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Direktor: Univ. -Prof. Dr. med. Joachim Windolf

Enhancement of human bladder carcinoma cell chemosensitivity to Mitomycin C through quasi-monochromatic blue light (λ=453nm)

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Lisa Hegmann

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„The most courageous act is still to think for yourself. Aloud.”

Coco Chanel

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Zusammenfassung

In der Medizin wird in den verschiedensten Bereichen nach immer neuen Therapieoptionen gesucht. Ob in der Immunologie, Rheumatologie oder auch der Chirurgie – konstant wird nach neuen Erkenntnissen geforscht, die es möglich machen, Erkrankungen früher zu erkennen, effektiver zu behandeln oder im besten Falle heilen zu können. Ein Fachgebiet, auf dem dabei wohl am meisten Forschung betrieben wird, stellt das weite Feld der Karzinomerkrankungen dar. Besonders in der westlichen Welt stellen die ‚bösartigen Tumorerkrankungen' einen beachtlichen Anteil der Todesursachen von Patienten dar. Diese Arbeit befasst sich mit Tumorerkrankungen aus dem Urogenitaltrakt. Weltweit wurden im Jahr 2020 etwa 573 000 Neudiagnosen allein des Blasenkarzinoms verzeichnet. Auf Grund dieser beachtlichen Anzahl sowie der insgesamt relevanten Tendenz des Tumorrezidivierens bzw. -progresses im Verlaufe der Erkrankung gibt es das klare Bestreben nach effizienteren Therapieoptionen. Neben bereits etablierten Verfahren wie der chirurgischen Tumorentfernung und chemotherapeutischen Behandlung gibt es diverse neuere immunmodulatorische Ansätze, die Patienten einen Überlebensvorteil gewähren sollen. Diese Arbeit beschäftigte sich mit der Kombination zweier Therapieoptionen mit dem Ziel eine effizientere Zelltoxizität zu erreichen. Dazu wurde das bereits etablierte und für die Therapie des Urothelzellkarzinoms zugelassene Chemotherapeutikum Mitomycin C verwendet, dessen Effektivität durch die Kombination mit einer zeitlich vorgeschalteten Phototherapie gesteigert werden sollte. In den durchgeführten *in-vitro* Experimenten erfolgte zunächst die Behandlung der Karzinomzellen mit einer Bestrahlung durch blaues Licht der Wellenlänge von 453 nm. Ein für sich betrachtet nicht toxischer Stimulus, welcher über die Entkoppelung der mitochondrialen Atmungskette zu einer vermehrten Produktion von reaktiven Sauerstoffspezies führte und zusätzlich durch eine signifikant erniedrigte Produktion von ATP charakterisiert wurde. Anschließend wurden die Zellen mit der oben erwähnten, DNS interkalierenden Chemotherapie versetzt. Wir konnten zeigen, dass durch die Vorschädigung und die damit einhergehende reduzierte regenerative Kapazität der Tumorzellen eine gesteigerte zytotoxische Kapazität der Chemotherapie mit Mitomycin C erreicht werden konnte. Anhand verschiedener, den Zellstoffwechsel abbildender Experimente konnten zudem genauere Aussagen bezüglich der erreichten additiven Toxizität der Kombinationstherapie aus Photo- und Chemotherapie getroffen werden.

Summary

In medicine, new therapy options are always sought in a wide variety of areas. Whether in the field of immunology, rheumatology or surgery, new findings are constantly being searched that make it possible to detect diseases earlier, treat them more effectively or, in the best case, to cure them. One field in which most research is probably carried out is the wide field of carcinoma diseases. Especially in the Western world, these, summarized under the widespread term of malignant tumor diseases, represent a considerable proportion of causes of patients' death. This work deals with tumor diseases originating from the genitourinary tract, the urothelial cell carcinomas. Globally, approximately 573,000 new diagnoses of bladder cancer have been recorded in 2020.

Due to this relevant number of affected patients plus the condition's tendency of recurrence or tumor progress in the course of the disease, there is a clear desire for more efficient therapy options. In addition to established procedures such as surgical tumor removal and chemotherapeutic treatment, there are various newer therapeutic approaches in the field of immunological tumor defense that are intended to give patients a survival advantage. In this work, we dealt with the combination of two therapy options in order to achieve a more efficient cell toxicity. In these *in-vitro* experiments we used Mitomycin C, a chemotherapy which is already established and approved for the treatment of urothelial cell carcinoma, whose effectiveness was sought to be increased by combining it with phototherapy. First, the urothelial carcinoma cells were treated with irradiation of blue light, at a wavelength of 453 nm. Taken individually, a non-toxic stimulus, which leads to an increased production of reactive oxygen species through the decoupling of the mitochondrial respiratory chain. Subsequently, the cells were added up with the DNA alkylating chemotherapy, mentioned above. Due to the previous damage and the associated reduced regenerative capacity of the tumor cells, an increased cytotoxic efficiency of Mitomycin C could be detected. On the basis of various experiments depicting cell metabolism, more precise statements could be made about the achieved synergistic toxicity of the combined therapy of photo- and chemotherapy.

List of Abbreviations

5-ALA	5-amilolevulinic acid
°C	Degrees Celsius
A	Ampere
ATP	Adenosine triphosphate
BAX	Bcl-2-associated X protein
BCG	Bacillus Calmette-Guérin
CO_2	Carbon dioxide
(c)PARP	(cleaved) Poly(ADP-ribose) polymerase
DCFDA	Dichlorofluorescein diacetate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DNS	Desoxyribonuklein-säure
ECAR	Extracellular acidification rate
EDTA	Ethylene-diaminetetraacetic acid
EORTC	European Organisation for Research and Treatment of Cancer
FACS	Fluorescence activated cell sorting
FCCP	Cyanide-p-tri-fluoromethoxyphenyl-hydrazone
FDA	Fluorescein diacetate
h	Hours
H_2O_2	Hydrogen peroxide
J/cm²	Joule per square centimeter
LED	Light-emitting diode
mg/ml	Milligram per milliliters
µg/ml	Microgram per milliliters
mm	Millimeters
MMC	Mitomycin C
µm	Micrometers
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mW/cm²	Milliwatts per square centimeter
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
nm	Nanometers
NOX	NADPH oxidase family
Nrf2	Nuclear factor erythroid 2-related factor 2
O_2^-	Superoxide anion
OCR	Oxygen consumption rate
PBS	Phosphate buffered saline
PD-1	Programmed cell death 1 receptor
PDT	Photodynamic therapy
PTT	Photothermal therapy
PJ	Propidium iodide
ROS	Reactive oxygen species
RPMI1640	Roswell Park Memorial Institute internal medium code 1640
TURBT	Transurethral removal of bladder tumor
U	Electric voltage
uBC	Urothelial bladder carcinoma
UVA	Ultraviolet A
W	Watt
XF base medium	Xeno-free medium
XOR	Xanthine oxireductase

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Introduction

Cancer is, in addition to cardiovascular diseases, among the most common causes of death, especially in Western countries. In recent years to decades, these incidences have been even more increasing, whilst the exact cause of this phenomenon is not pretty clear. [2] Among others, rising age and a wide variety of environmental factors, as well as various combination of these factors play a role in this multifactorial disease. Regardless of the underlying mechanisms, the increasing numbers require the need to constantly re-evaluate and optimize diagnostics and therapies of carcinoma diseases. Due to heterogenicity of cancer diseases there is a need to take a look on every cancer entity individually. In this context, tumors of the genitourinary tract are a not inconsiderable part of tumor diseases. In addition to renal cell carcinoma, urothelial cell carcinoma is the second most common tumor entity of the genitourinary tract. With globally approximately 573,000 new diagnoses of bladder cancer and about 210,000 deaths in 2020, urothelial carcinoma is responsible for a relevant proportion of the total worldwide carcinoma-related deaths. [2-5]

The primary risk factors include age, as the most significant one, tobacco smoking, occupational exposure to aromatic amines, or exposure to ionizing radiation. A familial history is rather rare in relation to urothelial bladder carcinoma. These tumors typically become symptomatic through hematuria or irritation syndromes as frequent urination. A subsequent diagnosis is mostly made through a cystoscopy and biopsy sampling. [6]

In general, therapy of urothelial carcinoma contains – depending on the tumor stage and localization – of organ preserving surgery as first line therapy if possible. Mostly, it is followed by a so-called adjuvant drug therapy. Usually it consists of single or combined chemotherapy, applied in local administration as a bladder instillation after surgery, or systemically. [7]

Another therapeutic option is a bladder instillation using Bacillus Calmette-Guerin (BCG), specifically derived from tuberculin bacteria, which triggers a bacterial cystitis with granuloma formation. Leading to the attraction of granulocytes and lymphocytes, it thereby unfolds its effect through inducing an anti-tumor immune response. [3, 8-10]

Nevertheless, 31 – 78% of affected patients develop recurrence over a period of 5 years after diagnosis. [11-13] This includes new manifestations at other locations of the genitourinary tract on the one hand, as well as recurring tumors in the same, already treated areas.

Additionally, there is the risk of tumor progression in the sense of a local spread of tumor masses as well as developing distant metastases, often in organs like lung, liver or bones. [14] Local growing of tumor masses as well as metastasized tumors have extensive effects on patient's morbidity and prognosis in the sense of long-term survival, as the median survival of patients with recurrent or metastatic bladder carcinoma remains about 14 – 15 months. [15] Taking this into account, it illustrates the need for additional therapy options.

A kind of obvious approach to avoid chemotherapy resistance is to combine existing therapeutic agents e.g. by using polychemotherapy. For urothelial carcinoma, polychemotherapy consists of methotrexate, vinblastine, doxorubicin and cisplatin, especially, for higher-grade tumors or more advanced diseases. Due to the high number of side effects such as fatigue, polyneuropathy up to bone marrow toxicity, including anemia, or infections up to septic diseases, the effect of polychemotherapy is limited through the morbidity of the treated patient. Here, it has to be considered, that most patients with urogenital carcinoma are part of the elderly population with already existing comorbidities. [3, 16] Again, there still remains desire for more effective carcinoma therapies with less side effects resulting in better compliance.

Furthermore, recent therapeutic approaches consider the need for more targeted therapy options for cancer in general. One recently tested option is Pembrolizumab, which belongs to the class of immune checkpoint inhibitors and inhibits PD-1. It shows complete responses in 46% for at least one year in circumscribed patient populations with high-risk BCG-unresponsive non-muscle-invasive bladder cancer. In general, blocking the interaction between the programmed cell death 1 receptor (PD-1) and its ligand has shown promising results in reactivation of anti-tumor immunity. [3] Nevertheless, overall, there remains a considerable residual risk for disease progression and recurrence of urothelial carcinoma.

A possible further approach to increase therapeutic effectiveness refers to the combination of an already established chemotherapy with other, non-primarily toxic therapy options. Similarly, there is an increasing desire for more specific therapy in the field of cancer treatment. In this context, photodynamic therapy (PDT) and photothermal therapy (PTT) have significantly grown in recent years. The objective is to specifically destroy the tumor tissue while avoiding damage to the surrounding healthy tissue. This idea is intended to represent a kind of advancement of the, locally-focused, non-selective chemotherapy. [17]

Phototherapy, a therapy widely used in other areas of medicine, such as dentistry or dermatology, would be a conceivable approach. In these fields, for example blue light irradiation is used for bleaching, polymerization of dental composites or as a clinical treatment of acne. [18-21]

In most cases of PDT, a special agent works as a photosensitizer which itself has no, or only a weak effect on cells. The photosensitizer is activated, by a catalyst e.g. locally applied light, absorbs light at a specific wave length and transfers the energy in order to convert it into a biologically active substance. While the photosensitizer is often applicated systemically, it is possible to activate the therapeutic effect only in a local manner to avoid systemic side effects. In the field of PTT, near infrared light is typically used to achieve localized photothermal effects. [17, 22]

In this study, we focused on blue light at a wave length of 453 nm, as it is part of daily and visible light which, unlike UVA light, is not cytotoxic itself. [23] Preliminary work has shown that light at this wave length is able to activate various photosensitizer. As Teixeira et al. could show, irradiation with blue light at a wave length of 470 nm is able to activate doxorubicin in MDA-MB-231 breast cancer cells. Regarding experimental setup, the breast cancer cells have been incubated with doxorubicin for two hours before blue light irradiation. After completed irradiation they could observe a time dependent increase of reactive oxygen species, accompanied by a decrease in cell viability in the combined treatment group. [24] Likewise results could be perceived as Lin et al. combined the dye acridine orange with blue light irradiation in human bladder cancer cells. When contemplated separately, both stimuli had almost no effect on the tumor

cells. However, if combined, a reduction in cell viability and an increased Caspase-3 and -7 activity could be measured. [25]

In addition, recent studies, including work from our own research group, could investigate that blue light on its own is able to inhibit proliferation, migration and differentiation of cells in promyelocytic leukemia, melanoma as well as colon carcinoma cells. [23, 26, 27] The underlying mechanisms of irradiation with blue light is based on an increase in reactive oxygen species resulting in a collapse of the mitochondrial respiratory chain through the interaction with flavin containing proteins. [22, 28-30]

Aim of this work was to investigate a possible additive or synergistic effect of blue light therapy in urothelial carcinoma cells, represent as an example by the cell line RT112, by combining it with the standard chemotherapeutic drug Mitomycin C. As an DNA alkylating drug, Mitomycin C leads to double-strand breaks and inhibits proliferation of cells. [31]

As blue light is part of visible light, it is easily accessible and can be applied locally. Because of its low penetration depth, it can be used safely without damaging the surrounding tissue. [32] Thus, in the absence of self-toxicity, we regard blue light as a useful therapeutic partner combined with Mitomycin C. By increasing cytotoxic effects of already applied chemotherapy without increasing the burden of side effects blue light irradiation could be a promising new adjuvant therapy option, which is highly needed in cancer therapy.

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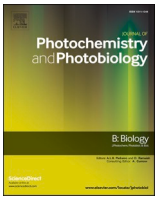

Enhancement of human bladder carcinoma cell chemosensitivity to Mitomycin C through quasi-monochromatic blue light (λ = 453 ± 10 nm)

Lisa Hegmann [a], Sofia Sturm [a], Günter Niegisch [b], Joachim Windolf [a], Christoph V. Suschek [a,*]

[a] Department for Orthopedics and Trauma Surgery, Medical Faculty and University Hospital Düsseldorf, Heinrich-Heine-University Düsseldorf, Germany
[b] Department of Urology, Medical Faculty and University Hospital Düsseldorf, Heinrich-Heine-University Düsseldorf, Germany

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ABSTRACT

Human urothelial bladder carcinoma (uBC) is the second most tumor entity of the urogenital tract. As far as possible, therapy for non-muscle invasive uBC takes place as resection of the tumor tissue, followed by intravesical chemotherapy or immunotherapy. Because of the high recurrence rate of uBC, there is a need for improved efficiency in the treatment. In the present in vitro study we have evaluated a new approach to enhance the cytotoxic efficiency of Mitomycin C (MMC), which is commonly used for intravesical treatment of uBC on the relevant urothelial cancer cell line RT112. For that we used quasi-monochromatic blue light (453 ± 10 nm) at its non-toxic dose of 110 J/cm^2 as an additive stimulus to enhance the therapeutic efficiency of MMC (10 μg/ml). We found, that blue light exposure of RT112 cells led to a very strong increase in intracellular production of reactive oxygen species (ROS) and to a significant reduction ($p < 0.05$) of all function parameters of mitochondrial respiration, including basal activity and ATP production. Although not being toxic when used as a single impact, together with MMC blue light strongly enhanced the therapeutic efficiency of MMC in the form of significantly enhanced cytotoxicity via apoptosis and secondary necrosis. Our results clearly show that blue light, most likely due to its ability to increase intracellular ROS production and reduce mitochondrial respiration, increased the cytotoxic efficiency of MMC and therefore might represent an effective, low-side-effect, and success-enhancing therapy option in the local treatment of bladder cancer.

1. Introduction

Urothelial bladder carcinoma (uBC) is the second most tumor entity of the genitourinary system. New cases in the year 2020 amount to more than 573.000 with a number of deaths of more than 210.000 patients worldwide. About 75% of newly diagnosed urothelial bladder carcinoma belong to the group of non-muscle-invasive tumors (Ta, T1, Cis) [1,2]. Of these, 20–25% progress to muscle-invasive disease during patients' lifetime [3]. Whereas muscle-invasive tumors normally have to be treated by radical cystectomy, therapy of non-muscle-invasive tumors is focused on retaining the bladder. According to the guidelines [1], first line therapy of locally limited tumor is resection of the tumor tissue as a transurethral removal of bladder tumor (TURBT). Although the tumor tissue can be eradicated by this, the recurrence rate is relatively high. About 31% - 78% of the patients will develop a recurrence or new occurrence of urothelial carcinoma within 5 years [4].

In order to delay progression of the tumor or reduce recurrence, resection is mostly followed either by chemotherapy as an intravesical infusion within 24 h post TURBT (so-called early instillation therapy). Most commonly, Mitomycin C (MMC) is used in this setting [5]. Combining these two treatments could significantly decrease the recurrence risk by 35% but nevertheless a significant part of patients, especially intermediate- and high-risk patients according to the EORTC classification will develop a relapse within 5 years [6]. Therefore, additional therapeutic approaches to reduce the rate of recurrence for these patients are either intravesical *Bacillus Calmette-Guérin* (BCG) or chemotherapeutic instillations post TURBT applied as several repetitions over a longer period of time [1,7–9]. Again, MMC is one of the chemotherapeutics most commonly applied in this setting. While notable side effects of the BCG instillation, for example cystitis in up to 90% of the patients, as well as fever, granulomatous inflammation of the prostate gland, pneumonitis, bladder contracture or even anaphylaxis have been described, MMC instillations are generally well tolerated [10,11]. However, retrospective analysis suggest that while both MMC and BCG significantly decrease recurrence rate, MMC seem to be inferior with regard to the prevention of tumor progression towards muscle

* Corresponding author at: Department of Orthopedics and Trauma Surgery, University Hospital Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany.
E-mail address: suschek@uni-duesseldorf.de (C.V. Suschek).

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invasion [12].

As a result, there is a clear desire to improve adjuvant therapy of non-muscle invasive uBC. This can be achieved by increasing the specificity or by increasing the effectiveness of the chemotherapy. As a result, lower doses of the chemotherapeutic agent could be used to achieve the desired therapeutic effect, which would both reduce treatment costs as well as therapeutic burden of the patient. However, even more important, this may retard tumor growth and reduce both the rate of cancer recurrence and progression.

A very simple but extremely effective adjuvant therapy would be the combination of a chemotherapeutic agent and phototherapy. Phototherapy on its own is already an established and widely used form of therapy [13]. Either the direct therapeutic effect of light is used, or the light effect is only achieved through the use of photosensitizers or is further intensified by them [13,14]. For example, doxorubicin was shown acting as a photosensitizer, if activated by blue light irradiation (450 nm), and leads to an increase in oxidative damage in the form of enhanced intracellular generation of reactive oxygen species (ROS) which in turn leads to increased cytotoxicity and decreased cell viability in the treated area [15]. Another example is the combined use of acridine orange, an DNA intercalating dye which can be activated by blue light at approximately 450 nm. As single stimuli both of them do nearly have no effect on the human bladder carcinoma cells, but combined, the treatment is able to significantly reduce tumor cell viability and enhance the activity of caspase-3 and caspase-7 [16]. In general, blue light of the above-mentioned wavelength of approximately 450 nm is a very interesting medium for modulating cell viability, whether used alone or in combination with other therapeutic approaches. Although blue light, in contrast to UVA light, is not toxic or cell-damaging even in very high doses of up to 400 J/cm^2, it can inhibit the proliferation, migration and differentiation of fibroblasts and keratinocytes from 80 J/cm^2 [17–19]. Additionally, an inhibition of cell proliferation, migration and a reduction of cell viability could be shown in melanoma, colon carcinoma and promyelocytic leukemia cells, if irradiated with blue light LED [20–22]. Furthermore, it could be observed that blue light exposure leads to a collapse of mitochondrial membrane potential due to increased ROS generation suggesting the induction of mitochondrial damage [23–25]. In general, it seems to be the case that blue light via the interaction with flavin residues of flavin-containing enzymes not only initiates the production of ROS, which in principle is damaging, but also substantially reduces or inhibits the energy metabolism of the cell through the interaction with corresponding enzyme systems [26].

In particular these properties of blue light that represent a very promising aspect of its therapeutic efficacy, especially when combined with another potent therapeutic agent. The study presented here evaluates whether and to what extent blue light has an additive or synergistic effect on the cytotoxic behavior of Mitomycin C towards urothelial bladder cell carcinoma cells. From a therapeutical point of view, the establishment of a novel therapy option for the treatment of urothelial bladder cell carcinoma, consisting of the use of an established chemotherapeutic agent such as mitomycin C and blue light would be a great success. We expect that the application of blue light will significantly strengthen or increase the cytotoxic effect and thus the therapeutic result of mitomycin C on the tumor cells. In addition to this highly relevant biological significance, this would mean that the therapeutic window would widen, making it possible to reduce the maximum dose of the therapeutic to achieve the same effect as otherwise only with the maximum dose of the drug. As a result, one could be able to significantly reduce the dose of chemotherapeutic agents that burden the patient, but also to individually adjust the patient's drug exposure according to the need for therapy.

2. Materials and Methods

2.1. Materials

If not otherwise indicated, all chemicals, antibodies, and assay kits were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). The human bladder carcinoma cell line RT112 was kindly made available by Dr. J. Fogh (New York, NY), Dr. M. A. Knowles (Leeds, UK) and Dr. B. Grossman (Houston, USA).

2.2. Cell Lines and Cell Culture

In our experiments we used the urothelial cancer cell line RT112 showing an epithelial-like morphology [27]. The RT112 cell line was cultivated in the form of adherent monolayer in RPMI 1640 Medium containing 1 g glucose/L (Life Technologies Ltd.; Paisley, UK), supplemented with 5% heat inactivated fetal bovine serum (Fetal Bovine Serum Gold, PAA Laboratories GmbH; Cölbe, Germany), 1% Penicillin/Streptomycin (PAN-Biotech GmbH; Aidenbach, Germany) and 1% sodium pyruvate (Life Technologies Ltd.; Paisley, UK) in T175 culture flasks (CELLSTAR® Cell Culture Flasks 175 cm^2 red filter cap, Greiner Bio-One GmbH; Kremsmünster Austria). Reaching a confluence of 70–80%, cells were sub-cultured. In order to detach cells, cultures were incubating with 1% trypsin/EDTA solution (PAN-Biotech; Aidenbach, Germany) for 5 min at 37 °C. Trypsin activity was neutralized by using a trypsin neutralizer (Life Technologies Ltd.; Paisley, United Kingdom). Cell cultures were maintained in a humidified incubator (95% air and 5% CO_2 at 37 °C).

2.3. Blue Light Emitting Source

The light source used was designed by Philips Research (Aachen, Germany) and the irradiance of the device was characterized using an Ulbricht sphere. The light source was a LED array in which 60 LEDs were evenly mounted on a flat rectangular circuit board of 12 × 10 cm. The electrical and optical device parameters were: $I = 1.75$ A, U = 40 V_{DC}, $P_{in} = 69$ W, optical output power = 13 W, LED maximum intensity was 0.21 W/nm at 453 nm, emission spectrum was 453 ± 10 nm.

Cell cultures in the current study were irradiated for 2820 s with an irradiance of 39 mW/cm^2 at a distance of 5 cm resulting in a fluence of 110 J/cm^2. Routinely, we tested the heat development during the irradiation of 2 ml PBS in a 6-Well cell culture plate (Greiner Bio-One GmbH; Kremsmünster, Austria) corresponding to our experimental setup. We observed an increase in temperature of 1–2 °C during the 10 min light exposure but sample temperature never exceeded 33 °C. The degree of evaporation we determined as the result of the irradiation was so low that the possible osmotic effects of the light-exposed sample were negligible. In order to achieve comparable conditions, cell culture plates containing the control samples were located in a windowed heating cabinet at 33 °C.

2.4. Chemotherapy

Mitomycin C (MMC; medac GmbH; Wedel, Germany), an alkylating chemotherapy which binds covalently to the DNA, leads to cross-linking and therefor inhibits the replication of the DNA [28] was obtained from the local university pharmacy at a concentration of 1 mg/ml. Due to data of preliminary works [29–31] and our own experiments we used MMC in a concentration of 10 μg/ml for all further experiments.

2.5. Experimental Setup

For experiments, cells were transferred to transparent multi-well culture plates and were cultivated in culture medium overnight at 37 °C and 5% CO_2 to achieve adherence and a nearly confluent layer. Prior irradiation with blue light (110 J/cm^2 with 453 nm) culture

medium was replaced by PBS (2 ml /well of the 6-well plates, 1 ml for 12-well plates, 0.5 ml for 24-well plates, 200 µl for 96-well plates). After light exposure, PBS was replaced by culture medium without or containing 10 µg/ml Mitomycin C. At time points indicated cells or cell samples were collected for the different analyses.

2.6. MTT Assay

At time points indicated cells were incubated in the dark with 100 µl MTT solution (Thiazolyl Blue Tetrazolium Bromide) reaching a final concentration of 0.5 mg/ml for 2 h at 37 °C. After aspiring the solution, 200 µl of DMSO was added and again incubated for 10 min. Afterwards 100 µl of this solution was transferred to a microtiter plate and absorbance was measured by a multilabel counter (Multilabel Counter VICTOR™ V Multilabel counter, Perkin Elmer; Waltham, United States) at a wavelength of 590 nm.

2.7. Detection of Apoptosis

Apoptotic events in the treated cell cultures in the form of relative amount of hypodiploid nuclei were quantified by FACS analysis (Flow cytometer FACS Calibur, BD Bioscience; Heidelberg, Germany) by the method described by Riccardi and Nicoletti [32]. As positive control we used the effective apoptosis-inducing agent Staurosporin at a concentration of 1 µg/ml.

2.8. Immunofluorescence-Based Quantification of Living, Apoptotic, and Necrotic Cell Death

In order to differentiate and quantify living, apoptotic, and necrotic cell death we used three analytic fluorescence dyes. Respective cell cultures were incubated for five minutes with fluorescein diacetate (FDA, 2 µg/ml; Santa Cruz Biotechnology; Texas, United States), Hoechst 33342 dye (0.5 µg/ml), and/or propidium iodide (PJ, 0.5 µg/ml). The non-fluorescent FDA enters into viable cells and after cleavage by active esterases FDA can be detected as a green fluorescent coloring of living cells, as [33], H33342 stains the chromatin of living cells by blue fluorescence and thus allows to evaluate nuclear morphology of apoptotic cells, whereas PJ only penetrates “leaky” membranes and thus is an excellent indicator for necrotic cells which glow in red fluorescence [33].

2.9. Cellular ATP Content

To explore the path of cell death due to the chemotherapy, influenced by the irradiation of blue light more detailed, we investigated the ATP level in the cells after treatment. In this case we used the ATP Assay (ATP Kit #LBR-T010, Biaffin GmbH&Co KG; Kassel, Germany) according to the manufacturer’s specifications.

2.10. Detection of Intracellular Generation of Reactive Oxygen Species

Cells were seeded in a density of 5×10^5 cells/cm^2. After 24 h medium was removed and cells were washed three times with PBS. Then cells were incubated with 50 µM DCFDA solution in PBS at 37 °C and 5% CO_2 for 1 h. DCFDA-solution was removed and cells were washed three times with PBS. Fluorescence was measured in a plate reader (Perkin Elmer VICTOR3™ V Multilabel Counter Model 1420, Waltham, Massachusetts) at an excitation of 495 nm and an emission of 529 nm.

2.11. Western Blot

Western Blot analysis of the respective protein expression patterns was perform exactly as described previously [34]. As primary antibodies we used antibodies for PARP (Rabbit anti PARP polyclonal antibody #9542; Cell Signaling Technology®; Leiden, Netherlands), Nrf2 (Rabbit anti Nrf2 monoclonal antibody #12721; Cell Signaling Technology®; Leiden, Netherlands), BAX (Santa Cruz Biotechnology; Texas, United States) and yH2AX (Santa Cruz Biotechnology; Texas, United States) and their corresponding second antibodies (Goat Anti-Mouse Immunoglobulin #P0447; Agilent Technologies; Santa Clara, United States; Goat Anti-Rabbit Immunoglobulin #D0487; Agilent Technologies; Santa Clara, United States). In order to normalize the results, the gel was visualized (ChemiDoc™MP Imaging System; Bio-Rad Laboratories GmbH; München, Germany) before and after the blotting process. The results were calculated as relatively intensities by the help of the software ImageLab™6.0 (Bio-Rad Laboratories GmbH; München, Germany).

2.12. Cell Preparation for Seahorse Assay

In order to detect mitochondrial respiration oxygen consumption rates (OCR), were used the Agilent Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA) exactly as described [35]. Cells were seeded in the respected cell culture plates at a density of 3×10^4 cells/well in 200 µl DMEM medium containing 10% FCS and cultures were maintained overnight in 37 °C incubator with 5% CO_2. In addition to a sham treated culture plate, in a second plate cells were irradiated in PBS buffer with blue light (453 nm) with dose of 110 J/cm^2. After irradiation PBS was discarded and cells were maintained in DMEM growth medium. One hour after light exposure in each well 150 µl of the medium was removed. After washing the plate two times 500 µl prewarmed XF base medium containing glucose (25 mM), glutamine (2 mM), and sodium pyruvate (1 mM) was added in each well making the final volume of 550 µl. The 96-well cartridge containing the cells was then automatically calibrated by the Seahorse XF24 analyzer. The following Mito Stress Test Assay was performed according to the protocol described by Butler et al [36] OCRs were detected under basal conditions followed by the sequential addition of oligomycin A (0.25 µM), an effective ATP synthase inhibitor, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, 10 µM), a potent mitochondrial oxidative phosphorylation uncoupler, and rotenone/antimycin A (10 µM each), rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. Thus we were able to estimate the contribution of blue light exposure for basal respiration, proton leak, maximal respiration capacity, spare reserve respiration capacity, nonmitochondrial respiration, and ATP-linked respiration of the irradiated tumor cells.

Additionally, in order to evaluate the impact of blue light exposure on the capacity of the glycolytic pathway after glucose starvation we used the Agilent Seahorse Glycolysis Stress Test. The assay was performed according to the manufacturer’s recommendations and protocols. The evaluation of the extracellular acidification rate (ECAR) as an indicator of glycolysis is a common strategy to address the conditions of this energy-producing metabolic pathway [5]. Cells are driven towards glycolysis and their ability to increase glycolytic activity is assessed in order to meet metabolic and bioenergetic requirements. Non-glycolytic acidification is assessed through glucose starvation. The assay measures basal glycolysis after addition of 10 mM D-glucose, glycolysis capacity upon blockage of mitochondrial ATP production using 0.25 µM oligomycin, and glycolytic reserve after addition of 2-Desoxy-D-glucose (100 mM).

2.13. Statistical Analysis

All values were reported as means ± SD and derive from the indicated number of independent experiments. The statistical analysis was done with GraphPad Prism 8.0. Data were analyzed using an unpaired *t*-test with $p < 0.05$ considered as to be significant.

3. Results

3.1. Impact of Blue Light (453 nm) on Mitomycin C Induced Cell Death of RT112 Cells

When using the MTT viability assay we found that compared to untreated RT112 control cultures, irradiating the cells alone with blue light (110 J/cm^2) or incubating the cells with Mitomycin C (10 μg/ml) each led to a clearly recognizable but not statistically significant decrease in the number of vital cells (Fig. 1A). However, a combined treatment of the RT112 cell cultures with blue light plus Mitomycin C led to a significantly increased ($p < 0.05$) toxicity compared to the treatments with blue light or Mitomycin C alone (Fig. 1A).

In addition to the MTT assay already mentioned, we also quantified the extent of the increase in cell toxicity induced by blue light with the help of fluorescence-based analysis of toxic events in general and, using FACS technology, the apoptotic cell death in particular. Using the fluorescent dyes used here, we were able to distinguish living cells from apoptotic and necrotic cells (Fig. 1C), to differentiate between necrotic and apoptotic cell nuclei (Fig. 1D) and to recognize different stages of apoptotic nuclei (Fig. 1E). In the quantitative evaluation of the necrotic cells marked by the propidium iodide, we were able to confirm the findings obtained with the help of the MTT assay. In RT112 cultures that were treated with blue light plus Mitomycin C, we were able to achieve a significantly ($p < 0.05$) higher proportion of necrotic cells than after treatment with the individual measures (Fig. 1B).

However, the increased cytotoxicity of Mitomycin C that we were able to achieve through the blue light was not only based on necrotic cell death, but also on apoptosis. In the RT112 cultures treated with Mitomycin C plus blue light, we were able to detect a strong and significant increase ($p < 0.05$) in the number of hypodiploid, apoptotic nuclei when compared with the frequency of corresponding apoptotic events in RT112 cultures treated with Mitomycin C or blue light alone (Fig. 2).

We also considered the influence of the additive treatment of tumor cells with Mitomycin C plus blue light on the expression of the proapoptotic protein Bax and cPARP, an important parameter for the activity

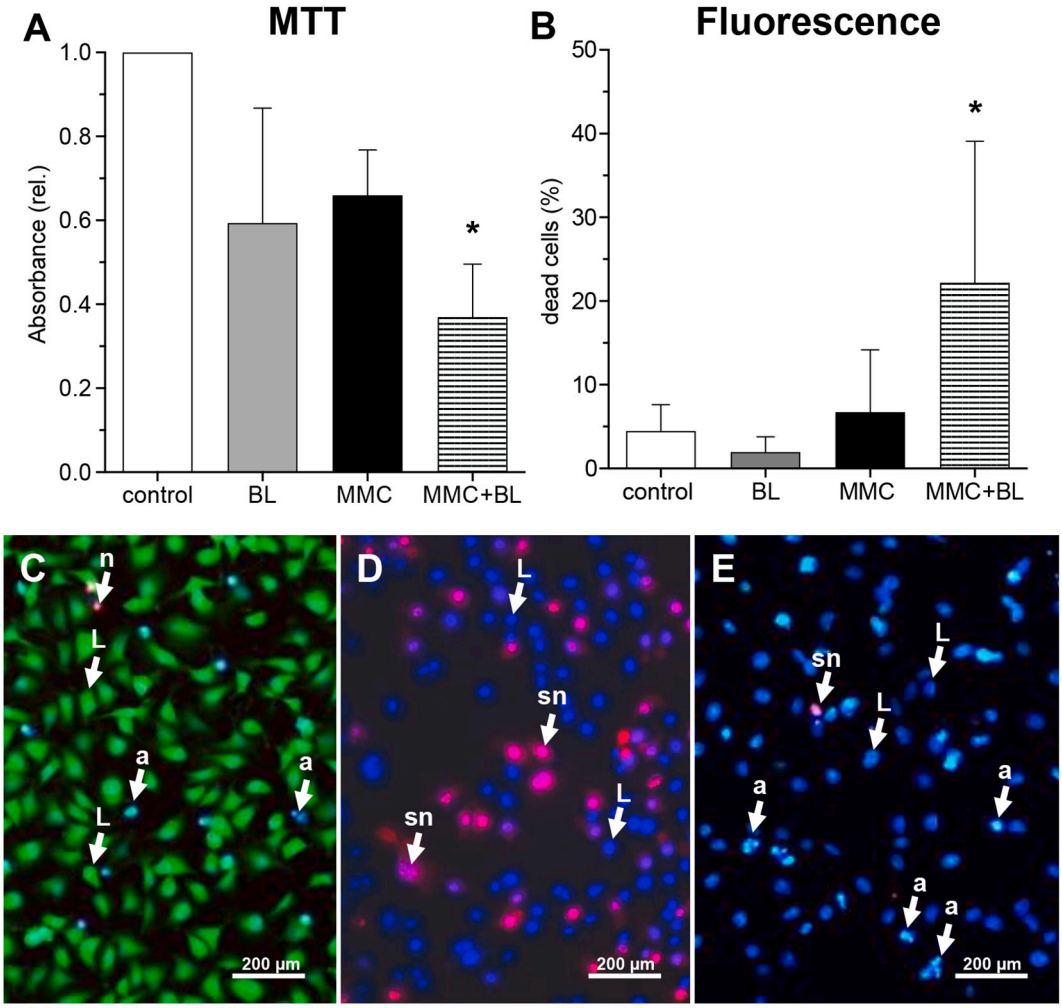

Fig. 1. Impact of blue light (453 nm) and Mitomycin C on cell death of RT112 cells. Bars shown in A and B represent mean ± SD of eight (A) or six (B) individual experiments. Shown are values of the non-treated control cultures (100%, white bar), blue light irradiated cultures (BL, 110 J/cm^2, grey bar), Mitomycin C treated cultures (MMC, 10 μg/ml, black bar), and MMC + BL treated cultures (striped bar). *, $p < 0.05$ as compared to the values of the other three bars. A, relative number of living cells, detected by the MTT assay, in RT112 cell cultures treated as indicated. B, relative number of dead cells in RT112 cell cultures treated as indicated by using different fluorochromes for the detection of living cells (fluorescein diacetate - FDA, 2 μg/ml), necrotic cells (propidium iodide - PI, 0.5 μg/ml) and visualization of the nuclear morphology of living or apoptotic cell nuclei (H33342 dye, 0.5 μg/ml). C, D, E: By choosing the appropriate fluorescence filter, it was possible to differentiate between the different vitality states of the cells. C, living cells (L, green colored by FDA), necrotic nuclei (n, red colored by PI), pyknotic and/or fragmented apoptotic nuclei (a, blue colored by H33342). D, Intact cell nuclei of living cells (L, blue colored by H33342), secondary necrosis (sn, red colored by PI). E, secondary necrosis (sn, red colored by PI), pyknotic and/or fragmented apoptotic nuclei (a, blue colored by H33342). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

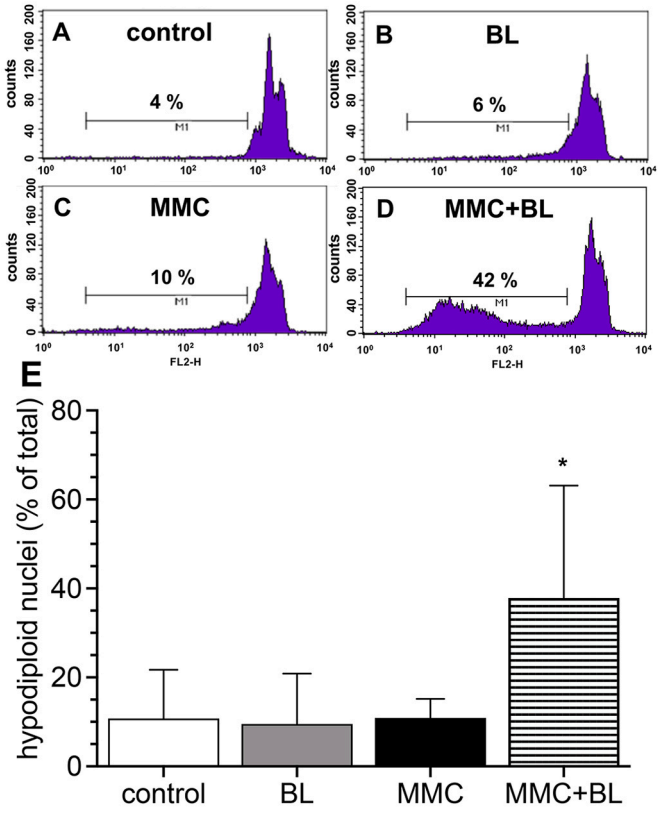

Fig. 2. Impact of blue light (453 nm) and Mitomycin C on apoptotic cell death of RT112 cells. With the help of FACS technology, we have detected the relative proportion of apoptotic cells with hypodiploid cell nuclei according to Riccardi and Nicoletti [32] in both untreated and treated RT112 tumor cell cultures. A-D, FACS-histograms of an individual representative experiment. A, non-treated control cultures; B, blue light irradiated cultures (BL, 110 J/cm^2); C, Mitomycin C treated cultures (MMC, 10 µg/ml); D, blue light irradiation of Mitomycin C treated cultures (MMC + BL). D, Quantitative evaluation of apoptotic cells with hypodiploid cell nuclei of the control cultures (control, white bar), blue light irradiated cultures (BL, 110 J/cm^2, grey bar), Mitomycin C treated cultures (MMC, 10 µg/ml, black bar), and blue light exposed Mitomycin C treated cultures (MMC + BL, striped bar). Bars represent the mean ± SD of four individual experiments. *, $p < 0.05$ as compared to the values of the other three bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of effector caspase-3, as key molecular parameters of apoptotic cell death. As we show in Fig. 3, blue light also produced a significant apoptosis-enhancing effect ($p < 0.05$) at the level of these two parameters. Thus, the light exposure of RT112 cultures treated with Mitomycin C led to an increased Bax protein expression as compared to the control cultures and cultures treated with Mitomycin C alone (Fig. 3A). With regard to the influence on PARP metabolism, we were able to determine that blue light had no influence on the level of PARP protein expression either alone or in combination with Mitomycin C (Fig. 3B). But the combined treatment of the RT112 cultures with blue light plus Mitomycin C induced a sharp increase in the production of cPARP, the active caspase-3 cleave product of PARP. This increase was statistically significantly ($p < 0.05$) higher than the sPARP generation in the control cultures and in RT112 cell cultures treated with blue light or Mitomycin C alone (Fig. 3B).

3.2. *Blue Light Significantly Modulates Molecular Key Parameters of Energy Metabolism and ROS Production in RT112 Cells*

In the search for further molecular mechanisms of the above-described effect of blue light on the RT112 tumor cells, we observed that blue light used alone or in combination with Mitomycin C significantly ($p < 0.05$) reduced ATP production, as compared to respective ATP values in control tumor cell cultures or Mitomycin C treated RT112 cultures (Fig. 4A). In parallel, we observed that exposure of RT112 tumor cells to blue light led to a strong and statistically significant ($p < 0.05$) increase in intracellular ROS production of more than eight-fold (Fig. 4B).

The modulative influence of blue light on the two last-mentioned parameters prompted us to evaluate whether and what influence blue light has on mitochondrial respiration and glycolysis of the light-exposed RT112 tumor cells. The results show that one hour after exposure to light, the respiratory chain was almost completely inoperative and no longer responded to the effects of the various function inhibitors (oligomycin, rotenone, antimycin A) and activators (FCCP) used (Fig. 5A). With regard to the individual mitochondrial function parameters, we observed a strong significant ($p < 0.05$) decrease in basal activity, ATP production, maximum respiration, spare respiration capacity, non-mitochondrial oxygen consumption and coupling efficiency after irradiation (Fig. 5B-5G).

We also observed similar results in the context of the evaluation of the effect of blue light on the glycolysis of the irradiated cells (Fig. 6A). Here we were able to observe a significantly ($p < 0.05$) reduced glycolysis rate in light-exposed RT112 tumor cell cultures after the addition of glucose, as well as a significantly ($p < 0.05$) lower maximum

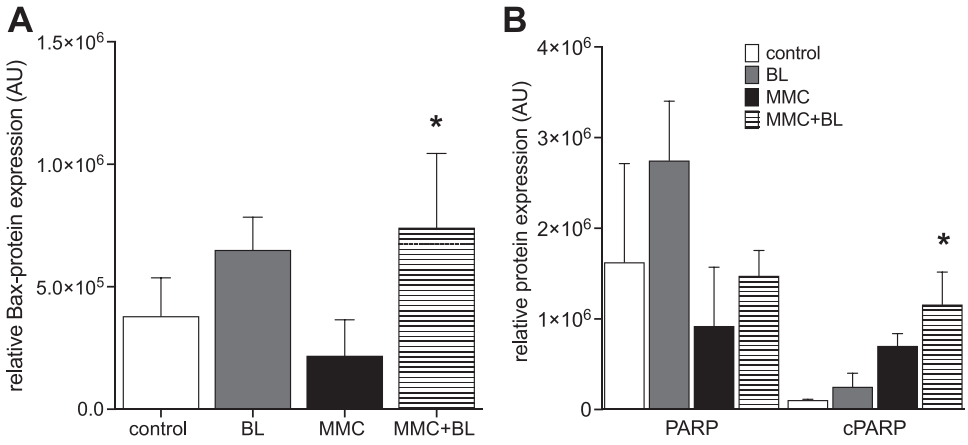

Fig. 3. Impact of blue light (453 nm) and Mitomycin C induced cell death of RT112 cells. Bars shown in A and B represent mean ± SD of six individual experiments. Shown are values of the non-treated control cultures (100%, white bars), blue light irradiated cultures (BL, 110 J/cm^2, grey bars), Mitomycin C treated cultures (MMC, 10 µg/ml, black bar), and blue light exposed Mitomycin C treated cultures (MMC + BL, striped bars). A, Bax protein expression. *, $p < 0.05$ as compared to the values RT112 tumor cell cultures treated only with Mitomycin C (MMC, black bar). B, PARP protein expression and caspase-3 dependent PARP-cleavage (cPARP).
*, $p < 0.05$ as compared to the cPARP values represented by the other three bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

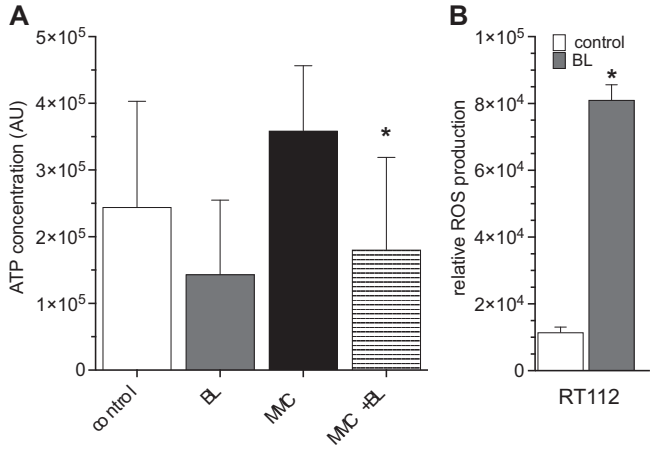

Fig. 4. Impact of blue light (453 nm) and Mitomycin C induced on ATP production and intracellular ROS formation of RT112 cells. Bars shown in A represent mean ± SD of six individual experiments. Shown are values of the non-treated control cultures (100%, white bar), blue light irradiated cultures (BL, 110 J/cm^2, grey bar), Mitomycin C treated cultures (MMC, 10 µg/ml, black bar), and blue light exposed Mitomycin C treated cultures (MMC + BL, striped bar). A, ATP concentration in RT112 tumor cell cultures. *, $p < 0.05$ as compared to the values RT112 tumor cell cultures treated only with Mitomycin C (MMC, black bar). B, Intracellular ROS generation in non-treated (control, white bar) and blue light irradiated (BL, grey bar, 110 J/cm^2) RT112 tumor cell cultures. *, $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

glycolytic capacity and glycolytic reserve (Fig. 6B-6E).

4. Discussion

For thousands of years, humans have been using the light emitted by the sun as a therapeutic agent against various skin diseases [37,38]. The therapeutic potential of light, in particular light, which alone or in combination with appropriate photosensitizers generates reactive intermediates with therapeutic potential, also including reactive oxygen species (ROS), is of steadily increasing interest in modern medicine [39]. In contrast to high-energy types of radiation such as ultraviolet radiation, blue light with a wavelength of 453 nm is not able to directly generate ROS by splitting molecules or applying energy to oxygen molecules due to its relatively low energy content. Nevertheless, the intracellular generation of reactive oxygen species (ROS) appears to be the relevant molecular mechanism for the effects of exposure to blue light [40–42].

Contrary to what is often assumed, the production of reactive oxygen species ensured by cells own enzyme systems represents a normal cell-physiological process for the control or induction of different physiological signaling pathways. Thus, ROS play an important role in immunity, cell growth and cell signaling. However, in excess, ROS are deadly to cells and the overproduction of these molecules leads to a wide variety of serious diseases [43,44]. The enzymes involved in ROS production are e.g. flavoenzymes of the mitochondrial respiratory chain, in particular NADH dehydrogenase (complex I) and isoenzymes from the NADPH oxidase family (NOX) but also other like 5-lipoxygenase or xanthine oxidoreductase (XOR) [45]. In the functioning of flavoenzymes, the natural substrates NADH or NADPH play the role of reduction equivalents and serve as electron donors for the reduction of the flavin residues. Only the reduced form guarantees a targeted and controlled transfer of electrons to an oxygen molecule and thus the generation of superoxide radical anions ($O_2^{\cdot-}$) and H_2O_2 [46,47]. In contrast to the physiological function of the flavoenzymes, the interaction with blue light leads to a photoreduction of the flavin content of the enzyme due to the absorption properties of flavin residues without

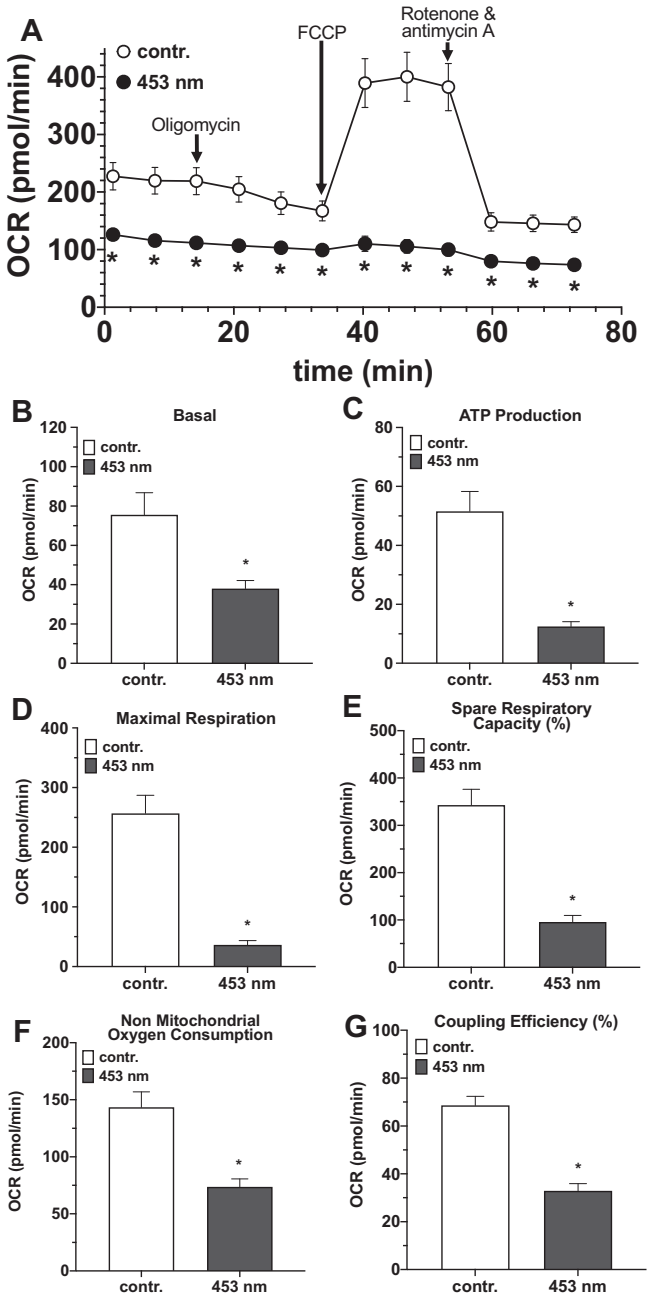

Fig. 5. Modulation of mitochondrial respiration of RT112 tumor cells by blue light. Oxygen consumption rate (OCR in pmol/min) of 3 × 10^4 RT112 tumor cells/well was measured under basal conditions (A, open circles; B-G, white bars) or one hour after irradiation with blue light (110 J/cm^2, A, black circles; B-G, grey bars) followed by the sequential addition of oligomycin (0.25 µM), FCCP (1 µM), and rotenone plus antimycin A (1 µM), as indicated. Each data point represents the mean ± SD of 12 individual OCR values. *, $p < 0.05$ as compared to the corresponding values obtained under basal conditions. B-G, the assay reports multiple key parameters, including basal respiration (B), ATP-linked respiration (C), maximal respiration (D), spare respiratory capacity (E), non-mitochondrial oxygen consumption (F), and coupling efficiency (G). Bars represent the mean ± SD of 12 individual OCR values obtained under basal conditions (white bars) or one hour after light exposure (grey bars). $p < 0.05$ as compared to the corresponding values obtained under basal conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

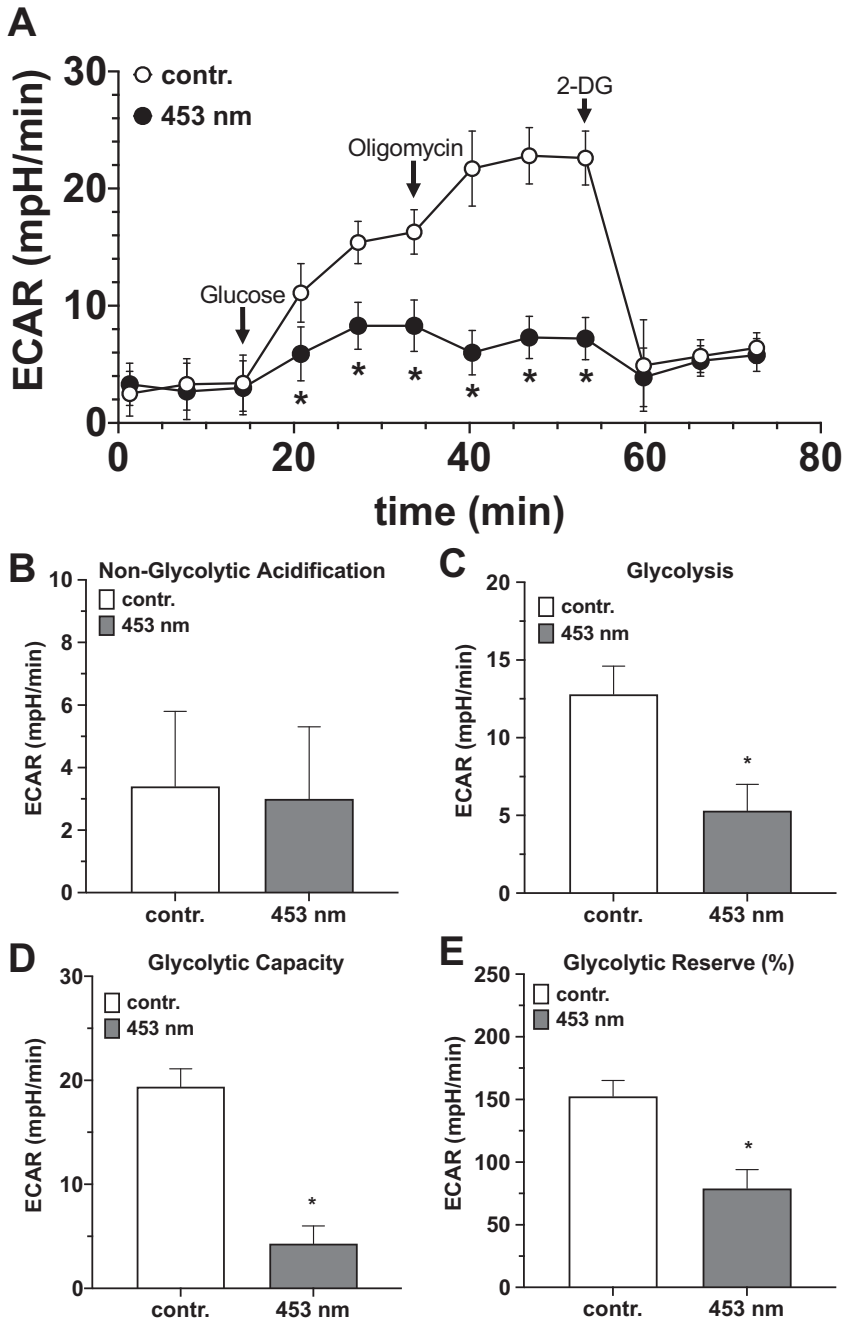

Fig. 6. Modulation of glycolysis of RT112 tumor cells by blue light. A, kinetic of extracellular acidification responses (ECAR in mpH/min) of 3×10^4 RT112 tumor cells/well to glucose (10 mM), oligomycin (0.25 μM), and 2-Desoxy-D-glucose (2-DG, 100 mM) was measured under basal conditions (open circles) or one hour after irradiation with blue light (110 J/cm^2, black circles. Each data point represents the mean ± SD of 12 individual ECAR values. *, $p < 0.05$ as compared to the corresponding values obtained under basal conditions. B-E, the assay reports multiple key parameters, including non-glycolytic acidification (B), glycolysis (C), glycolytic capacity (D), and glycolytic reserve (E). Bars represent the mean ± SD of 12 individual ECAR values obtained under basal conditions (white bars) or one hour after light exposure (grey bars). $p < 0.05$ as compared to the corresponding values obtained under basal conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

participating in the natural substrate NADH or NADPH [48]. Immediately after the light-induced flavin reduction, a process of flavin reoxidation begins. This process takes place in a light-independent reaction and, as under physiological conditions, leads to electron transport to oxygen molecules and thus also to the formation of ROS in the form of superoxide radical anions or hydrogen peroxide [46]. However, since the enzyme has no activity control via a feedback mechanism, e.g. the regulation of substrate consumption, the level of ROS production by the flavoenzyme is solely a function of the light dose. Depending on the light dose, very high amounts of ROS can be generated. So, it is not surprising that we were able to observe such a strong ROS-inducing effect of blue light also in the RT112 bladder cancer cell line used here.

The biological responses observed as a result of blue light increased ROS production include a reduction in migration, proliferation and differentiation of the various cell types exposed to blue light, and above a critical threshold of ROS production it can negatively affect cell viability and become cytotoxic [18,40,48,49]. In order to be able to better record additive or synergistic effects of blue light with the chemotherapeutic agent, we were very careful in the study presented here to use a light dose that, when applied alone, could not induce any significant cytotoxicity. It should of course not go unmentioned that blue light can also have a strong cytotoxic effect, depending on the dose used and the frequency of radiation. For example, Zhang et all were able to show that long irradiation of HL-60 myelogenous leukemia cells with blue light (456 nm) alone led to strong cytotoxicity in the light-exposed cell cultures. The predominant mode of induced cell death was apoptosis, accompanied by all the typical features of caspase-3 controlled apoptosis [21].

Nevertheless, using the chosen non-toxic light dose we observed a significant increase in Mitomycin C toxicity in RT112 bladder cancer cell

cultures exposed to blue light. We could not clearly determine the type of increased cell death. We originally assumed that the combined use of the chemotherapeutic agent plus blue light would increase cell death via apoptosis. We were able to partially confirm this expectation. We observed a significant increase in apoptosis, accompanied by a significant increase in the expression of the pro-apoptotic protein Bax and a significantly increased rate of the caspase-dependent degradation of PARP. Overall, this scenario indicates a significantly increased rate of caspase-3 mediated apoptosis. We attribute this increased rate of apoptosis to the greatly increased intracellular production of ROS, which are known to be very effective inducers of apoptosis [50,51].

On the other hand, we also observed a strong and significant increase in secondary necrosis. Apoptotic cell death is a finely tuned and programmed cell death which requires energy to be carried out successfully. If a cell carrying out the apoptotic program no longer has the required energy sources in the form of ATP, it stops this cell death program at the corresponding point in the mechanism and becomes necrotic (secondary necrosis) [52]. This is primarily the case when a damaged cell no longer has sufficient glycolytic or mitochondrial ATP synthesis or is unable to form ATP due to a lack of substrates. This finding of the increased rate of secondary necrosis prompted us to characterize the influence of blue light on the energy metabolism of the RT112 cancer cells used here. In fact, using SeaHorse technology, we were able to find that irradiation of the RT112 cultures with blue light induced a complete breakdown of the mitochondrial respiratory chain and a significant reduction in ATP synthesis. We consider this blue light-induced breakdown of the mitochondrial respiratory chain to be the causal mechanism for the increased rate of secondary necrosis in irradiated RT112 cultures. Of course, the question arises as to why the irradiation of the cells as a single stimulus led to a greatly increased ROS production and mitochondrial breakdown, but did not lead to increased toxicity of the cells. As we were recently able to show with human skin fibroblasts, the decoupling of the respiratory chain shown here is a reversible process after exposure to blue light. Depending on the light dose used, the mitochondrial respiratory chain recovers quite quickly and showed its original activity potential after just 18 to 24 h. This temporal course therefore makes sense that in the context of a combination therapy, the addition of the chemotherapeutic agent, as carried out by us, takes place shortly after the radiation, at the time of the greatest ROS exposure and ATP production inhibition.

With the results generated as part of our study, we show that the combined simultaneous or sequential local application of a chemotherapeutic agent and blue light can represent an effective treatment option for certain types of cancer. Due to the use of blue light, the penetration of which into biological tissue can be approx. 1.5 mm, clinical application would be limited to the treatment of superficial, epithelial and freely accessible tumors or tumors accessible with the help of special medical technology. However, the treatment of deeply invaded tumors would only make limited sense. In the case of bladder cell carcinoma, whose cells we have used here as an example, the existing modern endoscopy technology could be used. Corresponding technology could also be used in the treatment of epithelial tumors of the intracranial space. Such a light-assisted therapy option could be individually adapted to the respective patient through the use of higher light doses and further promote the success of the therapy. By using special templates that sharply delimit the area to be treated, healthy areas in the treatment area could also be protected from possible side effects of the therapy. Of course, it should not go unmentioned that as a pure in-vitro study, the results of the project presented here are of course only conditionally meaningful with regard to therapeutic clinical use. We see fewer problems in the medical-technical implementation of our propagated therapy option than at the level of the antioxidative equipment of different tumor cell types. For example, oxidative stress induced by blue light would induce less damage in tumor cells that have a higher antioxidant capacity, e.g. through increased expression of antioxidant enzymes, than in tumor types that do not have such antioxidant protection. Thus, the amount of light used therapeutically would have to be set individually for each type of tumor, or the efficiency of the light energy would have to be adjusted by adding additional photosensitizers.

Authors' Contributions

Lisa Hegmann: conception, collection and assembly of data, study design, data analysis and interpretation, manuscript writing

Sofia Sturm: conception and study design, collection and assembly of data

Günter Niegisch: data analysis and interpretation

Joachim Windolf: final approval of manuscript, data analysis and interpretation,

Christoph V. Suschek: conception and study design, data analysis and interpretation, manuscript writing

Ethics Approval and Consent to Participate

In the context of the project only commercially available cell lines were used, no samples from patients or voluntary donors were used, so that no special ethical-relevant aspects have to be observed and corresponding explanations have to be made.

Consent for Publication

The present manuscript does not contain any kind of individual person's data in any form.

Availability of Data and Material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Discussion

The source ‘light’ has been used in wide fields of medicine for millennia. [33-35] Even in early human history, the healing effect of sunlight was known and appreciated as for example Hippocrates (460 – 370 BC), considered as the ‘Father of Medicine’, stated the ‘sunnier side of the hill’ was the healthier place to live. [36] Throughout history and over the years, the subject of 'light' has been repeatedly addressed. Even if initially in a rudimentary way, as Florence Nightingale, known as ‘the Lady with the Lamp’ discovered that her patients in hospital rooms with windows facing the east recovered the fastest, light fascinated humanity and remained a constant part of research. [37]

For some time now, other therapeutic approaches of light are part of the research. One example is to use the irradiation of light in order to convert a substrate into a biological active substance. [38]

This present work can also be classified in this context, as we used monochromatic blue light at a wavelength of 453 nm and therefore part of the daily and visible light, to enhance the effect of a substrate – respectively of Mitomycin C (MMC). MMC is an already established, alkylating chemotherapy which is used after performed tumor surgery of urothelial bladder carcinoma. [31] By using the chemotherapy as an intravesical installation, remaining carcinoma cells, which could not be resected through surgery in order to protect the function of the bladder, can be killed. The achieved results of this study indicate reactive oxygen species (ROS) as a key player for the observed increased cell death when combining the treatment of chemotherapy with blue light irradiation. [23, 39, 40]

Blue light at a wavelength of 453 nm is part of the visible light and therefore light of lower energy, which is not able to directly interact with enzymes in order to transfer energy or split molecules to generate superoxide anions or other reactive oxygen species, as for example ultraviolet radiation does. [25, 29] Therefore, in this study we took a closer look on how blue light generates ROS.

As commonly known, cell signaling pathways as well as the process of cell proliferation or differentiation are finely tuned processes that occur every second in every cell of the body, involving a multitude of proteins, receptors or other

signaling molecules. Part of these are reactive oxygen species, playing a crucial role in essential metabolic processes of each cell. Nevertheless, as often is the case, it depends on the relative quantity and balance, because as vital as the substrates are in the right dosage, as soon as if the dosage of ROS exceed a certain amount, there is a toxic effect on the cells, potentially triggering a wide range of diseases. [41, 42]

The majority of the production of ROS stems primarily from essential enzyme complexes, more precisely from the flavoenzymes of the respiratory chain. In this group of enzymes, the NADH dehydrogenase, which forms the complex I of the respiratory chain, as well as its isoenzymes, the NADPH oxidase family (NOX) are the most important ROS producing enzymes. [43]

For a targeted and controlled electron transfer and to produce superoxide radicals and hydrogen peroxide, the reduced form of the flavin protein is needed. Furthermore, the substrates NADH or NADPH in this construct serve the role of reduction equivalents and as electron donors. [39, 44]

The flavoenzymes' function involves interacting with naturally incorporated regeneration properties. When exposed to blue light at a wavelength of 453 nm, the flavin contents absorb the light and undergo photoreduction without the involvement of the natural substrates NADH or NADPH. Following this photo-induced reaction, the cell attempts to restore the enzyme's original state, initiating a light-independent reoxidation of the flavin residue, resulting in the formation of reactive oxygen species like superoxide radical ions and hydrogen peroxide through electron transport to oxygen molecules. Normally, such enzyme reactions have various regulation processes, for example the amount of available substrate (e.g., NADH). [44, 45] In this case, the process is purely driven by the external stimulus, the applied light dose. Consequently, by increasing the light dose, very high levels of reactive oxygen species can be generated proportionally or perhaps even exponentially. In our experiments, we observed such an effect on the RT112 bladder carcinoma cell line.

As already described in the introduction, reactive oxygen species have been subject of current research for a long time. Inhibiting effects on cell division, migration and proliferation have been identified. There are also preliminary

studies regarding the inhibition of cell viability up to toxicity if the amount of ROS exceeds a critical value. [45-48]

This study sought to explore whether the efficacy of an established chemotherapeutic agent could be enhanced, either additively or synergistically, by combining it with exposure to blue light without having toxic effects of blue light itself.

That blue light itself can have cytotoxic effects could be demonstrated through other preliminary work: For example, Zhuang et al. applied blue light at a wavelength of 456 nm at a sufficient dose and frequency to HL-60 myeloid leukemia cells. The primary outcome observed was an increase in induced cell death, or apoptosis, along with the typical or characterizing features of the Caspase-3-controlled apoptotic pathway. [26]

Therefore, it should be noted again that this work aimed to use an irradiation dose below a significant cell toxicity level. Despite the use of a "non-toxic" light dose, a significant difference in the cumulative toxicity of mitomycin C in the light exposed RT112 bladder carcinoma cells could be observed compared to the non-exposed ones in the experiments carried out. Our hypothesis proposed that this observed phenomenon resulted from the amplified induction of apoptosis, in the group receiving combined therapy of blue light irradiation followed by mitomycin C chemotherapy. Our experiments partially substantiated this assumption, showing a significant increase in apoptosis, coinciding with a marked increase in the expression of pro-apoptotic proteins. We observed an enhanced level of BAX, which is a marker of mitochondrial injury, and degraded PARP, both caspase-dependent proteins. [49] This effect we assign to the enhanced level of intracellular ROS production, which is recognized for its potent role in driving apoptosis e.g. through inducing abnormal gene expression or because of the blockage of cell communication. [50-52]

However, this was not the only observed outcome; a substantial rise in secondary necrosis was also evident. While apoptotic cell death is a precisely regulated and programmed process, secondary necrosis occurs when a cell lacks the energy reserves, in the form of ATP, needed for apoptosis. This lack of ATP ends the programmed cell death, and the cell succumbs in the meaning of secondary

necrosis. [53] This deficiency of ATP can result from compromised mitochondrial or glycolytic metabolism or a lack of necessary substrates for synthesis.

To pinpoint the cause of the notably increased rate of secondary necrosis, we took a closer look at the energy metabolism of the RT112 bladder carcinoma cells. We were able to show that blue light irradiation at a wavelength of 365 nm causes a total collapse of the mitochondrial respiratory chain and thereby significantly diminishing ATP synthesis, using Seahorse technology.

This irradiation-induced decrease in ATP production is thought to underpin the established rise in secondary necrosis rates in our RT112 cell culture.

Initially, it is not immediately apparent why blue light irradiation alone does trigger an increase in ROS production and mitochondrial degradation, but not cell toxicity itself. In an experiment with human skin fibroblast, it could be demonstrated that the previously mentioned decoupling of the mitochondrial respiratory chain after blue light irradiation is a reversible process.

Following the termination of the light exposure, the respiratory chain has the capacity to recover rather quickly within 18 to 24 hours, regaining its original activity potential. The understanding of the temporal sequence is crucial for the application of the second stimulus, the chemotherapy. Therefore, the addition of chemotherapy was performed shortly after the completed irradiation unit, to make the most of the amplified ROS production and the resultant ATP production inhibition.

In summary, our research findings suggest that either simultaneous or in quick succession applicated combination of blue light irradiation at a wavelength of 453 nm and a chemotherapeutic agent could be an effective therapeutic approach for certain cancer types. Admittedly, this treatment option is limited to superficial, epithelial and freely accessible tumors or those accessible with the aid of specialized medical technology, as blue light has a penetration depth of about 1.5 – 2 mm in biological tissue. [32] At the same time, this fact also grants a certain level of safety for deeper cell layers which also implies a limited sense of irradiating deeper invading tumor masses on the other hand. An additional safety aspect could include the use of customized stencils that restrict the horizontal spread of the treatment area, consequently minimizing potential side effects for non-tumor-cells. Furthermore, as blue light effects the mitochondrial chain, one

can propose the hypothesis that cells which divide more often, such a tumor cells, depend more on the mitochondrial chain than healthy tissue cells. [54] Therefore, tumor cells are more vulnerable for the effects of blue light irradiation.

In our research, we used urothelial bladder carcinoma cells, which can be targeted with blue light irradiation through modern endoscopy technology. In this case, the relatively low penetration depth of the light would be advantageous as it could protect the underlying muscle cells from unnecessary damage.

For instance, blue light in conjunction with the amino acid 5-aminolevulinic acid (5-ALA) is already deployed for diagnostic purposes in brain tumor surgery. It is used as a dye taking advantage of an enzyme defect in tumor cells which leads to an accumulation of protoporphyrin IX. Under the application of blue light, the corresponding tumor cells then glow in a pink color. [55] Because of the surgical procedure, a superficial accessibility of the tumor is ensured during the surgical measure. Therefore, with adjustments to the light dose and technology, treating intracranial tumor cells could also be conceivable.

In conclusion, it should be noted that the presented results are derived from pure *in-vitro* experiments, which could limit their relevance to the actual therapeutic success regarding the combined therapy of blue light irradiation followed by sequential chemotherapy application. Whereas, nevertheless there are already some in vivo studies on the increase of reactive oxygen species after blue light irradiation. [56, 57]

Potential limitations to the success of the therapy are less likely to appear from the process of medical-technical implementation, but more regarding the individual antioxidant capability of various tumor cell types. For instance, tumor cells with enhanced antioxidant capacity, e.g., through increased expression of relevant repair mechanisms, would experience significantly less cell damage from blue light-induced oxidative stress, compared to tumor cells lacking such mechanisms or enzymes. This would imply an individual adjustment of the used light dose, necessary for each tumor cell type, also with potential additive use of additional photosensitizers or even subsequent radiotherapy, if needed.

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