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Combined toxicity of indirubins with cold physical plasma in skin cancer cells *in vitro*

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Cold physical plasma is a partially ionized gas that generates various components identified as potential anticancer compounds. Due to its topical application, cold plasmas are suitable, especially in dermatological applications. We, therefore, tested the cold plasma effects in skin cancer cells *in vitro*. An atmospheric pressure argon plasma jet was used as the plasma source. The plasma exposure alone reduced the metabolic activity and induced lethal effects in a treatment time-dependent fashion in both cell lines investigated. This was accompanied by executioner caspases 3 and 7, cleavage indicative of apoptosis and reduced cell migration and proliferation. Recent research also indicated roles of novel indirubin derivatives with potent anticancer effects. Three candidates were tested, and reduced metabolic activity and viability in a dose-dependent manner were found. Strikingly, one compound exerted notable synergistic toxicity when combined with plasma in skin cancer cells, which may be promising for future *in vivo* experiments. © 2022 The Author(s). Published on behalf of The Japan Society of Applied Physics by IOP Publishing Ltd

1. Introduction

Plasma technology is an enabler for many processes in industry and manufacturing. Its properties are especially beneficial for surface modifications and thermo-sensitive surfaces. The latter was possible due to the development of plasma systems operated at low temperatures. This spurred the inherent idea of investigating the potential of such sources for medical purposes, primarily of plasma sources operated at about body temperature.[1)] Throughout the past two decades, significant progress has been made in studying plasma technology for biomedical applications in terms of plasma medicine devices' design, construction, characterization, and biological effects. A few handfuls of plasma systems have even been tested on human probands and patients, and some have entered daily clinical practice in hundreds of medical centers, such as the atmospheric pressure argon plasma jet kINPen.[2)] Its main indication is the treatment of chronic wounds and ulcers. Strikingly, its experimental usage in palliative squamous cell carcinoma patients with ulcerating and infected tumor wounds showed a decline in tumor mass in some of the patients, albeit the intent-to-treat was antimicrobial and anti-fungal effects on the tumor surfaces.[3)]

Skin cancer is a widespread type of malignancy. While some types of skin cancer, such as actinic keratosis and basal cell carcinoma, have a modest malignancy, squamous cell carcinomas and especially melanomas in late stages are frequently causing tumor-related deaths.[4)] Since plasma is a topical treatment option, it appears plausible to test plasma treatment against skin cancer as a novel research line. A number of reports have been published in this regard already.[5)] In addition, plasma therapy could be combined with other compounds. Recently, a component of traditional medicine, indirubin and its derivatives have raised attention among chemists and oncologists to generate new sub-classes of compounds with potential anti-skin-cancer efficacy.[6)]

To this end, we tested here the toxicity of the kINPen argon plasma jet against skin cancer cells *in vitro*, especially with regard to metabolic activity, viability, and migration and motility. In addition, three indirubin compounds were investigated for their anti-skin-cancer properties and afterward combined with plasma treatment. Strikingly, one compound exerted notable synergistic toxicity when combined with plasma in skin cancer cells, which may be promising for future *in vivo* experiments.

2. Materials and methods

2.1. Cell culture

The human cancer cell lines A431 (squamous cell carcinoma; ATCC: CRL-1555) and A375 (skin malignant melanoma; ATCC: CRL-1619) were cultured in fully supplemented cell culture medium, i.e. Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum, 1% glutamine, and 1% penicillin and streptomycin (all Corning, Germany). Cells were cultured under standard conditions at 37 °C, 95% humidity, and 5% CO_2 in a cell culture incubator (Binder, Germany). The day before experiments, cells were seeded at a density of 1×10^4 in 96 well flat-bottom plates or 5×10^5 in 24 well flat-bottom plates (both Eppendorf, Germany) cells per well in 100 μl (96 well well plates) or 500 μl (24 well plates) of fully supplemented cell culture medium. In some experiments, catalase (5 μg ml^{-1}) was added to the culture medium. Therefore, catalase was present in cultures before cold plasma exposure was performed.

2.2. Plasma source and treatment

The atmospheric pressure argon plasma jet kINPen (neoplas, Germany) was employed for plasma treatment. As feed gas, argon (Air Liquide, Germany) was used at two standard liters per minute to ignite the plasma at about 1 MHz with a dissipated power of about 1 W.[7)] During the treatment, the jet was positioned for the indicated time over the geometrical center of each well at the same height, which was ensured by a programmed, motorized *xyz* stage (CNC, Germany) with

100 μm precision. The 24 well and 96 well plates were treated with a customized template. Argon gas alone (plasma = off) was used to control for any effects exerted by the inert gas, which was not the case throughout the study (data not shown). After the plasma treatment, evaporated water was compensated by adding predetermined amounts of double-distilled water to restore iso-osmolarity.

2.3. Small molecules and combination treatment

This study used three different Indirubin-derived small molecules to analyze potential additive effects in combination with cold plasma treatment. Therefore, Indirubin-3′-oxime (I-3oxime) was purchased from Sigma-Aldrich, and the drugs 3-[3′-Oxo-benzo[b]thiophen-2′-(Z)-yliden]−1-(β-D-manno-pyranosyl)oxindole (KD85) and (Z)-3-(3-Oxobenzo[b]thiophen-2(3H)-yliden)indolin-2-one (KD88) were provided externally (see acknowledgments). Briefly, glycosylated isatin was prepared by cyclizing the glycosylated aniline with oxalyl chloride and following condensated with thiaindan-3′-one.[8] Small molecules were initially solved in DMSO and stored at −20 °C. Drugs were further diluted in RPMI1640 medium and added to adherent cells. After incubation, tumor toxicity in response to treatment was determined by evaluating metabolic activity and cell viability. The drugs were left in the cultures across the entire cultivation period of 3 and 24 h, and in some cases, 48 h. For combination treatment, the cells were seeded one night before the start of the experiment, as above, before the drugs were added, or the wells were left untreated. One hour later, the wells were exposed to cold plasma or were left untreated. The drugs were not washed away in combination treatment regimens.

2.4. Metabolic activity

Metabolic activity was investigated by the alamar blue assay. Therefore, 100 μM of 7-hydroxy-3H-phenoxazin-3-on-10-oxid (resazurin; Alfa Aesar, Germany) was added to the cells 20 h after plasma exposure or drug application. Subsequently, cells were incubated for 3–4 h under standard conditions at 37 °C, 95% humidity, and 5% CO_2 in a cell culture incubator. In living cells, non-fluorescent resazurin is metabolized into fluorescent resorufin in a NAD(P)H/H^+-dependent reaction,[9] thereby indicating the cell's metabolic state. Resorufin fluorescence was determined at λ_{ex} 530 nm and λ_{em} 590 nm using a microplate reader (F200; Tecan, Switzerland). In some experiments, to determine the metabolic activity as early as 3 h after treatment, resazurin was added to all wells immediately after cold plasma treatment or drug addition.

2.5. Flow cytometry

Tumor-toxicity of cold plasma and small molecules was characterized 24 h after treatment in 24 well plates using flow cytometry. Cells were stained with sytox blue dead cell stain (ThermoFisher, Germany) and activated caspase 3 and 7 detection reagent (ThermoFisher, Germany) for 30 min at 37 °C. Cells were washed and acquired using flow cytometry (CytoFLEX S; Beckman-Coulter, Germany). Gating and quantifying mean fluorescence intensities were performed using Kaluza 2.1 analysis software (Beckman-Coulter, Germany).

2.6. Generation of GFP-expressing cancer cells by CRISPR-Cas9 genome editing

Prior to transfection of A431 cancer cells, GFP donor DNA was generated and cleaned up using the TrueTag DNA Donor Kit (ThermoFisher, Germany) according to the manufacturer's protocol. Based on general protocols for Alt-R CRISPR-Cas9 mediated genome editing, 6 μl $sgRNA_{ACTB}$ (1 μM; Integrated DNA Technologies), 6 μl Cas9 Nuclease V3 (1 μM; Integrated DNA Technologies), and 88 μl OptiMEM I were incubated for 5 min at room temperature. Subsequently, 100 μl of the formed sgRNA:Cas9 complexes as well as 500 ng of the synthesized N-puro-GFP donor DNA were added to 1×10^6 cells in 400 μl medium in a 24 well plate and were transferred into cells via lipofection with RNAiMax (ThermoFisher, Germany). After cells reached 80%–90% confluency, GFP-positive cells were single-cell sorted using a MoFlo Astrios^{EQ} (Beckman-Coulter, Germany) based on their fluorescence intensity ($\lambda_{ex} = 488$ nm; $\lambda_{em} = 525 \pm 25$ nm) into 96 well flat-bottom plates containing 100 μl of fully-supplemented cell culture media per well. For clone selection after 3–4 weeks of incubation, grown populations were tested for comparability with the A431 wildtype (WT) cell line and their GFP expression intensity using a microplate reader (F200; Tecan, Switzerland). Further, GPF protein expression in response to plasma application was monitored by high content imaging (Operetta CLS; PerkinElmer, Germany) using a 20× air objective (NA = 0.4). Images were acquired in brightfield and fluorescence channels ($\lambda_{ex} = 475$ nm; $\lambda_{em} = 515$ nm) before and 24 h after exposure using Harmony 4.9 software (PerkinElmer, Germany).

2.7. Scratch assay

To examine the impact of cold plasma on cell migration *in vitro*, 1×10^6 cells were seeded in a 24-well plate and incubated under standard conditions (37 °C, 95% humidity, 5% CO_2). After reaching confluence, scratches were performed across the well center using a 200 μl pipette tip. Detached cells were removed through medium exchange, and adherent cells were plasma-treated (60 s). After exposure, the gap closure was monitored with an Axio Observer Z.1 microscopy (Zeiss, Germany) before and 24 h and 48 h after exposure to evaluate cell migration and proliferation[10] within a pilot experiment with three technical replicates per sample.

2.8. Microscopy

High content imaging (Operetta CLS; PerkinElmer, Germany) was used to analyze plasma-triggered apoptosis induction 3 and 24 h after treatment. Briefly, cells were stained with Caspase 3/7 detection reagent (Thermo Scientific, Germany) for 30 min at 37 °C, and images were acquired in brightfield and fluorescence channel ($\lambda_{ex} = 475$ nm; $\lambda_{em} = 515$ nm) using a 20× air objective (NA = 0.4). The experiment and subsequent algorithm-based quantitative image analysis were achieved using Harmony 4.9 software (PerkinElmer, Germany).

2.9. Statistical analysis

Statistical analysis was performed using Prism 9.3 (GraphPad Software, USA). The determination of IC25 values was done using nonlinear regression analysis against log2 transformed exposure times. Data are mean ± standard deviation (SD) of two independent experiments unless otherwise indicated. Two-way analysis of variances (ANOVA) was used to compare difference between groups and statistically significant differences were marked (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

3. Results

3.1. Cytotoxicity in cold plasma-treated skin cancer cells *in vitro*

To evaluate the toxicity of cold plasma in skin cancer cells, two different cell lines were treated with kINPen-generated

plasma [Fig. 1(a)] for different exposure times. The number of viable and terminally dead cells was determined using flow cytometry [Fig. 1(b)]. A plasma exposure-time dependent viability reduction was observed 24 h after treatment in A375 melanoma cells and A431 squamous cell carcinoma cells *in vitro*, with A431 cells showing less susceptibility to plasma-induced cell death [Fig. 1(c)]. Furthermore, iterating the cell concentration (i.e. increasing the cell number while keeping the volume of cell culture medium constant) revealed notable differences in cell survival. While treatment of 5×10^4 and 1×10^5 cells per well reduced skin cancer cell viability similarly, 5×10^5 challenged cells showed no response to plasma-triggered toxicity as indicated by the calculated IC_{25} values [Fig. 1(d)]. Thus, the cell number used to perform experiments influences the plasma sensitivity of cancer cells. The previous results were validated by assessing the metabolic activity of cells with the alamar blue assay 3 and 24 h post plasma treatment [Fig. 1(e)]. A decline of metabolically active cells was found already 3 h after plasma application and was lower following 24 h incubation. ROS are considered primary mediators of plasma-induced biological effects,[11] and catalase was added (an enzyme catalyzing the decomposition of hydrogen peroxide to water and oxygen) as a control condition to confirm the effect of ROS. Catalase reduced the cytotoxic effects of plasma in treated skin cancer cells [Fig. 1(e)]. To understand the molecular basis of plasma-induced cytotoxicity, plasma-treated skin cancer cells tested for activation of executioner caspases 3 and 7, known to be involved in the onset of apoptotic regulated cell death. Caspase activation was found at early (3 h) and late (24 h) after plasma treatment [Fig. 2(a)], confirming apoptosis to be involved in plasma-induced skin cancer cell cytotoxicity. In addition, we wondered whether plasma treatment affects the general protein expression of gene sets required for cellular maintenance. To this end, a stably transduced GFP-positive skin cancer cell line was plasma-treated, and fluorescence microscopy was used to find similar GFP expression in viable GFP-expressing cells 24 h after treatment [Fig. 2(b)]. Next, the question was whether skin cancer cell migration and motility were affected by

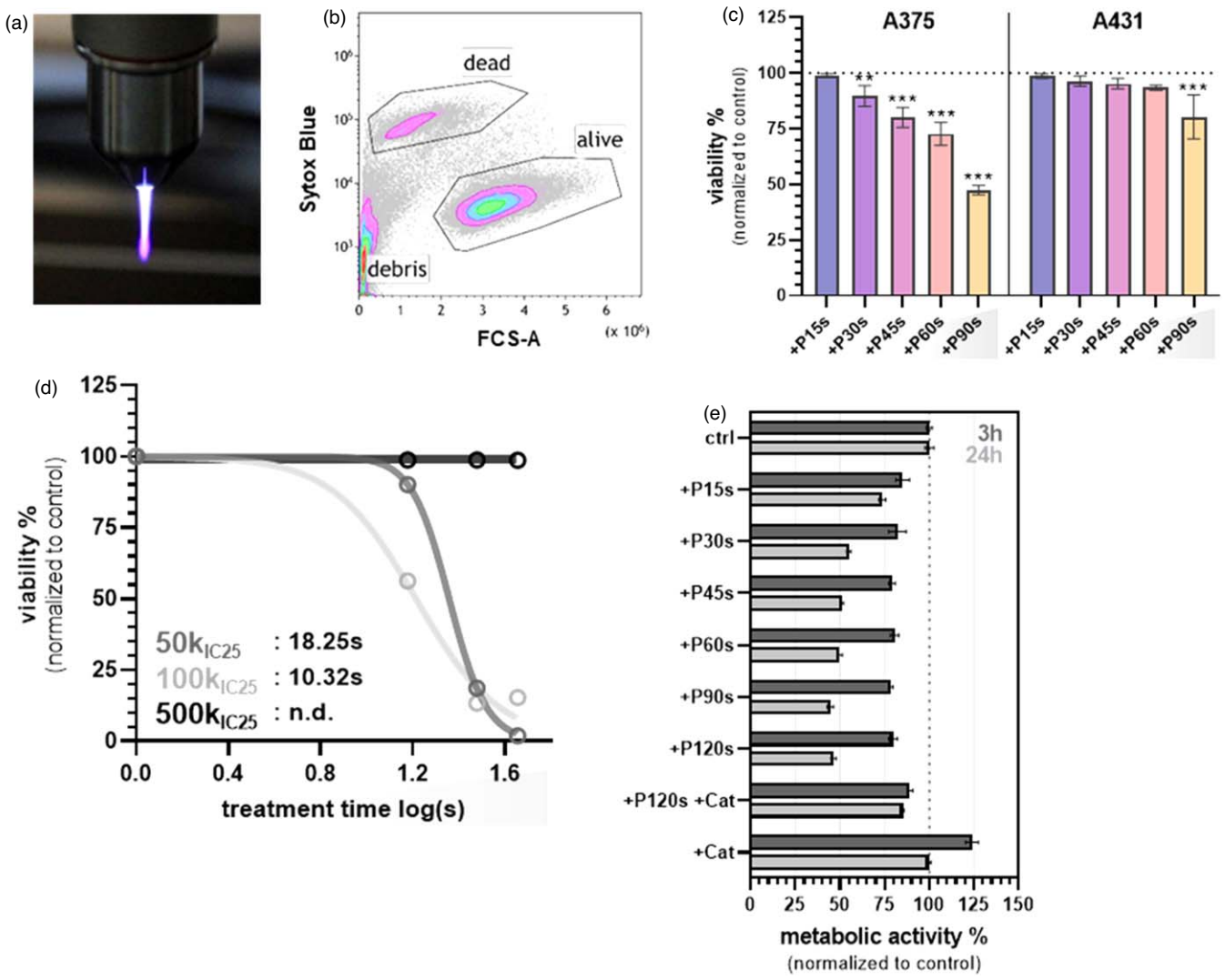

Fig. 1. (Color online) Plasma treatment reduces viability in skin cancer cells *in vitro*. (a) photographic image of the atmospheric pressure argon plasma jet kINPen; (b) representative flow cytometry dot plot showing forward scatter (FSC) against the fluorescent DNA dye sytox blue indicative of terminally dead cells; (c) relationship between plasma treatment time and reduction in viability in A431 and A375 skin cancer cells 24 h post plasma treatment as determined using flow cytometry; (d) relationship between plasma treatment time as well as the number of cells and reduction in viability in A431 skin cancer cells 24 h post plasma treatment and calculated IC_{25} values; (e) metabolic activity of A431 skin cancer cells 3 h and 24 h post plasma treatment and influence of catalase addition added to the cells prior to plasma exposure. Data are one representative of three independent experiments. Cat = catalase.

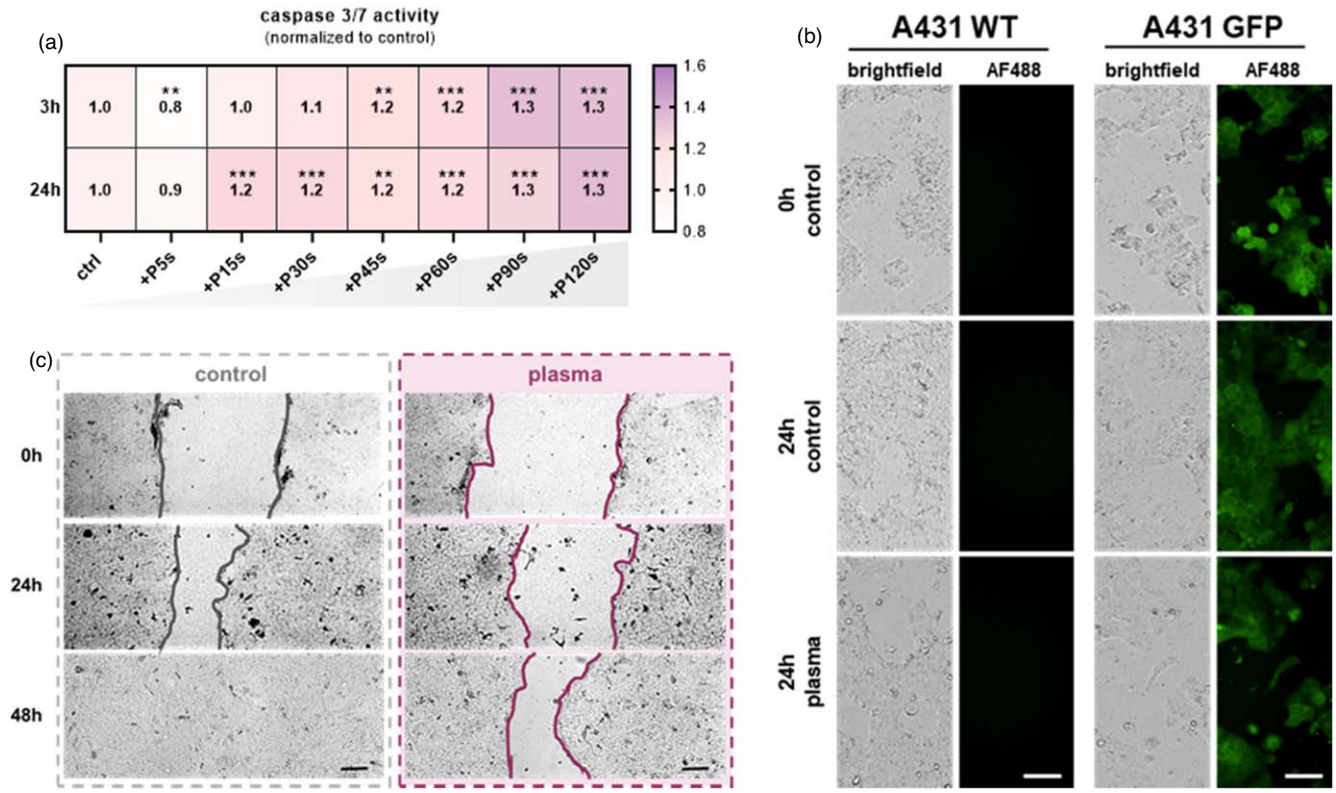

Fig. 2. (Color online) Plasma treatment induces apoptosis via caspase activation and decreases cell motility. (a) quantification of activated caspases 3 and 7 in A431 cells (at least 2000 individual cells per time point and condition analyzed) at 3 and 24 h post plasma treatment as determined using high content imaging analysis; (b) brightfield and fluorescence microscopy of wildtype and stably GFP-transduced A431 cells indicating no loss of GFP translation 24 h after plasma treatment; (c) scratch assay of A431 cells with or without plasma treatment, indicating loss in motility or proliferation. Data are median of one representative of three experiments (a), one representative of several GFP clones (b), and one technical replicate of three replicates within a pilot experiment (c).

plasma treatment. To this end, confluent cell cultures were scratched and exposed to plasma or not, followed by microscopy at different time points. Plasma treatment reduced the ability of skin cancer cells to migrate into and repopulate the gap [Fig. 2(c)].

3.2. Indirubin mono and combination treatment led to a declined

Indirubins were suggested as promising anticancer agents, and we tested three derivates for their cytotoxic effects in skin tumor cells by analyzing their metabolic activity and viability 3 h, 24 h, and 48 h after substance addition to different concentrations. At 3 h, I-3-oxime (I3O) and KD85 already showed some metabolic activity but not viability decline, while KD88 showed no effects [Fig. 3(a)]. This indicated that upon drug addition, toxicity was quickly established but that this was not due to necrotic cell death but rather regulated cell death because the former would have led to elevated numbers of terminally dead cells already at 3 h. At 24 h, IO3 and KD85 but not KD88 markedly reduced viability and metabolic activity [Fig. 3(b)]. Notably, IO3 was effective at concentrations between 10 μM and 100 μM, while KD85 had high tumor cell toxicity already between 1 and 10 μM. One hundred micromolar of KD85 killed all cells in the culture. A similar trend for all drugs and concentrations was observed 48 h after drug addition [Fig. 3(c)]. To test whether the three indirubin derivates showed combined toxicity with plasma treatment, skin cancer cells were plasma-treated, and afterward, the drugs were added at a clinically realistic low concentration of 1 μM. Cellular metabolic activity was assessed 3 and 24 h later. At 3 h, the toxicity of the drugs alone (shown at "ctrl") was modest, and combination effects with plasma treatment were not apparent for all compounds but KD85 (Fig. 4, upper panel). In addition, plasma treatment showed, in principle, a treatment time-dependent effect, as expected. At 24 h, the tendencies of the mono treatments (Figs. 1–3) and the combination treatment at 3 h were confirmed. KD88 alone showed no toxicity (Fig. 3). Hence, combination effects with plasma exposure were the lowest, resulting in the highest metabolic activity rates (Fig. 4, lower panel). Because plasma treatment without any drug even reduced metabolic activity to a greater extent than in the presence of KD88, it can even be speculated that KD88 has antioxidant or protective properties in the tumor cells. I3O alone showed toxicity at higher concentrations, while at a lower concentration in combination with plasma treatment, the resulting toxicity was overall modest and similar to that of the absence of any compound. Strikingly, KD85 showed a synergistic effect with only 5 s of plasma treatment and remained at baseline for longer plasma treatment times.

4. Discussion

The present work investigated the cytotoxicity of cold physical plasma and indirubin small molecules as single and combination treatments in human skin cancer cells. Besides dose-response relationships, we found a notable synergistic effect of plasma treatment with one of the indirubin compounds.

Plasma treatment is known to have anti-cancer properties in skin cancer and various other types of malignancies

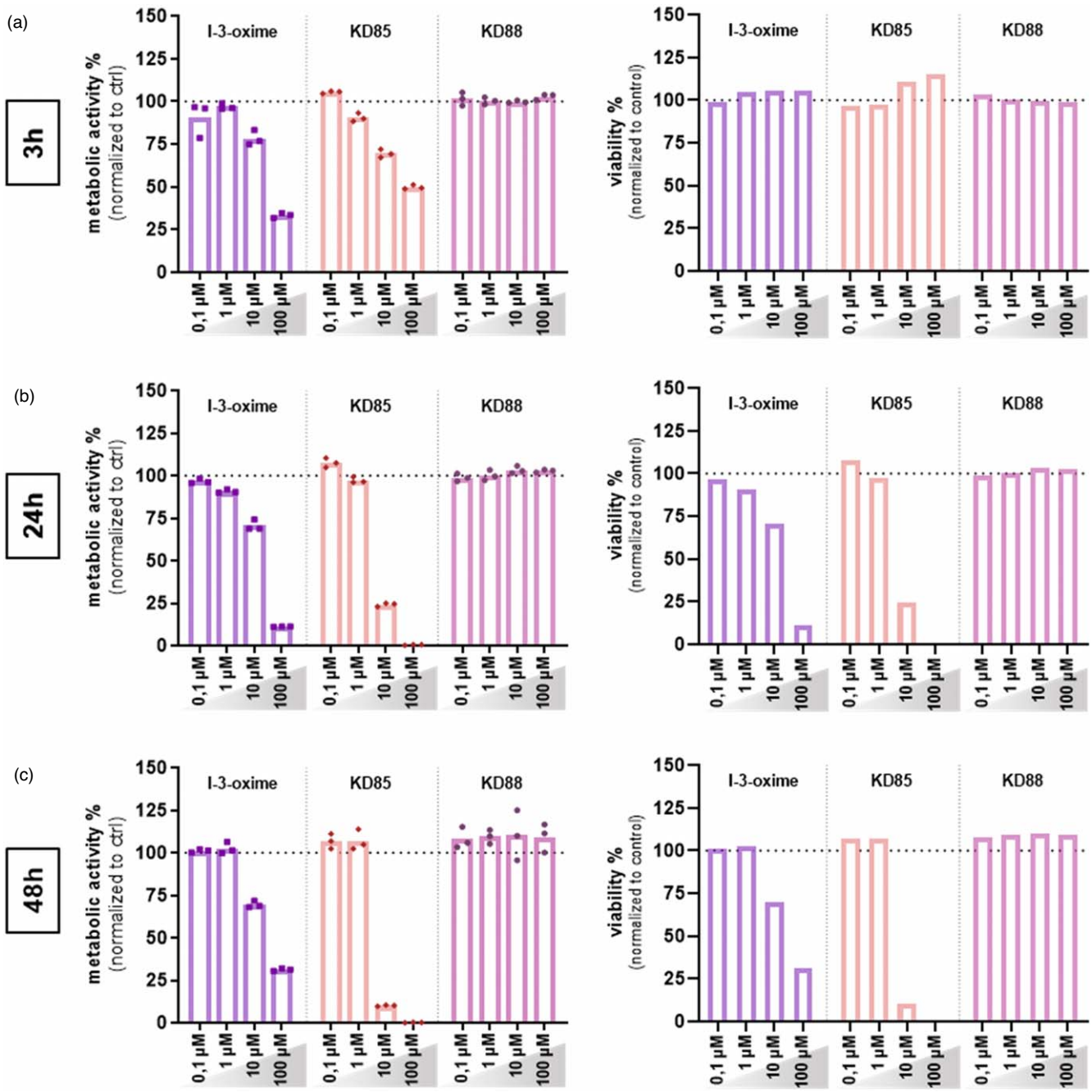

Fig. 3. (Color online) Dose-response relationship between three indirubin compounds and plasma treatment. (a) three indirubin compounds titrated in A431 cells and metabolic activity (left) and cell viability (right) 3 h later; (b) three indirubin compounds titrated in A431 cells and metabolic activity (left) and cell viability (right) 24 h later; (c) three indirubin compounds titrated in A431 cells and metabolic activity (left) and cell viability (right) 48 h later. Data are mean and SD of one representative of three independent experiments for the metabolic activity assays (left side results), while flow cytometry viability analysis (right side results) were done only once to confirm principal findings of metabolic activity analysis.

in vitro and *in vivo*.[5,12,13] Our *in vitro* work showed a decline in metabolic activity and cell viability in a plasma treatment time-dependent fashion, which is in line with previous reports using other plasma sources and skin cancer cell lines.[14,15] Moreover, we found induction of apoptosis to precede cellular demise in plasma-treated skin cancer cells. This cell death pathway is commonly observed in eukaryotic cells following plasma exposure,[16,17] independent of their malignancy status. Along similar lines, a hampered migration and motility in plasma-treated skin cancer cells in our study has been observed in previous reports in plasma medicine,[18,19] also for melanoma cell lines exposed to the cold plasma of the kINPen jet,[20,21] making our findings consistent with the literature. The relationship between cell number and plasma-induced toxicity has been less studied. At higher cell concentrations, the plasma exposure was non-toxic to the tumor cells, even at long treatment times. Considering the many millions of non-malignant and malignant cells present in very tiny tumors of, e.g. a few cubic millimeters only, it becomes clear that the *in vitro* models are limited in assessing the suitability of plasma sources for anticancer treatment in terms of stoichiometric application. More powerful plasma sources with higher energy densities *and* appropriate safety profiles are needed if a decrease in tumor mass is the goal. Alternatively, this could be achieved via multiple treatment sessions as done in the cancer patient

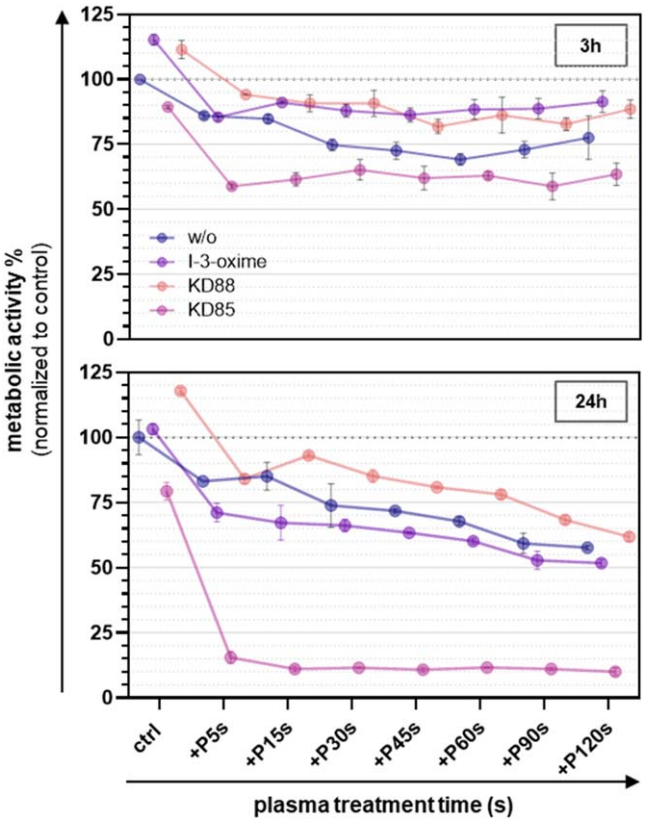

Fig. 4. (Color online) Combined toxicity of indirubins and plasma treatment. Metabolic activity 3 and 24 h after plasma treatment alone (w/o = without) or small molecules alone (indirubins at ctrl = control) or a combination of both in A431 skin cancer cells. Data are mean and SD of one representative of three independent experiments.

study with the kINPen,[3)] or plasma cancer treatment might be rather focused on promoting immunogenic effects,[22)] which was not investigated in the current study.

We identified synergistic toxicity of KD85 indirubin and plasma treatment. Several studies demonstrated combined anticancer toxicity of plasma and chemotherapy,[23–25)] radiotherapy,[26)] or pulsed electric fields.[27,28)] The exact mode of action of KD85 is so far not known. Since the toxicity of even higher KD85 levels was low at 3 h, the drug likely induces regulated cell death by sensitizing tumor cells to ROS as only 5 s plasma treatment already largely abrogated cell viability. We have observed a similar phenomenon previously in melanoma cells but not non-malignant HaCaT keratinocytes using ADDA 5, a potent inhibitor of cytochrome C oxidase (complex IV in mitochondrial membranes). After cytochrome IV blockade, which by itself was only of low toxicity, the tumor cells became exceedingly sensitive to plasma-induced cytotoxicity by endogenous ROS amplification and ATP crisis.[29)] Therefore, it can be speculated that KD85 interferes with mitochondrial pathways. Future studies will shed more light on molecular details of the combined effects of cold plasma and the indirubin KD85 in cancer cells.

There was a notable discrepancy between gas-treated and 120 s plasma-treated cells compared to untreated control cells when catalase was added before treatment [Fig. 1(e)]. Our previous studies have shown that catalase fully scavenges plasma-derived hydrogen peroxide in treated liquids.[30)] This treatment fully protected eukaryotic cells from plasma-induced cytotoxic effects,[31)] which was not the case in the current study as catalase abrogated most but not all plasma-mediated metabolic activity reduction. There has been speculation that cold plasma treatment leads to enzyme inactivation, explaining the lower protection. For instance, RNAse, a principally stable enzyme, was shown to be terminally inactivated using extended cold plasma exposure.[32)] Yet, we do not believe in having rendered catalase fully inactive, as partial protection was still achieved. Instead, other species may have contributed to cold plasma-mediated cytotoxicity and were targeted by the antioxidant enzyme catalase. These species could have also led to potential modifications to the indirubin compounds, thereby affecting their action on the cells. For the plasma jet kINPen, it was recently shown that its short-lived reactive species repertoire could oxidatively modify amino acids,[33,34)] which also affected cellular metabolic activity and cytokine release.[35)] In addition, plasma-derived reactive species were shown to modify lipids, also from human and mouse-derived samples.[36,37)] Moreover, plasma-treated protein, such as the model protein ovalbumin, was rendered more immunogenic following oxidative post-translational modifications (oxPTMs).[38)] With regard to the action of the drug, we do not believe the plasma to have rendered its structure significantly less cytotoxic. Already 3 h post-exposure, indirubins showed toxic effects in cells [Fig. 3(a)], suggesting a quick intracellular action and onset of regulated cell death. Nevertheless, it would be interesting to investigate potential drug modifications and their functional consequences after plasma treatment in future studies.

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Conflict of Interest

The authors have no conflict of interest to declare.

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