

Dual Hyaluronic Acid and Folic Acid Targeting pH-Sensitive Multifunctional 2DG@DCA@MgO-Nano-Core-Shell-Radiosensitizer for Breast Cancer Therapy

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Abstract

Background: Globally, breast cancer (BC) poses a serious public health risk. The disease exhibits a complex heterogeneous etiology and is associated with glycolysis and oxidative phosphorylation (OXPHOS) metabolic reprogramming pathways which fuel proliferation and progression. Due to the late manifestation of symptoms, rigorous treatment regimens are required following diagnosis. Existing treatments are limited by a lack of specificity, systemic toxicity, temporary remission, and radio-resistance in BC. In this study, we developed CD44 and folate receptor-targeting multi-functional dual drug-loaded nanoparticles (DDM). These contained a hyaluronic acid (HA) and folic acid (FA) conjugated to a dichloroacetate (DCA) shell linked to a 2-deoxy glucose (2DG) and magnesium oxide (MgO) core to enhance localized chemo-radiotherapy for effective BC treatment.

Methods: The physicochemical properties of nanoparticles, including stability, selectivity, release in response to pH, cellular uptake, and anticancer efficacy were comprehensively examined. Mechanistically, we identified multiple component signal pathways as important regulators of BC metabolism and mediators of inhibitory effects toward DDM.

Results: Nanoparticles exhibited sustained DDM release properties in bio-relevant media, which was responsive to acidic pH providing flexibility to the control of drug release from nanoparticles. DDM-loaded and HA-FA-functionalized nanoparticles exhibited increased selectivity and uptake by BC cells. Cells study indicated that functionalized DDM significantly suppressed cancer cell growth and radiotherapy (RT) improvement via cell cycle arrest, apoptosis enhancement, and modulation of glycolysis and OXPHOS pathways.

Conclusions: By highlighting DDM mechanisms as an antitumor and radio-sensitizing reagent, our analysis also revealed glycolysis and OXPHOS modulation via PI3K/AKT/mTOR/NF- κ B/VEGF_{low} and P53_{high} signal pathways. In conclusion, we indicated that multi-functionalized DDM depletion-mediated metabolic reprogramming via multiple signal pathways in BC cells is a promising targeted metabolic therapy.

Introduction

Breast cancer (BC) is one of the most common malignant tumors with the highest mortality-rate in women. Therapeutic options for BC pharmacotherapy have several limitations, such as a lack of therapeutic efficacy, toxicity toward healthy tissues, and high rate of metastasis and recurrence [1, 2]. Accordingly, new therapeutic strategies must be developed target and inhibit BC growth, which is characterized by higher glycolytic activity, glutamine consumption, glutaminolysis levels generated via aerobic conditions (Warburg effect) and mitochondria oxidative phosphorylation (OXPHOS), and the overexpression of CD44 receptors and folate receptors- α (FR- α) [3–5]. Recent studies indicated that the tumor suppressor, P53, phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), mammalian target of rapamycin (mTOR), nuclear factor- κ light chain enhancer of activated B cells (NF- κ B), and vascular

endothelial factor (VEGF) pathways are mutated and related to a wide variety of cancer cell phenotypes, including uncontrolled proliferation, genomic instability, and metabolic reprogramming [6]. Therefore, suggesting that aerobic glycolysis, mitochondrial metabolism, CD44 and FR- α have considering being particularly important for selection and targeting by anticancer drugs. The exploration of drug therapy combinations is a promising strategy; as synergistic effects from multiple drugs potentially lead to better therapeutic outcomes and a reduced probability of drug resistance in cancer cells [7]. 2-Deoxy glucose (2-DG) and dichloroacetate (DCA) are drug combinations currently used in cancer clinical trials [8]. Both 2-DG and DCA are established as BC chemotherapeutic treatments and have been combined with radiotherapy (RT) in clinical trials. However the clinical use of DCA is restricted due to mild toxicity in hematologic, hepatic, renal, and cardiac systems [9, 10]. Similarity, these treatments are also still limited due to rapid relapse issues, systemic toxicity, and non-specific drug delivery, leading to low therapeutic outcomes [11]. Also, poor survival rates in patients with BC may be attributable to radio-resistance stemming from increased cell DNA repair capabilities after RT [12]. Therefore, the development of strategies to overcome these limitations and provide targeted and controlled release for efficient chemo-radiotherapy in BC patients is essential [13]. Nanomedicines have served as vital drug delivery systems and improved cancer therapy with reduced systemic toxicity of anticancer drugs in healthy tissues [14]. Drug encapsulated in nanocarriers are promising modalities as they increase drug accumulation at tumor tissues due to enhanced permeability and retention (EPR) effects, thereby increasing cancer therapeutics efficacy [15]. Because of these advantages, we developed a stimuli-reacting core-shell nanoparticle system conjugated with hyaluronic acid (HA) and folic acid (FA), consisting of two different agents for multi-targeted BC therapy. Moreover, the DDM were surface conjugated with HA and FA to target CD44 and FR- α receptors, which are overexpressed in BC cells. Similarity, CD44 and folate receptors have a high affinity for HA and FA; which are usually captured to nourish fast-dividing BC cells [16]. Therefore, our design innovation was underpinned by a novel biodegradable multi-target nanocarrier that targeted overexpressed CD44 and FR- α receptors in BC cells. This was followed by the release of three therapeutic agents for the effective chemo-radiotherapy of BC.

Materials And Reagents

Synthesis of DDM

1. Preparation of MgO nanoparticles

Firstly, MgO nanoparticles (MgO-NPs) were prepared by the method reported by Diana et al [17]. Briefly, urea was added to an aqueous solution of magnesium nitrate (0.25 M) under constant stirring at 70°C until gel formation. Then, MgO-NPs were formed by placing the gel at 500°C using muffle furnace for 3 hr. Finally, formed MgO-NPs were washed with deionized water (D.I.W) and dried at 70°C for 2 h (Fig. 1a).

2.1.2. Preparation of MgO@DCA@2DG

This novel composite structure was prepared by a simple impregnation method. Firstly, prepared MgO-NPs (from step 1) were dispersed in 50 ml ethanol using water bath sonication for 45 min. Then, aqueous

solutions of previously prepared DCA and 2DG (10 mM) were added to the dispersion under constant stirring for 2 h, at room temperature (25°C). Finally, the formed powder was collected, washed, and dried (Fig. 1a).

2. Preparation of MgO@DCA@2DG conjugated with HA and FA

Formed composite structure (from step 2), was dispersed in D.I.W by water bath sonication for 30 min. Then, aqueous solutions of HA and FA (20 mM) were added into the solution, followed by sonication of the mixture for 30 min. Third, mixture was left to stirrer for 2 h at constant stirring. Finally, the resulted powder was collected using centrifugation and dried at 80°C for 1h (Fig. 1a).

Characterization of DDM

Firstly, the stoichiometry of the synthesized DDM sample is examined via employing the energy dispersive X-ray spectra (EDX), JEOL JSM-5600 LV, Japan. In order to confirm the formation of the exact sample with detected functional groups, Fourier transform infrared (FT-IR) spectroscopy (NICOLET iS10 model instrument) is conducted over a wide range (400–4000 cm^{-1}). The crystal structure of the samples is investigated via x-ray diffraction technique (XRD; Shimadzu XRD-6000). XRD patterns are obtained in the range of 2θ from 17° to 90° at room temperature. Cu K α is used as a radiation source of wavelength $\lambda = 0.15408$ nm, scan rate 0.8°/min, operation voltage 50 kV and current 40 mA [18, 19]. Information on the surface morphology of the samples' particles is obtained using scanning electron microscopy (SEM), JEOL JSM-5600 LV, Japan). Finally, the shape and size of the synthesized samples were obtained by a high resolution Transmission electron microscopy (HRTEM), JEOL JSM-5600 LV, Japan).

Stability of DDM

To determine stability, DDM charge and sizes were studied on incubation with PBS and 10% fetal bovine serum (FBS; pH 7.4) at body temperature (37°C) using DLS for 6 days [5].

Cell culture

The cell lines used in this study were purchased from the Cell Culture Department, VACSERA (Cairo, Egypt). Normal cells (MCF-10A), MCF-7 and MDA-MB-231 BC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin-streptomycin (100 U/ml) and 10% fetal bovine serum (FBS) in a 5% CO₂ humidified chamber at 37°C. For different study treatments, cells were used at 100% confluence.

Cytotoxicity/morphology assay

The cytotoxic effects of dual drug-loaded nanoparticles (DDM) were analyzed using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma) assay [20]. Briefly, BC cells (1×10^5 cells/ml (100 μl /well) were seeded in 96-well plates and cultured for 24 h. The medium was then replaced

by 5 mg MTT in 20 μ l DMEM. Cells were further incubated for 4 h, the DMEM/MTT mixture removed, and 150 μ l dimethyl sulfoxide added to dissolve formazan crystals. Then, the absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (BioTeck, Bad Friedrichshall, Germany) at 570 nm. The half-maximal inhibitory concentration (IC_{50}) was calculated using SPSS one-way ANOVA (IBM Inc., Chicago, IL, USA). Graphs were drawn using Graph-Pad Prism software.v.8.0 (Graph-Pad Prism Inc., USA). Cell morphology was recorded using a phase-contrast inverted microscope fitted with a digital camera (Nikon, Japan). All studies were performed in triplicate.

DDM Release

To study drug release, the DDM suspensions were subjected to pH 3, 7 and 9 at 37°C conditions. At pre-determined time points, the particles were collected using an external magnet (1.3 Tesla), and the supernatant saved for analysis after 24 h incubation. MgO release was quantified at 380 nm absorbance using UV-vis.

Cell selectivity and DDM uptake

Normal (MCF-10A), MCF-7, and MDA-MB-231 cells were seeded in 24-well plates with round coverslips at 2×10^4 cells/well. The next day, cells were incubated with medium containing an IC_{50} DDM dose. After a 24 h incubation, the cells were washed three times in phosphate buffer saline (PBS) and divided in to four aliquots for different methods; the first and second methods were investigated DDM cell selectivity via measurement of CD44 expression by flow cytometry, as described in flowcytometric analysis part, and FR- α expression using qRT-PCR as described in real time PCR part. The third method was investigated MgO levels in normal and cancer cells using atomic absorption spectrophotometry (AAS) AAS model (Al-1200), was used with an air-acetylene burner (slot length = 11 cm). Instrument settings: Wavelength = 285.2 nm, lamp current = 5 mA, slit width = 0.2 nm, air-flow = 1.8 L/min, and ignition-flow = 2.4 L /min. A standard MgO solution was prepared by serial diluting 1000 mg/L MgO stock solution (Scharlau Chemie) in D.I.W. After 24 h incubation, cells were washed three times in PBS, then centrifuged at 3000 rpm for 5 min, and supernatants aspirated into bottles. Supernatants and pellets were then diluted in D.I.W and homogenized before MgO determination [21]. The forth method was investigated DDM cell uptake using TEM Imaging in normal and cancer cells. In brief, cells were fixed in in 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4 (Electron Microscopy Sciences, Hatfield, PA, USA) in plates for 1 h at room temperature. After this, cells were scraped from plates, centrifuged at a low speed, and suspended in 2.5% glutaraldehyde. Samples were processed at the Egyptian atomic energy Authority by post-fixation in 1% osmiumtetroxide, rinsing in distilled water, and dehydration through a graded acetone series. Samples were embedded in epoxy resin, cut into 70 nm sections, and analyzed and photographed using a JEOL 100CXII TEM [22].

γ -Ray irradiation

Cells were irradiated with γ -rays using a ^{137}Cs source (Gamma-cell-40 Exactor; NCRRT, AEA, Cairo, Egypt). The dose rate was 0.012 Gy/s. Dosimetry was used for all the studies to ensure dose uniformity and dose rates were delivered using a Fricke reference standard dosimeter [23].

Multi-MTT assay

MCF-7 and MDA-MB-231 cells were plated at of 1000 cells/well in 6-well tissue culture plates and treated with DDM IC₅₀ dose of 281.9 and 192.8 µg/ml, respectively for 24 h. Cells were then exposed to 3 Gy (single dose) or 6 Gy (fractionated dose, 3 Gy every 3 days) of ¹³⁷Cs-radiation (Fig. 2a). During the study period, drug-free medium was replaced every 3 days. Finally, cells were incubated with MTT, and cell survival cells measured as described. Cell morphology was also recorded as described. Survival curves were calculated, to derive the Dose-Modifying Factor (DMF); this was calculated at the iso-effect of survival fraction (SF) = 50% with radiation treatment alone respect to combined treatments [24]. To calculate of the proliferation-survival, only the early exponential phase of cell growth was used in the following equation; survival = 2^{- (t delay/t doubling time)}, t delay = the time- to reach a specific absorption value of irradiated cells vs. control, and t doubling time = the time required for cells to double [25].

Protocol of study

To evaluate anti-proliferation and radio-sensitizing effects of DDM, six study groups were designed (Figure. 2a): 1) cells without treatment (Control), 2) cells treated with DDM only (DDM), 3) cells exposed to a fractionated dose (6 Gy) of irradiation alone (FDR), 4) cells treated with DDM 24 h before FDR (DDM + 6Gy-FDR). Cell survival was assessed at 24 h, 48 h and 72 h compared with the control group, to study some important hypotheses on the mechanisms of actions of DDM with or without FDR.

Cell cycle, apoptosis and CD44 analysis using flow cytometry

MCF-7 and MDA-MB-231 cells (3 × 10⁵ cells/well) were either untreated (control group) or treated with an IC₅₀ DDM dose (DDM) for 24 h and exposed to 6Gy-FDR (RT). After 24 h incubation, cells were harvested, washed twice in ice-cold PBS, and fixed overnight in 70% ethanol at 4°C. Then, cells were washed in PBS, collected by centrifugation, and stained with propidium iodide (PI) (50 µg/ml) for cell cycle analysis. An annexin-V fluorescein isothiocyanate (FITC) kit (Beckman Coulter, Marseille, France) was used to measure apoptosis. An FITC-conjugated anti-CD44 antibody (1:400, Cat. No: YKIX337.8, eBioscience) was incubated with cells for 30 min at 4°C to assess CD44 levels. All staining was performed using a FACSCanto II flow cytometer followed by analysis using BD Accuri-C6 Plus software (Biosciences, CA, USA) [26].

Determination of glucose, lactate, and hyaluronic acid (HA) metabolism

After treatment with DDM for 24 h and exposure to RT, glucose, lactate, and HA metabolism in MCF-7 and MDA-MB-231 cells was measured. Cell supernatants were harvested and indices measured using commercial kits (Cat. No: GAGO20 and MAK064 (Sigma-Aldrich) for glucose and lactate, respectively) and HA (Cat. No: 029 – 001, Corgenix, Inc. UK) on a spectrophotometer (V-630 Bio UV-Vis, JASCO, USA). Glucose, lactate, and HA concentrations were determined at 540 nm, 570 nm, and 450 nm, respectively.

Analysis of Hexokinase (HK) and pyruvate dehydrogenase (PDH) activities

Intracellular HK and PDH activity was evaluated with a spectrophotometer (V-630 Bio UV-Vis: JASCO: USA) using Quantification Kit, Cat. No: MAK091-1KT and MAK183-1KT respectively, according to the manufacturer's instructions (Merck KGaA, Darmstadt, Sigma-Aldrich, Germany). The HK and PDH concentrations were determined with OD values at 450 nm.

RNA isolation and real time PCR analysis

RNA extraction and qRT-PCR Total RNA from MCF-7 and MDA-MB-231 cells in each group was extracted by Trizol Reagent (Thermo Fisher Scientific). cDNA was obtained from total RNA using the PrimeScript™ RT (Table. 1) reagent kit (Takara Bio, Inc., Otsu, Japan). The expression of mRNA was assessed by qRT-PCR, which was carried out in triplicate by an SYBR Premix Ex Taq™ kit (Takara Bio, Inc.) and an ABI 7900HT Real-Time PCR system (Thermo Fisher Scientific). The primers used are presented in Table 1. GAPDH was used to normalize the results of qRT-PCR and the comparative cycle threshold values ($2^{-\Delta\Delta Ct}$) were adopted to analyze the final results.

Table 1
The primers for quantitative real-time PCR

Genes	Forward Primers	Reverse Primers
FR- α	5'-CTGGCTGGTGTGGTAGAACAG-3'	5'-AGGCCCCGAGGACAAGTT-3'
PKM2	5'-GAGGCCTCCTTCAAGTGCTG-3'	5'-CATGGCAAAGTTCACCCGGA-3'
GAPDH	5'-GTCAAGGCTGAGAACGGGAA-3'	5'-AAATGAGCCCCAGCCTTCTC-3'

Measurement of intracellular PKM2, HIF-1 α , PDK1, NF- κ B, VEGF and ROS levels by ELISA assay

Pyruvate kinase isozymes M2 (PKM2), Pyruvate Dehydrogenase Kinase 1 (PDK1), Hypoxia-inducible factor 1-alpha (HIF-1 α), NF- κ B, VEGF and reactive oxygen species (ROS) levels were determined by using the markers assay kit (MyBiosource; Cat. No: MBS2505089, MBS078206, MBS282197, MBS2089167, MBS355343 and MBS166870 respectively), following a modification of the manufacturer's protocol. Cells were seeded in 150 mm plates, treated as described above, collected and homogenized (2×10^6) in 100 μ L of ice-cold water. Reactions were carried out following manufacture's protocol and absorbance was measured at 570 nm using an automatic micro-plate reader (Quant, BioTek Instruments, Inc., Winooski, VT, USA).

Western Blotting Analysis

MCF-7 and MDA-MB-231 cells were seeded at 4×10^5 /well in 6-well plates. After treatments, cells were lysed in lysis buffer plus 10 μ l PMSF (100 mM added to 1 ml buffer, Solarbio, Beijing, China) on ice for 30 min. Cells lysates were separated using 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gels, blotted using polyvinylidene difluoride membranes which, and blocked in 5% skim milk in PBS plus 0.1% Tween 20 (TBST). Membranes were then incubated overnight with primary antibodies at 4°C for 12 h.

The following antibodies were used: PI3K, AKT, mTOR total and phosphorylated, P53, SIRT1, and SIRT3 rabbit polyclonal antibodies (1:1000). A β -actin rabbit polyclonal antibody (1:4000, Proteintech, USA) was used as a loading control for normalization. A secondary anti-rabbit antibody conjugated to horseradish peroxidase (1:4000; Proteintech) was incubated with membranes for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescent reagent (Thermo Fisher Scientific). Band images were obtained and quantified using the Protein Simple Digital imaging system (Fluor Chem R, USA).

Statistical Analysis

All experiments were carried out at least in triplicate and the results were expressed as the mean \pm standard error (SEM). The statistical software package (SPSS Inc., Chicago, IL) was used for analysis. Statistical significance between all groups was analyzed by using the $p < 0.05$. Statistical analyses graphs were drawn using Prism, version 8 (GraphPad Software, La Jolla, CA).

Results

Characterization of DDM

XRD displays a true detection of the crystallinity and the combination of the exposed DDM sample (Fig. 1a), because it explains the status of the atoms, size, and axes. XRD results of the DDM powder was presented in (Fig. 1b); many peaks were recognized for MgO-NPs. Diffraction characteristics are displayed inside 2θ (degree) as 24.20, 32.60, 37.50, 58.93, 62.03 and, 72.18 where some peaks represent the Bragg's appearances (001), (111), (20), (220), (311), and (222) extensions in that position sequentially, which can be recorded to the levels of cubic MgO (JCPDS 75-0447).

This suggests that the MgO-NPs (core structure) were regularly crystal in fact and produced the face-centered cubic (fcc) crystalline configuration. It must be stated that the amorphous peak at 17.35 (*) was due to outer organic shells (DCA, 2DG, HA, and FA). The composition of the synthesized DDM sample is analyzed by EDX (Fig. 1c), where the presence of O, C, Cl, and Mg is confirmed, where the existence of Mg, and O atoms is confirmed for the core MgO-NPs. Moreover the presence of O, C, and Cl is attributed to the DA, 2DG, HA, and FA multi-shells structures in the synthesized sample.

In order to further illustrate the structural features of the samples, element mappings have been carried out selectively to the synthesized DDM sample and the images are depicted in (Fig. 1d). It is evident from these images that the elements Mg, C, Cl, and O exist, which agreed with preceding EDX results. Further, those elements are homogeneously distributed. From the images we can conclude that both Mg (blue color), and O (pink color) atoms are located in the same places which confirms the core structure.

The SEM image of the synthesized DDM sample is shown in (Fig. 1e). The surface behavior reveals dark layers represents the outer shells (HA and FA) with remarkable smooth agglomerates can be observed due to the occupation of a large quantity of layers at the grain boundary which could control the grain

growth. Also, the bright particles represented the MgO-NPs core which confirms the promising core-shell structure.

HR-TEM image of the core-shells structure of the synthesized DDM is shown in (Fig. 1f). The synthesized composite possesses semi-spherical structure with diameter sizes ranging from (165.21 nm to 88.94 nm) with an average size of (99.87 nm). It must be noted that, the condensed particles were attributed to the core MgO-NPs (yellow circles), while the faint layers were corresponding to the shell layers DDM which are entirely validated by color in mapping/SEM images.

(Fig. 1g) shows the FT-IR spectra of the synthesized DDM samples. For the present nanocomposite the characteristic vibration peak at 680 cm^{-1} was assigned to the stretching mode of MgO (in the core) and other assigned peaks were for the shells formed and was in a good agreement with the literature.

DDM stability

To better understand nanoparticle stability, nanoparticle sizes were monitored by dynamic light scattering (DLS) (Fig. 1h). The average hydrodynamic diameter of DDM remained essentially stable; nanoparticles did not aggregate over 6 days in PBS plus 10% FBS. DLS data indicated that DDM had a hydrodynamic diameter range of $237.8 \pm 17\text{ nm}$ to $484 \pm 26\text{ nm}$. Also, with increased incubation time, the ζ -potential of nanoparticles was stabilized at values around -4.3 mV and -5.06 mV . Nanoparticle interactions with media cationic constituents may have resulted in the neutralization of negative surface charges on nanoparticles, leading to less negative ζ -potential values. Importantly, interactions did not generate nanoparticle aggregation even after a prolonged 6-day incubation.

The anti-proliferative effects of DDM on BC cell growth

Anticancer activity results from MCF-7 and MDA-MB-231 cells indicated that DDM demonstrated anti-proliferative activities after 24 h. DDM proved efficient at most concentrations. The IC_{50} was $281.9\text{ }\mu\text{g/ml}$ for MCF-7 cells and $192.8\text{ }\mu\text{g/ml}$ for MDA-MB-231 cells. The inhibitory effects of DDM were stronger in MDA-MB-231 cells than MCF-7 by less than half. Thus, DDM exhibited higher anticancer activities especially toward triple-negative MDA-MB-231 cells (Fig. 2a). Phase-contrast images showed cell morphological changes in a DDM dose-dependent manner (Fig. 2b). Apoptosis cell shrinkage, cell fragmentation, membrane blebbing, and detachment traits were observed. Similarly, cell numbers were decreased concomitant with increasing DDM concentrations.

DDM Release

To determine pH-dependent drug-releasing properties, the drug release behavior in vitro was studied by UV – vis in pH original, 3, 7 and 9 phosphate buffer solutions (PBS) containing DMSO 0.1% to simulate a neutral environment of normal cells and acidic conditions in cancer cells. As shown in (Figure. 2c). There

is more than 30 % release within 24 h in pH 3. In sharp contrast, about less 7 % and 0.5 % DDM was released at pH 7 and 9 respectively in 24 h, due to protonation and solubility of DDM in acidic environments.

Selective delivery and cellular uptake of DDM in BC cells

We confirmed the selective delivery efficiency of DDM (IC₅₀ doses) into cancer cells by investigating HA, CD44, and FR- α expression levels. As indicated (Fig. 2d,e) normal cells (MCF-10A) treated with DDM showed non-significant differences in these levels when compared with untreated normal cells, indicated that DDM non-selective to normal cells. As expected, higher HA, CD44, and FR- α expression levels (11-, 4.4-, and 5.3-fold, respectively) were observed in DDM-free MCF-7 cells, and 5.5-, 4.4-, and 5.3-fold higher levels (respectively) in DDM-free MDA-MB-231 cells when compared with DDM-free normal cells. After DDM treatment, lower HA, CD44, and FR- α expression levels were observed in MCF-7 cells + DDM (64.6%, 54.6%, and 55.9%, respectively), and in MDA-MB-231 cells + DDM (43.7%, 53.8%, and 60.4%, respectively) when compared with DDM-free cancer cells. These observations suggested HA and FA conjugated to DDM was a useful ligand in targeting overexpressed CD44 and FR- α receptors on BC cell membranes.

The cell uptake and localization of DDM in MCF-10A (normal cells), MCF-7, and MDA-MB-231 cells was quantified using AAS (Fig. 2f). Quantitative data indicated a higher uptake of DDM by MCF-7 and MDA-MB-231 cells (17.7- and 17.4- fold, respectively) than normal cells, suggesting an increased DDM selective uptake into BC cells than normal cells.

To visualize the internalized nanoparticles and assess their distribution in relation to subcellular compartments, we performed TEM analysis. In TEM images, numerous high electron density-staining nanoparticles were observed inside the cells treated with DDM, while not observed in cells that were not exposed to DDM (Fig. 2g). Normal MCF-10A cells treated with DDM showed a much weaker uptake of nanoparticles than the cancer MCF-7 and MDA-MB-231 cells. In contrast, the uptake nanoparticles from cancer cells treated with DDM were the strongest with significant difference ($P < 0.001$). Because the size primarily influences the composite nanoparticles uptake, the uptake of DDM composite particles was calculated based on the intracellular DDM concentration. When these values are considered against the estimated number of composites introduced to the MCF-7 and MDA-MB-231 cells, ~ 56 and 81 % of nanoparticles respectively from IC₅₀ dose are more efficiently internalized than normal cells (Fig. 2f,g).

DDM inhibited tumorigenesis and enhanced radiosensitivity of human breast cancer cells

We performed multi MTT assays to determine the radio-sensitizing (RS) ability of DDM on BCC exposed to 3Gy-SDR or 6Gy-FDR following 24, 48, and 72 h (Fig. 3b,c). Treatment with IC₅₀ dose DDM only, significantly decreased the survival of MCF-7 cells to 59.14, 55.12, and 47.83%, and in MDA-MB-231 cells by 61.85, 55.34, and 48.57 % at 24, 48 and 72 respectively, compared to untreated cells. In the cells

exposed to 3Gy-SDR or 6Gy-FDR only, there is no significant difference between the cells survival rate, compared to untreated cells, while significant increase was observed in MCF-7 cells by 1.64, 1.81 and 2.1 fold, and in MDA-MB-231 cells by 1.6, 1.8 and 1.9 fold, at 24, 48 and 72 respectively, compared to DDM group. Moreover, we assessed the influence of DDM as radio-sensitizer on cells survival. As shown in (Fig. 3b,c), DDM + 3Gy-SDR induced a statistically significant reduction in MCF-7 cells survival to 64.6, 48.4 and 22.6 %, and in MDA-MB-231 cells to 62.2, 41.2 and 28.4 % at 24, 48 and 72 respectively, compared to DDM group. While, DDM + 6Gy-FDR induced a highly significant reduction in MCF-7 cells survival to 31.9, 17.2 and 19.3 %, and in MDA-MB-231 cells to 22.6, 23.9 and 20.4 % at 24, 48 and 72 respectively, compared to DDM group. Moreover, DDM + 3Gy-SDR induced a significant reduction in MCF-7 cells survival when compared with 3Gy-SDR only to 39.2, 26.7 and 10.8 %, and in MDA-MB-231 cells to 38.5, 22.8 and 14.6 % at 24, 48 and 72 respectively. While, DDM + 6Gy-FDR induced a highly significant reduction in MCF-7 cells survival when compared with 6Gy-FDR only to 19.8, 9.8 and 9.3 %, and in MDA-MB-231 cells to 13.9, 14.4 and 10.8 % at 24, 48 and 72 respectively. Additionally, DDM + 6Gy-FDR markedly reduced survival rate in MCF-7 cells to 49.5 and 35.7 % at 24 and 48 h respectively, and in MDA-MB-231 cells to 36.3 % at 24 h, compared with DDM + 3Gy-SDR (Fig. 3b,c). According to our results, generally the MDA-MB-231 cells showed to be the most sensitive to the radio-sensitizer DDM than MCF-7 cells which showed more early sensitive to the radiotherapy, whereas MDA-MB-231 has proved to be later sensitive (Fig. 3d). (Fig. 3e,f) indicates the results of dose modifying factor (DMF) for all treatments at 24, 48, and 72 h. According to this figure, the dose-response rate in both the 3Gy-SDR and 6Gy-FDR groups combined with DDM was significantly higher than the single treatments, while the DDM + 6Gy-FDR group was also higher than DDM + 3Gy-SDR. This results indicated that DDM induced radio-sensitizing-modified effect with 6Gy-FDR higher than the single treatments and DDM + 3Gy-SDR group, therefore, the 6Gy-FDR at 24 h was selected for further analysis.

DDM/or RT induced cell cycle arrest and apoptosis in human breast cancer cells

To investigate the mechanism behind the anticancer activity of IC50 dose DDM (DDM) and increased sensitivity to 6Gy-FDR (RT) in breast cancer cells, we analyzed cell cycle distribution and cell apoptosis by flow-cytometry. As shown in (Fig. 4a,b,c), the majority of control cells were blocked in the G1 phase before treatments. However, treatment with DDM induced a marked increase in the proportion of cells in the G2/M and sub G1 phases (apoptosis) compared with the control or RT alone. Also, we found the majority of RT-treated MCF-7 cells were blocked in the S phase, and the MDA-MB-231 cells were blocked in the G1 phase compared with the control. While, combination treatment induced a higher proportion of cells in the G2 phase and sub G1 phases, and simultaneously a decrease in the percentage of cells in the G1 phase and the S phase compared with the control or RT alone. Furthermore, combination treatment induced a marked proportion of cells in the sub G1 phase compared with DDM only. Cell apoptosis is one of the important determinants of anticancer and radiosensitivity. As shown in flow-based images of cell apoptosis (Fig. 4d,e,f), the percentage of apoptotic cells (including early, late and necrotic cells) of the

DDM or RT groups was increased after 24 h treatment without significant change for RT group in MCF-7 cells compared with the control, and apoptotic cells were significantly increased after treatment with DDM + RT compared to control and cells treated with or DDM or RT only. These results suggest that combinatorial treatment of DDM + RT has a potential synergistic effect on the regulation of cell cycle arrest and apoptosis in BCC.

DDM was a dual target of glycolysis and OXPHOS pathway and improved radio-sensitivity of breast cancer cells

To verify the mechanism of the inhibition effect of DDM on glycolysis and OXPHOS, we tested the expression of glucose, hexokinase, lactate, mRNA-PKM2 (mPKM2) and protein-PKM2 (pPKM2) for glycolysis metabolic pathway, and HIF-1 α , PDK1, PDH, SIRT1 and SIRT3 for mitochondria metabolic pathway, after treatment with DDM/ or RT for 24 h in breast cancer cells (Fig. 5). The results showed that DDM treatment down-regulated significantly the levels of glucose, hexokinase, lactate, mPKM2 and pPKM2 than control or RT only in MCF-7 (Fig. 5a,c) and MDA-MB-231 (Fig. 5b,c) cells. While, the results showed that breast cancer cells treated with RT alone, did not display a significant effect on glycolysis pathway compared to the control group. To understand the potential mechanisms of DDM-mediated radio-sensitization, we examined the effects of DDM with RT on MCF-7 (Fig. 5a,c) and MDA-MB-231 (Fig. 5b,c). In both cells, treatment with DDM + RT down-regulated strongly the levels of glucose, hexokinase, lactate, mPKM2 and pPKM2 than all treatments, except, levels of hexokinase, lactate and pPKM2 in MDA-MB-231 cells in compared with DDM only.

We observed that traditional anticancer drug treatment, killed cancer cells, but that some cells survived through ATP induction by OXPHOS activation, and that the surviving cancer cells later acquired drug aggressive growth and resistance. Therefore, the anticancer effect and radio-sensitization of primary therapeutic drugs may be potentiated with OXPHOS inhibitors by DDM. To test this hypothesis, we used breast cancer cell lines (MCF-7 and MDA-MB-231), and we analyzed the expression of HIF-1 α , PDK1, PDH, SIRT1, SIRT3 and ROS for mitochondrial metabolic pathway (Fig. 5). After treatment with DDM for 24h, we showed a significant decreased in levels of HIF-1 α , PDK1, PDH, SIRT1, ROS and an increased in SIRT3 level in MCF-7 (Fig. 5d,f,g) and in MDA-MB-231 cells (Fig. 5e,f,g) compared with control or RT only. Whilst, the results showed that RT alone did not display a significant effect on mitochondrial metabolic pathway compared to the control group in both cell lines. The combination of DDM + RT showed a significant decreased in levels of HIF-1 α , PDK1, PDH, SIRT1, ROS and an increased in SIRT3 level in MCF-7 (Fig. 5d,f,g) and in MDA-MB-231 cells (Fig. 5e,f,g) compared with all treatments except PDK1, PDH, SIRT1, SIRT3 and ROS in both cells, indicated that DDM has a dual effect an anti-proliferative and radio-sensitization.

DDM/or radiotherapy regulated metabolic glycolysis and OXPHOS through novel PI3K/AKT/mTOR/P53/NF- κ B/VEGF

signal pathway in breast cancer cells

To further explore the underlying mechanism of metabolic glycolysis and mitochondrial pathway, we investigated a series of relative key proteins involved in the Warburg effect and cell energy metabolism regulation, including PI3K, AKT, mTOR, P53, NF- κ B and VEGF. DDM significantly down-regulated the levels of PI3K, AKT, mTOR, NF- κ B, VEGF and up-regulated the P53 level in MCF-7 (Fig. 6a,b,c) and MDA-MB-231 cells (Fig. 6d,e,f) compared with control or RT only except VEGF level in MCF-7-treated with RT only. Also, the results showed that treatment with RT alone induced a moderately effect on the regulated-pathway of metabolic glycolysis and OXPHOS by reduced PI3K, AKT, mTOR, NF- κ B, VEGF levels and up-regulated the P53 level in both cells except VEGF in MDA-MB-231 compared with untreated cells. Further, in an attempt to elucidate the mechanism of DDM in the enhancement of radiation effect on regulated-pathway of metabolic glycolysis and OXPHOS, the result showed that upon radiation treatment following DDM significantly down-regulated the level of PI3K, AKT, mTOR, NF- κ B, VEGF and up-regulated the P53 level in MCF-7 (Fig. 6a,b,c) and MDA-MB-231 cells (Fig. 6d,e,f) compared with all treatments except NF- κ B and VEGF when compared with DDM only, suggesting that DDM increased radiation effect by inhibition of glycolysis and OXPHOS through modulation of the PI3K, AKT, mTOR, P53, NF- κ B, VEGF signaling.

Discussion

Our findings agreed in part with observations made by Warburg *et al.*, termed the Warburg effect. The Warburg premise states that cancer cells switch from mitochondrial energy to glycolysis for ATP production, which is blocked by 2DG via HK targeting [27]. But not have been sufficient to discontinued cancer progression. As reported by a previous study, cancer stem cells metabolize predominantly via OXPHOS rather than glycolysis [28]. Furthermore, DCA promotes pyruvate influx into mitochondria via targeted pyruvate dehydrogenase kinases [27]. However, glycolytic and mitochondrial bioenergetics suppression by multiple cancer development mediators by DDM could function as potential therapeutic options against BC. The innovative DDM core-shell exhibited several characteristics. Firstly, it consisted of an MgO core for enhancing of a potent radio-sensitizer–2DG and DCA with a pH-sensitive degradable polymer shell for controlled release of a targeted-drug-DDM in response to acidic tumor conditions. Recently, MgO-NPs, as potential candidates for drug delivery, anticancer, magnetic resonance imaging, and hyperthermia systems have been studied [29]. Secondly, the possible incorporation of contrast agents such as MgO into nanoparticles could help track drug uptake by TEM imaging and IR quantitative measurements. Thirdly, it allows for the simultaneous delivery of chemotherapeutic reagents (2DG and DA) and radio-sensitizers (e.g., MgO). Finally, HA and FA molecules on the DDM surface help target overexpressed CD44 and FR- α receptors in BC cells thereby facilitating site-specific targeted therapy.

Diffraction characteristics are displayed inside 2θ (degree) as 24.20, 32.60, 37.50, 58.93, 62.03 and, 72.18 where some peaks represent the Bragg's appearances (001), (111), (20), (220), (311), and (222) extensions in that position sequentially, which can be recorded to the levels of cubic MgO (JCPDS 75–0447) [30]. This suggests that the MgO-NPs (core structure) were regularly crystal in fact and produced the face-centered cubic (fcc) crystalline configuration. It must be stated that the amorphous peak at

17.35 (*) was due to outer organic shells DDM. The presence of O, C, Cl, and Mg is confirmed, where the existence of Mg, and O atoms is confirmed for the core MgO NPs by EDX. Moreover the presence of O, C, and Cl is attributed to the DA, 2DG, HA, and FA multi-shells structures in the synthesized sample [31, 32]. It is evident from these images that the elements Mg, C, Cl, and O exist, which agreed with preceding EDX results. Further, those elements are homogeneously distributed. From the images we can conclude that both Mg (blue color), and O (pink color) atoms are located in the same places which confirms the core structure. The surface behavior reveals dark layers represents the outer shells (HA and FA) with remarkable smooth agglomerates can be observed due to the occupation of a large quantity of layers at the grain boundary which could control the grain growth [33]. Also, the bright particles represented the MgO-NPs core which confirms the promising core-shell structure. The synthesized composite possesses semi-spherical structure with diameter sizes ranging from (165.21 nm to 88.94 nm) with an average size of (99.87 nm). It must be noted that, the condensed particles were attributed to the core MgO-NPs (yellow circles), while the faint layers were corresponding to the shell layers DDM which are entirely validated by color in mapping/SEM images. For the present nanocomposite the characteristic vibration peak at 680 cm^{-1} was assigned to the stretching mode of MgO (in the core) and was in a good agreement with the literature [34–36].

In FTIR results, The characteristic IR absorption peaks at 1608 , 1776 and 1501 cm^{-1} are observed in the spectrum which assigned to FA, and due to N-H bending vibration of CONH group, C = O amide stretching of the α - carboxyl group and absorption band of phenyl ring, respectively [37]. The presence of a band at 3696 cm^{-1} is attributed on OH and NH stretching region. The band at 2965 cm^{-1} can be attributed to stretching vibration of C – H in HA. The band at about 1776 cm^{-1} corresponds to the amide carbonyl and the band at 1440 cm^{-1} can be attributed to the stretching of COO⁻, which refers to the acid group of molecule HA. The absorption band at 1089 cm^{-1} is attributed to the linkage stretching of C – OH in HA [38]. DCA normally exhibits a peak at around 1836 cm^{-1} assigned to the C = O stretching vibration for the COCl group. On the other hand, the spectrum of DCA has a peak at 1776 cm^{-1} assigned to the C = O of the COOH group and peaks at around 1341 , and 1272 cm^{-1} assigned to the O–H and C–O, respectively. In addition to these, the 1608 cm^{-1} peak assigned to the COO⁻ antisymmetric stretching vibration were also observed in the spectrum of DCA [39]. Peaks located at 1272 cm^{-1} for O-H blending of 2DG and 1089 , and 930 cm^{-1} for C-O stretching of 2DG indicating the presence of 2DG in the synthesized nanocomposite [40]. Finally, peak at 680 cm^{-1} was assigned to the stretching mode of MgO in the MgO-NPs core.

In this study, DDM was improved by applying a more effective pegylation strategy using a functionalized core-shell nanoparticle, in contrast to nanoparticles used in previous studies [41]. This approach increased stability in bio-relevant media and improved functionalization with HA and FA-targeting ligands. Our DDM had an average hydrodynamic diameter size of approximately 330 nm over 6 days, which was appropriate for tumor tissue accumulation and adhering to EPR principles [5]. The zeta potential value of DDM demonstrated good stability, perhaps because electrostatic repulsion between

particles was dominated by van der Waals attraction forces, ensuring stability in solution. The DDM did not aggregate and was adequately stable in bio-relevant media, suggesting a good retention of optimum size characteristics in the blood circulation. This enhanced stability may be attributed to the novel core-shell design which could reduce interactions between nanoparticles and bio-relevant media constituents, e.g., proteins. *In vivo* protein binding to nanoparticle surfaces could lead to rapid uptake by the reticuloendothelial system and nanoparticle removal from the general circulation, reducing or eliminating accumulation at tumor sites [5, 42].

We evaluated DDM cytotoxicity in BC cells using the MTT assay and showed DDM inhibited BC cell proliferation in a dose-dependent manner. Previous studies reported that the suppression effect of 2-DG or DCA by itself was limited since the cytotoxic dose did not significantly increase the inhibition ratio [43, 44]. Also, cytotoxicity curves indicated that MDA-MB-231 cells were more affected by DDM than MCF-7 cells. This was probably because DDM was targeted more to cells which were characterized by more mesenchymal (drug resistance), and relying on glycolysis for ATP production (Warburg effect) under both normoxic and hypoxic conditions. Also, MDA-MB-231 cells grew considerably faster ($\sim 1.5\times$) than MCF-7 cells [45].

Cell surface-specific markers have been extensively used for cancer-targeted therapies [46]. Among these, CD44 and FA- α receptors have been studied for selective nanoparticle drug delivery to BC sites. According to previous research, nanoparticle size primarily influences composite nanoparticle uptake [47]. In this study, selective cell uptake was used to calculate and image DDM in BC cells. Cells treated with DDM showed much stronger fluorescent signal uptake than normal cells, suggesting synergic effects from HA/FA-mediated endocytosis. Hence, dual-receptor-mediated synergic internalization significantly improved drug system selectivity and targeted efficiency to BC cells. Additionally, DDM cellular uptake was significantly decreased in normal cells, suggesting the CD44 and FR- α dependent uptake, which facilitated the enhanced intracellular drug concentration toward both CD44 and FR- α receptors which overexpressing in cancer cells. Our findings agreed with a previous study showing that improved cytotoxic effects for drugs in cancer cells was attributed to drug release under acidic conditions [48]. The drug release kinetics of DDM under acidic (pH 4) conditions demonstrated the feasibility of the DDM formulation in BC cells cytoplasmic drug release after CD44 and FR- α receptor-mediated endocytosis. Thus, our qualitative and quantitative data showed that DDM system specifically and selectively bound to overexpressed FR- α and CD44 receptors in cancer cells via CD44/FR- α receptor-mediated endocytosis, and response to release in the acidic condition. These findings, associated with the mechanism of action, indicated DDM is a promising platform for BC therapy.

Although tumor cell radio-sensitization is considered a promising strategy to combat cancer, it is equally important to reduce IR mediated toxic effects in healthy tissues surrounding tumors. Thus, using drug/RT combinations could permit increased tumor control even for radio-resistant triple-negative BC tumors. The main impact of RT is reactive oxygen species (ROS) generation which damages DNA and causes cell death via apoptosis, and importantly, ROS production also contributes to malignancy. Nevertheless, various tumors acquire radio-resistance that is accountable for the failure of RT and recurrence of tumor

or metastasis, due to HA overexpression and down oxygen-levels (hypoxia) in tumor-microenvironment [49–51]. In spite of the effectiveness of DDM only at all intervals time than RT only in reduction of cells survival. Induce radio-sensitization with ROS reduction by DDM, may represent a plan to overcome tumor radio-resistance and improve therapeutic outcomes. Also, our combined chemo-radiotherapy at 6Gy-FDR showed increased radio-sensitivity in BC cells, especially MDA-MB-231 cells, in agreement with Simona *et al.* and Jalil *et al* [51, 52]. Our findings showed that when the RT dose was increased to 6Gy-FDR + DDM, cell death and DMF rates were significantly higher than other treatments. These findings indicated the effectiveness of DDM + 6Gy-FDR toward cell death and induced modified effects. When compared with 3Gy, the energy transferred to cells at 6Gy-FDR was sufficient to cause cell death, in agreement with Ebrahimi *et al* [53]. It appeared that 6Gy-FDR + DDM exerted significant effects on cell survival reduction and rapidly growing cells (MDA-MB-231) when compared with MCF-7 cells. Analysis of the radio-modifying-effects of DDM in BC cells showed that the DDM administration time with respect to RT was important in determining effects. Stimulation is generally higher after exposure to FDR for 24 h. Since the majority of induced damage response pathways shortly following RT, function optimally for a few hours after RT, as indicated by Jalil *et al* [54], therefore, the 6Gy-FDR (RT) was selected for further analysis.

Cell cycle regulation and cell death signaling pathways have important roles in cancer development, therefore they serve as potential cancer therapeutic targets [55]. Indeed, compounds that induce cell cycle arrest and apoptosis may function as radio-sensitizing reagents and valuable strategies for cancer drug discovery [56, 57]. We determined the effects of DDM/or RT on cell cycle progression and apoptosis in BC cells and showed the cell cycle was arrested at the G2/M phase, and apoptosis induced by DDM treatment in BC cells. As confirmed by previous research, the radio-sensitizing effects of anticancer drugs are due to cell cycle alterations and apoptosis progression. 2-DG is one such anticancer drug and was shown to enhance radio-sensitivity by blocking cells in the G2/M phase of the cell cycle, and enhancing apoptosis [58]. Our results indicated that DDM treatment before RT increased radio-sensitivity in BC cells via G2/M arrest. More interestingly, we observed an accumulation in the S phase for MCF-7 cells and G1 phase for MDA-MB-231 cells, with no significant differences in Sub G1 when exposed to radiation only, also, induce slight apoptosis in all cells compared with control cells, may be due to that AKT decrease leads to G1/S arrest in various cancer cells when exposed to RT as reported by Jia Luo *et al* [59]. Radiation-induced cell cycle checkpoints are believed to provide cells with additional time to repair DNA damage before further engagement with the cell cycle. Delays at G1 after exposure to radiation are characteristic of cells expressing a wild-type P53 pathway [60]. Therefore, our results showed that the simultaneous exposure of BC cells to DDM, with or without RT, accelerated the cell cycle through G1/S and arrested it in G2/M phase. Apoptosis is a major cancer suppression mechanism and is characterized by morphological and ultra-structural changes associated with biochemical processes [61]. DDM incorporates promising complex agents including 2DG, DCA, and MgO which induce apoptosis and act against BC cells [62, 27, 29], but do not show strong cell death when used separately. Our annexin-V data indicated that DDM induced apoptosis in BC cells and showed potential anti-proliferative and apoptogenic effects when agents were combined. Also, low apoptosis levels mediated by RT in MDA-MB-231 cells may have been related to induced resistance in these cells; this observation agreed with

previous work confirming that radio-sensitivity in MDA-MB-231 cells was significantly lower than MCF-7 cells [63]. While FDR following DDM improved cell death when compared with RT, perhaps DDM induced CD44 suppression and FA-receptor overexpression which contributed to poor radio-responses and chemo-resistance. However, our results indicated that DDM followed by RT strongly enhanced cell death in BC cells.

Multiple genes are involved in the Warburg effect (aerobic glycolysis) and mitochondrial metabolism controlled processes. Thus, modulating one gene may be insufficient to suppress tumors and potentially facilitates cancer progression and drug resistance [64]. Glucose, hexokinase [65], PKM2 [59], lactate [66], PDH, PDK1, SIRT1, and SIRT3 [65], are essential glycolytic and mitochondrial metabolism enzymes controlling tumor progression or regression. Previous studies reported that blocked glycolytic and mitochondrial metabolic enzymes constituted major targets in preventing cancer growth [67, 68], consistent with DDM effects in this study. HK2 is the isoform expressed in cancer cells and regulates the first step in glycolysis. It is regulated by P53 and HIF-1 and is reported as the target of 2DG. Another important kinase in glycolysis is pyruvate kinase M2; it is phosphorylated by PI3K/AKT, thereby promoting the Warburg effect and tumorigenesis [69]. Another kinase is pyruvate dehydrogenase kinase-1 (PDK1) which is reportedly a target of DCA. It regulates the transformation of pyruvate to mitochondria through pyruvate dehydrogenase (PDH) rather than convert to lactate [70]. Two mitochondrial sirtuins are SIRT1 and SIRT3. The latter is the major mitochondrial sirtuin which promotes ATP production by regulating tricarboxylic acid cycle enzymes [71].

Previous studies reported that blocked glycolytic and mitochondrial metabolism enzymes constituted major targets in preventing cancer [67, 68], consistent with the DDM effects seen in this study. Our results suggested that DDM inhibited the metabolic reprogramming of BC, perhaps due to the major targeting mediators inhibition in aerobic glycolysis and mitochondrial metabolism. To control the glycolysis pathway, DDM induces a significant decrease in glucose uptake, hexokinase, PKM2, and lactate levels, indicating potential effective of DDM in Warburg effect blockage, maybe by dephosphorylation of HK and PKM2 through PI3K/AKT inhibition thereby preventing the Warburg effect and cancer progress, while, for controlling of OXPHOS pathway, it induced down-regulation of ROS production, PDH phosphorylation, and PDK1 and SIRT1 activity, also up-regulation of SIRT3, activate several proteins in the apoptotic pathway as annexin-V and p53, and then induce cell death, indicating greater effective potency than anticancer drugs which targeted single pathway [72, 73]. Previous studies reported that DCA plus a SIRT inhibitor exerted anticancer activity via P53 acetylation in MCF-7 cells [65, 74]. Our study suggested that SIRT1, SIRT3, and ROS, as OXPHOS regulators at the mitochondria, SIRT1 overexpression and SIRT3 down-expression are associated with glycolysis and proliferation through dependent-P53 in MCF-7 and MDA-MB-231 cells.

Because cancer cells use the Warburg effect and OXPHOS for energy production, also the conversion of pyruvate into acetyl CoA is irreversible and the future therapy targeting dual cellular metabolism (glycolysis and OXPHOS) should be designed [22, 73]. These results demonstrated that although the anticancer efficacy of drugs is ROS dependent [75], the DDM/or RT approach is not, maybe due to P53

stimulation which induces the transcription of the TIGAR gene, which lowers the fructose-2,6-bisphosphate (F-6-bP) and thus decreases glycolysis, mitochondrial ATP production and overall levels of ROS, indicated that DDM/or RT a promising anticancer strategy through targeting of OXPHOS in BC cells. In our study, poor radiation responses observed in BC cells may have been due to the tumors use of the cellular environment through a hypoxic cellular environment, enhanced glycolysis or OXPHOS and ROS elevation, which results in the radio-resistance in agreement with Pajak et al [76]. Multiple studies demonstrated that ROS may have a role as a Warburg effect stimulant via HIF-1 in response to hypoxia, in agreement with our research [77, 78]. In our study, changes in gene and cellular levels indicated that hexokinase, PKM2, lactate, PDH, PDK1, SIRT1, and SIRT3 promoted the Warburg effect and OXPHOS mechanisms in MCF-7 and MDA-MB-231 cells. We observed that DDM added prior to RT enhanced radiation-induced cell death via modulated metabolic reprogramming. This observation agreed with previous research demonstrating that 2-DG added prior to or immediately after IR, enhanced radiation-induced cell death by modifying energy-dependent cellular processes, e.g., DNA damage repair, cell cycle checkpoints, and apoptosis [27, 76]. We suggest DDM selectively blocks both glycolysis and OXPHOS pathways and enhances radio-sensitivity in BC cells via the down-regulation of mediators in these processes through PI3K/AKT/mTOR/P53NF- κ B/VEGF signaling pathways.

Conclusions

In this study, pegylated DDM was functionalized with HA and FA. These core-shell nanoparticles exhibited controlled 2DG, DCA, and MgO, even in bio-relevant media (cell culture medium), which was accelerated at acidic pH. The HA and FA-functionalized DDM only or as radio-sensitizer before RT increased cell cycle arrest, apoptosis, and cytotoxicity against BC cells. Also, DDM exhibited increased uptake and increased cytotoxicity against MDA-MB 231 cells when compared with MCF-7 cells. Thus, glycolysis/OXPHOS inhibition by DDM and FDR treatment may induce cancer metabolic reprogramming via a novel PI3K/AKT/mTOR/P53NF- κ B/VEGF pathway in BC cells. Therefore, the dual targeting of glycolysis/OXPHOS pathways is suggested as a promising antitumor strategy.

Declarations

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Authors' contributions

Mostafa A. Askar, Noura Magdy Thabet and Mohamed Khairy Abdel-Rafei, designed the study, carried out the search, study selection, data abstraction, analysis and drafted the manuscript. Omama E. El Shawi and Hamed Helal carried out the search, study selection. Gharieb S. El-sayyad, Ahmed I. El-Batal, Mohamed abd El kodous, Go Kawamura and Atsunori Matsuda participated in material science preparation, characterization and analysis. All authors reviewed and edited the manuscript and approved the final 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.

Ethics approval and consent to participate

Not applicable, and the cell lines used in this study were purchased from the Cell Culture Department, VACSERA (Cairo, Egypt). The materials were prepared and applied on the cell lines in the national center for radiation research and technology, atomic energy authority.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

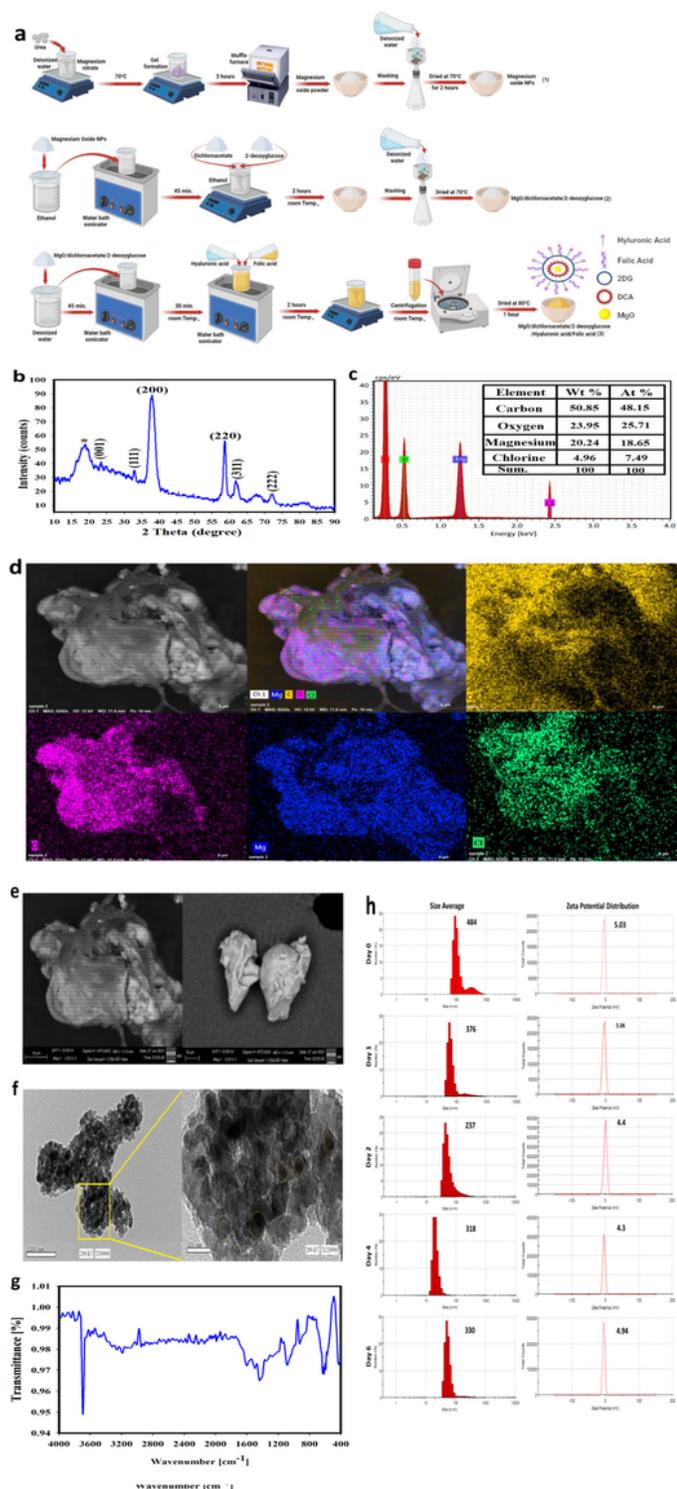


Figure 1

Characteristics of DDM. (a) Diagram of DDM synthesis. (b) The crystallinity behavior of MgO-NPs, and amorphous shape of organic shells in the synthesized DDM by XRD analysis. (c) EDX spectra of the synthesized DDM. (d) Elemental mapping images of the synthesized DDM. (e) SEM images of the synthesized core-shell DDM sample at different magnifications. (f) HRTEM images of the synthesized core-shell DDM at different magnifications. (g) FTIR analysis of the synthesized core-shell DDM. (h) Stability of DDM in PBS containing 10% FBS.

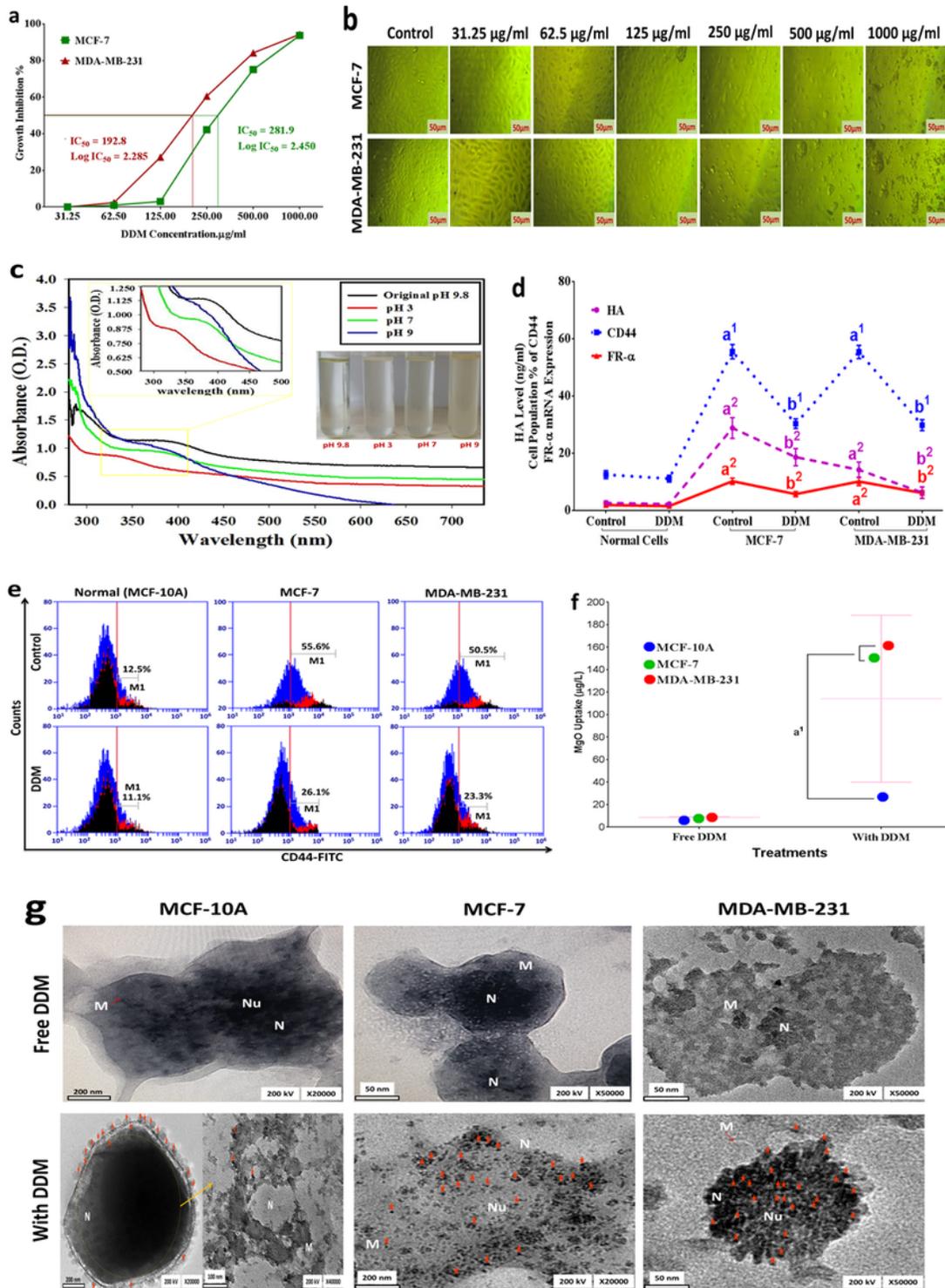


Figure 2

Inhibition of breast cancer (BC) cell proliferation, release, enhanced selectivity, and cell uptake by DDM. (a) MTT assay of BC cells treated with DDM at different concentrations. Values are represented as the mean \pm standard error of the mean (SEM) of triplicate samples from two independent experiments. (b) Phase-contrast images of MCF-7 and MDA-MB-231 cells. (c) Release of DDM. (d) Representative histogram of selective HA, CD44, and FR- α expression in BC cells. (e) Flow-cytometry images showing a single parameter histogram for CD44 in different groups. (f) Atomic absorption spectrophotometry intracellular MgO uptake data in MCF-10A, MCF-7, and MDA-MB-231 cells after a 24 h incubation with an IC50 DDM dose. (g) Transmission electron microscopy (TEM) images of MCF-10A, MCF-7, and MDA-MB-231 DDM uptake after a 24 h incubation with an IC50 DDM dose. Data are represented as the mean \pm standard error of the mean (SEM) (n = 3). a1p < 0.001, a2p < 0.01 vs. untreated normal cells; b1p < 0.001, b2p < 0.01 vs. untreated cancer

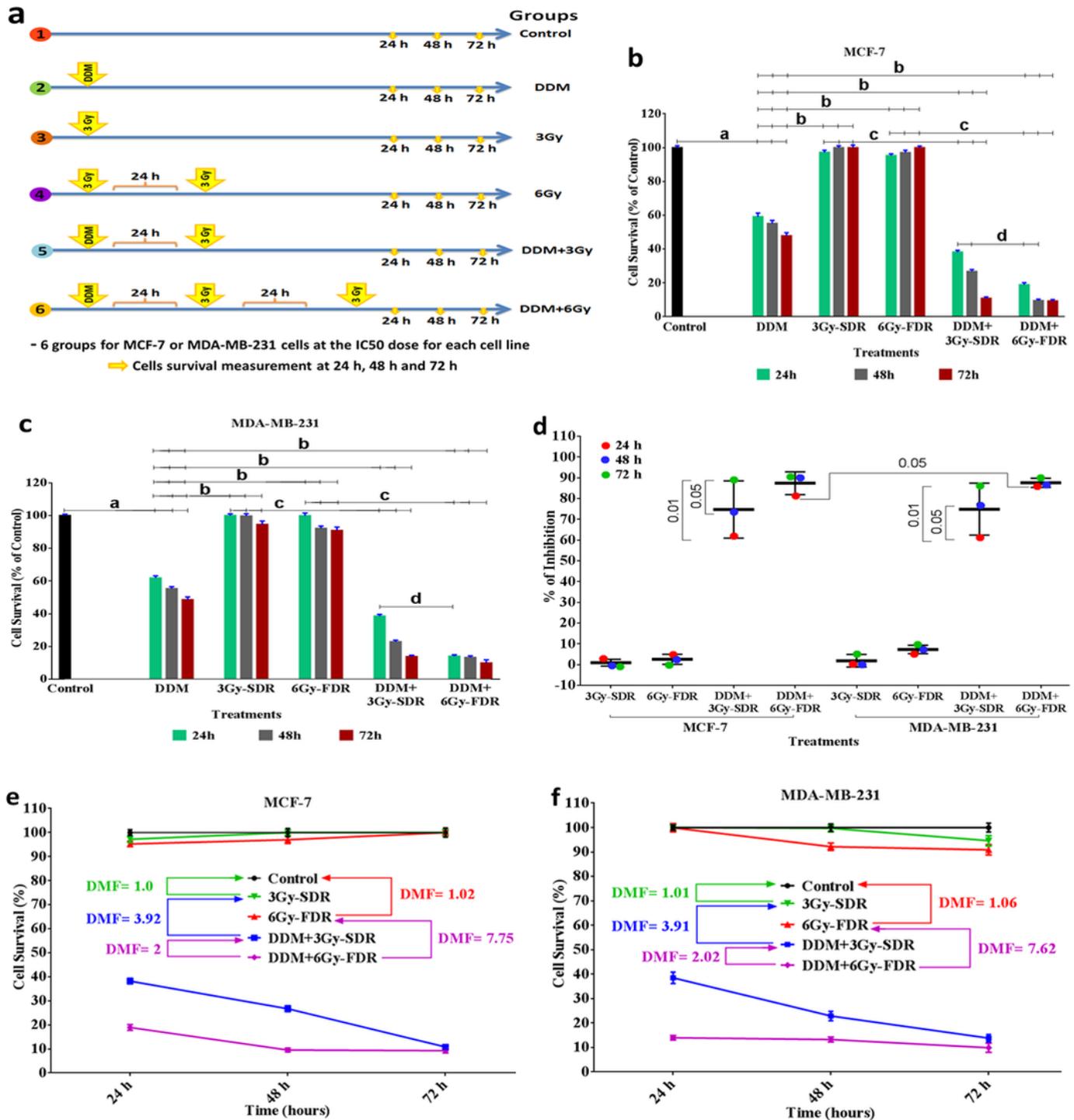


Figure 3

Cell survival and radio-sensitization rates using the multi-MTT assay. (a) Experimental schematic outline of the six different treatment groups. Evaluation of cell survival (b and c), percent inhibition (d), and dose-modifying effect curves (e and f) in response to DDM, 3Gy-SDR or 6Gy-FDR, and a combination of both at 24 h, 48 h, and 72 h using the multi-MTT assay. Data are the mean \pm standard error of the mean (SEM)

where control cells are 100% (n =3). ap < 0.01 vs. control; bp < 0.01 vs. DDM group; cp < 0.001 vs. 3Gy-SDR or 6Gy-FDR groups; dp < 0.05 vs. 3Gy-SDR+DDM group.

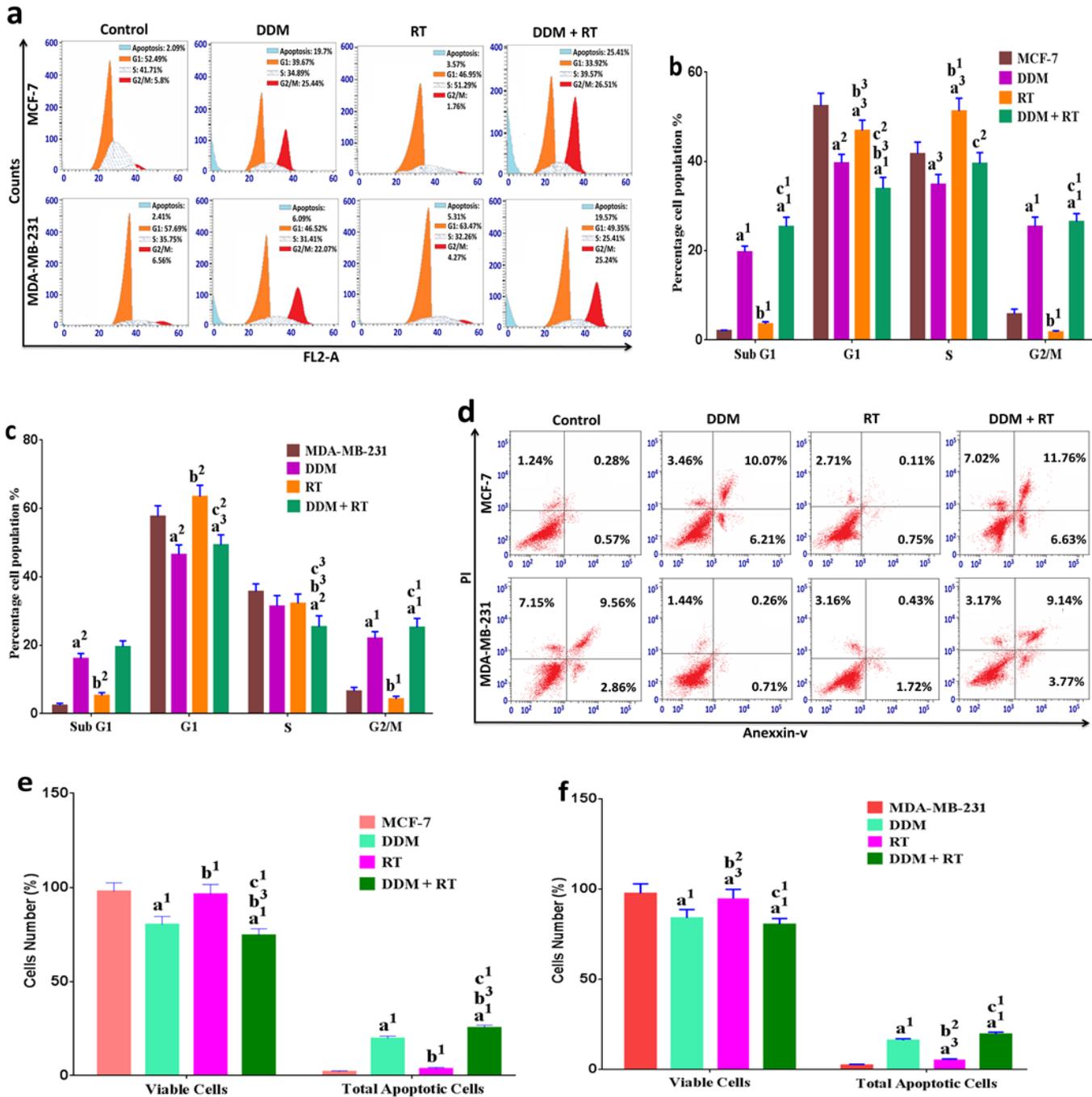


Figure 4

DDM/or RT induced cell cycle arrest and apoptosis in BC cells. (a, b, c) Representative cell cycle images of MCF7 and MDA-MB-321 cells at G2/M arrest after a 24 h treatment with DDM or DDM + RT, and cycle arrest at S phase in MCF-7 and G1 phase in MDA-MB-231 when treated with RT only. (d, e, f) A representative dot plot of annexin-V-FITC and PI staining in BC cells treated with DDM or DDM + RT for 24 h showing increased apoptotic cells when compared with controls or RT alone. Slightly increased

apoptotic cells are seen in the RT group compared to the control. Data are the mean \pm standard error of the mean (SEM). a1p < 0.001, a2p < 0.01, a3p < 0.05 vs. control; b1p < 0.001, b2p < 0.01, b3p < 0.05 vs. DDM group; c1p < 0.001, c2p < 0.01, c3p < 0.05 vs. RT group.

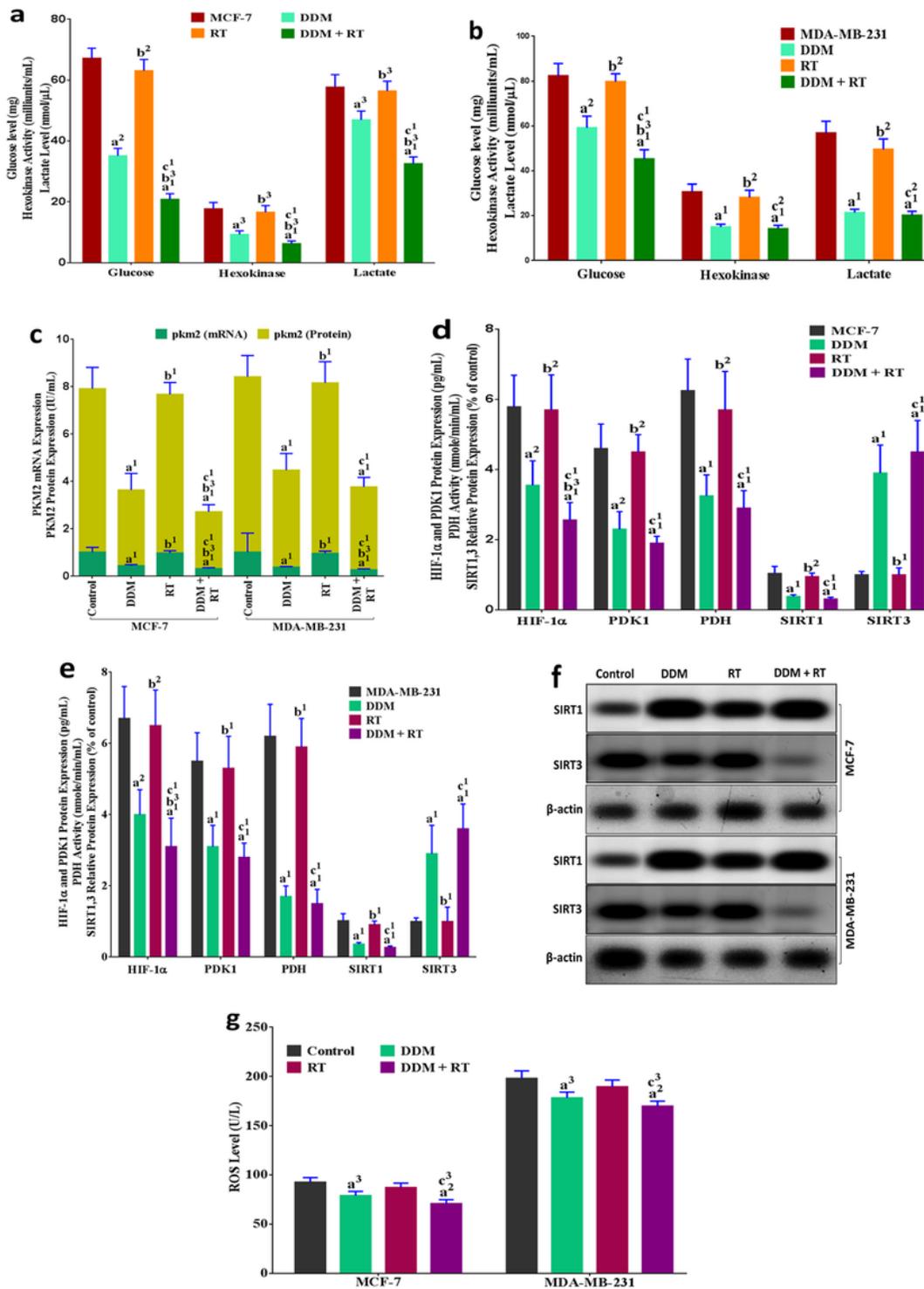


Figure 5

The influence of DDM/or RT on glycolysis and mitochondrial metabolic pathways in BC cells. (a, b) Glucose, hexokinase, and lactate levels were analyzed by colorimetric assay in all groups. (c) mRNA and

protein expression of PKM2 was rescued in all groups. (d, e) HIF-1 α , PDK1, SIRT1, and SIRT3 protein expression and PDH enzymatic activity was rescued in all groups. (f) Relative SIRT1, SIRT3, and β -actin protein levels in all groups. (g) ROS levels (ELISA) in all groups. All group values are given as the mean \pm standard error of the mean (SEM). a₁p < 0.001, a₂p < 0.01, a₃p < 0.05 vs. control; b₁p < 0.001, b₂p < 0.01, b₃p < 0.05 vs. DDM group; c₁p < 0.001, c₃p < 0.05 vs. RT group.

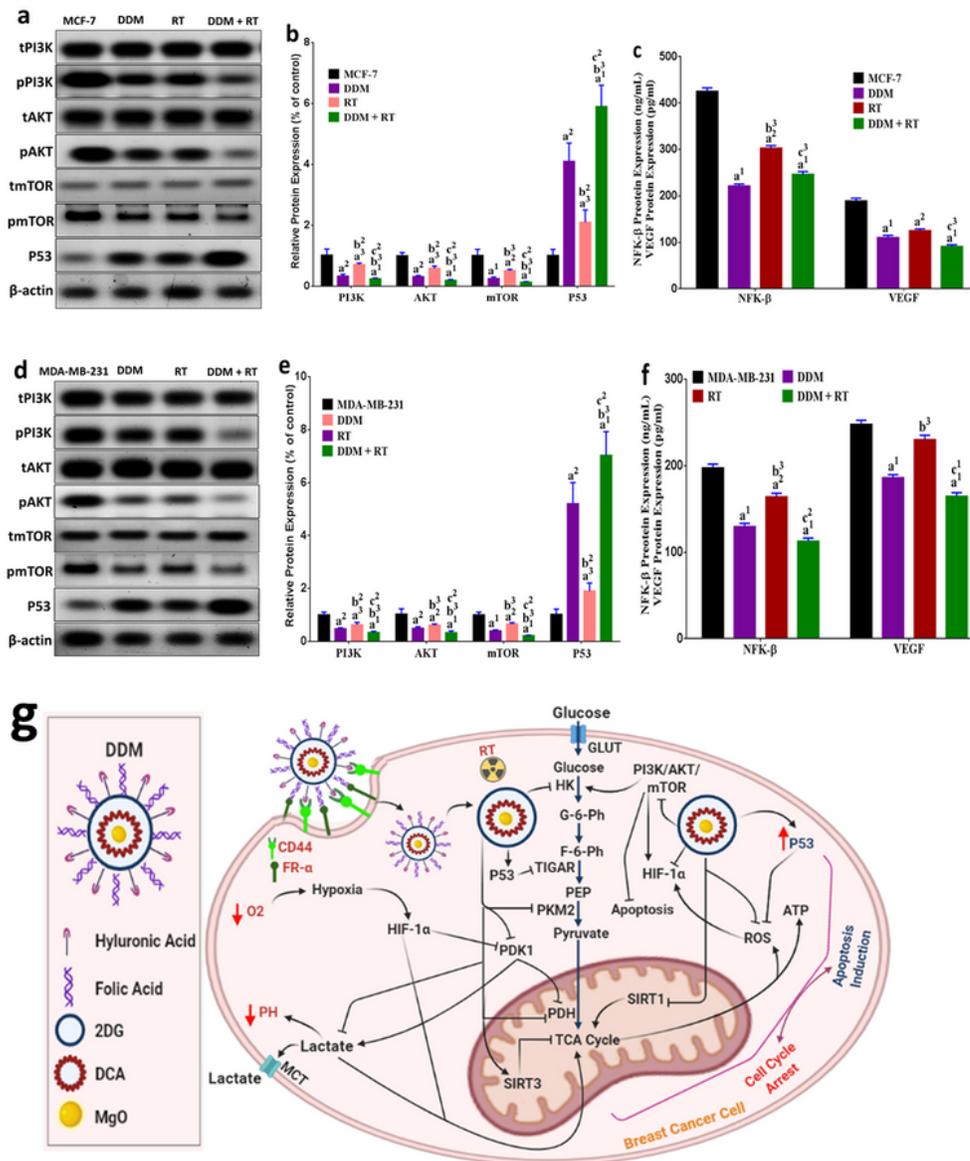


Figure 6. DDM/or RT modulates key proteins regulating glycolysis and OXPHOS pathways in BC cells. PI3K, AKT, mTOR, P53, and β -actin protein levels in MCF-7 cells (a, b) and MDA-MB-231 cells (d, e). NF- κ B and VEGF levels (ELISA) in both cell groups (c, f). (g) Schematic representing the mode of action of targeted multifunctional core-shell-nano (DDM) and RT. The DDM core-shell is a chemotherapy drug targeted to cancer cell surfaces via cancer cell-specific ligands. DDM binds to surfaces by recognizing specific receptors resulting in DDM internalization via endocytosis. Inside the cell, DDM undergoes endosomal escape leading to cytotoxic drug release. Treatment with DDM only or as a radio-sensitizer induces cancer cell death by modulating the metabolic reprogramming of BC cells via a novel PI3K/AKT/mTOR/P53/NF- κ B/VEGF pathway. (→) Indicates pathway direction and (—) indicates blocking functions. All group values are presented as the mean \pm standard error of the mean (SEM). a₁p < 0.001, a₂p < 0.01, a₃p < 0.05 vs. control; b₁p < 0.001, b₂p < 0.01, b₃p < 0.05 vs. DDM group; c₁p < 0.001, c₂p < 0.01, c₃p < 0.05 vs. RT group.

"See image above for figure legend."