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Metformin enhances response to chemotherapy combined with immunotherapy in a triple negative breast cancer *in vivo* model

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ABSTRACT

Triple-negative breast cancer (TNBC) is a clinically heterogeneous disease that is predominantly treated with chemotherapy. Although immune checkpoint inhibitors targeting the PD-1/PD-L1 axis, such as atezolizumab (ATZ), have expanded therapeutic options, their clinical benefit remains limited, emphasizing the need for strategies that improve efficacy without increasing toxicity. Metformin (MTF), a widely prescribed antidiabetic drug, has demonstrated anticancer properties and synergistic effects with chemotherapy and immunotherapy in several tumor models. This study evaluated whether adding MTF to a chemo-immunotherapy regimen could enhance outcomes in an in vivo TNBC model. In vitro experiments were performed to determine the IC50 values of MTF and doxorubicin (DOXO) in EMT6 breast cancer cells and to assess their combined effects. In vivo studies were conducted using BALB/c mice bearing subcutaneous EMT6 tumors. Treatment was initiated when tumor volumes reached 50–120 mm^3 . DOXO and ATZ were administered intraperitoneally for two weeks, with DOXO given twice weekly and ATZ once weekly. ATZ was injected at least three hours after DOXO, and MTF was provided via drinking water. Tumor growth was monitored daily and compared among treatment groups. The addition of MTF enhanced the antitumor efficacy of low-dose DOXO (0.3 mg/kg) combined with ATZ, producing tumor-growth inhibition equivalent to that achieved with a 20-fold higher DOXO dose plus ATZ. After fourteen days, tumor volumes in the triplet therapy group were reduced compared with DOXO plus ATZ alone. Importantly, mice receiving the MTF-containing regimen maintained body weight, indicating no added toxicity. These findings support metformin as an adjunct to chemo-immunotherapy.

1. Introduction

Triple-negative breast cancer (TNBC) is a heterogeneous group comprising several well-defined molecular subtypes [1]. Classical chemotherapy based on anthracyclines and taxanes with or without an ICI is the standard of care for early TNBC [2].

Atezolizumab (ATZ) is an immune checkpoint inhibitor targeting PD-L1 and is an effective and well-tolerated treatment option for TNBC [3–5]. In fact, it has been approved for use in combination with paclitaxel for the treatment of PDL1 + metastatic TNBC [6,7].

Metformin (MTF) is a prescribed drug for type 2 diabetes with well-tolerated side-effects. It has been reported to have anticancer effects. Data from *in-vitro* and *in-vivo* studies confirmed the anticancer activity of MTF against several types of cancer, including TNBC [8,9]. In a previous work, we have also shown that doxorubicin (DOXO) or docetaxel (DOCE) combined with MTF produces a strong synergistic effect in three TNBC cell lines [10]. MTF has been used to target cancer stem cells in combination with doxorubicin in breast cancer mouse models, showing

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a synergistic effect between these two drugs [11]. This synergistic effect has also been shown with paclitaxel and carboplatin in combination with MTF [12].

In addition, MTF has immunomodulatory effects [13]. It has been shown that MTF treatment of tumor-bearing mice inhibits oxygen consumption by tumor cells with consequent reduction of intratumoral hypoxia and alleviating hypoxia-associated immunosuppressive effects [14]. Furthermore, MTF affects tumor-infiltrating lymphocytes (TILs) status increasing the cytotoxic activity of CD8 + T cells by reducing the stability and membrane localization of PD-L1 [15]. Moreover, MTF shifts macrophages from an immunosuppressive to an immune-stimulatory phenotype, leading to down-regulation of tumor-infiltrating, immunosuppressive Treg cells, and up-regulation of Tmem cells [16,17]. Several previous studies suggested the possible synergy between MTF and ATZ in other neoplasia [18,19].

Therefore, the aim of this study is to assess the antitumor effects of ATZ in combination with MTF and chemotherapy in TNBC syngeneic mouse models.

2. Methods

2.1. Cell culture and reagents

EMT6 cells were cultured in Waymouth's MB 752/1 Medium with 2 mM L-glutamine, 15% heat-inactivated fetal bovine serum, 100 mg/mL penicillin (Gibco) and 100 mg/mL penicillin-streptomycin (Gibco), at 37° C in a humidified atmosphere with 5% (v/v) CO_2. EMT6 cells were obtained from ATCC (August 2021). They 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 (MTF; Sigma Aldrich D150959) and Doxorubicin (DOXO; Sigma Aldrich 44583) were diluted in distilled water and DMSO 10%, respectively. The human PD-L1-mouse IgG1 antibody anti-hPD-L1-mIgG1 (Invivogen), containing a variable region equivalent to that of Atezolizumab, and the constant region of mouse IgG1, were used for *in vivo* experiments.

2.2. Cell viability assays

EMT6 cells were treated with each drug or drug combination at a range of concentrations to establish an IC50. Concentration points were selected for each drug and cell line based on the IC50 and dose-response curves published previously by our group [8,10]. Approximately 5000 cells per well were seeded in 96-well plates. Drugs alone or in combination were added to the cells after 24 h. Cell survival was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) after 72 h of drug exposure, following the manufacturer's instructions. Untreated cells were used as controls. Three independent triplicate experiments for each condition were performed.

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 (IC50) values and were calculated using the Chou-Talalay method [20]. Briefly, IC50 for each drug and drug combination was calculated. The synergistic or antagonistic effect of the drug combinations was defined using the combination index (CI) theorem of Chou-Talalay, defining additive effects (CI = 1), synergism (CI < 1) and antagonism (CI > 1). For drug combination experiments, the drugs were combined at a constant ratio at concentrations of 4IC50, 2IC50, IC50, ½ IC50, ¼ IC50 (21). The data were analyzed with CompuSyn software [21], and dose-response curves were constructed with GraphPad Prism 6.

2.4. In vivo experiments

All experiments were done following guidelines and approval from Comunidad de Madrid Animal Welfare Ethical Committee was obtained (PROEX 280.8/21).

Ten mice were studied for each condition. 1×10^6 EMT6 cells in 100 μl of PBS or saline were subcutaneously implanted in the back of immune-competent BALB/c mice. Treatment began when tumours reached a desired volume size of 50–120 mm^3 (measured with a digital calliper). All drugs were administered intraperitoneally in 100 μL of sterile saline during two weeks except for MTF, which was administered in the drinking water, at 1 mg/mL beginning in the same day of the first drugs treatments. DOXO was administered twice per week whereas Anti-hPD-L1-mIgG1 was administered once per week. At least 1 h before DOXO administration, mice were dosed with tramadol 5 mg/Kg. Anti-hPD-L1-mIgG1 was applied intraperitoneally on the contralateral flank to which the chemo has been administered at least three hours after DOXO administration. The primary outcome was tumour response. Tumour size was measured using a digital calliper every day until mice are euthanized. Tumour size at the beginning of the treatment and just before euthanasia was compared. For tumour volume determination (mm^3), three measures were taken (x, y, z).

2.5. Statistical analysis

Comparisons between quantitative variables were done applying a non-parametric Kruskall-Wallis test. P-values are two sided and considered statistically significant under 0.05.

3. Results

3.1. Cell culture experiments

In vitro EMT6 IC50 concentrations were defined for DOXO and MTF individually, being 3.5 μM, and 12 mM respectively (Fig. 1A and B). Then, the combination of metformin with doxorubicin was tested. An antagonistic effect between MTF and DOXO was observed in this cell line (Fig. 1C).

3.2. In Vivo Experiments

We tested the effect of metformin when added to a chemotherapy-ICI combination, including a mouse-adapted anti-PD-L1 equivalent to Atezolizumab (mATZ), using a syngeneic mouse model harbouring an EMT6-derived tumor. Daily increase tumor volume, as indicator of treatment efficacy, and mice weight minus tumor weight, as a welfare indicator, for each treatment scheme were studied.

First, we tested the effect of adding MTF to a combination of DOXO+mATZ. MTF and mATZ were maintained at a constant dose in all the experiments whereas the combination DOXO plus mATZ was tested at two different doses of DOXO, 0.3 mg/kg and 6 mg/kg, which is the equivalent of the dose of DOXO that a human would receive. Follow-up in mice in the DOXO(0.3 mg/Kg)+m-ATZ and DOXO(6 mg/Kg)+m-ATZ arms was interrupted in two weeks due to extended tumour growth and quality of live criteria, respectively. Mice in the DOXO (0.3 mg/Kg)+m-ATZ+MTF arm were followed for three weeks.

Variations in tumor volume between the triplet DOXO (0.3 mg/kg) plus mATZ plus MTF, only mATZ plus MTF, DOXO (0.3 mg/kg) plus MTF and finally, DOXO (0.3 mg/kg and 6 mg/kg) plus mATZ were compared. Our results shown that MTF increases the efficacy of DOXO (0.3 mg/KG)+mATZ combination and this increased efficacy is equivalent to that of DOXO of 6 mg/kg plus mATZ (Fig. 2A). Moreover, the differences in tumor volume between DOXO (0.3 mg/kg) plus mATZ plus MTF and DOXO (0.3MG/KG) plus mATZ are statistically significant after fourteen days of treatment. Mean increase in tumor volume was 205, 1096 and 365 mm^3 in the DOXO(6 mg/Kg)+m-ATZ, DOXO

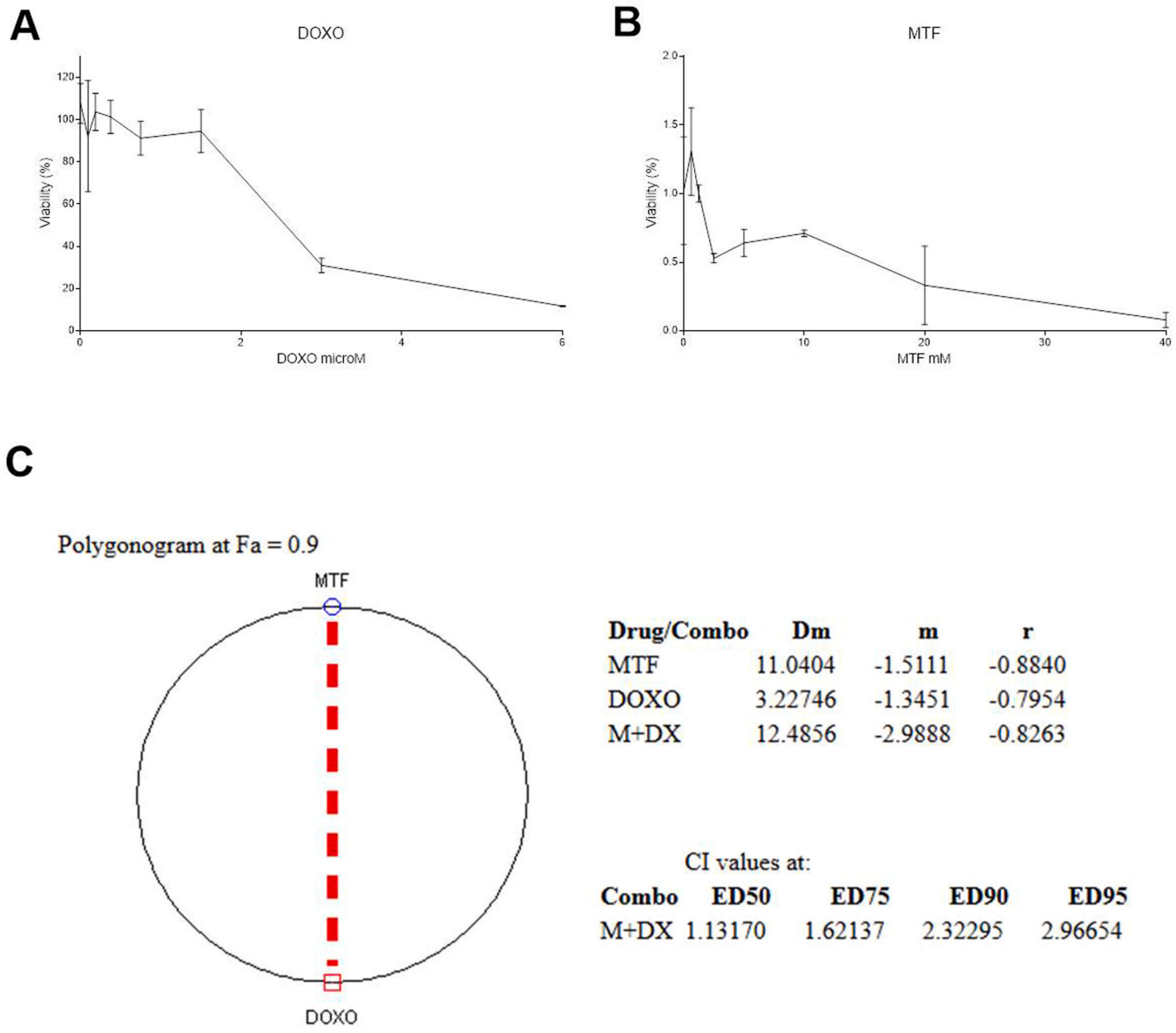

Fig. 1. EMT6 in vitro experiments. A. Dose-response curve of EMT6 treated with DOXO (0-6 μM). B. Dose-response curve of EMT6 treated with MTF (0-40mM). C. Polygonogram evaluating the equipotent combinations of the tested drugs. Green and solid line indicates synergy and red broken line indicates antagonism. The degree of boldness represents the degrees of synergism or antagonism. At the bottom table, Dm is the median-effect dose (in this case the IC50); m, the shape of the dose-response curve; and r, the measurement of the goodness of fit. The combination index (CI) is a quantitative measurement of the degree of drug interactions in terms of synergy or antagonism, being a CI>1 considered as antagonism, CI=1 additive effect, and CI<1 synergism.

(0.3 mg/Kg)+m-ATZ, and DOXO(0.3 mg/Kg)+m-ATZ+MTF arms respectively (Fig. 2C).

Additionally, the response observed to the DOXO (0.3 mg/kg)+mATZ+MTF combination is not only equivalent to when higher doses of DOXO (6 mg/kg) plus mATZ are administrated, but also has less impact in mice welfare, as shown by the maintenance in the mice weight with this treatment scheme (Fig. 2B). These differences in mice weight between the two treatment schemes are statistically significant after fourteen days of treatment. Mean weight at the beginning of the treatments was 19.8 gr for the complete mice population, whereas mean weight after 14 days of treatment was 15.4, 20.9 and 21.7 gr in the DOXO(6 mg/Kg)+m-ATZ, DOXO(0.3 mg/Kg)+m-ATZ, and DOXO (0.3 mg/Kg)+m-ATZ+MTF arms respectively, (Fig. 2D).

In addition, the response to DOXO (0.3 mg/KG)-mATZ was equivalent to mATZ-MTF response (Fig. 2A, 2C).

Finally, the efficacy of DOXO (0.3 mg/kg) plus mATZ plus MTF was compared to the efficacy of administering DOXO alone at a range of concentrations. Higher concentrations of DOXO cause a decrease in tumor volume. However, the triplet, with a dose of DOXO of 0.3 mg/kg, was still more effective than DOXO alone at a concentration of 2.4 mg/kg (Fig. 3).

4. Discussion

In this study, the antitumor effects of ATZ in combination with MTF and chemotherapy in TNBC syngeneic mouse models were characterized, showing that adding MTF to a combination of ATZ and DOXO increased therapy efficacy, with no added side effects. We used EMT6 cell line to growth tumors in syngeneic mouse models because it is well-known that EMT6 express PD-L1, making this cell line an useful model to study immunotherapies.

First, we tested the response of DOXO combined with MTF in EMT6 cells in vitro. Unlike the results obtained in our previous work [10], where a synergy between MTF and DOXO in some TNBC human cell

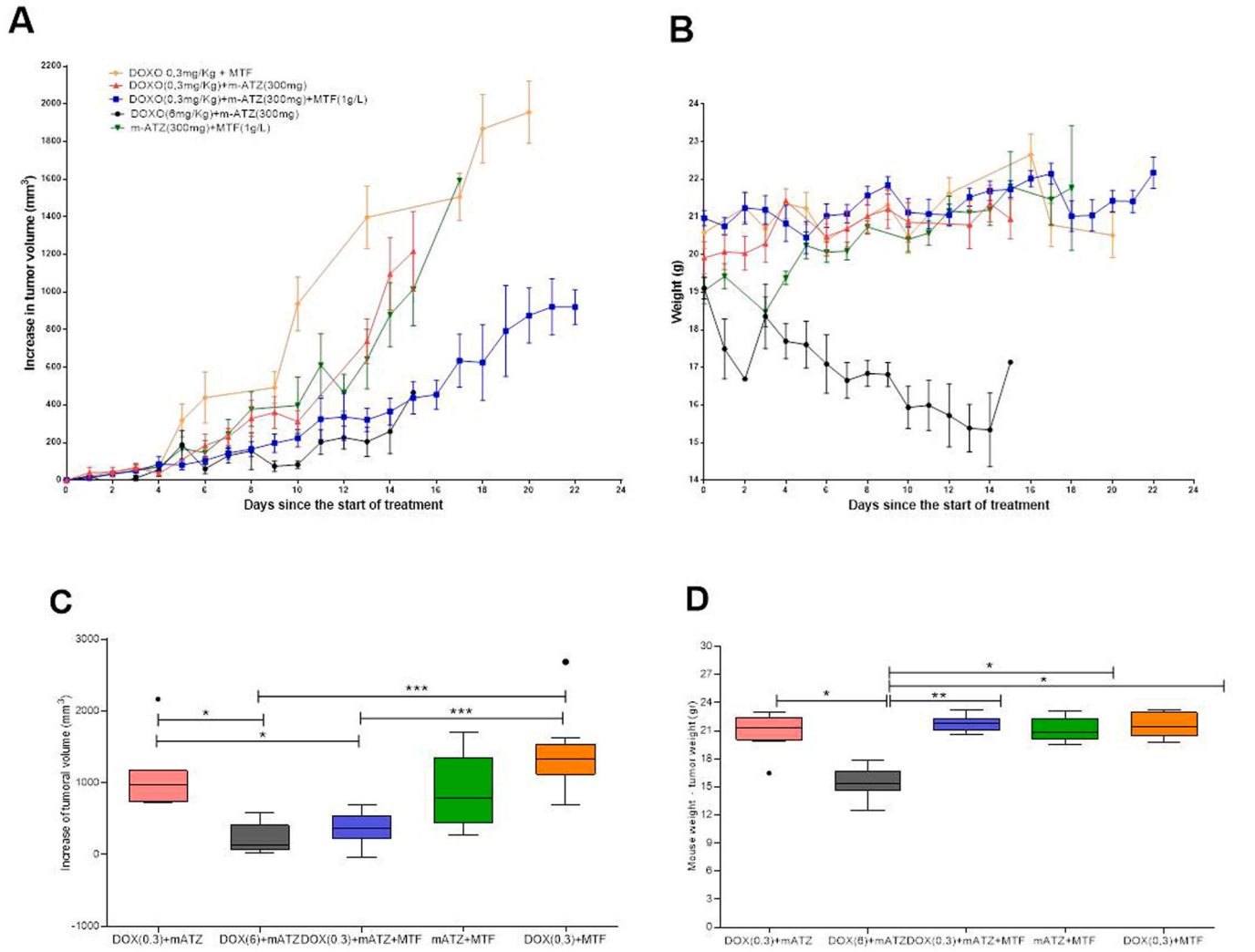

Fig. 2. EMT6 syngeneic mouse model assays. A) Daily increase of tumor volume after first treatment. B) Daily mice weight minus tumor weight after first treatment. C) Increase of tumor volume after 14 days of treatment; *p<0.05, **p<0.01, ***p<0.005. D) Mice weight minus tumor weight after 14 days of treatment; *p<0.05, **p<0.01. Each arm includes measurements from 10 mice.

lines was observed, EMT6 mouse cells showed an antagonistic effect between DOXO and MTF. A synergistic effect between DOXO and MTF has been also observed in a xenograft model derived from MCF10A ER-Scr cell line [11].

EMT6 injected in BALB/c mice is a widely used model for the study of immunotherapy and immunity in breast cancer [22–24]. EMT6 cell line was injected into immunocompetent BALB/c mice, with the aim of study the efficacy of DOXO, mATZ and MTF. Syngeneic mouse models as this one not only enables the investigation and development of immunotherapies, but also are useful to study the efficacy of other drugs due to the higher invasiveness of murine tumor cells and with metastasis showing similar characteristics to breast cancer patients [25,26].

The boosted effect of MTF in combination with different immunotherapies has been previously assessed using in vitro and in vivo models with promising results [27–29]. In this study, we characterized the efficacy of adding MTF to mATZ and DOXO in a syngeneic breast cancer murine model.

Remarkably, MTF increased the efficacy of the combination of ATZ plus DOXO at 0.3 mg/kg. Tumor reduction was equivalent to that obtained with DOXO 6 mg/kg plus ATZ. This data suggests that, although no synergy between DOXO and MTF has been observed in vitro, an improvement in efficacy occurs when combining mATZ with DOXO and MTF in vivo. This improvement in efficacy may be partially due to the immunomodulatory effects of MTF [13]. MTF has been reported to increase the cytotoxic activity of CD8 + T cells [15], which are essential for the benefit of immune checkpoint inhibitors [30]. Recently, Amato et al. observed significant decreased tumor cell viability with the combination of MTF and atezolizumab or pembrolizumab in co-cultures of non-small cell lung carcinoma cell lines with peripheral blood mononuclear cells from non-small cell lung carcinoma patients, which was superior than the decrease in viability observed in pembrolizumab or atezolizumab alone [18]. The proposed mechanism involve in this synergy effect is double. On the one hand, metformin has effects in metabolism [8]. TNBC is characterized by an elevated Warburg effect [31], metformin may reverse this Warburg effect and favours mitochondrial metabolism. Chemotherapy targets mainly proliferation, which is another characteristic of TNBC. Therefore, the existence of a synergy between these two drugs is reasonable. Regarding the immunomodulatory effects, it is described that MTF modifies PD-L1 expression [15]. This could be translated into a decrease of the immune evasion abilities of the tumor, favouring T cell infiltration and in consequence response to anti-PDL1 and anti—PD1 therapies (Fig. 4).

The combination of DOXO plus MTF did not shown a better efficacy than DOXO plus ATZ, as expected by the in vitro results showing that synergy does not exist between DOXO and MTF. However, ATZ plus MTF has the same effect than DOXO plus ATZ, supporting the hypothesis that

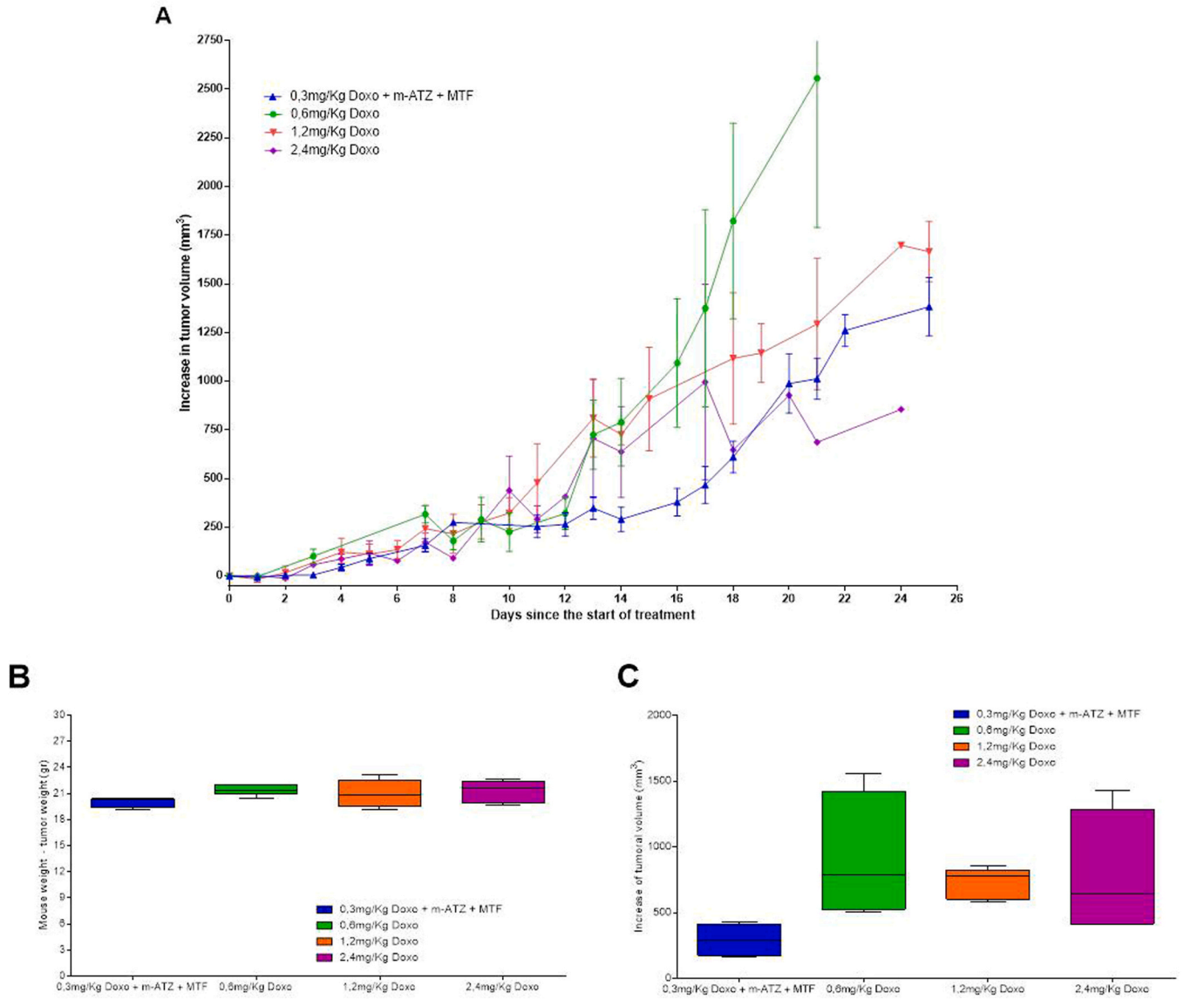

Fig. 3. EMT6 syngeneic mouse model assays during treatment with DOXO plus ATZ plus MTF A) Daily increase of tumor volume at different doses of DOXO and the triplet DOXO plus ATZ plus MTF. B) Increase of tumor volume after 14 days of treatment at different doses of DOXO and the triplet DOXO plus ATZ plus MTF. C) Mouse weight-tumor weight after 14 days of treatment at different doses of DOXO and the triplet DOXO plus ATZ plus MTF. *p<0.05, **p<0.01, ***p<0.005. D) Mice weight minus tumor weight after 14 days of treatment; *p<0.05, **p<0.01. Each arm includes measurements from 5 mice.

mATZ has a main role in the improvement in efficacy showed by the triplet DOXO + mATZ + MTF.

In addition, the combination of ATZ plus DOXO at 0.3 mg/kg plus MTF has the same effect in tumor volume reduction as DOXO 6 mg/kg plus ATZ, but without the significant reduction of weight in mice, indicating an improvement in animal welfare. This may translate into a reduced toxicity in humans. Adverse reactions are frequent in patients after DOXO administration, including fatigue, alopecia, nausea and vomiting, oral sores, tissue ulceration and necrosis, and significant cardiac toxicity [32]. The most severe adverse effect of MTF is lactic acidosis [33], which can be easily corrected.

Finally, higher doses of DOXO had an increased effect in tumor volume, proving that DOXO is effective in the model we used. However, the combination of ATZ and DOXO at a concentration of 0.3 mg/kg with MTF has better efficacy than DOXO 2.4 mg/kg. This strongly suggests that the dose of chemotherapy can be reduced by adding MTF and ATZ.

The study has some limitations. A larger study using other TNBC cells would be of great interest. In addition, the use of humanized mice models may shed light on the mechanistic effects involved in MTF, ATZ and DOXO synergy and how this synergy is affected by TNBC molecular heterogeneity. In line with this, future studies of immune infiltration or cytokine profiling may be useful.

5. Conclusions

Altogether, these results suggest a synergistic effect between DOXO, MTF and ATZ, mostly based on the benefit of adding MTF to ATZ. This may allow reducing the dose of DOXO, with the subsequent reduction of adverse effects.

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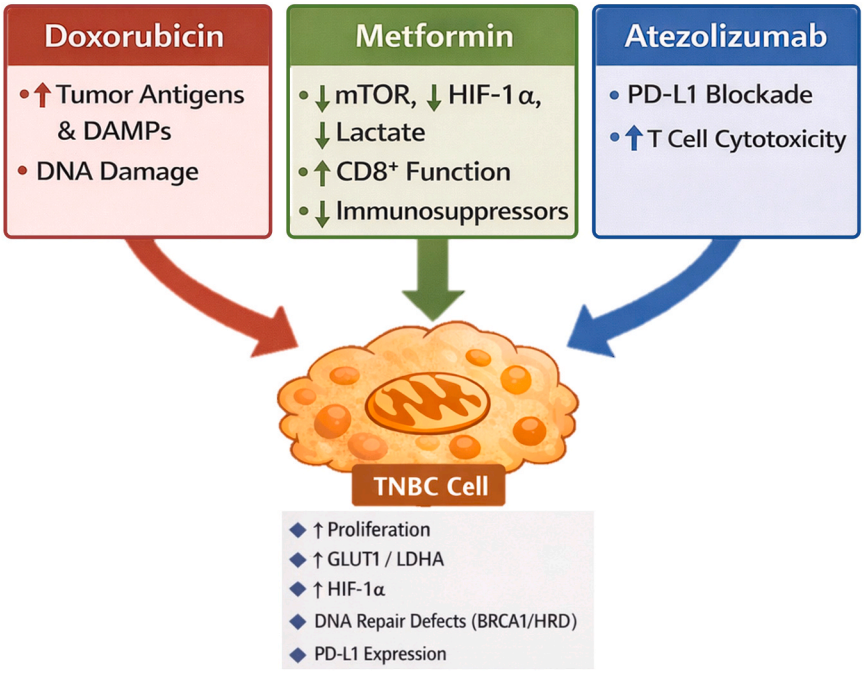

Fig. 4. Metformin enhances the efficacy of doxorubicin and atezolizumab in TNBC. Doxorubicin induces DNA damage and immunogenic cell death, increasing tumor antigen release. Atezolizumab blocks PD-L1–mediated immune inhibition, restoring $CD8^+$ T-cell cytotoxicity. Metformin suppresses mTOR/HIF-1α signaling, reduces lactate production and tumor-associated immunosuppression, and improves $CD8^+$ T-cell metabolic fitness. By reprogramming the tumor microenvironment, metformin amplifies chemotherapy-induced immunogenicity and potentiates immune checkpoint blockade, resulting in enhanced antitumor activity.

CRediT authorship contribution statement

Elena López-Camacho: Methodology. **Fernando Becerril-Gómez:** Methodology. **David Alonso-Martín:** Methodology. **Rocío López-Vacas:** Methodology. **Lucía Trilla-Fuertes:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Enrique Espinosa:** Writing – review & editing, Conceptualization. **Angelo Gámez-Pozo:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Luz Vega-Clemente:** Methodology. **Pedro Lalanda-Delgado:** Methodology. **Pilar Zamora:** Writing – review & editing, Conceptualization. **Mariano García-Arranz:** Investigation, Conceptualization. **Juan Ángel Fresno Vara:** Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Data availability

No data was used for the research described in the article.

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