Biomedicine & Pharmacotherapy 149 (2022) 112844

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Synergistic effect of antimetabolic and chemotherapy drugs in triple-negative breast cancer

ARTICLE INFO

Keywords
Triple-negative breast cancer
Antimetabolic drugs
Chemotherapy drugs
Synergism

ABSTRACT

The triple-negative breast cancer (TNBC) subtype comprises approximately 15% of all breast cancers and is associated with poor long-term outcomes. Classical chemotherapy remains the standard of treatment, with toxicity and resistance being major limitations. TNBC is a high metabolic group, and antimetabolic drugs are effective in inhibiting TNBC cell growth. We analyzed the combined effect of chemotherapy and antimetabolic drug combinations in MDA-MB-231, MDA-MB-468 and HCC1143 human TNBC cell lines. Cells were treated with each drug or with drug combinations at a range of concentrations to establish the half-maximal inhibitory concentrations (IC_{50}). The dose-effects of each drug or drug combination were calculated, and the synergistic or antagonistic effects of drug combinations were defined. Chemotherapy and antimetabolic drugs exhibited growth inhibitory effects on TNBC cell lines. Antimetabolic drugs targeting the glycolysis pathway had a synergistic effect with chemotherapy drugs, and antiglycolysis drug combinations also had a synergistic effect. The use of these drug combinations could lead to new therapeutic strategies that reduce chemotherapy drug doses, decreasing their toxic effect, or that maintain the doses but enhance their efficacy by their synergistic effect with other drugs.

1. Introduction

The triple-negative breast cancer (TNBC) subtype is a heterogeneous group in which various molecular subtypes have been described [1]. The common feature of TNBC is a lack of estrogen receptors and progesterone receptors and no human epidermal growth factor receptor 2 (HER2) amplification. It comprises approximately 15% of all breast cancers and is associated with poor long-term outcomes compared with other types of breast cancer. The lack of drug-targetable receptors makes conventional chemotherapy the standard treatment [2]. Adjuvant chemotherapy should include an anthracycline and a taxane [3]; however, toxicity and chemotherapy resistance are major limitations. Also, blockade of the PD-1/PD-L1 axis has emerged as a promising therapeutic option to enhance antitumor immunity [4–6], although new therapeutic options to maximize the benefits and minimize the toxicity of chemotherapy are still needed.

Metabolic reprogramming, such as the Warburg effect, is required for both malignant transformation and tumor development, including invasion and metastasis [7], and it is considered one of the hallmarks of cancer [8]. TNBC is a high metabolic group, with a particular dependency on glucose metabolism, [9] and it can be distinguished from other breast cancers based on its metabolic profile [10]. We had previously shown that the activity of metabolism-related molecular processes is increased in TNBC based on microRNA and protein expression profiles obtained from estrogen receptor-positive and TNBC samples [11]. TNBC cells show upregulated expression of key genes within the glycolysis and glutamine metabolic pathways [12]. Antimetabolic drugs targeting these metabolic pathways are effective in inhibiting TNBC cell growth [13], and other potential metabolic strategies to target TNBC have been described [14]. In addition, targeting cellular metabolism is a promising strategy to overcome drug resistance in cancer therapy [15]. Combining antimetabolic drugs with chemotherapy could increase the effect of the chemotherapy drugs while allowing dose reductions to reduce treatment toxicity. In this study, we analyzed the combined effect of chemotherapy and antimetabolic drug combinations in TNBC cell lines.

2. Materials and methods

2.1. Cell culture and reagents

We used TNBC cell lines MDA-MB-231, MDA-MB-468 and HCC1143, classified as basal B, basal A, and basal A, respectively, according to the molecular classification by Neve et al. [16]. The cells were cultured in RPMI-1640 medium with phenol red (Biological Industries), supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 mg/ml penicillin (Gibco) and 100 mg/ml streptomycin (Gibco). In L-γ-glutamyl-p-nitroanilide (GPNA) experiments, which need an acid pH for total dissolution, 50 mM of HEPES (Gibco) was added to the medium as a buffering agent. All the cell lines were cultured at 37 °C in a humidified atmosphere with 5% (v/v) CO_2 in the air. The MDA-MB-231 cell line was kindly provided by Dr. Nuria Vilaboa (La Paz University Hospital), previously obtained from the American Type Culture

Abbreviations: TNBC, triple-negative breast cancer; HER2, human epidermal growth factor receptor 2; MTF, metformin; 2DG, 2-D-deoxy-glucose; AOA, aminooxyacetic acid; GPNA, L-γ-Glutamyl-p-nitroanilide; DOX, doxorubicin; EPI, epirubicin; DOCE, docetaxel; PTX, paclitaxel; AAT, aspartate aminotransferase; SNP, single nucleotide polymorphism.

https://doi.org/10.1016/j.biopha.2022.112844
Received 20 January 2022; Received in revised form 16 March 2022; Accepted 17 March 2022
Available online 23 March 2022

Collection (ATCC) in January 2014. The MDA-MB-468 and HCC1143 cell lines were also obtained from ATCC (July 2014). The cell lines were routinely monitored in our laboratory and authenticated by morphology and growth characteristics, tested for mycoplasma, frozen, and were passaged for fewer than 6 months before the experiments. Metformin (Sigma Aldrich D150959), 2-D-deoxy-glucose (2DG) (Sigma Aldrich D8375), aminooxyacetic acid (AOA) (Sigma Aldrich C13408), GPNA (Sigma Aldrich G6133), doxorubicin (Sigma Aldrich 44583), epirubicin (Sigma Aldrich E9406), docetaxel (Sigma Aldrich 01885) and paclitaxel (Sigma Aldrich T7402) were obtained from Sigma-Aldrich (St. Louis, MO, USA). MTF, 2DG and AOA were diluted in distilled water. GPNA was diluted in acidified distilled water. Chemotherapy drugs were diluted in DMSO 10%. The percentage of DMSO present in the culture medium at the highest concentration for each drug was $\leq$ 0.00064%. Previous literature indicates that this concentration will not provoke any DMSO derived toxicity [17].

2.2. Cell viability assays

Cells were treated with each drug or drug combination at a range of concentrations to establish an IC_{50} for each cell line. Concentration points were selected for each drug and cell line based on the IC_{50} and dose-response curves published in the literature [13, 18–20] and from the National Cancer Institute-60 (NCI-60) cell panel web information (https://tpwb.nci.nih.gov/GeneExpressionNCI60/GI50.html). For drug combination experiments, the drugs were combined at a constant ratio at concentrations of IC_{50}x4, IC_{50}x2, IC_{50}, ½ IC_{50}, ¼ IC_{50}, 1/8 IC_{50} and 1/16 IC_{50}, following the Chou-Talalay recommendations for this type of experiment [21]. Approximately 5000 cells per well were seeded in 96-well plates. After 24 h, an appropriate concentration of drug was added to the cells, which were incubated for a total of 72 h. Untreated cells were used as controls. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) kit was used for the quantification of cell survival after 72 h of drug exposure following the manufacturer's instructions. Absorbance was measured on a microplate reader (Multiskan Sky Microplate Spectrophotometer, Thermo Fisher Scientific). Showed results included pool data from at least 3 independent triplicate experiments for each condition.

The anthracyclines doxorubicin and epirubicin and the taxanes docetaxel and paclitaxel were combined with antimetabolic drugs targeting the glycolysis pathway (MTF and 2DG) and the glutamine metabolism (AOA). (Table 1a). Also, various antimetabolic drug combinations were tested. First, antiglycolysis drugs were combined (MTF + 2DG); second, each antiglycolysis drug was combined with the antiglutamic drug AOA (MTF + AOA and 2DG + AOA) and lastly, we used a combination of 2 antiglutamic drugs: AOA and GPNA (Table 1b).

2.3. Dose-effect and drug combination analyses

The dose effects of each drug or drug combination were defined by the half-maximal inhibitory concentration (IC_{50}) values and were calculated using the Chou-Talalay method [21]. The IC_{50} for each drug and drug combination was calculated, and the synergistic or antagonistic effect of the drug combinations was quantified. Drug combination effects were defined by the resulting combination index (CI) theorem of Chou-Talalay, which offers a quantitative definition for additive effects (CI = 1), synergism (CI < 1) and antagonism (CI > 1). The dose-reduction index (DRI) is defined by the DRI equation of Chou TC. and it is a measure of how many folds the dose of each drug in a synergistic combination may be reduced at a given effect level when compared with the doses of each drug alone [22]. The data were analyzed with CompuSyn software [23], and dose-response curves were constructed with GraphPad Prism 6.

2.4. DNA extraction and single-nucleotide polymorphism genotyping

DNA was extracted from untreated cells using the ISOLATE II RNA/DNA/Protein Kit (BIOLINE) following the manufacturer's instructions. We used TaqMan OpenArray technology on a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems) with a custom single-nucleotide polymorphism (SNP) array format, which allows simultaneous genotyping of 180 SNPs in major drug metabolizing enzymes and transporters (PharmArray). Information on the pharmacogenetic variants associated with drug responses was gathered from the variant and clinical annotations in the Pharmacogenomics Knowledge Base (PharmGKB; www.pharmgkb.org).

3. Results

3.1. Chemotherapy and antimetabolic drugs exhibited growth inhibitory effects on the three analyzed TNBC cell lines, with varying susceptibility to each drug

First, the response of 3 TNBC cell lines treated with drugs targeting 2 routes of cell metabolism was evaluated: the glycolysis pathway (MTF and 2DG) and the glutamine pathway (AOA and GPNA). Cell viability was relativized according to the total cell viability of the untreated control (Fig. 1). All antimetabolic drugs showed growth inhibitory effects on the three TNBC cell lines. Antiglutamic drugs showed lower IC_{50} than drugs targeting glycolysis (Table 2a). A heterogeneous response to antimetabolic drugs was observed among the 3 TNBC cell lines. Metformin treatment showed a broad effect on cell proliferation, with HCC1143 cells the most resistant and MDA-MB-468 the most sensitive to this treatment. In contrast, HCC1143 cells were very sensitive to 2DG (Table 2a).

A heterogeneous cell response to anthracyclines and taxanes was also observed among the 3 TNBC cell lines (Fig. 2). The MDA-MB-231 and MDA-MB-468 cell lines were more sensitive than HCC1143 to both chemotherapy drug families, and specifically, MDA-MB-468 cells showed high sensitivity to anthracyclines (Table 2b).

Table 1
Chemotherapy and antimetabolic drug combinations (A) and antimetabolic drug combinations (B).

(A)			Antimetabolic drugs	
Chemotherapy drugs	Anthracyclines	Doxorubicin (DOX) and Epirubicin (EPI)	antiglycolysis drugs	Metformin (MTF)
				2-deoxy-D-glucose (2DG)
			antiglutamic drug	Aminooxyacetic acid (AOA)
	Taxanes	Docetaxel (DOCE) and Paclitaxel (PTX)	antiglycolysis drugs	Metformin (MTF)
				2-deoxy-D-glucose (2DG)
			antiglutamic drug	Aminooxyacetic acid (AOA)
(B)			Antimetabolic drugs	
Antimetabolic drugs	antiglycolysis drugs	Metformin (MTF)	antiglycolysis drug	2-deoxy-D-glucose (2DG)
			antiglutamic drug	Aminooxyacetic acid (AOA)
		2-deoxy-D-glucose (2DG)	antiglutamic drug	Aminooxyacetic acid (AOA)
	antiglutamic drug	Aminooxyacetic acid (AOA)	antiglutamic drug	L-γ-glutamyl-p-nitroanilide (GPNA)

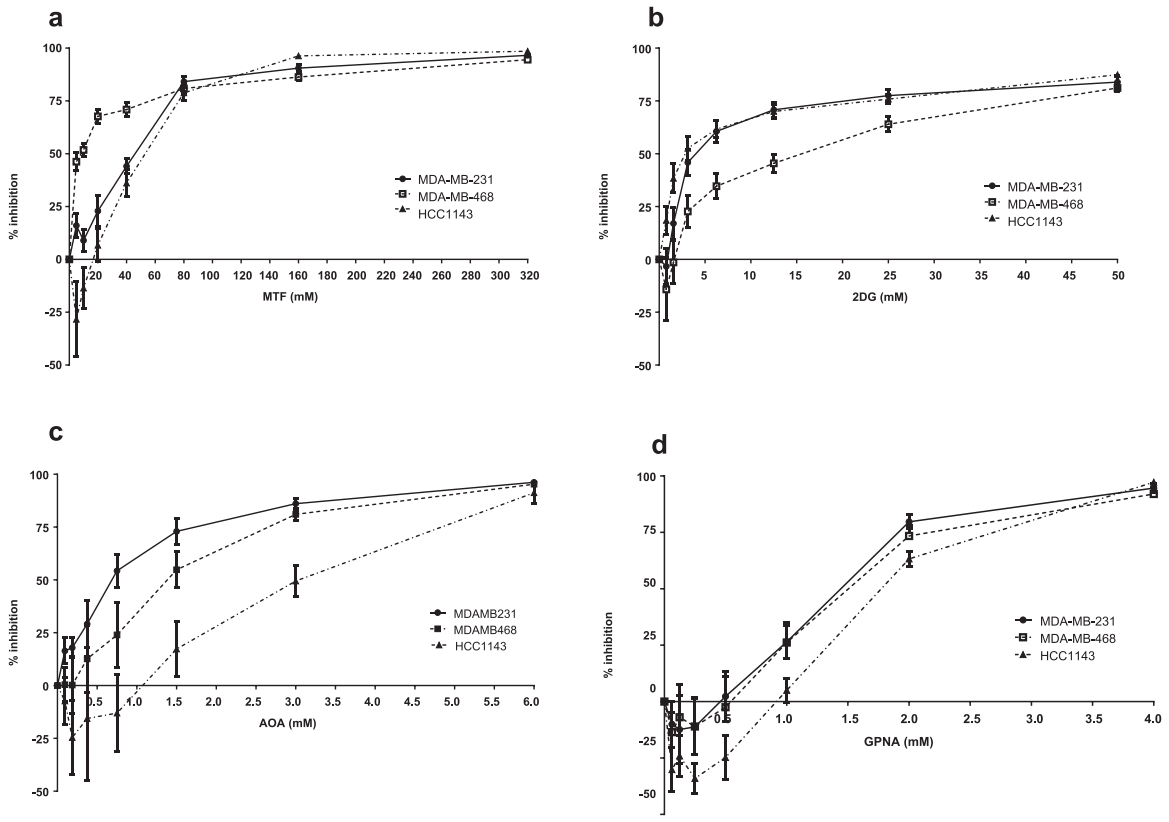

Fig. 1. Inhibition (%) of MDA-MB-231, MDA-MB-468 and HCC1143 when exposed to the antimetabolic drugs: (a) MTF, range 0–320 mM, (b) 2DG, range 0–50 mM, (c) AOA, range 0–6 mM and (d) GPNA, range 0–4 mM. Values are mean ± SD of 9 or more experiments.

Table 2
MDA-MB-231, MDA-MB-468 and HCC1143 half-maximal inhibitory concentration (IC_{50}) values of antimetabolic (A) and chemotherapy (B) drugs.

A.				
	IC_{50} MTF (mM)	IC_{50} 2DG (mM)	IC_{50} AOA (mM)	IC_{50} GPNA (mM)
MDA-MB-231	33.10	16.70	0.63	1.73
MDA-MB-468	7.99	28.73	2.29	2.49
HCC1143	77.72	3.81	4.21	2.58

B.				
	IC_{50} DOX (nM)	IC_{50} EPI (ng/ml)	IC_{50} DOCE (nM)	IC_{50} PTX (nM)
MDA-MB-231	127.44	57.04	9.61	32.39
MDA-MB-468	32.10	23.94	2.02	44.29
HCC1143	252.98	181.52	15.46	51.30

Metformin (MTF), 2-deoxy-D-glucose (2DG), aminooxyacetic acid (AOA), L-γ-glutamyl-p-nitroanilide (GPNA)
Doxorubicin (DOX), epirubicin (EPI), docetaxel (DOCE), paclitaxel (PTX)

3.2. Antimetabolic drugs targeting the glycolysis pathway had a synergistic effect with chemotherapy drugs

Combinations of each antimetabolic drug (metformin, 2DG and AOA) with each chemotherapy drug (doxorubicin, epirubicin, docetaxel and paclitaxel) were tested. Metformin and 2DG showed a broad synergistic effect with doxorubicin, epirubicin and docetaxel, except for the 2DG+docetaxel combination in the HCC1143 cell line, which showed antagonism, whereas paclitaxel combinations only showed synergism in the MDA-MB-231 cell line (Table 3 and Supplementary tables 1 and 2). On the other hand, the antiglutamic drug AOA showed antagonism with all chemotherapy drugs with one exception: the AOA + epirubicin combination in the HCC1143 cell line, which showed synergism (Table 3).

3.3. Antiglycolysis drug combinations had a synergistic effect, but not when combined with antiglutamic drugs

Antimetabolic drug combinations were tested. Antiglycolysis drug combinations (MTF+2DG) showed synergistic effects for the 3 TNBC cell lines, whereas the combination of each antiglycolysis drug (MTF or 2DG) with the antiglutamic drug AOA showed synergistic or antagonistic effects depending on the cell line (Table 4).

Antiglutamic drug combinations (AOA+GPNA) were also tested, showing an antagonistic effect for the 3 TNBC cell lines, in contrast to antiglycolysis drug combinations (Table 4).

3.4. SNP genotyping of breast cancer cell lines

SNP genotyping was performed to evaluate the association between polymorphisms, metformin and chemotherapy treatment response. Polymorphisms previously related to the sensitivity of these drugs were studied using a custom expression array.

Regarding the response to metformin, polymorphism rs2282143 in SLC22A1 was detected in homozygosis in MDA-MB-468 cells. On the other hand, the rs628031 polymorphism, also in SLC22A1, was found in homozygosis in HCC1143 cells and in heterozygosis with a possible duplication in MDA-MB-468 cells. Regarding the response to chemotherapy drugs, polymorphisms in the doxorubicin transport encoding genes *ABCB1* (rs2032582 and rs1045642), *ABCC2* (rs3740066) and *SLC22A1* (rs2282143), and in the doxorubicin drug metabolism-related

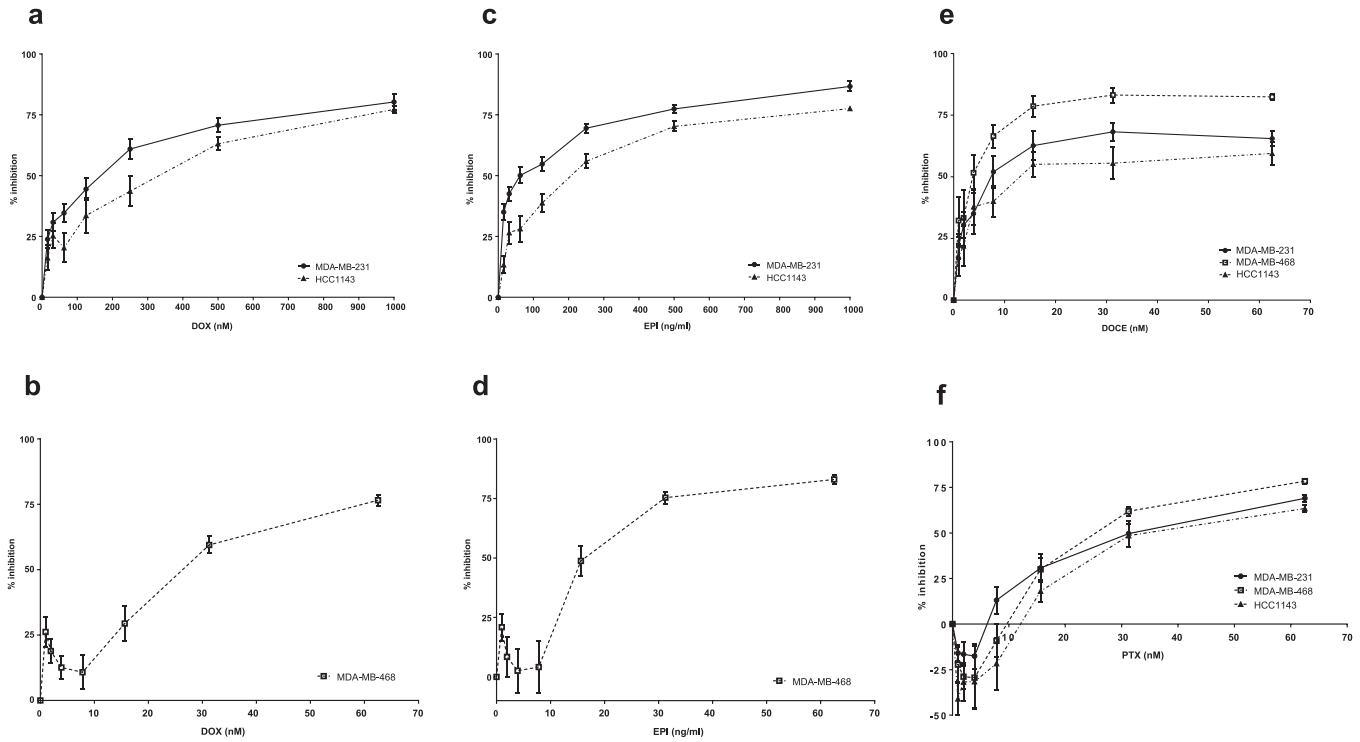

Fig. 2. Inhibition (%) of MDA-MB-231, MDA-MB-468 and HCC1143 when exposed to chemotherapy drugs: (a) doxorubicin, range 0–1000 nM for MDA-MB-231 and HCC1143, (b) doxorubicin, range 0–62.5 nM for MDA-MB-468, (c) epirubicin, range 0–1000 ng/ml for MDA-MB-231 and HCC1143, (d) epirubicin, range 0–62.5 ng/ml for MDA-MB-468, (e) docetaxel, range 0–62.5 nM, and paclitaxel, range 0–62.5 nM. Values are mean ± SD of 9 or more experiments.

Table 3
Antimetabolic and chemotherapy drug combination effects defined as CI (combination index), and DRI (doses-reduction index) values for synergistic combinations.

		CI [1]			DRI [2] (antimetabolic drug)			DRI (chemotherapy drug)		
		231	468	HCC	231	468	HCC	231	468	HCC
MTF	DOX	0.14	0.22	0.41	7.00	4.51	2.43	7076.07	4476.46	2475.05
	EPI	0.10	0.36	0.22	9.69	2.78	4.61	5659.01	1371.68	2716.94
	DOCE	0.28	0.27	0.21	3.59	3.74	4.67	3987.42	4665.53	4600.27
	PTX	0.65	1.88	7.27	1.55	---	---	1589.40	---	---
2DG	DOX	0.38	0.36	0.61	2.66	2.79	1.65	2645.63	2803.16	1663.43
	EPI	0.54	0.21	0.84	1.86	4.70	1.20	1070.42	2351.27	696.52
	DOCE	0.62	0.45	1.47	1.62	2.21	---	1772.19	2783.85	---
	PTX	0.94	1.00	2.31	1.06	---	---	1075.43	---	---
AOA	DOX	1.66	3.82	1.38	---	---	---	---	---	---
	EPI	3.86	3.95	0.71	---	---	1.41	---	---	803.75
	DOCE	2.24	8.72	1.15	---	---	---	---	---	---
	PTX	2.39	13.82	2.30	---	---	---	---	---	---

Metformin (MTF), 2-deoxy-D-glucose (2DG), aminooxyacetic acid (AOA), doxorubicin (DOX), epirubicin (EPI), docetaxel (DOCE), paclitaxel (PTX).
(1) **CI: Combination index**; synergism (CI < 1, light grey squares), additive effect (CI = 1, medium grey squares) and antagonism (CI > 1, dark grey squares).
(2) **DRI: Dose-reduction index**: quantify how many folds the dose of each drug in a synergistic combination may be reduced at a given effect level when compared with the doses of each drug alone. DRI values were shown only for synergistic combinations (CI<1).

Table 4
Antimetabolic drug combination effects defined as CI (combination index).

	CI [1]		
	MDA-MB-231	MDA-MB-468	HCC1143
MTF + 2DG	0.02	0.005	0.14
MTF + AOA	0.63	1.71	0.29
2DG + AOA	1.60	0.35	1.61
AOA + GPNA	2.37	1.67	1.33

[1]**CI: Combination index**; synergism (CI < 1, light gray squares), and antagonism (CI > 1, dark gray squares). Metformin (MTF), 2-deoxy-D-glucose (2DG), aminooxyacetic acid (AOA), L-γ-glutamyl-p-nitroanilide (GPNA).

encoding gene *CYP2C19* (rs12248560) were detected. In addition, the rs11572103 (*CYP2C8 *2* allele: 805 A>T, Ile269Phe) polymorphism observed on the *CYP2C8* gene and related to paclitaxel response was detected (Table 5). Detailed SNP results are shown in Supplementary Table 1.

4. Discussion

In this study we showed that chemotherapy and antimetabolic drugs exhibited growth inhibitory effects on TNBC cell lines, but the response to both drug types among the 3 TNBC cell lines was heterogeneous. Also, we showed that antimetabolic drugs targeting the glycolysis pathway had a synergistic effect with chemotherapy drugs and that antiglycolysis drug combinations had a synergistic effect too.

Metformin and 2DG target the glycolysis pathway in different ways. Metformin attenuates the expression of several important genes involved in glucose metabolism, including glucose transporters and glycolysis enzymes [24], whereas 2DG is a non-metabolized glucose analog that acts as a competitive inhibitor of glycolysis [25]. Metformin interferes cellular metabolism at different levels. The mechanism of action for metformin is the stimulation of AMPK. Inhibiting mitochondrial respiratory chain complex I by metformin can activate AMPK by reducing ATP production, leading to an increase in the AMP:ATP ratio [26]. AMPK activation can not only suppress the transcription of gluconeogenic genes but also inhibit lipogenesis and improve insulin sensitivity. Activated AMPK switches cells from an anabolic to a catabolic state, shutting down the ATP-consuming synthetic pathways and restoring energy balance. This regulation involves phosphorylation by AMPK of key metabolic enzymes and transcription factors/co-activators modulating gene expression [27]. As a result, glucose, lipid and protein synthesis as well as cell growth are inhibited whereas fatty acid oxidation and glucose uptake are stimulated [27].

AOA and GPNA target the glutamine pathway at different levels. AOA is an inhibitor of the aspartate aminotransferase enzyme [18], whereas GPNA is an inhibitor of the glutamine transporter ASCT2 [19]. In our study, the 3 TNBC tested cells displayed high sensitivity to glutamine-targeting agents, in accordance with previous results finding that TNBC primary tumors and cell lines had elevated glutaminase expression as well as a high dependence on extracellular glutamine for growth [28].

All the antimetabolic and chemotherapy drugs tested in this study induced TNBC cell death (Figs. 1 and 2), although the response was heterogeneous depending on the cell line. In a recent study, metabolic heterogeneity of TNBC tumors has been described by using a multi-omics database [29]. This fact could explain the heterogeneous cell response to the antimetabolic drugs that we observed. With the aim of studying polymorphisms that could also explain this heterogeneous cell response, an SNP array was used to analyze polymorphisms in genes involved in drug transport or metabolism [30]. The high sensitivity of MDA-MB-468 cells to metformin could be partly due to rs2282143 SNP in the SLC22A1 carrier, which is related to decreased clearance of metformin. This SNP appears with a frequency of 8% in the black population, which is the population origin of this cell line. In addition, SLC22A1 rs628031, which have been previously associated with a poor response to metformin, was homozygotic in the HCC1143 cell line [13]. A heterogeneous cell response to anthracyclines and taxanes was also observed (Table 2). The high sensitivity of MDA-MB-468 cells to doxorubicin and epirubicin could be related to polymorphisms in the doxorubicin transports encoding genes ABCB1 (rs2032582 and rs1045642), ABCC2 (rs3740066) and SLC22A1 (rs2282143), and with the doxorubicin drug metabolism-related encoding gene CYP2C19 (rs12248560). Genetic polymorphisms in ABCB1 have been linked to altered tumor responses to docetaxel and doxorubicin [31]. Regarding taxane treatment responses, the MDA-MB-231 and MDA-MB-468 cell lines were more sensitive to docetaxel and paclitaxel treatments, which could be associated with the polymorphism rs11572103 (CYP2C8 *2 allele: 805 A>T, Ile269Phe) observed on the CYP2C8 gene. This gene codifies the cytochrome P450 2C8, the main metabolizing enzyme of paclitaxel. This allele variation might reduce the ability of the enzyme to metabolize paclitaxel and decrease drug clearance [32].

Differences in cell responses to the various antimetabolic drug combinations were observed. With a few exceptions, depending on the TNBC cell line, the antiglycolysis drugs metformin and 2DG had a synergistic effect with chemotherapy drugs, whereas the antiglutamic drug

Table 5
MTF and chemotherapy drugs response related SNPs.

			HCC1143				MDA-MB-231				MDA-MB-468			
SNP	Gene	Drug	A1	A 2	GN	IC_{50}	A 1	A 2	GN	IC_{50}	A1	A2	GN	IC_{50}
rs2282143	*SLC22A1*	MTF	C	C	CC	77.7 mM	C	C	CC	33.1 mM	T	T	TT	7.9 mM
rs628031	*SLC22A1*		A	A	AA		G	G	GG		A	G	AAG	
rs2032582	*ABCB1*	DOX	C	C	CC	252.9 nM	C	C	CC	127.4 nM	na*	na	na	32.1 nM
rs1045642	*ABCB1*		A	A	AA		A	A	AA		G	G	GG	
rs3740066	*ABCC2*		C	T	CT		C	T	CT		C	C	CC	
rs2282143	*SLC22A1*		C	C	CC		C	C	CC		T	T	TT	
rs12248560	*CYP2C19*		C	C	CC		C	C	CC		T	T	TT	
rs11572103	*CYP2C8*	PTX	T	T	TT	51.3 nM	T	T	TT	32.3 nM	A	A	AA	44.2 nM

*na: not amplified; A: allele; GN: genotype; IC_{50}: half-maximal inhibitory concentration; Metformin (MTF), doxorubicin (DOX), paclitaxel (PTX).

AOA had antagonism with chemotherapy. The antagonistic effect with AOA was unexpected, given that previous results had shown that other antiglutamic agents, such as the CB-839 and 968 molecules (both with antiproliferative activity in various TNBC cell lines) produced increased cell sensitivity when combined with doxorubicin and paclitaxel, respectively [28, 33]. This result suggests that each molecular type could possess a specific metabolic phenotype, and the mechanism by which chemotherapy alters the metabolic regulation of TNBC cells is not fully understood.

Chemotherapy had the most powerful synergism with metformin. The only exception was for paclitaxel, which showed antagonism in the case of MDA-MB-468 and HCC1143 cell lines. It has been shown that metformin leads to a variety of molecular mechanisms alterations when combined with chemotherapeutic drugs [34], and its treatment efficacy varies between TNBC subtypes [35]. Molecular differences between mechanisms involved in chemotherapy drug combinations in these TNBC cell lines could explain the differences in terms of synergism or antagonism for each drug combination.

2DG also had a synergistic effect with chemotherapy drugs. There was more synergism in 2DG plus anthracycline combinations. Our results are in accordance with previous studies indicating that 2DG acts synergistically with those chemotherapeutic drugs that cause DNA damage, such as anthracyclines [36].

Potential mechanisms involved in the synergisms of metformin and anthracyclines/taxanes have been described [34]. Metformin potentiates the anticancer efficacy of anthracyclines. The enhancing cytotoxicity of doxorubicin is related with mechanisms involved in increasing doxorubicin cellular uptake via inhibiting ABCB1 function, which encode for drug resistant protein P-glycoprotein (Pgp) and cell cycle arrest at G1/S transition. Moreover, metformin could elevate DOX-induced apoptosis [37–39]. Epirubicin is one of the isomers of doxorubicin and both drugs have comparable anti-tumor activity and similar mechanisms, so mechanisms involved in metformin enhancing epirubicin cytotoxicity should be similar. Mechanisms involved in the synergism of metformin and taxanes were related to the metformin specific inhibition of ERCC1 that enhances the taxanes-induced cytotoxic effect [40]. Also, blocking the MAPK signalling pathway can potentiate the anti-tumoral activity of metformin and docetaxel [41]. In addition, it has been described that metformin potentiates the anti-tumor activity of docetaxel through down-regulation of lipoprotein or cholesterol synthesis [42].

The antiglycolysis drug combination (MTF + 2DG) showed a strong synergistic effect on the three TNBC cell lines. Both drugs inhibit glycolysis, and metformin also inhibits mitochondria oxidative phosphorylation [43]; thus, the combination synergistically reduces ATP production and induces cell death. Previous studies have shown that treating breast cancer cells with 2DG and metformin combinations resulted in a significant decrease in cell viability and an increase in apoptosis [44–46].

Antiglycolysis and antiglutamic drug combination, (MTF + AOA) showed synergistic effects but depending on the cell line. The synergistic effect could be explained by the metformin mediated inhibition of the complex I electron transport chain in mitochondria that causes energetic stress in cells. This causes a decrease in nicotinamide adenine dinucleotide (NADH) oxidation and tricarboxylic acid (TCA) flux, leading to low levels of TCA metabolites. Cells react by rewiring metabolic flux. This includes the increased glutamine utilization to provide alternative sources of ATP as well as metabolites. The inhibition of the glutamine pathway by AOA hinders this alternative.

AOA and GPNA target glutamine metabolism, but GPNA interferes with glutamine uptake targeting the glutamine transporter ASCT2 [19], whereas AOA interferes with glutamine metabolism, targeting the aspartate aminotransferase enzyme [18]. Both alone showed a decrease in TNBC cell viability, but the combination of AOA and GPNA was not synergistic. Although there are other glutamine transporters, the ASCT2 transport is critical for TNBC cells [20], so ASCT2 inhibition results in significant cell glutamine deprivation. For this reason, the inhibition effect in glutamine metabolism by AOA is not perceptible.

Glucose and glutamine metabolism in cancer cells is markedly elevated relative to normal cells, specifically in TNBC cells [12]. In this study, we showed that antimetabolic drugs induce TNBC cell death. This finding is in accord with previous results showing that antimetabolic drugs targeting glycolysis and glutamine pathways are effective for inhibiting TNBC cell growth [13].

Metabolic drugs might also have drastic effects on the immune cells. However, the energetic interplay between tumor and immune cells leads to metabolic competition in the tumor ecosystem, limiting nutrient availability and leading to microenvironmental acidosis, which hinders immune cell function. Metformin has been investigated in this context. The effects of metformin on the immune system are dependent on the pathological context, yielding opposite effects depending on whether they occur in the context of cancer or other pathologies. Metformin has immunostimulatory effects when used for the treatment of cancer in animal models while having immunosuppressive effects in other pathologies. The immunostimulatory effects of metformin treatment in cancer are indirect and mediated by tumor cells through different mechanisms while the immunosuppressive effects of metformin in other pathologies are the direct consequence of its action on immune cells [47]. In fact, concerning the immunosuppressive effects of metformin in non-cancer pathologies, no untoward immunological consequences were observed in the large number of subjects with type 2 diabetes that have been treated over the decades with metformin.

Immunostimulatory effects of metformin treatment in cancer were well characterized [48–51]. Many of them were mediated by intratumoral hypoxia. Metformin treatment of tumor-bearing mice inhibits oxygen consumption by tumor cells with consequent reduction of intratumoral hypoxia. Hypoxia has immunosuppressive effects and alleviation of hypoxia in the tumor microenvironment contributes to relieving immunosuppression. Moreover, it has been shown that metformin can increase the efficacy of immunotherapy. The combination of metformin with PD-1 and CTLA4 blockade resulted in improved intratumoral T-cell function and tumor clearance in mice cell-derived xenografts (CDX) models [52, 53].

Further studies using animal models are necessary to establish the appropriate combinations of synergistic drugs. Some combinations with metformin and doxorubicin have been studied using cell-derived mouse xenografts, with promising results [54]. The combination of metformin and 2DG also suppressed tumor growth in TNBC xenograft models in vivo [55].

The glycolysis inhibitors metformin and 2DG have low toxicity when administered either alone or in combination with chemotherapy [34, 44, 56]. Moreover, it has been demonstrated that pre-treatment with metformin produced significant ($P < 0.05$) cardiac protection in doxorubicin induced cardiotoxicity in rats [39].

The higher concentration of antimetabolic drugs used in this work was 320 mM for MTF, 50 mM for 2DG, 6 mM for AOA, and 4 mM for GPNA. Safe doses and pharmacodynamics of MTF and 2DG were well known. Diabetic patients receiving typical doses of 1.5–2.5 g of metformin per day (~30 mg/kg) have plasma levels in the 10 μM range [57]. All metformin concentrations used in this work were far below this plasma concentration. 2DG is considered a safe agent for clinical use. Clinical trials confirmed that administration of 2DG alone or combined with other anticancer therapies, such as chemotherapy and radiotherapy, was safe and well-tolerated by patients [58]. The dose of 45 mg/kg (Cmax=277 μM) was the recommended 2DG dose in phase II clinical trials [59]. The 2DG concentrations used in this work were also far below the maximum plasma concentration reached this dose. GPNA has been used only in preclinical models, were it is well-tolerated, but human safety is still unknown [60]. High concentrations of AOA has been used in humans for the treatment of other pathologies, like tinnitus, with undesired side effects [61], although it is possible to reduce toxicity with a dose adjustment in the context of cancer

combined treatment.

All this together open the possibility of employing these combinations to increase TNBC treatments efficacy, with few adverse effects.

5. Conclusions

The use of chemotherapy and antimetabolic drug combinations could lead to reduced chemotherapy drug doses, thus decreasing their toxic effect, or to maintaining the doses but enhancing their efficacy due to drug synergism. Clinical trials could be performed to test therapeutic alternatives based on antimetabolic and chemotherapeutic drug combinations in patients with advanced TNBC.

Funding

María I. Lumbreras-Herrera and Andrea Zapater-Moros are supported by Consejería de Educación e Investigación de la Comunidad de Madrid (IND2018/BMD-9262). Elena López-Camacho is supported by the Spanish Economy and Competitiveness Ministry (PTQ2018–009760). This work is supported by an unrestricted grant from Roche. The sponsors were not involved in the study design, in data collection or analysis, in the decision to publish, or in the preparation of this manuscript.

CRediT author statement

Elena López-Camacho and **Lucía Trilla-Fuertes**: Conceptualization, investigation methodology, formal analysis, writing - original draft. **Angelo Gámez-Pozo**, **Juan Ángel Fresno Vara:** Conceptualization, investigation. **María Isabel Lumbreras-Herrera, Irene Dapía, Rocío López-Vacas and Pedro Arias:** Methodology. **Enrique Espinosa and Pilar Zamora**: Conceptualization, writing - review and editing.

Data availability

The collected data in this study are available from the corresponding author on reasonable request.

Conflict of interest statement

Enrique Espinosa, Angelo Gámez-Pozo and Juan Ángel Fresno Vara are shareholders of Biomedica Molecular Medicine SL. Elena López-Camacho, and Andrea Zapater-Moros are employees of Biomedica Molecular Medicine. The other authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgment

The graphical abstract has been created using figures from BioRender.com.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112844.

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