolism and therefore, is characterized by its high acidity and hypoxic state. Nanoparticle-mediated Drug Delivery Systems (DDS) have emerged as a promising tool in this direction, as they can be utilized for the treatment of various diseases by circumventing healthy body tissues, thus causing minimal cytotoxicity and cell death in healthy tissues while targeting only diseased tissues [5]. Many nanoparticles have anticancer properties on their own, whereas others are best described as nanocarriers used for ferrying the hydrophobic drugs selectively to the site of neoplasia [6].

Nowadays, reliance on natural products as a source of new drugs represents the core of new research in the field of drug discovery. Amygdalin, a naturally occurring vitamin B_{17} that could be found in the seeds of many plants in the Prunus Rosacea family (apricots, apples, bitter almond, black cherries, plums, and peaches), is one of those natural products that have received great attention [7]. Amygdalin has many great properties, especially its anticancer activity. Mechanistically, amygdalin function as an anticancer agent by inducing apoptosis and cell cycle arrest through releasing toxic Hydrogen Cyanide (HCN) only after its hydrolysis and thereby destroying cancer cells. Unfortunately, the cytotoxic HCN can also

permeate normal cells which results in cyanide poisoning and eventual cell damage [8].

Folic Acid (FA), improves the targeting efficiency of cancer therapy due to its preferential binding to the Folate Receptor-α (FR-α) overexpressed on cancer cells. FR-α receptors are known to be over expressed in BC cells and to have a high affinity for FA, which are typically captured to feed the fast-dividing BC cells [9]. Utilizing FA in therapeutic formulations has many advantages as FA could be considered as stipulated nutrient that has good stability, biocompatibility, as well as its biodegradability by Tumor Microenvironment (TME) allows for the release/availability of anticancer drugs at the tumor site [10]. Drugs encapsulated in nanocarriers are promising therapeutic modalities because they offer drug accumulation potential in tumor tissues thanks to the Enhanced Permeability and Retention (EPR) effects of their nanocarriers, which in turn augments cancer therapeutic efficacy [11]. Based on the aforementioned advantages of FA-functionalized nanocarriers, we have developed a stimuli-responsive FA-functionalized nanoparticle system consisting of amygdalin for targeted BC therapy.

Here, we report the synthesis, characterization, physiochemical properties, and cytotoxic profile of Amy-F nanoparticles against BC cells. We extended our goal to gain insight into the effect of novel Amy-F nanoparticle on tumor regulatory mediators role through modulation of tumor promoting factors/immunosuppressive modulators to restrain BC promotion *in vitro*.

LITERATURE REVIEW

Synthesis of Amy-F nanoparticles

The amygdalin solution was sonicated at 25°C for 15 min to form an aqueous solution (10 mM) of amygdalin (vitamin B_{17}). In a separate glass vial, 25 mg of folic acid was dissolved in 25 mL of Dimethyl Sulfoxide (DMSO) while stirring. The folic acid solution was then added to the amygdalin solution under vigorous stirring for 3 h. The resultant solution was then washed using MilliQ water and allowed to dry in an oven at 80°C. The resultant Amygdalin-Folic acid Nanoparticles (Amy-F NPs) was stored at room temperature for further work.

Characterization of Amy-F nanoparticle

High Resolution Transmission Electron Microscopy (HR-TEM) (JEOL JSM-5600 LV, Japan) was used to obtain the size and shape of the synthesized Amy-F nanoparticles. Using the X-ray diffraction technique (XRD; Shimadzu XRD-6000) the amorphous structure of the synthesized Amy-F nanoparticle samples was inspected. XRD spectra were acquired in the range of 2θ from 17°C to 90°Cat 25°C. Copper K-α is a radiation source of scan rate 0.8o/min, wavelength λ=0.15408 nm, current 40 mA and operation voltage 50 kV. Information on the surface morphology of the samples' particles is obtained using Scanning Electron Microscopy (SEM), JEOL JSM-5600 LV, Japan). Finally, examination using the Energy Dispersive X-ray spectra (EDX) (JEOL JSM-5600 LV, Japan) was performed to confirm nanoparticle formation.

Cell lines and culture media

Breast tumor cell lines (MCF-7 and MDA-MB-231) and normal cell line (MCF-10A) were obtained from the cell culture department, VACSERA, Cairo, Egypt. The cells were cultivated by the distributor's instructions in high glucose RPMI 1640 medium (Thermo Fisher Scientific Inc., USA) in addition to 10% FBS (Thermo Fisher Scientific Inc., USA), penicillin (100 U/mL) and streptomycin (100 µg/mL) and incubated at 37°C in a humid environment (5% CO_2).

Cytotoxicity assay

Cellular viability and morphological assay were analyzed to indicate the cytotoxic profile of Amy-F nanoparticles using the 3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, USA) assay according to Van-de, et al. [12]. Amy-F was dissolved in propylene glycol (Sigma-Aldrich, USA). Before use, dilution of the Amy-F stock solution was carried out to the indicated concentration using a culture medium, and the final concentration of propylene glycol was 0.1% (v/v) in each well. The control wells contain cells that received the vehicle (propylene glycol) treatment only. In a tissue culture plate (96-well), the wells were inoculated with 1 X 10^5 cells/mL (100 µL/well) followed by incubation for 24 hours at 37°C to generate a complete monolayer sheet. Following the formation of a confluent cell sheet, decantation of the growth medium was carried out, and washing of the cell monolayer was performed twice using washing media. Right after that, the Amy-F was diluted twice in RPMI medium enriched with 2% maintenance serum medium. A volume of 0.1 mL of each dilution was then examined in different wells, with three control wells that contain only maintenance medium. After incubation, the plate was examined under microscope for any signs of physical toxicity (cell shrinkage, rounding or shrinkage, monolayer loss entirely or partially). An MTT solution (5 mg/ml in PBS) was prepared, and each well received 20 µL of the solution. The plate was then placed on a plate shaker (150 rpm/5 mins) to ensure the homogeneous distribution of MTT into the medium. The cells were then incubated at 37°C and 5% CO_2 for 1-5 h to ensure MTT metabolization. Each well was then treated with 200 µL of DMSO after removing the media in order to solubilize the resultant formazan crystals (MTT metabolic product). The absorbance of each well was then recorded at 570 nm using ELISA plate reader (BioTeck, Bad Friedrichshall and Germany). Using SPSS (IBM Inc., USA) one-way ANOVA, the half-maximum Inhibitory Concentration (IC50) was calculated. GraphPad Prism software (v.8.0) (GraphPad Prism Inc., USA) was used to create the graphs. Cell morphology was recorded using an inverted microscope with a digital camera (Nikon, Japan). All studies were carried out in triplicate.

In vitro Amy-F release

The *in vitro* drug release study was carried out as demonstrated by Askar, et al. [13]. At 37°C, the Amy-F suspensions were subjected to different pH at 6, 7, and 9. After 24 h of incubation, under UV irradiation using a 350 W mercury-vapor lamp endowed with an optical filter UG5 that allow selecting the 200–380 nm spectral range, where there is the most intense Hg spectral lines at 253 nm. The FA release in phosphate buffer PB solutions was measured using Lambda 950 model UV-VIS-NIR spectrophotometer (Perkin Elmer) [14].

Cellular selectivity and Amy-F uptake

Normal (MCF-10) and BC cells (MCF-7 and MDA-MB-231) were sowed at 2×104 cells/well density in 24-well plates using round coverslips. The cells were incubated with 100 µg/mL Amy-F for 24 h. Following incubation for 24 h, the cells were washed in Phosphate Buffer Solution (PBS) three times and divided into two aliquots for divergent methods; the first method was used to investigate Amy-F cellular selectivity according to Askar, et al. by determining the expression of FR-α expression *via* qRT-PCR, as demonstrated later in the real-time PCR part.

The second method was to employ UV-VIS-NIR spectrophotometer in estimating Folic Acid (FA) concentration in normal and cancer cells as well as through uptake quantity of FA that reflects the Amy-F uptake in normal and BC cells. Prior to use, a standard

solution of FA was prepared by serially diluting a 1000 mg/L stock solution (Scharlau Chemie, Barcelona, Spain). After 24 h, the cells were rinsed three times with PBS, centrifuged at 3000 rpm for 15 min, and the supernatant was aspirated into another plain bottle using a Pasteur pipette. Supernatant and pellet cell samples were diluted with MilliQ water and homogenized before the determination of FA concentration using Lambda 950 model UV-VIS-NIR spectrophotometer (Perkin Elmer) at 200–380 nm spectral range.

Radiation facility

All the MCF-7 and MDA-MB-231 cells that underwent irradiation were irradiated with Gamma rays (γ-rays) at 85 % confluency as a single shot (single exposure to 4 Gy) at a dose rate of 0.012 Gy/Sec using a 137Cs source (Gamma-cell-40 Exactor; NCRRT, EAEA, Cairo, Egypt). The dosimetry was applied in all experiments to ensure dose uniformity and dose rate employing a Fricke reference standard dosimeter [13].

Cell culture models and the study protocol

Amy-F's anti-proliferative and radiosensitizing effectiveness was studied by dividing MCF-7, and MDA-MB-231 cell cultures into four distinct groups as described. After 24 h of incubation following the radiotherapy dose, cells were harvested for further investigations to reveal Amy-F's antitumor effect.

Cell cycle, apoptosis, CD4, CD8, CD80, and TGF-β analysis by low cytometry

After 24 h of incubation following the last dose of radiotherapy, the 3 x $c10^5$ cells/well were harvested with trypsin, washed twice in ice-cold PBS, then fixed with 70% ethanol at 4°C overnight for all groups of both MCF-7 and MDA-MB-231 cell lines. Afterwards, the cells were washed with PBS and centrifuged before being stained with Propidium-Iodide (PI) (50 µg/mL) for cell cycle analysis (Cat. No: ab139418) and apoptosis markers (BCl-2, Caspase-3, CD4, CD8, CD80, and TGF-β) were measured using FITC Kits (Cat. No: 340575, 550480, 557767, 557766, 567442, and 562962, respectively; Beckman Coulter, Marseille, France). The staining was evaluated using a FACSCanto-II flow cytometer, and the data were analyzed using BD Accuri-C6 Plus software (Biosciences, CA, USA) [15].

Determination of MAPK, P38, Fe, and ROS

The levels of phospho-MAPK (Ser-93), p38 (Phospho-Tyr-323), Fe, and intracellular ROS in treated and vehicle-treated MCF-7 and MDA-MB-231 cells were measured. Cells were seeded in a 6-well plate (2×10^6 cells/well) overnight in a complete medium, then harvested and homogenized in 100 µL ice-cold water. The levels of MAPK, P38, Fe, and ROS were measured using MyBioSource ELISA kits, Cat.No: MBS629151, MBS9404731, MBS267375, and MBS039665, respectively (MyBiosource, Inc. Southern California, San Diego, USA). Reactions were carried out following the manufacturer's protocol and absorbance was determined using an automatic microplate reader.

RNA isolation and real-time qRT- PCR analysis

Total RNA was extracted from MCF-7 and MDA-MB-231 cells utilizing Trizol Reagent (Thermo Fisher Scientific). The Prime-Script™ RT reagent kit was then used to extract cDNA from total RNA (Takara Bio Inc., Otsu, Japan). Quantitative Reverse Transcription polymerase chain reaction (qRT-PCR) was used to estimate the mRNA expression, which was done in triplicate utilizing a SYBR Premix Ex Taq TM kit (Takara Bio, Inc.) and an ABI 7900HT Real-Time PCR system (Thermo Fisher Scientific). The primer sequence is as follows: for Homo sapiens Folate Receptor alpha, transcript variant 7, mRNA (FR-α) expression: Forward primer; 5'-CTGGCTGGTGTTGGTAGAACAG-3' and reverse primer; 5'-AGGCCCCGAGGACAAGTT-3' (Genecode:NM_016724.3) and for Homo sapiens Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), transcript variant 6 as a housekeeping gene: forward primer; 5'-GTCAAGGCTGAGAACGGGAA-3' and reverse primer; 5'-AAATGAGCCCCAGCCTTCTC-3 (Gene code: NR_152150.2)'. The comparative cycle threshold values (2–ΔΔCt) were applied to assess the final results, as the GAPDH gene expression was utilized to normalize qRT-PCR results [15].

Statistical analysis

All experiments were carried out in triplicate, and the results were stated as the mean ± Standard Error (SEM). The Kolmogorov-Smirnov (KS, $p>0.10$) test verified data normality, and all data was found to be normally distributed. ANOVA and Tukey multiple comparison post hoc tests was used for data analysis. Statistical analyses were carried out using prism, version 8 (GraphPad Software, La Jolla, CA). The $p<0.001$, $p<0.01$, and $p<0.05$ statistical significance levels were applied to indicate the difference between groups [16].

RESULTS

Structural characterization of Amy-F nanoparticles

As illustrated in XRD diffractogram, the synthesized Amy-F nanoparticle revealed the amorphous nature of the organic vitamin B_{17} and folic acid. The generated XRD models agree with the original standard card for both amygdalin and folic acid. The following two unique peaks are located at 11.14°, and 24.84° corresponding to folic acid amorphous structure [17]. There are other important peaks noted at 2Θ=5.25°, and 16.19°, which corresponding to amygdalin XRD pattern [18]. The most significant diffraction peaks were slightly changed due to the new construction of Amy-F form.

EDX spectrum of Amy-F nanoparticles showed the coexistence of O, C, and N which are accredited to the organic nature of both amygdalin and FA. EDX elemental mapping revealed the uniform distribution of O, C, and N throughout the Amy-F nanoparticles.

The SEM analysis showed an irregular structure of the synthesized Amy-F nanoparticles with remarkable smooth agglomerates due to the occupation of a large number of layers at the grain boundary, which could control grain growth [19]. Furthermore, HR-TEM image confirms the irregular structure of Amy-F nanoparticles with diameters ranging from 80-215 nm and an average diameter of 155.58 nm.

FT-IR spectroscopy was carried out to investigate the surface functional groups of the synthesized Amy-F nanoparticles. The FT-IR spectrum of the synthesized Amy-F nanoparticles. Concordantly with the literature, the characteristic IR absorption peaks at 1691, 1608, and 1515 cm^{-1} are observed in the FTIR spectrum assigned to folic acid, and are caused by N-H bending vibration of the CONH group, C=O amide stretching of the α-carboxyl group, and absorption band of the phenyl ring, respectively [20]. A band at 3005 cm^{-1} is attributed to the OH and NH stretching regions. The presence of narrow peak bands at 1759 cm^{-1} in amygdalin IR spectra is due to aldehyde and ketone C=O stretching. The position of the C=O stretching indicated the hydrogen bonding and conjugation within the molecules. High intensity peak, followed by peaks at 2900 cm^{-1} and 2773 cm^{-1}, are attributed to O-H stretching (carboxylic acid) vibrations and aldehyde C-H stretching. This O-H stretching vibration may be due to carboxylic compounds in the polymer protein matrix. Finally, the absorption band at 1409

cm^{-1}, 1325 cm^{-1}, and 700 cm^{-1} were assigned to amide II, amide III, and amide IV, respectively. Depending on the comparison achieved between the FTIR data of bare FA and amygdalin, it is worth mentioning that the connection type between the FA and amygdalin was by intermolecular hydrogen bonding (weak bond) as described previously, which was not present in bare FA and amygdalin, that indicated the incorporation behavior between FA and amygdalin as demonstrated by a weak bond. In our FTIR results, the incorporation behavior was detected as new peaks formed in the synthesized nanoparticles (weak physical bond; van der Waals forces). Presented the chemical structure of FA and amygdalin, and explained the type of connection between FA and amygdalin.

Cytotoxicity assay

The IC_{50} of Amy-F on MCF-10A was 180.3 µg/mL. Whereas, the data of the anticancer effect of Amy-F on BCCs (MCF-7 and MDA-MB-231) was showed an anti-proliferative activity against the cancer cell lines after 24 h. The IC50 of Amy-F was revealed at 79.8 µg/mL and 94.9 µg/mL for MCF-7 and MDA-MB-231 cells, respectively. Based on the above data, it could be concluded that Amy-F exerts no cytotoxic effect on normal cells. Amy-F con-centration-dependent cell morphological alterations were shown in phase-contrast images. There was evidence of cell fragmentation, apoptotic cellular shrinkage, membrane blebbing, and disengage characteristics observed in MCF-7 and MDA-MB-231 cells treated with Amy-F nanoparticle compared to the intact MCF-10A-nor-mal cells.

Amy-F release

The pH-dependent drug-releasing properties of Amy-F was studied *in vitro* by using UV–Vis at different pH (6, 7 and 9) in Phosphate Buffer Solutions (PBS) containing DMSO 0.1%, to simulate the neutral environment of normal cells and acidic conditions in cancer cells, at pH 6, there is more than 55 % release of Amy-F. Whereas, at pH 7 and 9 the release of Amy-F was less than 10% and 1%, respectively, after 24 h. The elevated release of Amy-F at acidic environments could be ascribed to the protonation and high solubility of Amy-F at such environments.

Cellular selectivity and uptake of Amy-F

To test the selectivity of Amy-F towards cancer cells, FR-α gene expression was evaluated in MCF-10A cells, MCF-7 and MDAMB-231. Treating MCF-10A with Amy-F showed a non-significant change in the levels of FR-α compared to untreated MCF-10A cells. On the other hand, a significant elevation in FR-α expression was observed in untreated MCF-7 and MDA-MB-231 (by 8.3 and 8.6 fold, respectively) compared to untreated MCF-10A. Whereas, a significant reduction in the expression of FR-α was observed in MCF-7 and MDA-MB-231 after treatment with Amy-F nanoparticles (by 80.9% and 77.3%, respectively) compared to untreated MCF-7 and MDA-MB-231, respectively. Based on this data, it could be concluded that Amy-F is only selective to FR-α receptors that are over-expressed on breast cancer cell membranes due to FA moiety which is incorporated to the Amy-F nanoformulaion.

The data of UV-VIS-NIR spectrophotometer show the cellular uptake of Amy-F in normal cells and both cancer cells. Quantitative data showed an elevated uptake of Amy-F by MCF-7 and MDAMB-231 cells (7.2 and 7.6- fold, respectively), than normal cells. These results confirmed a selective uptake of Amy-F by BCCs compared to normal cells.

In contrast, the uptake of nanoparticles from cancer cells treated with Amy-F was the higher with significant difference at $p<0.001$. The uptake of Amy-F particles was calculated depend on the intracellular concentration of Amy-F. These values are measured against the estimated number of Amy-F introduced to the MCF-7 and MDA-MB-231 cells, ~66.5 and 84.6% of nanoparticles, respectively, from IC_{50} dose are more efficiently internalized than normal cells.

Amy-F and/or RT-induced cell cycle arrest and apoptosis in BC cells

To evaluate the role of Amy-F as anticancer and radio-sensitizer in BCCs, cell cycle distribution and cell apoptosis by flow-cytometry were carried out.

The cell cycle analysis of untreated BCCs showed accumulation in the G_2/M phase. After treatment with Amy-F, remarkable elevation in G_1 and sub-G_1 phases was observed in BCCs when compared to groups of BCCs and 4 Gy of each BCC. Whereas, the cells exposed to 4 Gy displayed arrest at the G_1 phase with remarkable elevation in sub G_1 phase compared to the control cells. Furthermore, the combination group Amy-F+4 Gy revealed a higher proportion in G_1 and sub-G_1 phases in both cell lines as compared to BCCs, 4 Gy, and Amy-F of each BCC.

The analysis of apoptotic markers showed a significant elevation of caspase-3, and a significant decrease in the percentage of Bcl-2 in the Amy-F group in both cell lines as compared to BCCs and RT groups, except caspase-3 in MDA cells. Furthermore, the RT group exhibited a slight elevation in the percentage of caspase-3 and reduction of the Bcl-2 percentage in both cell lines as compared to untreated BCCs groups. Additionally, it was observed that treating BCCs with Amy-F+RT induces an increase in caspase-3 percentage, and a decrease of Bcl-2 compared to BCCs, Amy-F, and RT of each type.

Modulatory effect of Amy-F and/or RT on tumor promoting factors

To further elaborate on how the combined treatment of BCCs with Amy-F and RT induced anticancer activity and improved the radio sensitivity, we examined the tumor promoting factors expression, as well as the MAPK/P38/Fe/NO/ROS signaling axis. After 24 h, the control and treated cells were harvested and subjected to mediators analysis. Cells treated with Amy-F showed a significant reduction in MAPK, p38, Fe, and NO expression by (MCF-7; 39.7, 30.7, 28.7, and 53.3%, respectively), and (MDA; 15.4, 48.5, 22.1, and 48.1%, respectively), along with a significant elevation of ROS expression by 2.2 fold for MCF-7 and MDA compared to the control group. Moreover, cells treated with Amy-F induced significant reduction in the MAPK, p38, Fe, and NO levels by (MCF-7; 36.5, 27.7, 25.3, and 48.8%, respectively), and (MDA; 12, 45.8, 15.2, and 42.5 %, respectively) in comparison with the RT group.

Exposure to 4 Gy induced a significant increase in the levels of ROS by 2 fold for MCF-7 and MDA compared to the control group.

In contrast, the combination of Amy-F+RT elicited a profound reduction in the MAPK, p38, Fe, and NO levels in MCF-7 and MDA-MB-231 cells (~50%) as compared to control and RT groups, and (~20%) as compared to Amy-F group. Furthermore, the ROS levels were significantly elevated by 2 and 2.5 fold for MCF-7 and MDA cells, respectively, compared to control group. Whereas, the combined treatment with Amy-F+RT results in a significant increase in ROS levels by ~1.5 fold for MCF-7 and MDA cells when compared with Amy-F and RT groups, suggesting that the preemptive treatment with Amy-F prior to RT could hinder the excessive metabolic activity associated with ROS overproduction in both subtypes of BC cells.

Amy-F and/or RT down-regulated CD4 expression, synergized CD8-mediated suppression of CD80, and activated NKG2D expression in human BC cells

To further elaborate on how the combined treatment of BCCs with Amy-F nanoparticle and RT induced anticancer activity and improved the radio-sensitivity, we examined the CD expression of the key regulatory protein, CD4, CD8, CD80 as well as the NKG2D.

MCF-7 and MDA-MB-231 cells were treated with Amy-F nanoparticle at IC50 doses for each cell line and/or exposed to 4Gy (RT). After 24 h, the control and treated cells were harvested and subjected to flow cytometry and ELISA analysis of the target proteins. MCF-7 cells treated with Amy-F and/or RT showed a significant reduction in CD4 expression by 13.7, 10, and 34.9%, respectively, along with a significant reduction in CD4 expression in MDA-MB-231 cells by 54.5, 42.1, and 65.6%, respectively, for Amy-F, RT, and Amy-F+RT groups, respectively, compared to the control group. In turn, this is coupled with a pronounced elevation in CD8 expression ratios, by 1.6, 1.3, and 1.8 folds, respectively, for Amy-F, RT, and Amy-F+RT groups, respectively in MCF-7 cells, and by 1.9, 1.3, 2.6 folds, respectively, for Amy-F, RT, and AF+RT groups, respectively in MDA-MB-231 cells compared to the control cells. Whereas, a marked reduction in CD80 expression levels was observed by 33.3, 47.4%, respectively, in MCF-7 cells and by 26.2, 36.9%, respectively, for Amy-F and AF+RT groups, respectively, in MDA-MB-231 cells as compared to control cells. More importantly, NKG2D protein expression was augmented by 1.7, 1.1, and 2.7 folds in MCF-7 cells from the Amy-F, RT, and Amy-F+RT groups, respectively, and by 2.9, 1.5, 4 folds in MDA-MB-231 cells from the Amy-F, RT, and Amy-F+RT groups, respectively, compared to the control group. Cancer cells treated with Amy-F showed a considerable reduction in the CD4, CD80 expression, whereas, induced elevation in CD8 and NKG2D ex-pression was observed in Amy-F treated BCCs when compared to the RT group. In contrast, the combination of Amy-F+RT results in a profound decrease in the CD4 and CD80 expression, and an increase in the CD8 and NKG2D expression in MCF-7 and MDA cells compared to Amy-F and RT groups, suggesting that the preemptive treatment with Amy-F prior to RT could hinder the excessive metabolic activity associated with immunomodulatory in both subtypes of BC cells.

Amy-F and/or RT down-regulated TGF-β and blocked VEGF-induced angiogenic tendency through inhibiting INF-γ activation and reducing IL-2/IL-6 levels in human BC cells

The crosstalk between TGF-β and VEGF as well as INF-γ, IL-2, and IL-6 protein levels was examined to gain insight into the underlying mechanism by which Amy-F nanoparticle and/or RT alone or in combination can modify the pro-angiogenic and metastatic capacity of BC cells. The data showed that Amy-F markedly down-regulated TGF-β protein expression by 38.1 and 38.9% in MCF-7 cells and MDA-MB-231 cells, respectively, paralleled by a significant reduction in VEGF, INF-γ, IL-2, and IL-6 protein levels by (MCF-7: 41.5, 38.8, 39.5, and 50.5%, respectively, and MDA: 32.5, 44.6, 61.5, and 57.7%, respectively) compared to control. Similarly, but to a lesser extent, MCF-7 and MDA-MB-231 cells that are exposed to 4 Gy (RT) alone exhibited a reduction in TGF-β and VEGF protein expression (MCF-7: 18.8 and 14.9%, respec-tively, and MDA: 22.4 and 13.3%, respectively) compared to the control cells. Whereas, breast cancer cells exposed to RT alone displayed an augmentation in TGF-β, VEGF, INF-γ, IL-2, and IL-6 levels by 31, 45.5, 36.5, 51, and 88.6%, respectively, for MCF-7 cells, and by 27.1, 28.5, 68.6, 136, and 124.2%, respectively, for MDA cells when compared with Amy-F group.

Surprisingly, when BC cells were challenged by Amy-F prior to RT a remarkable suppression in TGF-β protein expression (56.6%) accompanied by a much more reduction in VEGF, INF-γ, IL-2, and IL-6 levels by 60.6, 60.2, 74.1 and 68.5%, respectively, was observed in MCF-7 cells, whereas a comparable impact was detected in MDA-MB-231 cells as indicated by reduced TGF-β expression (49.3%) as well as a significant decrease in VEGF, INF-γ, IL-2, and IL-6 levels by 66.3, 61.9, 83.3 and 75.6%, respectively, compared to control. Overall, the combination of Amy-F with RT robustly regulated the TGF-β signaling via the down-regulation of VEGF, INF-γ, IL-2, and IL-6 levels in treated BCCs and exhibited superior influence when compared to control, Amy-F, and RT groups, and thus abolished the pro-angiogenic and metastatic ability of human breast cancer cells.

CONCLUSION

Overall, according to the obtained results, we could suggest that Amy-F nanoparticles could ideally target two types of breast cancer cells (MCF-7 and MDA-MB-231) alone and could also augment the influence of RT when administered preemptively. Outer surface annex (FA) provided Amy-F with successful targeting machinery that enabled the nanocomposite to anchor into the cell membrane, facilitating its entry into the cell while in the acidic environment of cancer cells (pH 6). The physiochemical characteristics of NPs, including stability, selectivity, cellular uptake, and responsive release to pH as well as anticancer efficiency, were examined thoroughly. The FA-functionalized Amy-F alone or prior RT abrogated proliferation, induced cell cycle arrest at pre-G_1 and G_2/M, and increased apoptosis as revealed by lower BCl-2 levels and higher caspase-3 levels of breast cancer cells. Cell based assays revealed that Amy-F and/or RT reduced the tumor promoting factors and immunosuppressors of BCs by inhibiting MAPKlow, P38low, Felow, NOlow, ROShigh, CD4low, CD8high, CD80low, NKG-2Dhigh, TGF-βlow, VEGFlow, INF-γlow, IL-2low, and IL-6low expression. The exposure of both breast cancer cells to Amy-F before RT enhanced their response and augmented radiosensitivity, suggesting the emerging role of Amy-F nanoparticle as a potential radiosensitizer in therapeutic regimens for the treatment of breast cancer cells.

LIMITATIONS

We did not apply the material to animals for some limitations, including financial support and the abundance of data in the manuscript, and we need to apply the material in the future.

AUTHOR CONTRIBUTION

MAA, GSE-S, MSG, EK, E-SS, and IYA: Conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, writing–review and editing. All authors have read and agreed to the published version of the manuscript.

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AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this manuscript.

INFORMED CONSENT STATEMENT

Not applicable.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. Wu P, Sun Y, Dong W, Zhou H, Guo S, et al. Enhanced anti-tumor efficacy of hyaluronic acid modified nanocomposites combined with sonochemotherapy against subcutaneous and metastatic breast tumors. Nanoscale. 2019; 11:11470-83.
2. Bai DP, Zhang XF, Zhang GL, Huang YF, Gurunathan S, et al. Zinc oxide nanoparticles induce apoptosis and autophagy in human ovarian cancer cells. Int. J. Nanomedicine. 2017; 12:6521-35.
3. Shi R, Tang YQ, Miao H. Metabolism in tumor microenvironment: Implications for cancer immunotherapy. MedComm. 2020; 1:47-68.
4. Han X, Guo B, Li Y, Zhu B. Tissue factor in tumor microenvironment: A systematic review. J. Hematol. Oncol. 2014; 7:1-8.
5. Sullivan SP, Murthy N, Prausnitz MR. Minimally invasive protein delivery with rapidly dissolving polymer microneedles. Adv. Mater. 2008; 20:933-8.
6. Torchilin VP. Multifunctional, stimuli-sensitive nanoparticulate systems for drug delivery. Nat. Rev. Drug. Discov. 2014; 13:813-27.
7. Liczbinski P, Bukowska B. Molecular mechanism of amygdalin action *in vitro*: Review of the latest research. Immunopharmacol. Immunotoxicol. 2018; 40:212-8.
8. El-Desouky MA, Fahmi AA, Abdelkader IY, Nasraldin KM. Anticancer Effect of Amygdalin (Vitamin B-17) on Hepatocellular Carcinoma Cell Line (HepG2) in the Presence and Absence of Zinc. Anticancer Agents. Med. Chem. 2020; 20:486-94.
9. Liu M, Wang B, Guo C, Hou X, Cheng Z, et al. Novel multifunctional triple folic acid, biotin and CD44 targeting pH-sensitive nano-actiniaes for breast cancer combinational therapy. Drug. Deliv. 2019; 26:1002-16.
10. Jurczyk M, Jelonek K, Musial-Kulik M, Beberok A, Wrzesniok D, et al. Single-versus dual-targeted nanoparticles with folic acid and biotin for anticancer drug delivery. Pharmaceutics. 2021; 13:326.
11. Golombek SK, May JN, Theek B, Appold L, Drude N, et al. Tumor targeting *via* EPR: Strategies to enhance patient responses. Adv. Drug. Deliv. Rev. 2018; 130:17-38.
12. van de Loosdrecht A, Beelen RH, Ossenkoppele GJ, Broekhoven MG, Langenhuijsen MM. A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. J. Immunol. Methods. 1994; 174:311-20.
13. Askar MA, Thabet NM, El-Sayyad GS, El-Batal AI, Abd Elkodous M, et al. Dual Hyaluronic Acid and Folic Acid Targeting pH-Sensitive Multifunctional 2DG@ DCA@ MgO-Nano-Core–Shell-Radiosensitizer for Breast Cancer Therapy. Cancers. 2021; 13:5571.
14. Baibarac M, Smaranda I, Nila A, Serbschi C (2019) Optical properties of folic acid in phosphate buffer solutions: The influence of pH and UV irradiation on the UV-VIS absorption spectra and photoluminescence. Sci. Rep. 9:1-11.
15. Thabet NM, Abdel-Rafei MK, El-Sayyad GS, Elkodous MA, Shaaban A, et al. Multifunctional nanocomposites DDMplusAF inhibit the proliferation and enhance the radiotherapy of breast cancer cells *via* modulating tumor-promoting factors and metabolic reprogramming. Cancer Nanotechnol. 2022; 13:1-31.
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402-8.
17. Ruman U, Buskaran K, Pastorin G, Masarudin MJ, Fakurazi S, et al. Synthesis and characterization of chitosan-based nanodelivery systems to enhance the anticancer effect of sorafenib drug in hepatocellular carcinoma and colorectal adenocarcinoma cells. Nanomaterials. 2021; 11:497.
18. Marian E, Duteanu N, Vicas L, Rusu G, Jurca T, et al. Synthesis, characterization of inclusion compounds of amygdalin with β-cyclodextrin and sod-like activity and cytotoxicity on hela tumor cells. Arab. J. Chem. 2020; 13:6828-37.
19. Zipare K, Bandgar S, Shahane G. Effect of Dy-substitution on structural and magnetic properties of MnZn ferrite nanoparticles. J. Rare. Earths. 2018;36:86-94.
20. He Y, Wang XC, Jin PK, Zhao B, Fan X, et al. Complexation of anthracene with folic acid studied by FTIR and UV spectroscopies. Spectrochim. Acta. Mol. Biomol. Spectrosc. 2009; 72:876-9.