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Original Article

Tumor Treating Fields enhance chemotherapy efficacy by increasing cellular drug uptake and retention in mesothelioma cells

Rosy Amodeo[1,2], Lavinia Morosi[1], Marina Meroni[3], Ezia Bello[3], Sara Timo[4], Roberta Frapolli[3], Maurizio D'Incalci[1,2*], Monica Lupi[1*]

[1]Laboratory of Cancer Pharmacology, IRCCS Humanitas Research Hospital, Rozzano, Milano, Italy; [2]Department of Biomedical Sciences, Humanitas University, Pieve Emanuele, Milano, Italy; [3]Laboratory of Cancer Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano, Italy; [4]Metabolomics and Pharmacokinetics Unit, IRCCS Humanitas Research Hospital, Rozzano, Milano, Italy. [*]Equal contributors.

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Abstract: Tumor Treating Fields (TTFields) applied with standard chemotherapy have been approved for the first-line treatment of unresectable pleural mesothelioma (PM), an aggressive malignancy with limited effective therapy options. In this study, we demonstrated that the simultaneous exposure to TTFields and doxorubicin or vinorelbine enhanced treatment efficacy in patient-derived PM cells by increasing intracellular drug concentrations. This was achieved by modulating several genes that encode transport proteins, such as the downregulation of P-glycoprotein (P-gp). Using specific, sensitive and quantitative analytical techniques, we observed a more than 70% increase in intracellular concentrations of doxorubicin and vinorelbine in samples treated with TTFields, and a greater than 50% increase in drug uptake in cells exposed to TTFields and pemetrexed. This result indicates that the increased drug concentration observed in TTFields treated cells is significant not only for drugs that are P-gp substrates but also suggests that TTFields could potentially affect other efflux pumps. However, the co-exposure to the drug and TTFields was critical to increasing intracellular drug levels, highlighting the necessity of concurrent use with drugs to enhance the antiproliferative effects of treatment. The *in vitro* findings were further corroborated by *in vivo* pharmacokinetic experiments in mice subcutaneously injected with epithelioid PM tumors. Indeed, a 30% increase in intratumor concentrations was observed when vinorelbine was administered with TTFields. Our findings suggest that TTFields could be a well-tolerated approach for enhancing intratumoral drug levels and potentially achieving a more significant therapeutic impact on PM treatment.

Keywords: Pleural mesothelioma, Tumor Treating Fields (TTFields), cellular drug uptake, pharmacokinetics, combination treatment, cancer therapy

Introduction

Pleural mesothelioma (PM) is a rare tumor, with asbestos exposure being the main cause [1]. Its prognosis is very poor, and the use of surgery for treatment is still controversial [2]. Therefore, chemotherapy and more recently immunotherapy [3, 4] are the main treatment options. However, overall survival of PM patients is still limited, and there are no approved therapies once the tumors progress [5].

Given these circumstances, locoregional treatment with Tumor Treating Fields (TTFields), low-intensity intermediate-frequency electric fields, was approved by the FDA as a valuable therapeutic alternative for unresectable, locally advanced PM when applied with standard chemotherapy [6].

TTFields disrupt the formation of the mitotic spindle, inducing mitotic arrest or aberrant mitosis that lead to cell death [7, 8], but recently, a complex scenario of many other effects induced by this treatment has been described [9-11], such as increased cell membrane permeability and enhanced intracellular concentration of membrane-penetrating drugs [12]. In clinical practice, TTFields therapy is always

used concomitantly with standard systemic therapy, improving patient overall survival without eliciting severe toxicity additional to that expected by chemotherapy alone [6, 13-16]. The clinical data are supported by preclinical evidence suggesting that the simultaneous administration of TTFields with some anticancer drugs has a synergistic cytotoxic effect [17, 18]. However, only some of the mechanisms underpinning this effect have been investigated so far [10, 17, 19].

Recent transcriptomic studies on epithelioid CD473 and sarcomatoid CD60 patient-derived PM cell lines showed that TTFields modulate a significant number of genes, including some involved in xenobiotic transport in cancer cells [20]. One of the genes downregulated by TTFields is *ATP binding cassette subfamily B member 1* (*ABCB1*), which encodes P-glycoprotein (P-gp), known to be responsible for low intracellular drug retention, leading to cellular resistance to many anticancer drugs. These data suggest that one possible explanation for the synergism of TTFields with anticancer drugs may be attributed to the enhanced drug retention by cancer cells exposed to treatment.

This study aimed to test the hypothesis that TTFields affect the uptake and retention of anticancer agents with different structures and modes of action, and to investigate the biological mechanisms underlying these effects.

Materials and methods

Cell lines

PM cell lines CD473 and CD60, representative respectively of the epithelioid and sarcomatoid PM histotypes, were isolated from pleural effusions and/or lavage of patients' thoracic cavity, before administration of therapeutic treatments with the collaboration of the SS. Antonio e Biagio e Cesare Arrigo Hospital in Alessandria (Italy) [21]. All the cell lines were cultured in HAM's F10 medium (Euroclone, Milan, Italy) supplemented with 10% Fetal Bovine Serum (Euroclone) and 2 mM L-Glutammine (Lonza, Basel, Swiss) and periodically tested for mycoplasma contamination by PCR testing.

Dose-response experiments

Cells were seeded at about 40,000 cells/well in 12-well plates or on Thermanox coverslips (Nunc #174977 - Thermo Fisher Scientific, Waltham, Massachusetts, USA) and put inside the Inovitro™ ceramic dishes (Novocure Ltd., Haifa, Israel) as previously described [22]. After 72 h, when in exponential growth, culture medium was replaced by different drug concentrations (100, 30, 10, 3 or 1 nM doxorubicin (DOX) - TargetMol, Wellesley Hills, Massachusetts, USA; 1000, 300, 100, 30, or 10 nM vinorelbine (VNR) - Sigma Aldrich, St. Louis, Missouri, USA). After 72 h of incubation with the drug alone or drug and TTFields (150 kHz frequency; 0.76 V/cm intensity for epithelioid CD473 cells or 1.12 V/cm intensity for sarcomatoid CD60 cells) cells were detached and counted by LUNAII cell counter (Logos Biosystems, Anyang-si, South Korea). Cell survival was calculated as percentage of control samples, and dose-response curves were obtained by fitting experimental data with Hill function. The additive effect was calculated by multiplying the effects observed for the individual treatment in accordance with Bliss independence principle [23], which was also used to determine the interaction index. This parameter allowed the quantification of the magnitude of TTFields-drug interaction at different levels of treatment efficacy. Specifically, the predicted mortality for an additive effect between TTFields and drug was divided by the measured mortality for the co-treatment [24]. The treatment was considered additive when the 95% confidence interval (CI) included 1, synergistic when the 95% CI was less than 1, and antagonistic when the 95% CI was greater than 1.

Western blot analysis

After different times of exposure to TTFields, cells were lysed in RIPA Lysis buffer (Sigma Aldrich) supplemented with phosphatase and protease inhibitor tablets (PhosSTOP™ and cOmplete™, EDTA-free Protease Inhibitor Cocktail - Roche, Basel, Swiss) and prepared as previously described [20]. The primary antibodies used were P-gp (MA5-13854, RRID, AB_10979045; diluted 1:500 - Thermo Fisher Scientific), actin (C-2) (#sc-8432, RRID, AB_626630; diluted 1:1000 - Santa Cruz Biotechnology, Dallas, Texas, USA) and GAPDH (6C5) (#sc-32233, RRID, AB_627679; diluted 1:1000 - Santa Cruz Biotechnology) followed by incubation with an anti-mouse secondary antibody horseradish peroxidase (HRP)-conjugated

(RRID, AB_11125547; diluted 1:2500 - Bio-Rad, Hercules, California, USA). Blotted membranes were incubated with Clarity Western ECL substrate or Clarity Max Western ECL substrate (Bio-Rad) and signal detected with ChemiDoc MP imaging system (Bio-Rad). Densitometric analysis of the obtained bands was done with ImageJ software (https,//imagej.nih.gov/ij/). Normalized signals to GAPDH were expressed as fold change relative to control.

Immunofluorescence

Immunofluorescence analysis of DOX internalization was performed in epithelioid CD473 cells treated for 24 h with 1 μM DOX alone or applied with TTFields.

Actin depolymerization was monitored in both cell lines after 24 h and 48 h of TTFields exposure. Depolymerization of actin in sarcomatoid CD60 cells was induced by treating the cells with 500 nM of latrunculin B (Sigma Aldrich) for 2 h.

At the end of treatment cells were fixed with 4% paraformaldehyde for 10 min, washed twice with PBS and permeabilized with 0.1% TritonX-100 in PBS (Sigma Aldrich) for 4 min; after two washes with PBS cells were blocked with 1% Bovine Serum Albumin (Sigma Aldrich) in PBS for 30 minutes. After blocking, cells were incubated for 20 min with Alexa Fluor 488-Phalloidin (BK 8878; dilution 1:40 - Cell Signaling Technology, Danvers, Massachusetts, USA) and then washed three times with PBS, dried and mounted in Fluoroshield mounting medium (F6182 - Sigma Aldrich) or Fluoroshield mounting medium with DAPI (ab104139 - Abcam, Cambridge, UK). Cells were analyzed by confocal microscopy (TCS SP8 SMD microscope - Leica, Nussloch, Germany) using 488 nm excitation and 520 nm emission for phalloidin-Alexa488 and 530 nm emission for DOX. Several fields of view were collected for each experimental condition (ranging from seven to nine) using an oil immersion 63× objective. The instrument setting was consistent during sample acquisition.

Quantification of cellular drug uptake and efflux

For drug uptake analysis, cells in exponential growth were treated for 24 h with 1 μM DOX, 1 μM VNR or 100 μM pemetrexed (PEM) with or without simultaneous exposure to TTFields (150 kHz frequency; 0.76 V/cm intensity for epithelioid CD473 cells or 1.12 V/cm intensity for sarcomatoid CD60 cells), unless differently detailed in the figure legends. After treatment, the cells were detached with cold reagents and cell pellets were maintained at -20°C.

For drug efflux analysis, cells were treated for 1 h with 10 μM DOX (with or without previous 24-h exposure to TTFields at the same conditions used for drug uptake experiments) or for 24 h with 100 μM PEM with or without simultaneous exposure to TTFields. At the end of treatment cells were washed with cold cell culture medium and maintained at 37°C in pre-warmed medium for different time points. At each time point the cells were trypsinized and the suspensions were maintained at -20°C.

Intracellular DOX and VNR were quantified by a High-performance liquid chromatography (HPLC) method with fluorimetric detection (Vanquish™ Core HPLC systems - Thermo Fisher Scientific). Cell pellets treated with DOX were resuspended in 50 μL of distilled H_2O and then added with daunorubicin, as internal standard. Extraction was performed adding chloroform: isopropanol (1:1), after centrifugation at 15000 g, the organic phase was evaporated to dryness under N_2 flux. Extracts were injected into the HPLC with fluorescence detection at an excitation wavelength of 475 nm and an emission of 550 nm. Chromatographic separation was done under isocratic conditions with a mobile phase composed of water, acetonitrile, 0.1 M phosphoric acid on an Acclaim C18 5 mm 4.6×150 mm column (Thermo Fisher Scientific).

Cells pellets treated with VNR were added with vinblastine as internal standard and then with acetonitrile plus formic acid to precipitate protein. After centrifugation at 15000 g, the supernatant was evaporated to dryness under N_2 flux. Extracts were injected into the HPLC with fluorescence detection at an excitation wavelength of 280 nm and an emission of 360 nm. Chromatographic separation was performed on a Hypersil Gold CN 5 μm 4.6×150 mm column, using CH_3COONH_4 0.02 M pH 3 (mobile phase A) and CH_3CN (mobile phase B) under gradient conditions as follows, 30-80% B (0-5 min), 80%

B (5-7 min), 80-30% B (7-9 min), 30% B (9-12 min).

Intracellular PEM was quantified by LC-MS/MS detection on a TSQ Altis™ Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific) operating in positive ion mode monitoring the transitions m/z 428.45>163.05 (quantitation) and m/z 428.45>281.19 (confirmation) for PEM and m/z 433.50>163.12 (quantitation) and m/z 433.50>281.12 (confirmation) for deuterated PEM as internal standard. Cell pellets treated with PEM were added with deuterated PEM as internal standard and then with ice cold methanol to precipitate protein. After centrifugation at 15000 g, the supernatant was evaporated to dryness under N_2 flux. Chromatographic separation was achieved on a Gemini-C18 column (50 mm×2.0 mm, 5 µm particle size - Phenomenex Inc., Torrance, California, USA) at 40°C under isocratic conditions with water:acetonitrile = 85:15 plus formic acid 0.1%.

Amimals

Procedures involving animals and their care were conducted in conformity with the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate - UNI EN ISO 9001,2008 - Reg. N° 86121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The experimental protocol has been reviewed and approved by the Istituto di Ricerche Farmacologiche Mario Negri Ethical committee and approved by the Italian Ministry of Health (Aut.Min n°489/2016-PR).

Seven-week-old, female, athymic nude mice were engrafted with tumor fragments coming from CD473 cells. The growing tumor masses were monitored at least twice a week with a Vernier Caliper and the tumor volume calculated as $d^2 \times D/2$ were d and D were the width and the length of the tumor, respectively.

Pharmacokinetics

When mean tumor volume reached 300-400 mm^3, mice were randomized into three treatment groups (drug alone, drug and sham arrays, drug and TTFields). After 72 h of exposure to TTFields (or sham) (Inovivo™ - Novocure Ltd.), mice were treated i.v. with 10 mg/kg of VNR, and four animals per group were euthanized at 1, 6 and 24 h after drug treatment. At sacrifice, plasma, tumor and liver were collected and stored at -80°C for mass spectrometry quantifications of drug.

Ten microliters of 500 ng/mL vinblastine were added as internal standard to 100 µL of plasma, tumor or liver homogenate 1:10 (w/v) in ammonium formiate 5 mM+HCOOH 0.1%. The samples were vortexed and added with 500 µL of CH_3CN+HCOOH 0.1% to precipitate proteins. Samples were then centrifuged at 15000 g for 10 min at 4°C. The supernatants were transferred into clean tube and then evaporated under gentle N_2 flux. The residues were reconstituted in 200 µL of ammonium formiate 5 mM+HCOOH 0.1%:CH3CN+HCOOH 0.1% = 9:1, vortexed and centrifuged at 15000 g for 10 minutes at 4°C. The supernatants were transferred into an autosampler glass vial and 5 µL were injected into HPLC-MS/MS system. The calibration curve was built with a nude mice blank plasma or tissue homogenate in the range 5-1000 ng/mL. VNR was quantified by LC-MS/MS detection on a TSQ Altis™ Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific) operating in positive ion mode monitoring the transitions m/z 779.40>323.21 (quantitation) and m/z 779.40>658.37 (confirmation) for VNR and m/z 811.45>522.32 (quantitation) and m/z 811.45>751.45 (confirmation) for vinblastine as internal standard. Chromatographic separation was achieved on a Kinetex EVO C18 column (2.6 µm, 110 A, 2.1×100 mm - Phenomenex Inc.) at 30°C under isocratic conditions with water:acetonitrile = 85:15 plus formic acid 0.1%.

Statistical analysis

All experimental data were displayed as the mean ± standard error, unless differently claimed in the figure legend. The number of replicates considered for each experiment was reported in the respective figure legend. Un-

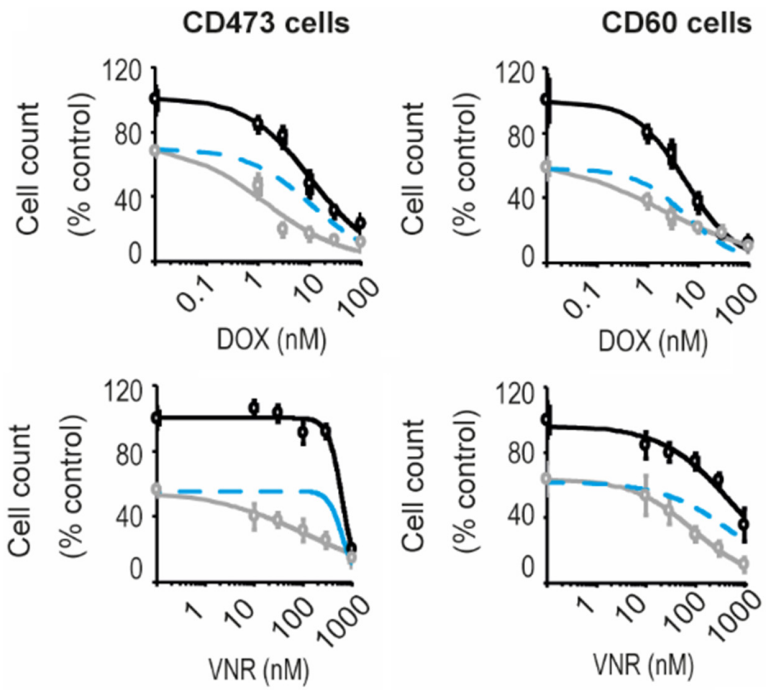

Figure 1. Efficacy of co-treatments. Dose-response curves for DOX and VNR, either alone (black line and symbols) or with TTFields (grey line and symbols), in epithelioid CD473 and sarcomatoid CD60 cells after 72 h exposure. Each symbol represents the mean of four replicates ± standard error. Data were calculated as percentage of controls. The co-treatment was considered as synergistic when its effect value/trendline was below that of the predicted additive effect (dashed light blue line).

paired two-tailed Student's t test was used to compare the differences between two groups of data and statistical significance was set at $P<0.05$. A one-way ANOVA test was used to compare the overall differences among the experimental groups, with statistical significance set at $P<0.05$.

Raw data are available in the open repository Zenodo in the "IRCCS Humanitas Research Hospital & Humanitas University" community (DOI: 10.5281/zenodo.13373988).

Results

Antiproliferative effects of TTFields applied with anticancer drugs

Previous data, obtained by exposing PM cell lines derived from pleural effusion and/or lavage of patients' thoracic cavity to TTFields, have shown that the epithelioid CD473 and the sarcomatoid CD60 cells have different sensitivity to treatment, with epithelioid cells being the most sensitive. RNA-Seq analysis of both cell types revealed that exposure to TTFields resulted in differential expression of several genes encoding transport proteins. Among the genes significantly downregulated in CD473 cells after 24 h of exposure, there was *ABCB1*, which encodes P-glycoprotein (P-gp) [20].

Based on this finding and the fact that TTFields are used in clinical trials with standard chemotherapy, we assessed the antiproliferative effect of 72-h co-treatments with TTFields and doxorubicin (DOX) or vinorelbine (VNR), both of which are P-gp substrates [25, 26]. VNR, in combination with gemcitabine, is used in second-line chemotherapy of PM [27], while DOX was chosen in this study because of its fluorescent properties, which facilitate monitoring of cellular internalization.

Cells were exposed to equi-effective TTFields conditions (150 kHz frequency and 0.76 V/cm intensity for CD473; 1.12 V/cm for CD60), and dose-response curves at different drug concentrations were obtained by counting cells at the end of treatment. **Figure 1** shows dose-response curves of co-treatments compared to the drug alone, along with the calculated additive effects, while **Table 1** presents the interaction indexes, with those indicating synergistic treatments highlighted in bold. These analyses confirmed a synergistic interaction between TTFields and VNR in both cell lines, whereas treatment with DOX was synergistic in CD473 cells and additive in CD60 cells, as the 95% CI overlaps with 1 in the sarcomatoid PM.

Effect of TTFields on cellular drug uptake

The intracellular drug concentration during or after TTFields exposure was studied by high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS/MS) analyses to determine if the enhanced

Table 1. Interaction index calculated for co-treatments with TTFields and various DOX or VNR concentrations

DOX concentrations	Interaction index [mean (95% CI)]	
	Epithelioid cells CD473	Sarcomatoid cells CD60
3 nM	**0.59** (0.53-0.65)	0.87 (0.64-1.09)
10 nM	**0.68** (0.72-0.91)	1.00 (0.95-1.06)
30 nM	**0.91** (0.87-0.95)	1.10 (0.96-1.25)
VNR concentrations	Interaction index [mean (95% CI)]	
	Epithelioid cells CD473	Sarcomatoid cells CD60
10 nM	**0.70** (0.46-0.95)	1.25 (0.15-2.35)
100 nM	**0.74** (0.51-0.96)	**0.75** (0.64-0.86)
300 nM	**0.67** (0.53-0.79)	**0.76** (0.64-0.89)

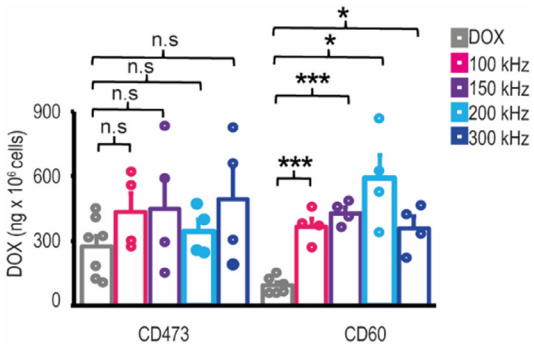

Figure 2. Impact of TTFields frequencies on cellular drug uptake. Quantification of intracellular DOX concentration obtained by HPLC analysis after 24 h of treatment with 1 μM DOX, or 24 h of simultaneous exposure to 1 μM DOX and TTFields at different frequencies (100, 150, 200 or 300 kHz). Each column represents the mean of at least four replicates ± standard error (Student's t test *P<0.05; ***P<0.005; n.s: not significant). None of the observed differences among the cells treated with different TTFields frequencies was statistically significant (Student's t test P>0.05. One-way ANOVA test P>0.05).

efficacy seen in co-treated PM cells was consistent with higher intracellular drug concentrations and downregulation of P-gp in TTFields treated cells.

In order to avoid misinterpretation caused by cell death and consequent cell membrane disruption, cellular drug uptake assessment was performed at times of treatment shorter than those used in the efficacy experiments described above.

Preliminary experiments were performed to compare intracellular drug uptake after 24 h of exposure to 1 μM DOX and TTFields at different frequencies (100, 150, 200 or 300 kHz) (**Figure 2**). Since we did not observe any significant difference between the frequencies, we decided to treat the cells with TTFields at 150 kHz, the frequency used in clinical PM treatment. Therefore, cells were incubated for 24 h with 1 μM DOX or VNR following two different schedules of treatment involving TTFields (**Figure 3A**).

In both cell lines, intracellular drug concentrations were higher when the samples were exposed to TTFields compared to those treated with the drug alone. Specifically, TTFields exposure increased intracellular DOX concentration by approximately 80% in CD60 cells and 70% in CD473 cells, while for VNR treated cells the increase was around 190% and 140%, respectively. Confocal microscopy of CD473 cells qualitatively confirmed that TTFields increased cellular drug uptake, as indicated by higher DOX fluorescence signals in cells treated with both agents. More importantly, these observations allowed us to appreciate that TTFields did not alter the cellular distribution of DOX, which remained mainly nuclear in all treated cells (**Figure 3B**).

Further reducing the incubation time did not alter the results, in fact, for the epithelioid subtype, a 2-h treatment with DOX and TTFields was sufficient to achieve a 50% increase in intracellular drug concentration. Whereas, a longer treatment duration of at least 4 h was required for CD60 cells to obtain results comparable to those observed in the more sensitive CD473 cells (**Figure 3C**).

Simultaneous co-exposure to drug and TTFields was necessary for the observed effects on intracellular drug levels. Cells exposed sequentially to drug and TTFields, with or without 24-h interval in between treatments, only showed a small, non-significant increase in intracellular DOX concentration (**Figure 3D**). These findings suggest that some of the effects induced by TTFields that led to increased intracellular drug concentration were transient.

Effect of TTFields on P-glycoprotein modulation of drug efflux

In light of the fact that intracellular drug levels result from a balance between drug uptake and

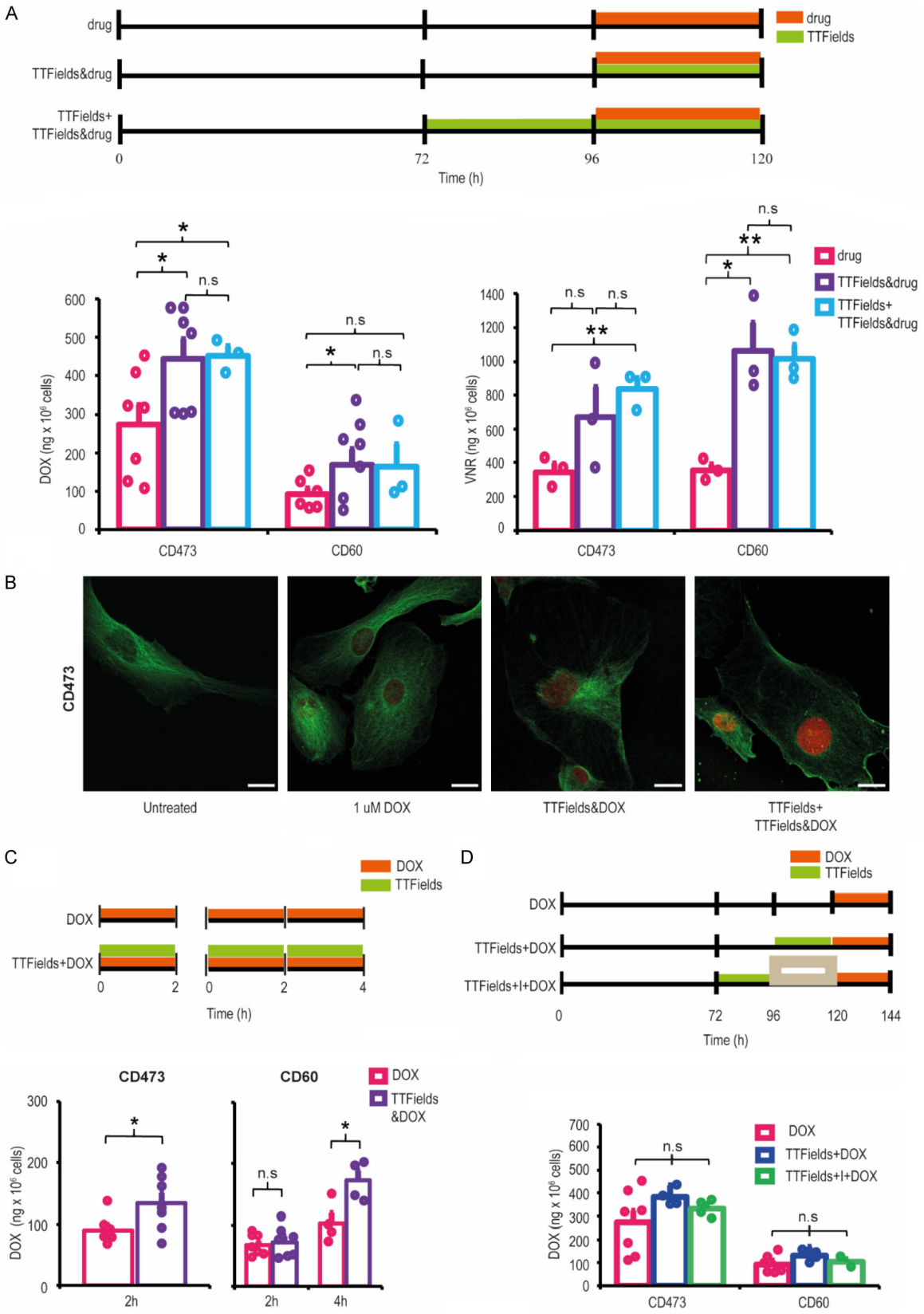

Figure 3. Cellular drug uptake. A. Design of experiment and analysis of intracellular drug concentrations after 24-h exposure to DOX or VNR alone or to two different schedules of treatment with drug and TTFields. Each column

represents the mean intracellular drug concentration ± standard error obtained from at least three independent experiments, while symbols represent the single replicates (Student's t test *P<0.05; **P<0.01. One-way ANOVA test P<0.05 for CD473 cells and P>0.05 for CD60 cells treated with DOX; P>0.05 for CD473 cells and P<0.01 for CD60 cells treated with VNR). B. Representative confocal images of epithelioid CD473 cells labelled with Alexa488-phalloidin (green fluorescence) and untreated or treated with 1 μM DOX (red fluorescence) or DOX and TTFields; scale bar, 20 μm. C. Quantification of intracellular DOX concentration after short treatments with 1 μM DOX or with the simultaneous exposure to DOX and TTFields (2 h of exposure to 150 kHz frequency and 0.76 V/cm intensity for CD473; 2 h and 4 h of exposure to 1.12 V/cm for CD60). Each column represents the mean intracellular drug concentration ± standard error obtained from at least two independent experiments, while symbols represent the single replicates (Student's t test *P<0.05). D. Quantification of intracellular DOX concentration after different schedules of treatment, DOX (drug alone); TTFields+DOX (sequential exposure); TTFields+I+DOX (sequential exposure with 24-h interval between the two treatments). Each column represents the mean intracellular drug concentration ± standard error obtained from at least three independent experiments, while symbols represent the single replicates (Student's t test P>0.05).

efflux, and considering that TTFields induce transcriptional downregulation of many genes coding for protein pumps (including *ABCB1*) [20], we decided to investigate P-gp modulation and its contribution to drug retention.

Western blot analyses of P-gp levels were performed in PM cells to evaluate the effect of TTFields on its expression. As shown in **Figure 4A**, P-gp expression was downregulated during TTFields exposure in both cell lines, with a greater effect in the epithelioid subtype CD473. However, the effect was temporary and, 24 h after TTFields discontinuation, P-gp levels returned to those measured before treatment even in the most sensitive cells (**Figure 4B**). This result further suggests that the simultaneous (rather than sequential) exposure to TTFields and anticancer drugs is crucial for optimal efficacy of the treatment.

In addition to exploring the expression of P-gp, we investigated whether its downregulation in TTFields treated cells resulted in higher drug retention inside the cells treated with DOX. Cells were treated for 1 h with 10 μM DOX with or without 24 h pre-treatment with TTFields. After treatment, DOX was removed and cells were left in drug-free medium for different periods. As expected, the downregulation of P-gp expression contributed to a significant increase in drug retention, especially in the epithelioid subtype (**Figure 4C**). In TTFields-treated CD473 cells, the reduction of DOX levels was about 15% compared to the 38% decrease observed in cells treated with DOX alone. In CD60 cells, the downregulation of P-gp expression caused by TTFields was insufficient to limit cellular drug efflux. Nevertheless, during the first 10 minutes after drug removal, the cellular DOX amount measured in TTFields treated cells was significantly higher than that observed in drug-only treated ones.

Effect of TTFields on the role of actin depolymerization in drug uptake

Disruption of actin networks has been shown to increase the uptake of different anticancer drugs and membrane-impermeable molecules in several cell lines [28-30]. Based on this observation, we tested the hypothesis that TTFields enhance cell membrane permeability by affecting actin polymerization [31].

Confocal microscopic analysis of TTFields treated cells stained with phalloidin showed actin depolymerization only in CD473 cells, where the enhancement of intracellular drug concentration was higher (**Figure 5A**). Furthermore, western blot analysis also suggested a slight and transient downregulation of actin expression in CD473 cells after 48 h of exposure to TTFields, although the effect was not statistically significant (**Figure 5B**). This finding was consistent with previous transcriptional analyses performed on these cell lines during TTFields exposure, which demonstrated that a panel of genes related to RHO GTPases pathway was differentially expressed in CD473 cells but not in the sarcomatoid subtype [20]. Additionally, western blot quantification of actin levels under basal conditions indicated that CD60 cells exhibited a higher expression of this protein (almost three times that observed in CD473 cells), which likely contributed to the reduced effect of TTFields on the actin meshwork in this cell line.

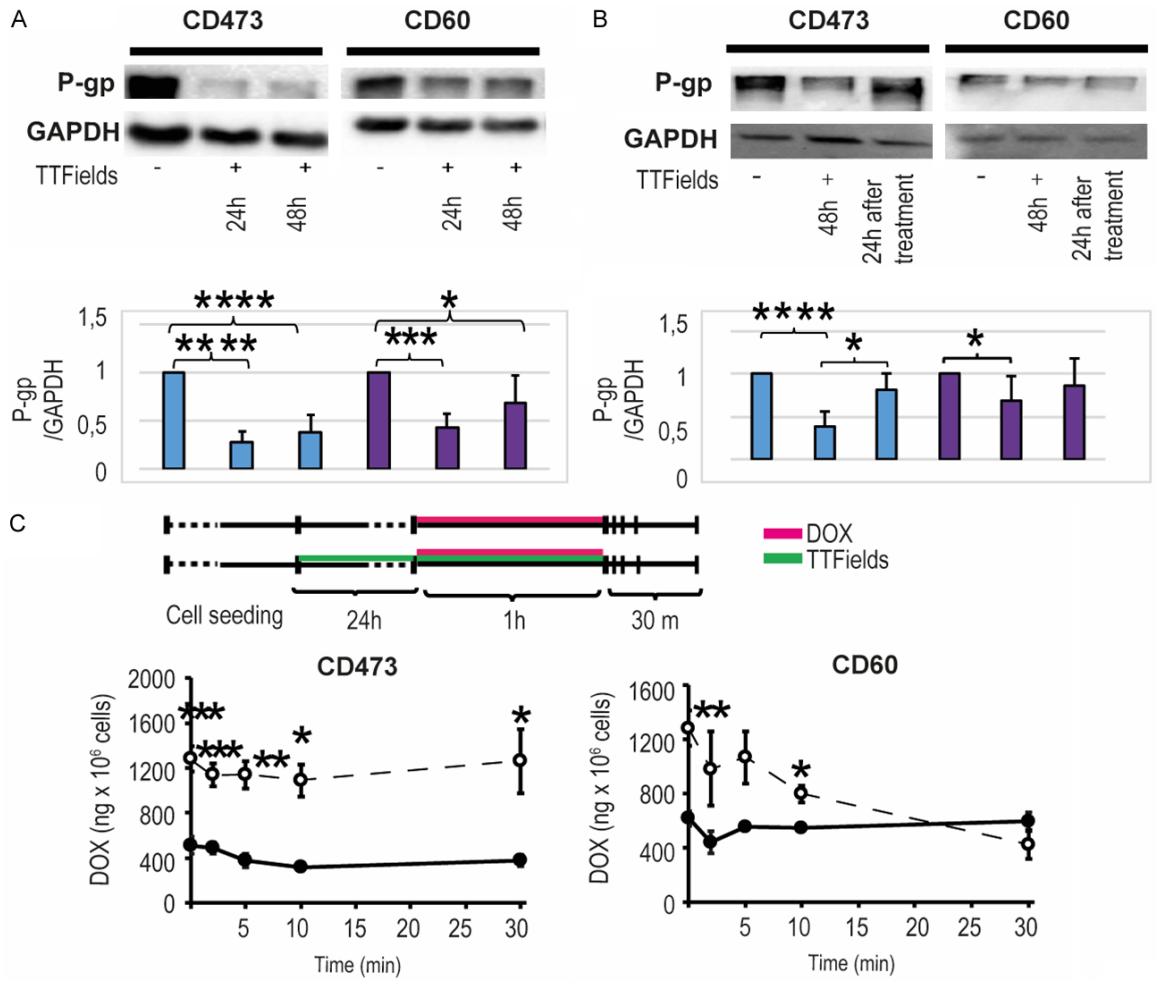

Figure 4. P-gp modulation and its role in drug efflux. A. Western blot of P-gp expression in epithelioid CD473 cells and sarcomatoid CD60 cells exposed for 24 h and 48 h to TTFields. B. Western blot of P-gp expression after 48 h of TTFields and 24 h after the end of treatment. A and B. The relative density of the band is reported as mean ± standard deviation of at least three independent experiments (Student's t test *P<0.05; ***P<0.005; ****P<0.001). C. Comparison of drug efflux in epithelioid and sarcomatoid PM cells after 1 h of exposure to 10 μM DOX (black line and symbols) or DOX preceded by 24 h TTFields (dashed line and white symbols). Each symbol represents the mean of at least four replicates ± standard error (Student's t test *P<0.05; **P<0.01; ***P<0.005). When not visible, error bars are smaller than symbols.

To further investigate the role of actin depolymerization in enhancing cell membrane permeability and increasing intracellular drug concentration, we treated CD60 cells with DOX and latrunculin B (LB), a known inhibitor of actin polymerization [32]. The quantification of intracellular DOX concentration in cells exposed to LB served as a proof of principle that the treatment with agents disrupting actin networks may lead to increased membrane permeability and thereby a higher intracellular drug concentration. CD60 cells treated with both DOX and LB showed a higher amount of intracellular DOX compared to cells treated with DOX alone (**Figure 5C**). Confocal microscopy of phalloidin labeled cells (**Figure 5D**) showed that the effect of LB on the actin meshwork in CD60 cells was similar to that observed in CD473 cells exposed to TTFields (**Figure 5A**). This result hints the possibility that the effects induced by TTFields on the cytoskeleton might indeed contribute to the increase in cellular drug internalization, especially in CD473 cells.

Effect of TTFields on drug uptake and efflux in pemetrexed treated cells

In order to further investigate the role of TTFields in drug uptake and retention, we treat-

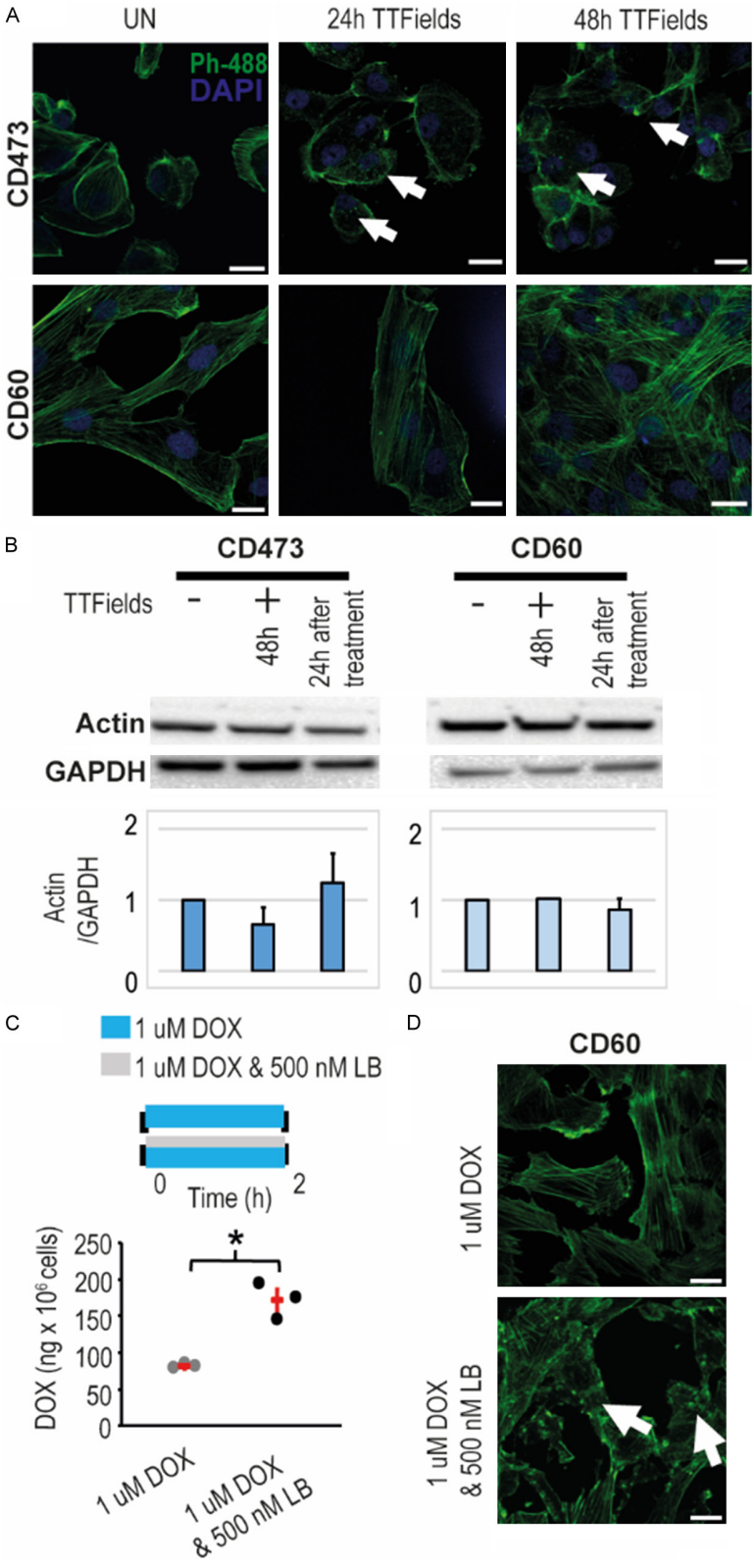

Figure 5. Effects of TTFields on actin expression and polymerization. A. Representative confocal images of epithelioid CD473 and sarcomatoid CD60 cells labelled with Alexa488-phalloidin before (UN, unstimulated) and after the stimulation with 24 h and 48 h of TTFields; white arrows pointed at actin depolymerization; scale bar, 20 µm. B. Western blot of actin levels in CD473 and CD60 cells in basal conditions, after 48 h of TTFields or 24 h after the end of treatment. The relative density of the band is reported as mean ± standard deviation of two independent experiments. Any of the observed differences was statistically significant (Student's t test $P>0.05$). C. Quantification of intracellular DOX concentration after 2 h of treatment with 1 µM DOX or with the simultaneous exposure to DOX and 500 nM latrunculin B (LB). The red symbols represent the mean of three replicates (gray and black circles for 1 µM DOX and 1 µM DOX & 500 nM LB, respectively) ± standard deviation (Student's t test *$P<0.05$). D. Representative confocal images of sarcomatoid CD60 cells labelled with Alexa488-phalloidin (green fluorescence) after treatment with DOX or DOX and LB. White arrows indicate actin depolymerization; scale bar, 20 µm.

ed both cell types with the antifolate agent PEM, used in combination with platinum compounds in PM first-line therapy. In contrast to DOX and VNR, PEM is not a P-gp substrate, while other ATP-driven efflux pumps, such as breast cancer resistance protein (BCRP/ABCG2) and multidrug resistance (MDR) proteins (MRP/ABCC) [33], are involved with its cellular efflux.

Quantification of intracellular drug concentrations after 24 h of incubation with 100 µM PEM, with or without simultaneous exposure to TTFields, demonstrated that the amount of drug internalized by the cells treated with PEM and TTFields was significantly higher than that of the samples exposed to the drug alone. The increase was about 50% in either cell type (**Figure 6A**), and drug reten-

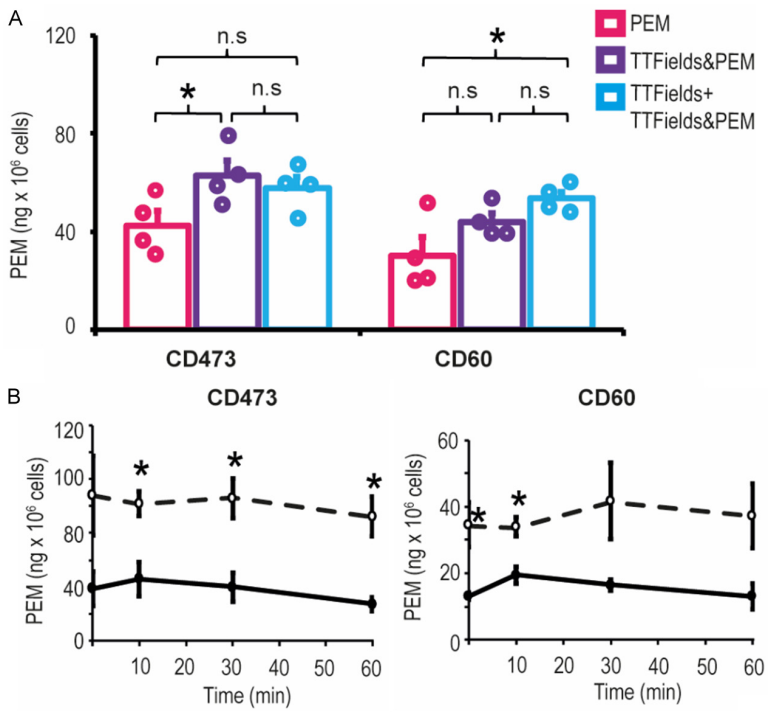

Figure 6. Drug uptake and efflux in PEM treated PM cells. A. Intracellular PEM concentrations after different schedules of treatment. Each column represents the mean of four values ± standard error (Student's t test *P<0.05. One-way ANOVA test P>0.05 for CD473 cells and P<0.05 for CD60 cells). B. Analysis of drug efflux after 24-h treatment with PEM (black line and symbols) or PEM and TTFields (dashed line and white symbols). Each symbol represents the mean of at least three replicates ± standard error (Student's t test *P<0.05).

tion remained higher in the samples that were treated with TTFields (**Figure 6B**).

This result indicates that the increased drug concentration observed in TTFields treated cells is not only significant for drugs that are P-gp substrates but also suggests that TTFields could potentially affect other efflux pumps. However, due to the resistance to PEM seen in CD473 and CD60 cells [21], we were unable to determine if the highlighted intracellular drug concentration resulting from TTFields exposure was linked to an enhanced sensitivity to the treatment.

Effect of TTFields on drug pharmacokinetics

We exploited the ability of our cellular models to develop tumors when transplanted in nude mice to perform *in vivo* pharmacokinetic experiments. Tumor fragments from epithelioid CD473 cells were subcutaneously transplanted, and at the time of treatment, mice were randomly divided in three groups: one treated with VNR alone, one with sham, and one with TTFields. Sham arrays were used as a control for the TTFields arrays, which might increase the stress level experienced by the animals and cause local heating (approximately 38.5°C). Mice treated with sham or TTFields were exposed to treatment for 72 h and then treated intravenously with VNR. At 1 h, 6 h and 24 h after VNR injection, mice were sacrificed, and plasma, liver, and tumor samples were collected for LC-MS/MS quantification of VNR (**Figure 7A**).

As shown in **Figure 7B**, both the sham group and the mice subjected to TTFields treatment showed elevated levels of the drug in the tumor. However, the pharmacokinetic profiles in plasma and liver were similar across all groups, including those that received VNR only. These findings suggest that the heating effect induced by TTFields was effective in increasing drug levels in the tumor, resulting in a 30% increase in the area under the curve (AUC) representing the tumor drug concentration vs time after administration, as compared to mice treated with VNR alone (88.6±1.1 μg h/g, 115.4±2.7 μg h/g, and 119.4±1.6 μg h/g for VNR, VNR+Sham, and VNR+TTFields treated mice, respectively).

Discussion

Acquired multidrug resistance, which often occurs during cancer chemotherapy, has been a longstanding issue in oncology. Despite numerous strategies tested over the years to address this problem, the potential to reverse drug resistance in anticancer therapy remains unresolved. The severe side effects of high drug doses, which can downregulate efflux pumps in cells and reduce resistance, present challenges in clinical settings [34]. A few years ago, Schneiderman et al. [35] demonstrated that

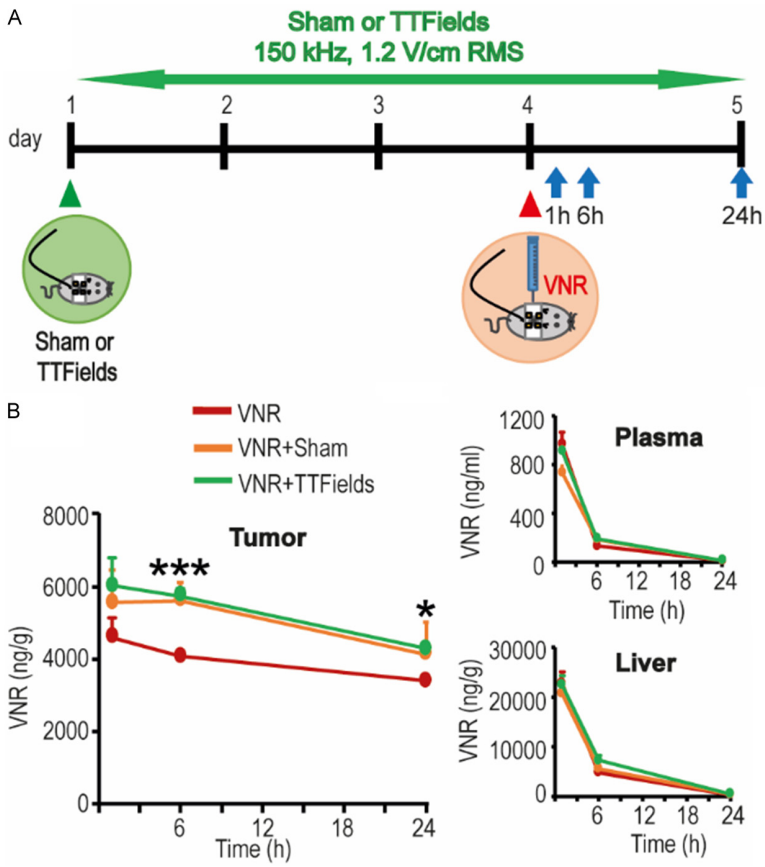

Figure 7. *In vivo* pharmacokinetics. A. Experimental plan for pharmacokinetic analysis. B. Quantification of VNR concentrations in plasma, liver and tumor. Four mice for each experimental group were sacrificed at 1, 6 and 24 h after drug treatment. Each symbol represents the mean ± standard deviation (Student's t test VNR vs VNR+TTFields *P<0.05; ***P<0.005).

TTFields treatment *in vitro* improved the sensitivity of multidrug resistant cells to paclitaxel and doxorubicin, hinting at its potential as a therapy for chemoresistant cells and as an effective adjuvant to enhance chemotherapeutic efficacy. However, no impact on intracellular drug levels was observed in either wild type or resistant cells in their study. In contrast, a more recent *in vivo* study [36] revealed that TTFields exposure increased the penetration of trastuzumab into breast cancer tissues, thereby enhancing its anticancer activity.

So far, only fluorescence-based methods have been used to quantify the impact of TTFields on cellular and tissue drug concentrations [37]. The use of specific and sensitive analytical methods allowed us to discover that the simultaneous *in vitro* exposure to TTFields and anticancer drugs increased intracellular drug concentrations, likely independently of the chemical characteristics of the drug molecule. One might speculate on the nature of the effects of TTFields, considering that they could potentially enhance drug penetration into cells and decrease drug efflux. The facilitation of drug penetration may be due to the formation of pores in the cell membrane, resulting from an electroporation-like effect [12], as well as the depolymerization of the actin cytoskeleton [31]. The reduction of drug efflux may be attributed to the ability of TTFields to modulate, at least transiently, the expression of some relevant transport proteins. These effects were observed in both investigated cell types, although they appeared to be stronger in the epithelioid cells, which were more sensitive to TTFields. The more significant downregulation of P-gp expression and the greater actin depolymerization observed in CD473 cells compared to CD60 cells may provide a potential explanation for the different levels of drug internalization, which contribute to a reduced efficacy of DOX and TTFields treatment on CD60 cell proliferation. Furthermore, previous findings have demonstrated that distinct cell cycle effects were induced by TTFields in the two cell lines, and that common transcriptional modulations (such as those of genes involved in DNA repair pathways and the DNA damage response) exhibited different kinetics and extents in the two models [20]. These may explain the specific efficacy of the co-treatment observed in CD473 and CD60 cells.

Our observations represent - to our knowledge - the first evidence suggesting that TTFields modulate P-gp expression, both at the transcriptional and protein levels. This is noteworthy, as P-gp is considered an important contributor to anticancer drug resistance in clinical settings. Recent results from preclinical studies have demonstrated that TTFields act on the

blood-brain barrier (BBB) by temporarily affecting its permeability in both *in vitro* and *in vivo* models [38]. Since P-gp plays a role in the BBB [39], it seems propitious to assume that the alteration of the BBB permeability by TTFields may involve de-regulation of P-gp. The results of this study also suggest that TTFields may alter the expression of other membrane transport proteins, but further experiments would be necessary to explore this aspect.

Our preliminary pharmacokinetic *in vivo* experiments demonstrated that TTFields exposure contributed to a 30% increase in intratumoral drug concentration. In the considered subcutaneous model, it was challenging to distinguish the specific contribute of TTFields from that of the raised temperature in the region of treatment. Further investigations in orthotopic tumor models, along with pharmacodynamics and imaging studies of intratumoral drug distribution, should be performed to achieve a better comprehension of the phenomenon. Nevertheless, TTFields may represent a well-tolerated strategy to enhance intratumoral drug levels, potentially increasing its therapeutic efficacy. From this perspective, no significant side effects were observed during *in vivo* studies. However, further investigations involving extended treatment durations may be necessary to clarify this issue.

In conclusion, the effects induced by TTFields, which enhance intracellular drug concentration, were shown to be transient. However, the possibility to treat the patients for long periods without significant side effects, but rather increasing the antitumor activity of TTFields [40], can easily overcome this limitation. Based on this observation, one might speculate that exposure to TTFields before chemotherapy administration could enhance tumor permeability and drug distribution, while simultaneous application of TTFields during anticancer drug administration may boost treatment efficacy by maintaining these transient effects.

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Disclosure of conflict of interest

None.

Address correspondence to: Monica Lupi, Laboratory of Cancer Pharmacology, IRCCS Humanitas Research Hospital, Via Manzoni 56, 20089 Rozzano, Milano, Italy. E-mail: monica.lupi@humanitasresearch.it

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