

Article

Synergistic Toxicity of Cold Gas Plasma and Cisplatin in Bladder Cancer Cells

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Simple Summary

Cold physical plasma (hereafter referred to as plasma) was evaluated for its potential to enhance cisplatin therapy in bladder cancer models. Using the cell lines RT-112, T24, and SCaBER, the plasma–cisplatin combination showed synergistic effects in SCaBER, additive effects in RT-112, and additive to mildly synergistic effects in T24 cells. Plasma and cisplatin displayed opposing monotherapy sensitivity profiles, favoring combination treatment. Hydrogen peroxide was identified as a key mediator of plasma- and combination-induced cytotoxicity in SCaBER cells. In the *in ovo* tumor chorioallantoic membrane (TUM-CAM) model, plasma and cisplatin monotherapies comparably reduced tumor burden by inhibiting tumor growth, enhancing tumor immunogenicity, and modulating cytokine secretion. By contrast, the combination treatment had limited effects on tumor mass and vascularization over monotherapies but induced distinct, cell line-specific changes in cellular markers, PD-L1, and secretion profile. Overall, these findings support further preclinical evaluation of plasma as an adjunct to cisplatin therapy in bladder cancer.

Abstract

Background/Objectives: Bladder cancer remains a therapeutically challenging malignancy due to high recurrence rates, progression to muscle-invasive disease, and frequent resistance to cisplatin-based chemotherapy. Cold physical plasma (hereafter referred to as plasma) has emerged as a locally applicable modality that generates reactive oxygen species (ROS) and shows preclinical antitumor activity, offering a potential strategy to enhance cisplatin efficacy while enabling dose reduction. Here, we investigated combination treatment with cisplatin and argon plasma generated by the clinically approved kINPen jet in human bladder cancer models. **Methods:** Three bladder cancer cell lines representing distinct entities were used, namely the urothelial carcinoma lines RT-112 and T24, and the squamous cell carcinoma line SCaBER. IC₂₅ values for plasma and cisplatin monotherapy were established by resazurin assay and used to design combination regimens. Treatment interactions were quantified by coefficient of drug interaction (CDI) analysis and monitored kinetically by long-term live-cell imaging. Plasma-derived ROS were measured in PBS and DMEM, and their functional relevance was assessed in SCaBER cells using catalase and N-acetylcysteine. *In ovo* validation was performed in the tumor chorioallantoic membrane (TUM-CAM) model, where tumor mass, vascularization, cellular marker expression, and cytokine secretion were analyzed. **Results:** Plasma and cisplatin exhibited opposing monotherapy sensitivity profiles across cell lines, creating a favorable basis for



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combination treatment. CDI analysis revealed clear synergy in SCaBER at intermediate cisplatin concentrations, additive effects in RT-112, and additive to mildly synergistic effects in T24. ROS profiling and scavenger experiments identified hydrogen peroxide as a key mediator of plasma and plasma–cisplatin cytotoxicity in SCaBER. In the TUM-CAM model, plasma and cisplatin monotherapies showed notable antitumoral potential. At the same time, plasma–cisplatin combination therapy elicited only modest effects on tumor growth and vascularization compared to monotreatments but induced distinct, cell line-specific alterations in cytokine and marker expression. Conclusions: These findings demonstrate that plasma can potentiate cisplatin cytotoxicity in bladder cancer cells and reshape tumor-associated molecular signatures, supporting further optimization and preclinical evaluation of plasma–cisplatin combination therapy.

Keywords: bladder cancer; urothelial carcinoma; cisplatin; kINPen; TUM-CAM model

1. Introduction

Bladder cancer is the tenth most common cancer worldwide, with an incidence nearly threefold higher in men than in women [1], and remains a major clinical challenge due to its high recurrence rates, substantial risk of progression, and pronounced biological heterogeneity [2]. Most cases are urothelial carcinomas, whereas squamous cell carcinoma and adenocarcinoma are less frequent and associated with distinct etiologies such as chronic irritation or parasitic infection [3,4]. Standard therapies, including drug-based chemotherapy and intravesical immunotherapy, constitute central pillars of bladder cancer management, with cisplatin-based combination approaches remaining a key component of systemic therapy in advanced disease [5]. Its antitumor activity is mediated by the formation of DNA–platinum adducts that disrupt DNA replication and transcription and activate apoptotic signaling pathways [6]. In addition, cisplatin induces oxidative stress, mitochondrial dysfunction, and complex DNA damage responses, which together contribute to its cytotoxic efficacy [7,8]. However, these largely non-selective mechanisms also give rise to substantial systemic toxicities [9], including nephrotoxicity, neurotoxicity, and myelosuppression, which frequently limit treatment eligibility or necessitate premature treatment discontinuation [10]. As a consequence, many patients are unable to receive or complete optimal cisplatin-based combination therapies, contributing to suboptimal outcomes and, in non-responders, the need for radical cystectomy [8,11]. These limitations highlight the urgent need for new therapeutic strategies that improve therapy efficacy while minimizing adverse effects. Strategies that enhance cisplatin sensitivity or permit dose reduction without compromising treatment effectiveness could therefore offer significant clinical benefit.

Cold physical plasma (hereafter referred to as plasma) has emerged as an innovative therapeutic modality that is gaining increasing attention in oncology [12] and is approved for clinical applications in dermatology [13]. Generated at near-physiological temperatures through partial ionization of a noble gas, plasma produces a rich mixture of reactive oxygen species (ROS) capable of modulating cell signaling, inducing oxidative stress, and triggering regulated cell death in cancer cells [14,15]. Plasma has shown antitumor activity in various malignancies, including melanoma [16], sarcoma [17], and head-and-neck cancers [18], and recent studies suggest promising applicability in bladder cancer as well [19–27]. Importantly, plasma can be applied locally and has shown a favorable safety and tolerability profile in preclinical and clinical studies, with no serious or clinically relevant adverse events reported [28–31]. This renders plasma a promising adjuvant oncological tool for

potential multimodal approaches, which have become a cornerstone of modern cancer treatment. Combining agents with complementary or convergent mechanisms may lead to synergistic tumor toxicity, allow dose reduction of individual components, and reduce the risk for resistance development [32]. Given the overlapping ability of cisplatin and plasma to induce oxidative stress and disrupt cellular homeostasis, their combined application represents a particularly compelling therapeutic concept.

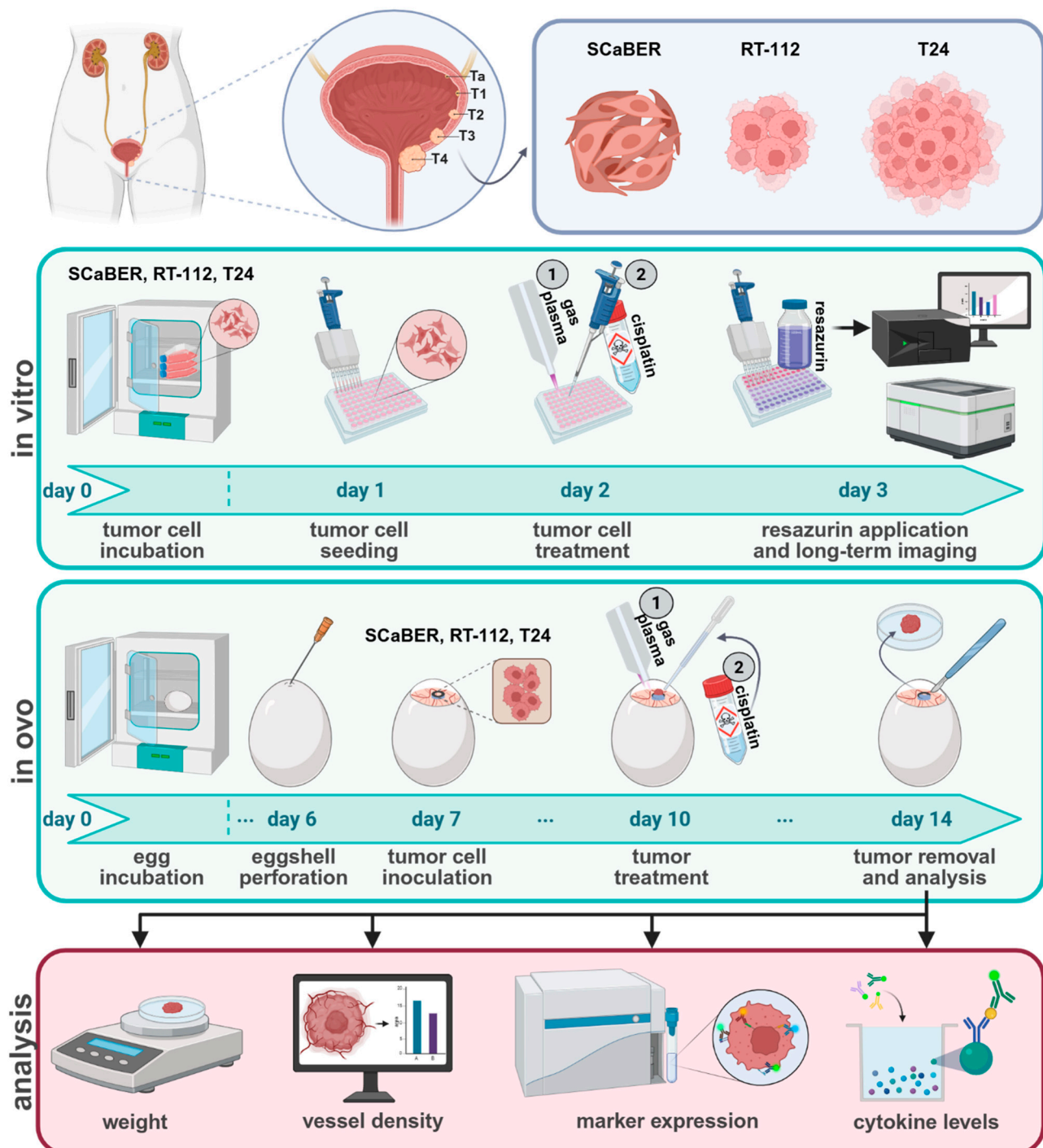


Figure 1. Schematic overview of the experimental design and analyses performed in vitro and in the TUM-CAM in ovo model.

The present study first aimed to directly compare cisplatin treatment with plasma generated by the well-characterized and clinically approved atmospheric-pressure argon

plasma jet kINPen [33] in bladder cancer models. Furthermore, we investigated the therapeutic potential of combining cisplatin with plasma treatment. Three human bladder cancer cell lines representing distinct histopathological entities were selected. RT-112 and T24 are urothelial carcinoma cell lines, with RT-112 derived from a grade 2 tumor exhibiting a more differentiated phenotype, whereas T24 originates from a high-grade, invasive carcinoma with a more aggressive biology. SCaBER, in contrast, is a squamous cell carcinoma line reflecting a rarer but clinically relevant bladder cancer subtype often associated with chronic irritation and squamous metaplasia [34]. We first established IC₂₅ values for plasma and cisplatin monotherapy, then assessed treatment interactions through metabolic activity measurements and coefficient of drug interaction analysis *in vitro*. To explore underlying mechanisms, we quantified plasma-generated reactive species and evaluated their functional relevance using ROS scavengers. Finally, treatment responses were examined *in ovo* using the tumor chorioallantoic membrane (TUM-CAM) model [35], assessing tumor mass, tumor cell count, vascularization, cellular marker expression, and cytokine secretion (Figure 1).

Collectively, this work evaluates the feasibility and therapeutic potential of plasma–cisplatin combination therapy and provides experimental groundwork for further translational development.

2. Materials and Methods

2.1. Cell Culture

Three human bladder cancer cell lines were used in this study. The epithelial squamous cell carcinoma line SCaBER and the urothelial carcinoma cell lines RT-112 and T24 (all ATCC, Manassas, VA, USA) were cultured under identical conditions in Roswell Park Memorial Institute (RPMI) 1640 medium (Pan Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum, 1% penicillin, 1% streptomycin, and 1% L-glutamine (all Corning, Berlin, Germany). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and passaged at sub-confluent densities according to standard procedures.

2.2. Plasma and Cisplatin Treatment

For gas plasma and cisplatin treatments, cells were seeded into 96-well microtiter plates (Greiner, Pleidelsheim, Germany) and allowed to adhere for 24 h at 37 °C. Before treatment, RPMI medium was replaced with Dulbecco's Modified Eagle Medium (DMEM; Pan Biotech, Aidenbach, Germany). Cells were then exposed to plasma, cisplatin dissolved in 0.9% sodium chloride solution, or a sequential combination (plasma treatment followed by cisplatin application), followed by incubation for 24 h. Plasma treatment was performed using the clinically approved and extensively characterized atmospheric-pressure argon plasma jet kINPen [33] (neoplas, Greifswald, Germany) operated at a gas flow rate of one standard liter per minute and a frequency of 1 MHz. Using a computer-controlled and motorized xyz stage (CNC, Geldern, Germany), the kINPen was hovered over the center of each well at 10 mm distance between the nozzle and the liquid surface. For gas plasma treatment *in ovo*, the jet was positioned in direct contact with the target surface (conducting mode). Gas controls using only non-ionizing argon gas were conducted under identical conditions to exclude potential gas flow effects. Liquid evaporation due to plasma exposure was compensated for by adding predetermined amounts of double-distilled water immediately following the treatment.

2.3. Resazurin Assay

Following treatment and 21 h of incubation, resazurin (Cayman Chemical, Ann Arbor, MI, USA) was added to each well to a final concentration of 100 µM, and plates were incu-

bated for 3 h. Viable cells metabolize non-fluorescent resazurin to fluorescent resorufin in a NADPH/H⁺-dependent reaction, which serves as a measure of cellular metabolic function and as an indirect indicator of cell viability. Fluorescence was measured using a microplate reader (F200 Infinite Pro; Tecan, Switzerland) at $\lambda_{\text{ex}} = 530\text{--}570$ nm and $\lambda_{\text{em}} = 590$ nm. Background fluorescence was subtracted, and values were normalized to untreated controls. The coefficient of drug interaction (CDI) was calculated to assess interactions between plasma and cisplatin using the formula

$$CDI = \frac{AB}{(A \times B)}$$

where AB is metabolic activity after combination treatment, and A and B represent metabolic activity after single plasma and cisplatin treatments, respectively. $CDI < 1$ indicates synergy, $CDI = 1$ additive effects, and $CDI > 1$ antagonism. CDI analysis guided the selection of optimal plasma-cisplatin combinations for downstream experiments.

2.4. Long-Term Live-Cell Imaging

Cells were seeded and treated as described above, followed by immediate staining with a caspase-3/7 activity reporter (ThermoFisher, Bremen, Germany) and DAPI (BioLegend, Amsterdam, The Netherlands) at final concentrations of 1 μM each. This approach allowed for discrimination between dead and viable cells, providing a measure of cell death. Continuous imaging was performed over 40 h using an Operetta CLS high-content imaging system (PerkinElmer, Springfield, IL, USA). Cells were maintained under standard culture conditions throughout acquisition. Images were captured every 1.5 h in non-confocal bright-field, digital phase contrast, and fluorescence channels for activated fluorophore-linked caspase-3/7 ($\lambda_{\text{ex}} = 475$ nm; $\lambda_{\text{em}} = 525 \pm 25$ nm) and DAPI ($\lambda_{\text{ex}} = 365$ nm; $\lambda_{\text{em}} = 465 \pm 35$ nm) using a 10 $\times$ air objective. The experiment and subsequent algorithm-based quantitative image analysis were achieved using Harmony 4.9 software (PerkinElmer, Germany).

2.5. ROS Detection and Scavenging

To quantify plasma-generated reactive species, hydrogen peroxide was measured using the Amplex UltraRed assay (ThermoFisher, Germany), and nitrate/nitrite concentrations were determined using the Griess assay (Cayman Chemical, USA) according to the manufacturer's instructions [36]. Briefly, 250 μL plasma-treated (1 slm; 5 s, 10 s, and 25 s) PBS or DMEM was analyzed alongside appropriate standards, and signals were recorded using a multimode plate reader. ROS scavenging experiments were performed in SCABER cells using 2 mM N-acetylcysteine (NAC) and 20 $\mu\text{g}/\text{mL}$ catalase. Cells were pre-treated with ROS scavengers and subsequently subjected to either plasma or cisplatin monotherapy or the combinatory approach. Following incubation for 24 h, metabolic activity was quantified using the resazurin assay.

2.6. TUM-CAM Model

The tumor chorioallantoic membrane (TUM-CAM) model was performed as previously described [37]. Briefly, fertilized, specific-pathogen-free (SPF) chicken eggs (VALO BioMedia, Osterholz-Scharmbeck, Germany) were incubated for 6 days at 37 $^{\circ}\text{C}$ and 60% relative humidity before the eggs' pointed pole was punctured at day 7. After 24 h, the eggshell was fenestrated, and tumor cells were seeded in a silicone ring on the chorioallantoic membrane (CAM). On day 10, developing tumors were treated with plasma, cisplatin, or the combination approach. On day 14, CAM vascularization was imaged prior to tumor excision and weighing. Vessel density was quantified using the vessel analysis tool from ImageJ (version 1.54r), determining vessel area relative to total area.

2.7. Cellular Marker Expression by Flow Cytometry

Tumors harvested from the TUM-CAM model were enzymatically and mechanically dissociated into single-cell suspensions using the gentleMACS Octo Dissociator and corresponding reagents (Miltenyi Biotec, Bergisch Gladbach, Germany). Supernatants of digested tumors were collected for secretion profile analysis. Following red blood cell lysis, two filtering steps, and washing, cells were resuspended in PBS and transferred into FACS tubes. Cells were stained with DAPI for live–dead cell discrimination and counterstained with anti-human antibodies targeting extracellular calreticulin (CRT; Alexa Fluor (AF) 594; BioTechne, Wiesbaden, Germany), CD324 (allophycocyanin (APC)-Fire750; BioLegend, The Netherlands), epithelial cell adhesion molecule (EpCAM; Brilliant Violet (BV) 605; BioLegend, The Netherlands), heat shock protein (HSP) 70 (APC; BioTechne, Germany), HSP90 (phycoerythrin; EnzoLifeScience, Lörrach, Germany), major histocompatibility complex (MHC) 1 (AF700; BioLegend, The Netherlands), programmed cell death ligand (PD-L) 1 (BV650; BioLegend, The Netherlands) and one anti-chicken antibody targeting MHC1 (fluorescein isothiocyanate; Biozol, Hamburg, Germany). After incubation and washing, samples were acquired by flow cytometry (CytoFLEX LX; Beckman-Coulter, Krefeld, Germany).

2.8. Quantification of Cytokines and Chemokines

Cytokines and chemokines of in ovo tumor supernatants were quantified using a bead-based multiplex immunoassay (BioLegend, The Netherlands). Distinct bead populations conjugated to capture antibodies against 13 cytokines (CCL4, IL-1 β , IL-2, IL-6, IL-8, IL-10, IL-18, IFN- α 2, IFN- β , IFN- γ , IP-10, TGF- β 1, and TNF- α) enabled simultaneous detection in one step. The assay followed a sandwich immunoassay principle using capture beads, biotinylated detection antibodies, and streptavidin-phycoerythrin for fluorescence-based readout. After washing, samples were acquired by flow cytometry (CytoFLEX LX; Beckman-Coulter, Germany). Absolute concentrations (pg/mL) of respective analytes were calculated against a standard curve using specific data analysis software (BioLegend, The Netherlands) and subsequently normalized to 1 mg tumor weight.

2.9. Statistical Analysis

Raw data were processed in Microsoft Excel (Microsoft, Redmond, WA, USA) and visualized using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Statistical tests included unpaired *t*-test, one-way ANOVA, and nonlinear regression, as appropriate, and are specified in corresponding figure legends. Data are presented as mean $\pm$ standard deviation (SD) for normally distributed variables or as median $\pm$ 95% confidence interval (CI) for non-normally distributed variables. Statistical significance was defined as $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

3. Results

3.1. Combined Treatment with Gas Plasma and Cisplatin Shows Additive and Synergistic Effects on Metabolic Activity and Viability in Bladder Cancer Cells

To investigate the therapeutic potential of combining plasma with cisplatin, we performed a series of in vitro and in ovo experiments using the human bladder cancer cell lines SCaBER, RT-112, and T24. Initial plasma dose–response studies were conducted to define appropriate exposure times and seeding densities. Plasma reduced metabolic activity in all three cell lines in a treatment-duration-dependent manner. Live-cell imaging further showed that higher densities led to confluent monolayers, particularly in RT-112, which impaired automated algorithm-based image analysis, especially at late measurement time points of long-term monitoring (Figure A1). A seeding density of 5×10^3 cells/well

was therefore selected for all subsequent experiments. All tested cell lines displayed distinct sensitivity profiles, and plasma exerted the strongest inhibitory effect in T24 and the weakest in SCaBER. In contrast, cisplatin showed the opposite pattern, with SCaBER being most sensitive and T24 requiring higher concentrations to achieve comparable levels of inhibition (Figure 2a).

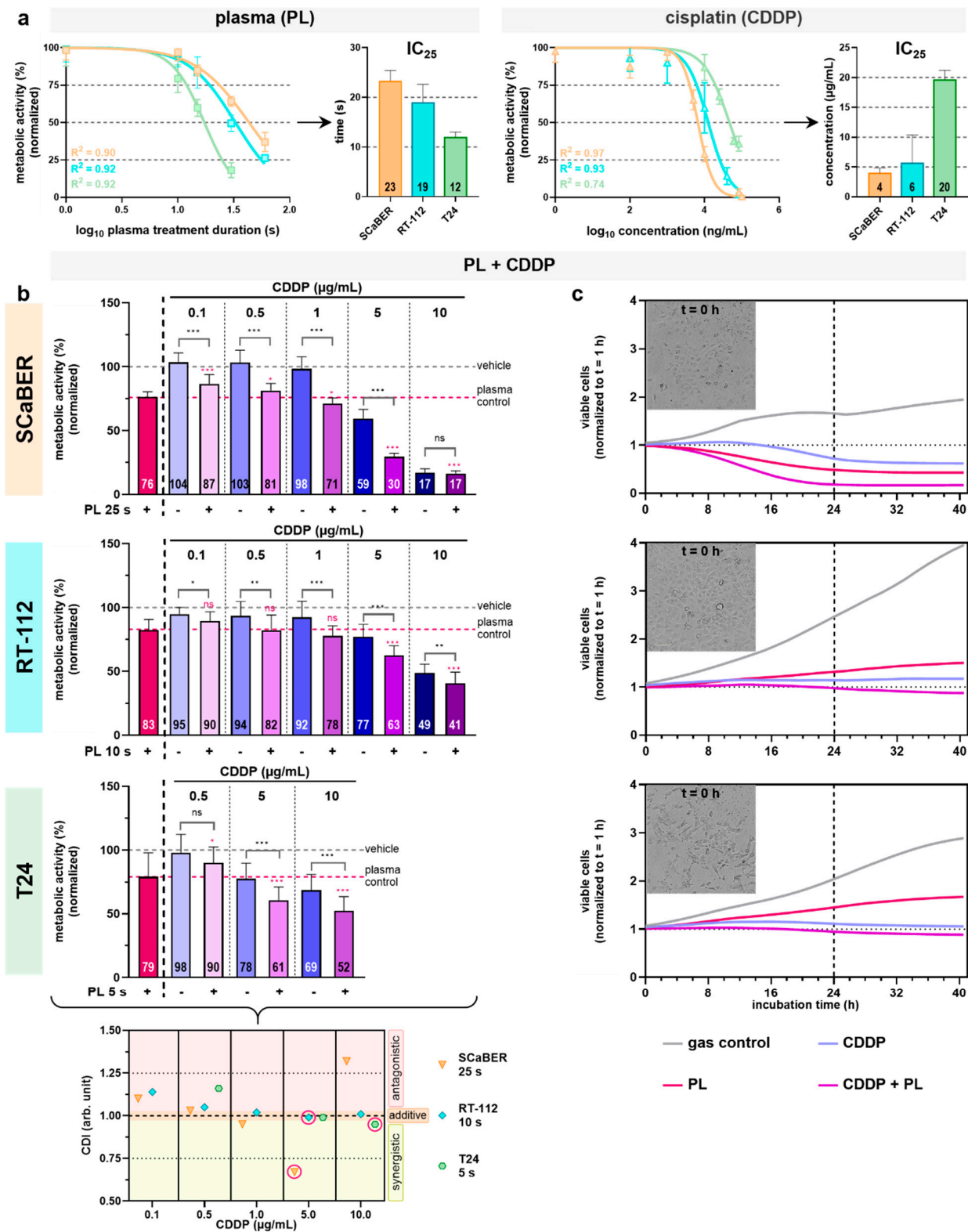


Figure 2. Mono- and combination therapy with plasma (PL) and cisplatin (CDDP) shows significant tumor-inhibiting effects on SCaBER, RT-112, and T24 cells. (a) Metabolic activity of cancer cells 24 h

post PL and CDDP monotreatment and calculated IC₂₅ values; (b) metabolic activity for combined PL and CDDP therapy (CDI—coefficient of drug interaction; red circles indicate most effective combination approaches); (c) number of viable cells (DAPI[−] and caspase3/7[−]) over 40 h for combined PL and CDDP treatment. Graphs show mean with standard deviation. Statistical analyses were performed using nonlinear regression (a) and one-way ANOVA followed by Dunnett's post hoc correction for gas plasma and combination treatment and unpaired *t*-tests for CDDP and combination treatment (b). (* *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, ns = non-significant).

The combination of plasma and cisplatin, based on the respective IC₂₅ values, reduced metabolic activity across all cell lines more effectively than cisplatin alone and, in cases of cisplatin concentrations above 1 µg/mL, even stronger than plasma monotreatment. Tumor toxicity was enhanced with increasing cisplatin concentrations in both mono- and combination treatments, with notable reductions beginning at ≥5 µg/mL. The coefficient of drug interaction (CDI) revealed cell line-specific interaction profiles. SCABER transitioned from antagonistic effects at low cisplatin concentrations to clear synergy at intermediate concentrations, followed by a shift back to antagonism at 10 µg/mL, indicating toxicity-driven saturation effects. RT-112 moved from antagonistic to additive interactions but did not exhibit marked synergy. T24 showed a similar pattern, transitioning from antagonistic to mildly synergistic interactions at higher cisplatin concentrations (Figure 2b).

To examine treatment kinetics, long-term live-cell imaging was performed over 40 h using the most effective plasma–cisplatin combinations identified for each cell line. Gas controls maintained the highest proportion of viable (DAPI- and caspase-3/7-negative) cells throughout the experiment. Across all three cell lines, the combination treatment resulted in the lowest number of viable cells. In RT-112 and T24, viability remained largely stable after treatment, whereas SCABER displayed a rapid and substantial decline within the first 16 h before stabilizing. Overall, SCABER was the most rapidly affected cell line, while RT-112 and T24 exhibited more gradual responses (Figure 2c). Together, these findings demonstrate that plasma enhances cisplatin efficacy in a concentration- and cell line-dependent manner, with distinct optimal combination conditions for each cell line, and SCABER being the most profoundly affected.

3.2. ROS Profiling and Scavenging Identifies Hydrogen Peroxide as One Major Mediator of Plasma-Induced Cytotoxicity in SCABER Cells

To characterize the plasma-generated ROS, we performed liquid analysis for quantification of the long-lived species nitrate (NO₃[−]), nitrite (NO₂[−]), and hydrogen peroxide (H₂O₂) in PBS and DMEM following plasma treatment. In PBS, nitrate and nitrite increased with longer treatment duration, whereas H₂O₂ accumulated to substantially higher concentrations. Similar H₂O₂ trends were observed in DMEM, although absolute concentrations differed due to medium-specific ROS-quenching components, e.g., pyruvate (Figure 3a). These results confirm robust and quantifiable ROS formation, with H₂O₂ as the predominant species. To assess the functional relevance of ROS for treatment efficacy, scavenger experiments were conducted in SCABER cells, which had shown the strongest combination effects. NAC exerted the strongest effect on the plasma–cisplatin combination, restoring about 50% of the combination-induced metabolic activity impairment, while having a measurable but only minor impact on cisplatin monotherapy and negligible effects on plasma alone. Vice versa, catalase predominantly increased metabolic activity in plasma-treated samples and produced a modest increase in the combination setting. As catalase showed no impact on CDDP monotreatment, cisplatin-triggered cytotoxicity is mainly H₂O₂-independent. As both antioxidative molecules are able to completely abrogate H₂O₂-mediated inhibition of metabolic activity, but neither catalase nor NAC fully abolished plasma-induced effects, additional ROS contribute to the observed tumor toxicity as well as enhanced effects in combination treatments. Nevertheless, these data

indicate H_2O_2 as a major contributor to plasma-induced cytotoxicity since catalase buffers about 50% of the provoked growth reduction (Figure 3b).

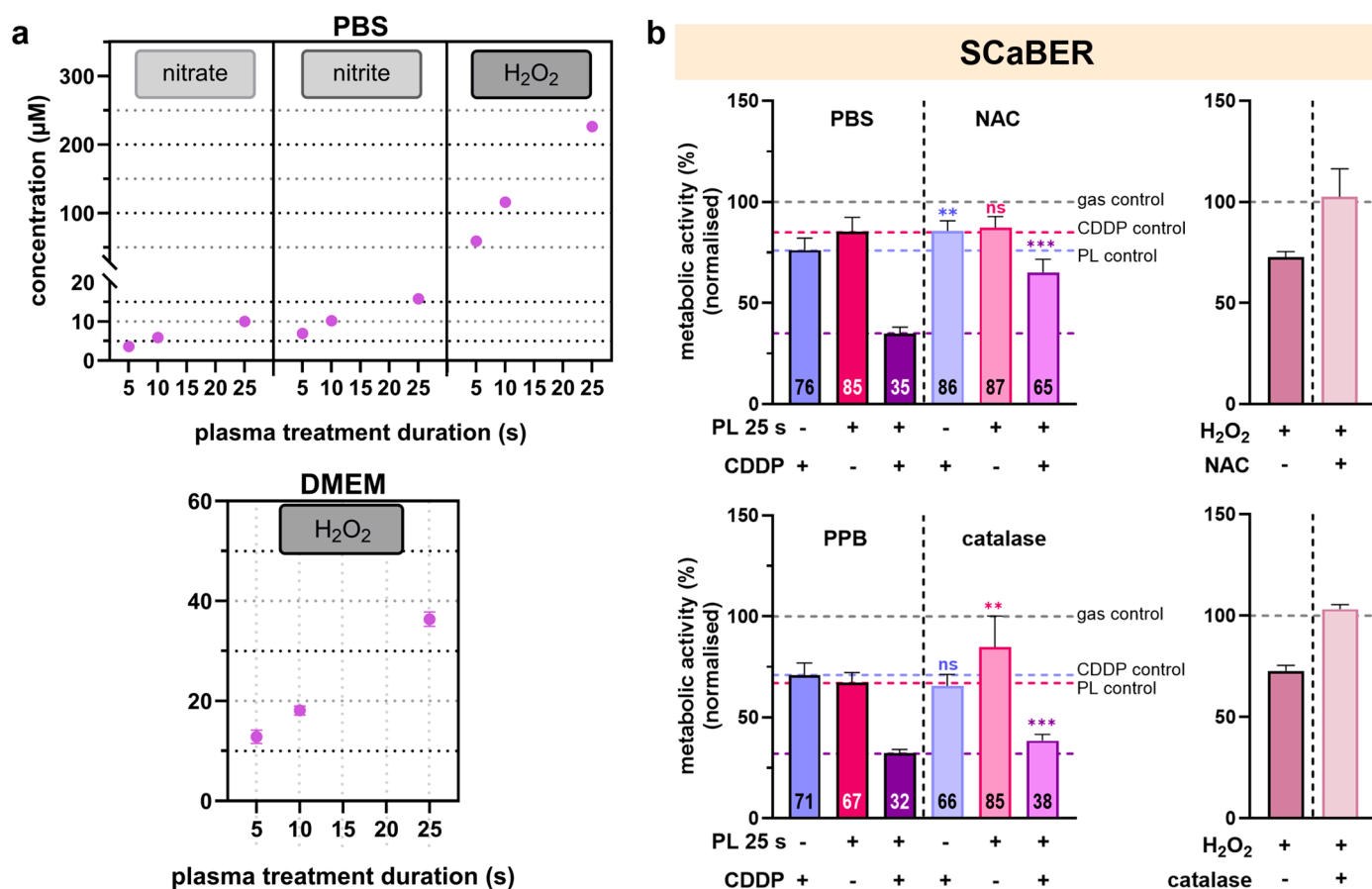


Figure 3. Reactive species profiling indicates hydrogen peroxide as major mediator of plasma-induced effects. (a) Quantification of reactive species in PBS and DMEM following plasma treatment; (b) metabolic activity of SCaBER cells in the presence or absence of N-acetylcysteine (NAC) in phosphate-buffered saline (PBS) and catalase in potassium phosphate buffer (PPB). Graphs show mean with standard deviation. Statistical analyses were performed using unpaired *t*-tests. (** *p* < 0.01, *** *p* < 0.001, ns = non-significant).

3.3. Cisplatin and Plasma Monotherapy Significantly Reduce Tumor Burden in a Bladder Cancer TUM-CAM Model in Ovo

To evaluate treatment responses in a more physiologically relevant setting, plasma and cisplatin efficacy were assessed in the in ovo tumor chorioallantoic membrane (TUM-CAM) model (Figure 4a). Representative tumor images demonstrated visible differences in tumor volume for cisplatin and plasma treatment groups compared to controls (Figure 4b). Evaluation of tumor weights revealed a strong tumor-inhibiting effect of both monotherapies (Figure 4c), emphasized by a strikingly diminished total tumor cell count (Figure 4d). Subsequent marker analysis indicated increased fluorescence intensities for HSP90 following both cisplatin and plasma treatment (Figure 4e). Quantitative evaluation confirmed a significant upregulation of calreticulin (CRT) and HSP90 on the tumor cell surface relative to vehicle control, whereas HSP70 showed only a non-significant upward trend. In contrast, the expression of the immunosuppressive marker PD-L1 remained unchanged across treatment groups (Figure 4f). Secretion profiling revealed distinct immunological modulation by the two monotherapies. The cytokines analyzed in this study play diverse roles in immune regulation, and alterations in their secretion profiles can reflect treatment-induced immunological responses. Plasma treatment resulted in the most prominent reductions in

CCL4, IL-6, IL-8, IL-10, TGF- β 1, and TNF- α . Cisplatin most effectively reduced IL-1 β and IL-18, although plasma also significantly decreased both cytokines compared with control. No significant changes were observed for IL-2, IFN- α 2, IFN- β , IFN- γ , or IP-10. Notably, secretion levels of CCL4, IL-10, and TNF- α were significantly lower after plasma treatment compared with cisplatin (Figure 4g). Collectively, these data indicate that plasma and cisplatin each modulate inflammatory signaling in ovo but in cytokine-specific patterns. Overall, monotherapy experiments demonstrate that both plasma and cisplatin inhibit tumor growth in the in ovo bladder cancer model, with plasma exerting generally stronger effects than cisplatin.

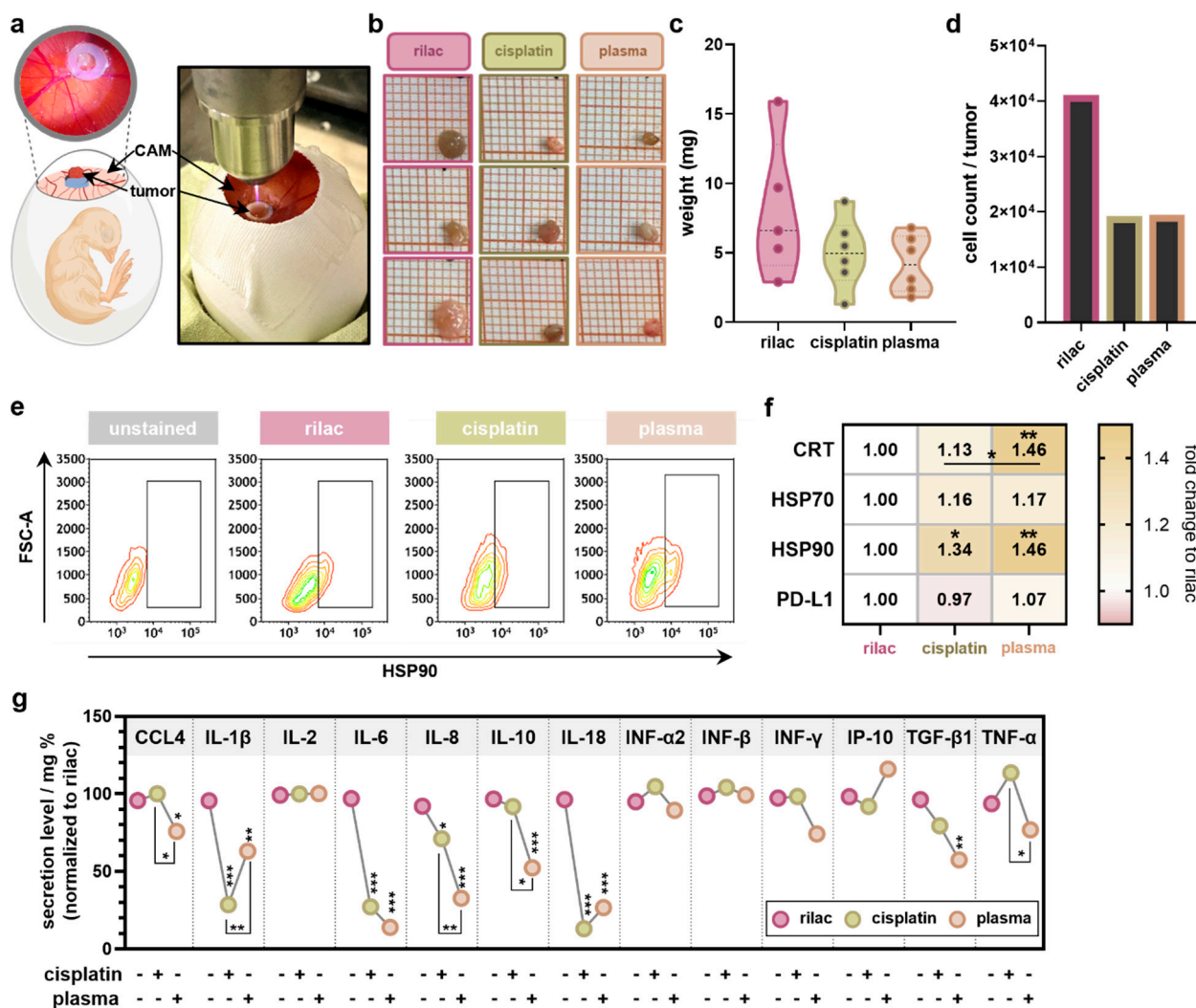


Figure 4. Cisplatin and plasma monotherapy demonstrate significant antitumoral potential in the TUM-CAM model. (a) Schematic illustration of the TUM-CAM model and plasma treatment with kINPen; (b) representative images of excised tumors; (c) tumor weight; (d) tumor cell count determined by flow cytometry; (e) representative flow cytometry contour plots for discriminating HSP90-positive cells (FSC—forward scatter); (f) expression levels of cellular markers; (g) cytokine secretion profiles. Graphs show mean with standard deviation (b–d) or median (e). Statistical analyses were performed using unpaired *t*-tests (* *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, ns = non-significant).

3.4. Combination Therapy Shows Limited Treatment Efficacy in the TUM-CAM Model

To validate in vitro combination outcomes, we examined the cisplatin–plasma combination therapy in the TUM-CAM model across all three bladder cancer cell lines. Based on the additive and synergistic effects observed in vitro, the analysis focused on comparisons between cisplatin monotherapy and cisplatin–plasma combination treatment. Tumor

weights exhibited substantial intra-group variability, and no statistically significant differences were detected between treatment conditions in any cell line. SCaBER consistently formed the largest tumors, whereas RT-112 generated the smallest (Figure 5a).

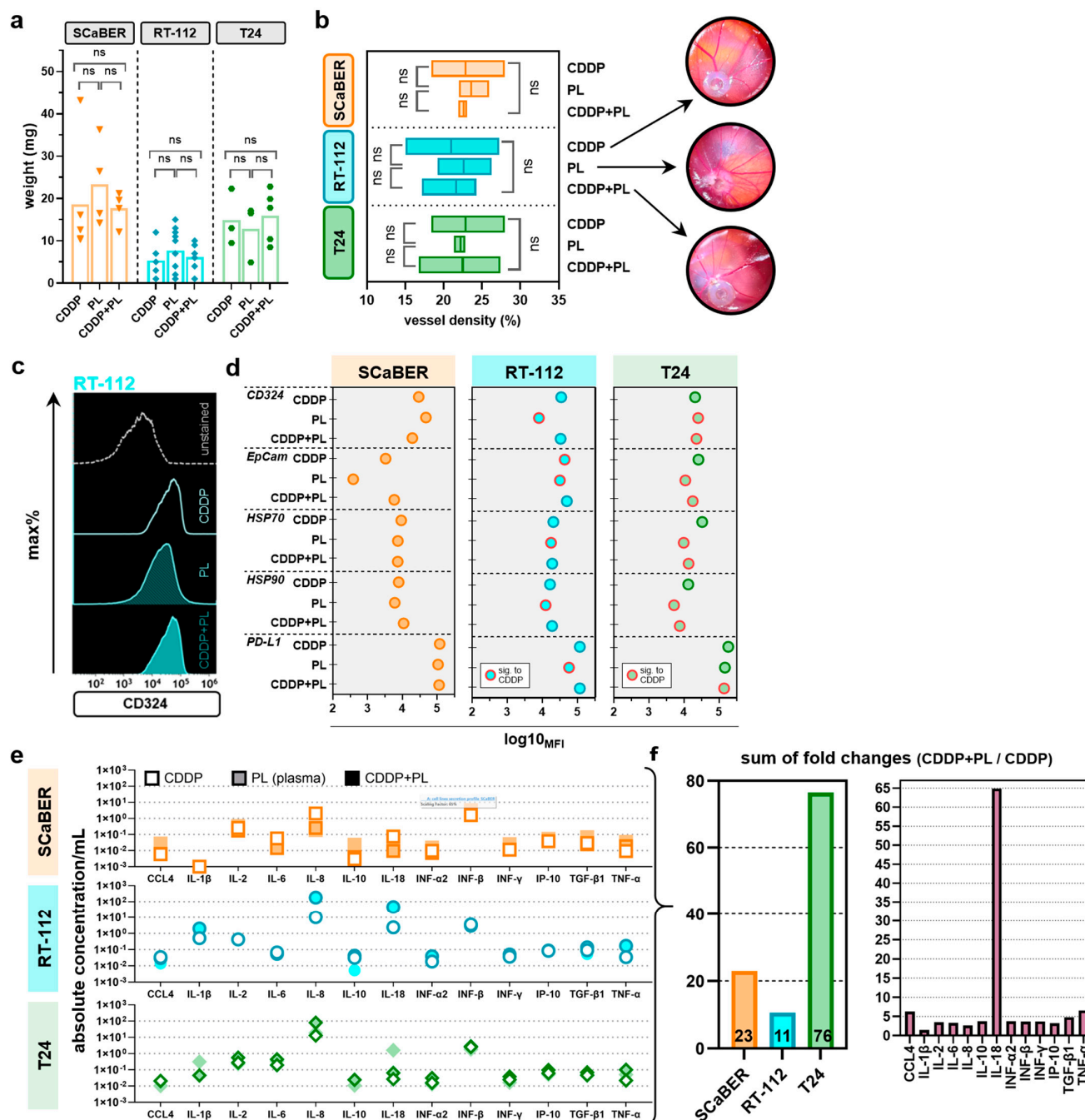


Figure 5. Addition of plasma (PL) to cisplatin (CDDP) treatment results in only limited enhancement of antitumor effects in the TUM-CAM model. (a) Quantification of tumor weight and (b) vessel density; (c) representative fluorescence intensities of CD324 in RT-112 tumors; (d) expression levels of cellular markers; (e) cytokine secretion profiles; (f) sum scores of fold changes for all cytokines sorted by cell line for all cytokines (left) or by cytokine for all cell lines (right). Graphs show mean with standard deviation. Statistical analyses were performed using unpaired *t*-tests (a,b,d) or ANOVA with Tukey's post hoc test (e) (ns = non-significant).

Vessel density measurements likewise revealed no significant differences between treatment groups, as representatively shown for RT-112 tumors (Figure 5b). Flow cytometric analysis of different surface-associated markers showed cell line-specific expression

modulation. CD324 expression was markedly decreased after plasma treatment in RT-112 (Figure 5c) but increased in SCaBER and T24. While no striking difference was observed between the CDDP mono- and plasma combination approaches in SCaBER and RT-112, CD324 expression was significantly increased in T24 upon cisplatin–plasma treatment. Likewise, EpCAM was notably downregulated in T24 upon combination therapy. EpCam exhibited the widest inter-line variability, with plasma treatment majorly reducing expression in SCaBER cells, while only moderately, but also significantly, decreasing it in RT-112 and T24. Although no significant changes were detected for expression of HSP70 under combination treatment in SCaBER and RT112, HSP90 was upregulated in both cell lines following CDDP-plasma administration compared to monotreatments. Intriguingly, the abundance of HSP70 and HSP90 was highest for CDDP monotherapy in T24 cells. PD-L1 differed minimally in SCaBER, decreased substantially in RT-112 after plasma monotherapy, and decreased significantly in T24 only after combination treatment (Figure 5d). Subsequently, released immunomodulatory molecules were quantified in supernatants of digested tumors, but IL-1 β concentrations were not reliably detectable in all groups of SCaBER since values did not exceed the limit of detection. Overall, cytokine and chemokine responses to combination therapy showed striking cell line-dependent regulation (Table 1). In SCaBER, the secretion of several signaling molecules was significantly affected under combination therapy compared with cisplatin alone. RT-112 showed no significant changes in the secretion profile between cisplatin and combination treatment. In T24, combination therapy significantly increased IL-1 β , IL-6, and IL-18, whereas other cytokines remained unchanged (Figure 5e). Summed fold-change analysis indicated that T24 was most affected by combination treatment, followed by SCaBER, with RT-112 showing the least changes. IL-18 displayed the strongest overall increase, followed by smaller increases in CCL4 and TNF- α (Figure 5f). Principal component analysis integrating all measurements derived from the TUM-CAM model revealed no distinct clustering by treatment or cell line. Most samples were broadly dispersed, although combination-treated RT-112 tumors deviated strongly along both principal components. While combination treatments displayed the highest divergence, being mainly separated along PC1, the T24 treatment groups and plasma-treated samples formed the tightest clusters (Figure A2).

Table 1. Statistical analysis of cytokine and chemokine quantification in SCaBER, RT-112, and T24 cells. Significance was assessed using one-way ANOVA followed by Tukey’s post hoc test.

		CDDP vs. PL	PL vs. CDDP + PL	CDDP vs. CDDP + PL
SCaBER	CCL4	0.93	<0.001	<0.001
	IL-1 β	-	-	-
	IL-2	0.08	0.002	0.07
	IL-6	<0.001	0.002	0.001
	IL-8	<0.001	0.7	<0.001
	IL-10	>0.99	0.04	0.04
	IL-18	<0.001	0.69	<0.001
	INF- α 2	0.28	<0.001	0.009
	INF- β	>0.99	<0.001	<0.001
	INF- γ	0.89	<0.001	<0.001
	IP-10	0.89	0.002	0.005
	TGF- β 1	0.84	<0.001	0.002
	TNF- α	0.05	0.08	0.001

Table 1. Cont.

		CDDP vs. PL	PL vs. CDDP + PL	CDDP vs. CDDP + PL
RT-112	CCL4	>0.99	0.31	0.27
	IL-1 β	0.004	0.005	0.88
	IL-2	>0.99	0.7	0.63
	IL-6	0.12	0.61	0.46
	IL-8	<0.001	<0.001	>0.99
	IL-10	0.48	0.06	0.36
	IL-18	<0.001	<0.001	>0.99
	INF- α 2	0.04	0.05	>0.99
	INF- β	0.96	0.66	0.51
	INF- γ	0.18	0.08	0.88
	IP-10	0.88	0.47	0.75
	TGF- β 1	0.23	0.03	0.41
	TNF- α	0.005	0.003	0.95
T24	CCL4	0.94	0.28	0.43
	IL-1 β	0.32	<0.001	<0.001
	IL-2	0.01	0.01	0.98
	IL-6	<0.001	0.006	<0.001
	IL-8	<0.001	<0.001	0.3
	IL-10	0.11	34	0.72
	IL-18	0.7	<0.001	<0.001
	INF- α 2	0.002	<0.001	0.69
	INF- β	0.86	0.09	0.19
	INF- γ	0.009	<0.001	0.06
	IP-10	0.004	0.001	0.59
	TGF- β 1	0.18	0.85	0.38
	TNF- α	0.001	0.02	-

4. Discussion

Cisplatin-based combination therapies remain a widely established component of systemic treatment for various malignancies [5], including bladder cancer [8]. In contrast, the therapeutic use of cold gas plasma in cancer is still under active investigation and has not yet entered clinical practice [12]. Nonetheless, preclinical evidence supports the antitumor potential of plasma-derived reactive species across multiple cancers [12,38], including early promising results in bladder cancer [19]. Furthermore, multiple preclinical studies emphasize the high tumor selectivity and great tissue tolerability of gas plasma treatment [19,39,40], underpinning the low side effect profile of this approach in first clinical trials [41,42]. These emerging findings provide a scientific rationale for evaluating plasma either as an independent modality or as part of combination strategies aimed at enhancing treatment efficacy. This study first demonstrates that plasma affects tumor growth similarly to cisplatin in human bladder cancer cell lines SCaBER, RT-112, and T24 in vitro, and even in a more complex in ovo tumor environment. Furthermore, it shows that plasma can enhance cisplatin efficacy in a concentration- and cell line-dependent manner and identifies reactive oxygen species (ROS), particularly hydrogen peroxide (H₂O₂), as functionally relevant mediators of these effects. At the same time, translation of the cisplatin–plasma combination into the in ovo TUM-CAM model revealed only modest and heterogeneous

treatment responses, underscoring both the promise and the current limitations of this combination approach.

In vitro, plasma and cisplatin displayed opposing sensitivity profiles across the three bladder cancer cell lines. T24 cells were most sensitive to plasma and least sensitive to cisplatin, whereas SCaBER cells showed the inverse pattern, with RT-112 occupying an intermediate position. The inverse sensitivity patterns observed here align with previous reports describing substantial heterogeneity in plasma responsiveness across cancer cell types. We previously demonstrated that plasma resistance correlates with factors such as aquaporin expression, membrane cholesterol content, and particularly the basal metabolic activity of tumor cells [38]. In addition, an increased antioxidative capacity, comprising highly maintained glutathione metabolism, augmented expression of detoxifying enzymes, and well-orchestrated regulation of proteasome pathways [43], has been associated with reduced sensitivity to plasma-derived reactive species [44]. Several bladder cancer cell lines, including RT-112, have been reported to exhibit elevated antioxidant buffering, such as higher glutathione, which may protect against plasma-induced oxidative stress [45,46]. Conversely, cisplatin resistance is often linked to diminished intracellular platinum accumulation through reduced uptake via copper transporter 1 (Ctr1) or enhanced efflux, as well as dysregulation of DNA damage-response pathways [47]. High glutathione levels can additionally detoxify platinum compounds, providing a mechanistic explanation for the remarkable scavenging effect of NAC. As a membrane-permeable cysteine precursor, it amplifies the intracellular bioavailability of cysteine for increased cellular GSH synthesis [48]. Thus, the CDDP and combination-induced impairment of metabolic tumor cell activity was partly abolished by NAC supplementation, suggesting GSH-mediated platinum detoxification as a potential mechanism of reduced CDDP sensitivity. These observations align with previous findings in bladder cancer showing that elevated glutathione correlates with increased cisplatin resistance relative to more chemosensitive tumor types, such as testicular cancer [45,46].

The combination of plasma and cisplatin led to stronger reductions in metabolic activity than at least one of the corresponding monotherapies in all three lines. CDI analysis further refined these observations, revealing clear synergy in SCaBER at intermediate cisplatin concentrations, additive interactions in RT-112, and predominantly additive to mildly synergistic effects in T24. Notably, the switch from synergy to antagonism at the highest cisplatin dose in SCaBER indicates a narrow therapeutic window where excessive toxicity may override combination benefits [49], as also observed in plasma–chemotherapy studies in melanoma [50]. These findings align with the general principles of combination therapy, where complementary mechanisms can yield additive or synergistic interactions and thereby improve treatment efficacy at lower individual doses [32]. Similar dose-dependent enhancement of tumor toxicity has been documented in other models combining plasma with chemotherapeutics, including pancreatic cancer treated with plasma-treated Ringer’s lactate [51] and Ewing sarcoma cells exposed to plasma together with doxorubicin or vincristine [17]. Consistent with these earlier studies, the combinations in the present work produced additive or synergistic antitumor activity once minimally effective cisplatin levels were reached. For example, in the case of T24, a combination of 5 µg/mL CDDP with plasma evoked a stronger antitumor effect than the 10 µg/mL CDDP monotherapy, halving the required concentration to achieve equal therapeutic efficacy. This impressively highlights the potential beneficial implementation of plasma in the treatment of urothelial cancer, as it could lower CDDP treatment dose, thereby minimizing side effects and improving patients’ quality of life. Together, these experiments underline that optimal plasma–cisplatin interactions occur within a defined concentration window and are shaped by the intrinsic biological properties of each cell line.

Mechanistically, ROS measurements and scavenger experiments point to hydrogen peroxide as a major effector species in plasma and plasma–cisplatin treatments, at least in SCaBER cells. The prominent role of H_2O_2 in plasma-treated liquids is consistent with previous work identifying long-lived plasma-derived species such as hydrogen peroxide as a key mediator of biological effects in liquid environments [52,53]. Catalase, which selectively converts H_2O_2 , partially hampered the antitumoral effects of plasma exposure and had a modest effect in the combination setting, indicating that H_2O_2 is a key mediator of plasma-induced tumor toxicity. This also suggests that other plasma-derived ROS, such as nitrate or nitrite, may contribute to tumor inhibition and are necessary for the full treatment response. In contrast, NAC had only minor effects on cisplatin alone and no measurable impact on plasma monotherapy, but restored metabolic activity in SCaBER subjected to combination treatment. These patterns suggest that oxidative stress becomes particularly consequential when plasma and cisplatin are applied together, and that species beyond H_2O_2 , potentially including nitrate, nitrite, or downstream derivatives, may contribute to combination-specific cytotoxicity. Collectively, these findings support a model in which plasma-derived H_2O_2 acts as a primary cytotoxic mediator that can amplify cisplatin-induced oxidative stress and DNA damage, thereby promoting enhanced cytotoxic effects in sensitive cell lines.

Despite the encouraging in vitro results obtained for plasma–cisplatin combination therapy, the TUM-CAM model revealed only modest additional effects. Although both monotherapies markedly diminished tumor growth in ovo, suggesting comparable antitumoral potential of plasma and cisplatin regarding proliferation inhibition and augmentation of tumor immunogenicity, combination treatment did not result in significantly higher reductions in tumor mass in any of the three bladder cancer models. Likewise, vessel density on the CAM remained largely unchanged across treatment groups. The absence of measurable tumor shrinkage in the present study may also reflect biological and technical factors, including differences in tumor hydration, three-dimensional tumor architecture, stromal interactions, and diffusion-limited exposure to cisplatin- and plasma-derived reactive species. The tumor microenvironment, including angiogenesis, extracellular matrix, and interactions with surrounding cells, can impact therapy response and may explain the divergent in vitro and in ovo findings. In addition, the single-application treatment scheme, optimized for in vitro assays, may be insufficient in ovo, where repeated or higher-intensity dosing may be necessary. Differences in baseline tumor size, growth kinetics, and cell line-specific biology may also shape the observed outcomes.

Although no major differences were observed for the assessed macroscopic tumor features, e.g., weight and vascularization, significant changes in tumor treatment response were detected on a molecular level by analysis of surface marker expression and secretion profile. Under monotherapy conditions, plasma treatment upregulated expression of CRT and HSP90, both established mediators of immunogenic cell death (ICD) [54], more strongly than cisplatin, underlining previously shown immunostimulatory effects of plasma by elevating the expression of damage-associated molecular pattern (DAMPs) [55,56]. Although changes were not significant, SCaBER and RT112 tumors exhibited higher HSP90 expression following combination treatment over CDDP monotherapy. This potentially higher abundance of ICD markers on the tumor cell surface could promote an antitumoral immune response, improving the therapeutic efficacy and proposing a beneficial impact of plasma in CDDP combination strategies. PD-L1 expression was variably affected, showing the lowest levels following plasma exposure in all three cell lines. Although combining plasma with CDDP had no major impact on PD-L1 abundance in SCaBER and RT112, significantly decreased expression compared to CDDP alone was detected in the high-grade cell line T24. This could be of major interest, especially with respect to advanced and aggressive stages

of urothelial cancer, as these frequently result in therapy failures due to high immunosuppressive capacity. Depletion of immune checkpoint inhibitors, such as PD-L1, could therefore impede the blocking and hijacking of cytotoxic T cells, fostering enhanced tumor recognition and improved antitumor immunity. These findings additionally emphasize that checkpoint inhibitors (e.g., pembrolizumab) could further boost plasma-complemented multimodal treatment approaches, delineating a potential prospective research avenue. Likewise, targeting the modulation of epithelial-to-mesenchymal transition (EMT) represents a promising strategy to hinder and reduce the metastatic potential of high-grade tumors. As associated markers are primarily expressed by aggressive phenotypes, levels of CD324 and EpCAM were not markedly altered in SCaBER and RT122 cells by any of the treatments. In contrast, plasma and combination treatment significantly elevated and diminished the expression of CD324 and EpCAM in T24 cells, respectively. Both surface proteins are engaged in the regulation of EMT, and loss or reduction in CD324 expression has been linked to an invasive phenotype in bladder cancer. At the same time, EpCAM overexpression is associated with high-grade urothelial carcinoma [57,58]. In this regard, plasma-combined CDDP therapy showed promising potential to alleviate EMT, being mainly beneficial in high-stage tumors.

Cytokine profiling further revealed distinct response patterns. Both plasma and cisplatin monotherapies significantly altered the cytokine landscape in the TUM-CAM model, albeit with largely nonoverlapping target profiles and comparable overall magnitudes of effect. Notably, monotherapy with either modality was associated with a general trend toward the reduced secretion of multiple cytokines. This likely reflects effective suppression of tumor cell activity and signaling capacity, potentially due to reduced viable tumor mass, metabolic shutdown, or transcriptional inhibition resulting from DNA damage, oxidative stress, or cell-cycle arrest. For example, both monotherapies significantly decreased the levels of IL-1 β , which represents a pro-inflammatory molecule frequently correlated with the progression of muscle-invasive bladder cancer [59]. Furthermore, IL-6 and IL-8, known as predictive biomarkers for advanced bladder urothelial carcinomas [60], were likewise downregulated, underpinning a substantial antitumoral effect of plasma and CDDP monotherapy.

In contrast, combination treatment induced pronounced cytokine increases in a cell line-dependent manner. In SCaBER tumors, the combination significantly elevated most measured cytokines compared with cisplatin alone, including CCL4, IL-10, and TNF- α . These mediators are highly pleiotropic: IL-1 β , IL-18, and TNF- α can promote either tumor-supportive inflammation or antitumor immunity [61], CCL4 can recruit immunosuppressive or cytotoxic immune cell subsets [62,63], and IL-10 can dampen inflammation [64], yet has been associated with poor outcomes in bladder cancer [65]. Collectively, this indicates a broadly enhanced inflammatory and immunomodulatory state under combination treatment, though net functional impact cannot be inferred from concentration changes alone. In T24 tumors, combination treatment selectively elevated IL-1 β , IL-6, IL-8, and IL-18 compared with cisplatin monotherapy. All four mediators are central regulators of inflammation, angiogenesis, and tumor cell behavior, and have been linked to both accelerated tumor growth and enhanced antitumor immunity, depending on timing, magnitude, and cellular source [66,67]. The selective induction of this pro-inflammatory cluster by plasma–cisplatin co-treatment suggests a reprogramming of the tumor-associated cytokine network in T24 that could, in an immune-competent host, substantially shape subsequent immune responses. In contrast, plasma alone primarily displayed higher levels of IL-8 and smaller changes in TGF- β 1 and IP-10, which are involved in proliferation, angiogenesis, and leukocyte recruitment [68–70]. Across all three cell lines, the most abundantly modulated soluble factors converged on a standard set of cytokines and chemokines, underscoring the

centrality of pathways controlling inflammation, immune cell recruitment, proliferation, and angiogenesis in the response to plasma and cisplatin.

In summary, the divergent cytokine responses observed under monotherapy versus combination therapy reflect fundamentally different modes of tumor stress and signaling rather than a simple linear amplification of treatment intensity. Reduced cytokine secretion under monotherapy is consistent with effective suppression of tumor-derived signaling. In contrast, increased cytokine levels under combination treatment likely represent stress-induced inflammatory reprogramming rather than enhanced tumor growth.

Several limitations of this study must be considered when interpreting these data. First, only three bladder cancer cell lines, one squamous and two urothelial, were examined, which may not capture the full molecular and clinical heterogeneity. While the inclusion of non-transformed urothelial cells could provide additional insights, our study focused specifically on therapeutic interactions in cancerous bladder cells. Second, mechanistic investigations were focused on ROS quantification and scavenging in a single cell line. We did not systematically dissect downstream cell death pathways, DNA damage responses, or the detailed functional consequences of the observed cytokine and marker changes. Third, plasma parameters and cisplatin doses were optimized for in vitro assays and then transferred to the TUM-CAM model without comprehensive dose- and schedule-finding, which likely contributed to the limited efficacy in ovo. In addition, the short experimental window inherent to the CAM assay limits the study of long-term tumor progression and chronic treatment responses, which are critical aspects of clinical bladder cancer. Despite these constraints, the TUM-CAM model recapitulates the complexity of the tumor pathophysiology to a high magnitude, eminently bridging the gap between poorly complex 3D in vitro platforms and costly and ethically critical in vivo models. To strengthen the scientific and translational relevance of our study, we employed the in ovo methodology to evaluate how 3D tumor growth and tumor microenvironment-associated aspects, for example, extracellular remodeling, vascularization, and gradual nutrient accessibility, may affect the treatment response. Such CAM-based approaches are increasingly used in preclinical bladder cancer research to study tumor growth, therapeutic response, and mechanisms such as chemotherapy resistance [19,71,72]. Nevertheless, no single model fully captures all aspects of human bladder cancer, with each model having specific strengths and limitations. From a translational perspective, the clinical application of a plasma gas jet to the bladder presents technical challenges, particularly with respect to homogeneous exposure of the urothelium and compatibility with standard clinical workflows [19,73]. However, a first clinical trial to deliver gas plasma-derived reactive species into the bladder via urethrocystoscopy has been conducted and demonstrated promising antitumoral potential [42]. Direct plasma jet treatment was used here as a reproducible tool to define and explore plasma–cisplatin interactions for the first time in a proof-of-concept study, and since it enables direct delivery of short-lived species to the tumor tissues. Clinically, indirect strategies like plasma-treated liquids represent a promising alternative by combining with established intravesical treatments and administered before, during, or after systemic cisplatin-based chemotherapy.

Despite these constraints, the present work provides several important insights. It confirms that cold gas plasma can potentiate cisplatin-induced cytotoxicity in bladder cancer cells in vitro, identifies hydrogen peroxide as a central mediator of plasma and combination effects in at least one cell line, and demonstrates that plasma–cisplatin treatment can reshape expression of key cellular markers and cytokines in a cell line- and context-dependent manner in the TUM-CAM model, promoting a pro-immunogenic and anti-metastatic phenotype. These findings support further exploration of plasma–cisplatin combination therapy with an emphasis on optimizing dosing and scheduling for in vivo

use, expanding mechanistic analyses to include immunogenic cell death markers, DNA damage and repair pathways, redox signaling, and evaluating efficacy in more advanced preclinical models with functional immune compartments.

In conclusion, cold gas plasma represents a promising adjunct to cisplatin-based therapy in bladder cancer, capable of enhancing cytotoxicity and modulating tumor-associated molecular phenotypes. Future studies should aim to refine combination regimens, clarify mechanistic underpinnings, and test therapeutic benefit in models that more closely reflect the clinical situation, with the long-term goal of enabling cisplatin dose reduction without compromising antitumor efficacy.

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Appendix A

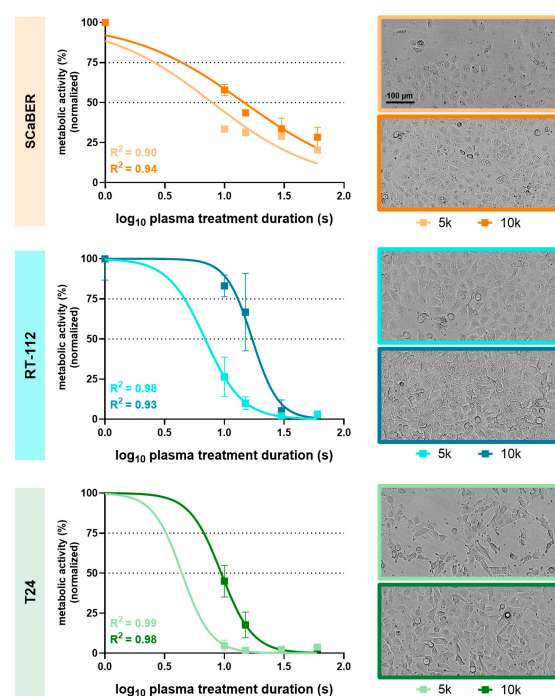


Figure A1. Metabolic activity of SCaBER, RT-112, and T24 cells in response to increasing gas plasma treatment times at different seeding densities.

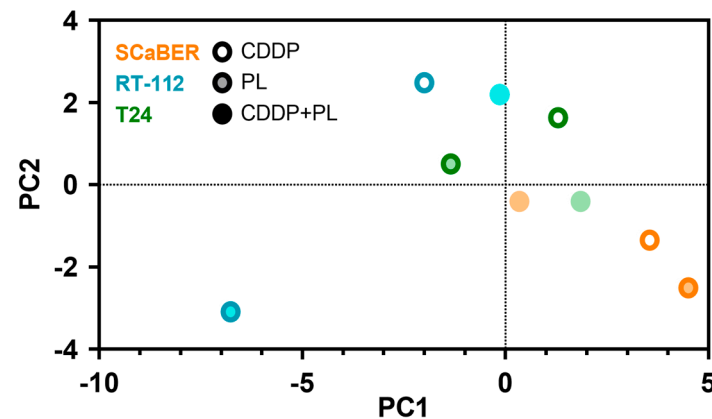


Figure A2. Principal component analysis (PCA) integrating all quantitative parameters obtained from the TUM-CAM experiments, including tumor mass, vessel density, cellular marker expression, and cytokine profiles.

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