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Modulation of Tumor Cell Metabolism by Laser Photochemotherapy with Cisplatin or Zoledronic Acid *In Vitro*

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Abstract. *Background/Aim: Laser photochemotherapy is a new approach in cancer treatment using low-level laser therapy (LLLT) to enhance the effect of chemotherapy. Materials and Methods: In order to evaluate the effect of LLLT on tumor cells, HeLa cells were treated with cisplatin or zoledronic acid (ZA) followed by LLLT. Cell viability was evaluated with 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay. Oxidative phosphorylation and glycolysis were measured using extracellular flux analysis. Immunocytochemistry of heat-shock protein 70 (HSP70) and western blot analysis were performed. Results: LLLT alone increased viability and was associated with lower oxidative phosphorylation but higher glycolysis rates. Cisplatin and ZA alone lowered cell viability, glycolysis and oxidative phosphorylation. This effect was significantly enhanced in conjunction with LLLT and was accompanied by reduced oxidative phosphorylation and collapse of glycolysis. Conclusion: Our observations indicate that LLLT may raise the cytotoxicity of cisplatin and ZA by modulating cellular metabolism, pointing to a possible application in cancer treatment.*

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Current knowledge in the area of cellular energy metabolism is largely based on experiments with differentiated non-malignant tissues. In the presence of oxygen, these cells primarily metabolize glucose through mitochondrial oxidative phosphorylation. Under anaerobic conditions, the same cells produce high amounts of lactate (anaerobic glycolysis) (1). In contrast, tumor cells have a significantly different metabolism. Otto Warburg was the first to recognize that cancer cells exhibit high rates of glycolysis even under aerobic conditions. This phenomenon is known as the Warburg effect or aerobic glycolysis (2). A large body of evidence indicates that increased glycolysis in conjunction with reduced mitochondrial respiration are the essential changes in metabolic reprogramming (1, 3, 4).

Today, chemotherapy plays a central role in cancer treatment. Zoledronic acid (ZA) and cisplatin are widely used chemotherapeutic agents. The *bis*phosphonate ZA is used for the treatment of several bone-related diseases, such as Paget's disease, postmenopausal osteoporosis and in patients with bone metastases (5). Additionally, several *in vitro* studies suggested that ZA is able to induce apoptosis and to inhibit cell proliferation (6, 7). Cisplatin is one of the most effective agents in the therapy of many solid tumor entities, such as lung, head and neck, ovarian, cervical and testicular cancer (8). Cytotoxicity of cisplatin can be increased by different laser-mediated applications. This was reported for laser thermal therapy (9), low-level laser therapy (LLLT) (10) and in combination with an additional photosensitizer for photodynamic therapy (11-18). Evaluating the concept of laser photochemotherapy, consisting of chemotherapeutic agents activated by LLLT, we showed that LLLT enhances the cytotoxicity of ZA and cisplatin towards primary human fibroblasts and head and neck squamous cell carcinoma (HNSCC) cells (10), as well as normal and malignant bone cells *in vitro* (19). However, the effect of laser-mediated

chemotherapy on cellular viability and metabolism of cancer cells is incompletely understood. Here, we investigated the impact of cisplatin and ZA on viability and metabolism of human cervical cancer cells, known to be bone-metastatic (20, 21), as well as sensitive to both these chemotherapeutic agents (22). Furthermore, we evaluated the influence of LLLT on these drugs, testing whether laser irradiation is able to raise the cytotoxicity of cisplatin and ZA by altering cellular metabolism in these cells.

Materials and Methods

Culturing and treatment of HeLa cells with laser photochemotherapy. HeLa cells were kindly provided by B. Matija Peterlin (University of California, San Francisco, CA, USA). Genotyping was performed by a commercial provider (DSMZ GmbH, Braunschweig, Germany) using nonaplex polymerase chain reaction, confirming the cell line to be identical with HeLa cells. Frozen stocks of genotyped HeLa cells were used for the studies presented here. Cells were incubated at 5% CO_2 (37˚C) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were plated at a density of 3×10^4 cells/well in 96-well polystyrene tissue-culture plates. After 24 h of cultivation, either 6.5, 13 or 26 μM *cis*-diamminedichloroplatinum(II) (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) or 50, 100 and 200 μM ZA (Zometa®; Novartis AG, Basel, Switzerland, stock: 4 mg/5 ml) was added according to published half maximal inhibitory concentration (IC_{50}) values for cisplatin (23) and ZA (24). Wells without agent served as controls. Twenty-four hours after incubation, cells were exposed to LLLT (λ=670 nm, 120 s, 100 mW/cm^2 at continuous wave mode with no additional filters) emitted from a non-thermal diode laser (Helbo, Bredent, Walldorf, Germany). For this, the designated laser tip for surface irradiation with a distance of 8 mm to the cell layer was used. The integrated spacer provided a constant tip-to-sample spacing and thereby guaranteed constant power density for laser treatment. Non-irradiated wells were used as normal controls. The whole experimental setup was carried out under sterile conditions in a vertical laminar flow cabinet. In order to avoid potential scattered irradiation, teflon separators were sited among the wells and the 96-well plate was placed on a black surface during LLLT. The lid of the plate was removed and all wells except for the one to be irradiated were covered. The hand piece of the laser was positioned with the plate by a fixed holder, which was moved from well to well for each irradiation step. After 24 h, a second round of irradiation was performed in the same manner as described above. Three hours after the last irradiation, cell viability was analyzed with sodium 3'-[1-(phenylaminocarbonyl)-3,4tetrazolium]-*bis* (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay. For metabolic analyses, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured by an Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA). Glycolysis, glycolytic reserve and the Warburg effect were calculated from these data (Figure 1). The influence of treatment on HeLa cells was studied in at least three independent triplicate experiments.

In order to evaluate a thermal impact of LLLT, on tumor cells, the expression of heat-shock protein 70 (HSP70) was evaluated by immunocytochemistry and western blot analysis. To induce HSP70 expression (positive control), cells were incubated for 6 h at 42.5˚C in six-well cell-culture dishes as described elsewhere (25). In a second group, cells were laser irradiated for 15 min and in a third group cells underwent heat shock for 15 min at 42.5˚C corresponding to the duration of the laser treatment.

Metabolic assays. OCR and ECAR of HeLa cells were measured in real-time using a Seahorse XF^{e}96 Extracellular Flux Analyzer (Seahorse Bioscience) using the XF Glycolytic Stress Test Kit as recommended by the manufacturer. If not stated differently, cells were plated at a density of 3×10^4/well (96 wells) and incubated overnight at 37˚C with 5% CO_2.

In order to measure glycolysis, the maximal ECAR after glucose injection was subtracted from the ECAR prior to glucose injection (Figure 1A). Glycolysis reserve was calculated using the measurement after oligomycin injection subtracted from the ECAR after glucose addition. To determine oxidative phosphorylation, the OCR was measured at baseline (Figure 1B). Values were BCA (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific) normalized prior to calculation of all parameters. According to the manufacturer's protocol, in order to estimate the Warburg effect, non-normalized samples were used by dividing ECAR after glucose injection by OCR values at baseline. After performing the measurement, the cell plate reading was normalized using BCA supplied by the manufacturer's as recommended. Normalization of XF96 raw data was achieved by dividing the values for each well by its respective protein concentration to correct for potential proliferative effects during incubation and loss of cell material during cell preparation.

The XTT cell proliferation assay kit was used according to the manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany). Formation of orange formazan salt by metabolic active cells was measured 3 h after incubation with the yellow tetrazolium salt [450 nm and 630 nm (reference), DTX880 Multimode Detector; Beckman Coulter, Inc., Mississauga Ontario, Canada].

Immunocytochemistry. HeLa cells were grown under standard cell culture conditions on coverslips in six-well cell-culture dishes. Cells were treated with LLLT or high temperature (42.5˚C) as described above. Immunocytochemistry was performed as described earlier using anti-HSP70 (1:250, mouse monoclonal, ab5349; Abcam; Cambridge, UK) (26). Secondary anti-mouse (sc-2010) fluorescein isothiocyanate (FITC)-coupled antibodies were from Santa Cruz Biotechnology, Inc (Heidelberg, Germany).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and western blot analysis. SDS-PAGE and western blot analysis were performed as previously described (26). In order to detect bands of interest, primary antibodies directed against HSP70 (Abcam ab5439, 1:1,000), proliferating cell nuclear antigen (PCNA) (mouse monoclonal, sc-56, 1:500; Santa Cruz Biotechnology, Inc.) or β-actin (mouse monoclonal, A5441, 1:2,000; Sigma-Aldrich®, St. Louis, MO, USA) were added to the nitrocellulose membranes followed by incubation (1:2,000) with an appropriate secondary goat anti-mouse (sc-2005) horseradish peroxidase-coupled antibody (Santa Cruz Biotechnology, Inc.). Bands were visualized with the enhanced chemiluminescence method (Amersham Biosciences, Buckinghamshire, UK) on X-ray film (Agfa, Cologne, Germany). Western blots were carried out in triplicate. All bands were quantified with the program ImageJ 1.48v (27) and were normalized to β-actin prior to statistical analysis.

Statistical analysis. For statistical analysis, IBM SPSS Statistics Version 22.0 (IBM Corp., Armonk, NY, USA) was used. Data are

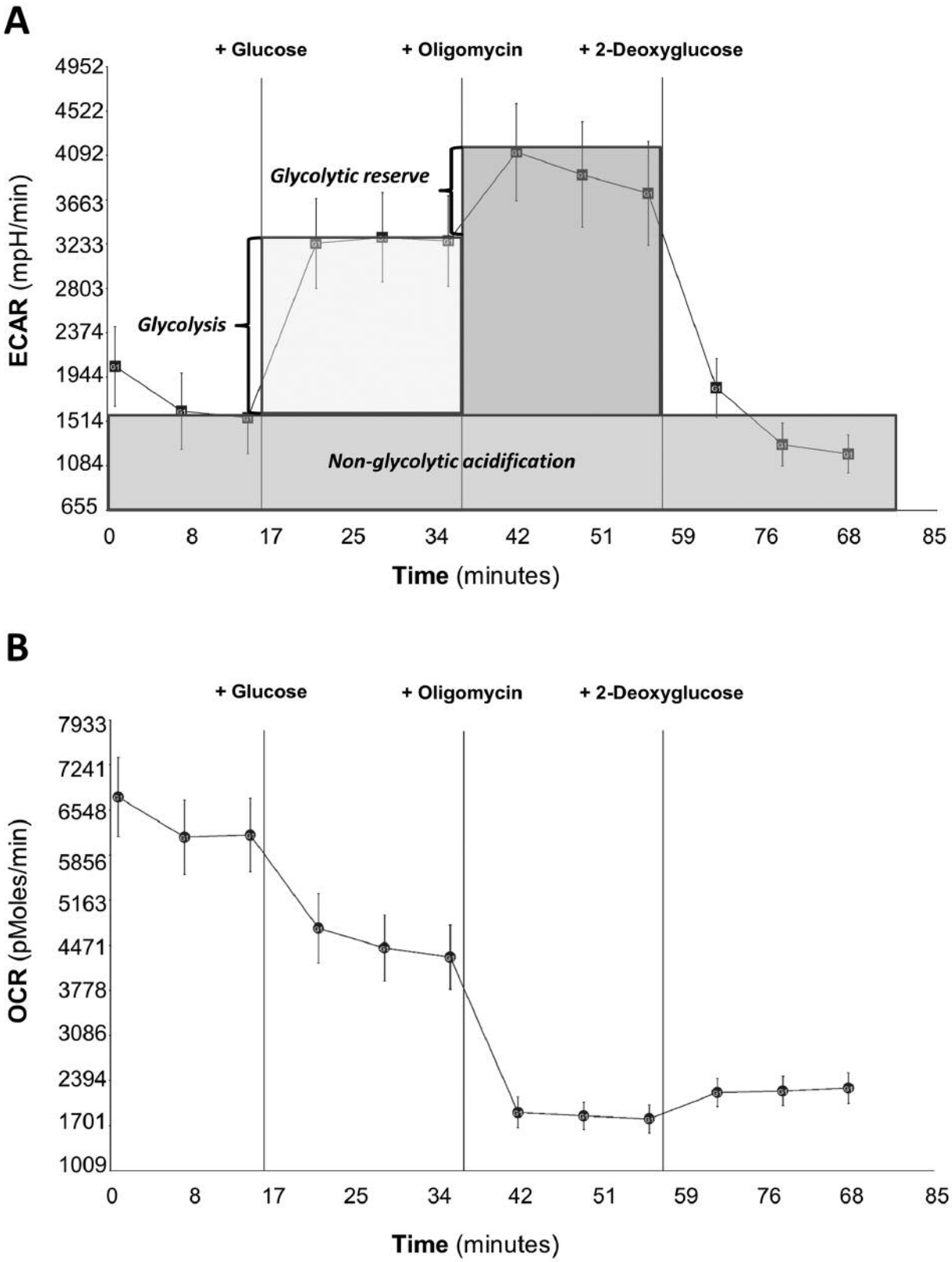

Figure 1. *Measuring extracellular acidification rate (ECAR) for glycolytic flux (A) and oxygen consumption rate (OCR) for oxidative phosphorylation (B) in HeLa cells. Metabolic response of HeLa cells to glucose (10 mM), oligomycin (5 μM) and 2-deoxyglucose (100 mM). Data are the mean±SEM, n=6.*

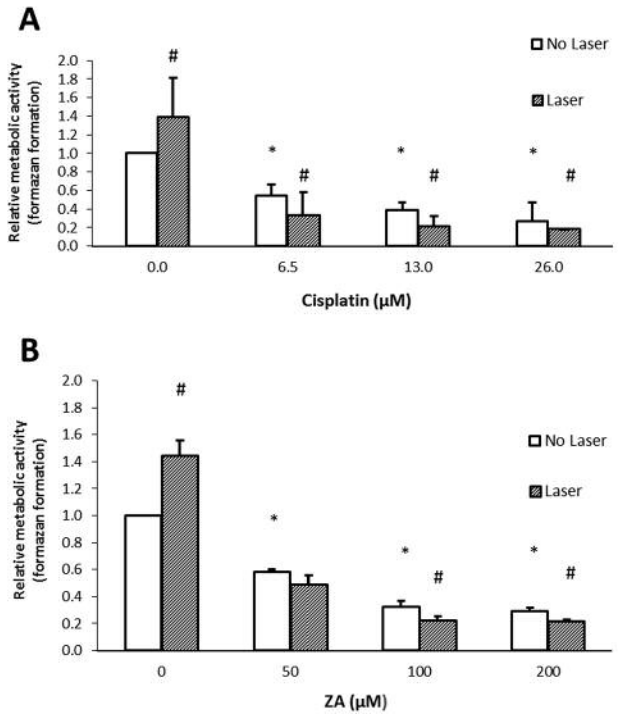

Figure 2. *Evaluation of the relative metabolic activity in HeLa cells after incubation with cisplatin (A), and zoledronic acid (ZA) (B) with and without low-level laser therapy (LLLT). Significantly different at *p<0.05 (chemotherapy vs. untreated), #p<0.05 (LLLT vs. untreated), data are presented as the mean of 3 experiments ±SEM.*

shown as the mean and standard error of the mean (SEM), with at least three independent replicates used for each data point. Normal distribution was assessed with the Shapiro–Wilk test. As data were not normally distributed, differences between the groups were analyzed with a non-parametric test (Kruskal–Wallis) and Mann–Whitney pairwise comparisons ($p<0.05$). Differences were considered as statistically significant at $p<0.05$. For comparison of western blot bands after quantification with ImageJ, values were calculated using the one-sided paired t-test.

Results

Effect of cisplatin and ZA on HeLa cell viability. Cisplatin significantly lowered viability of HeLa cells at all concentrations used (6.5, 13 and 26 μM) in comparison to the untreated controls ($p=0.001$) (Figure 2A). Similar results were obtained for ZA, cells treated with 50, 100 or 200 μM ZA demonstrated a significantly reduced cellular viability ($p=0.001$) (Figure 2B).

Laser therapy. Interestingly, LLLT alone significantly increased the viability of HeLa cells compared to non-irradiated control cells ($p=0.029$) (0 μM; Figure 2A and B). LLLT in conjunction with cisplatin treatment impaired cell viability at every cisplatin concentration tested compared with the control group (cisplatin only) (6.5, 13 and 26 μM: $p=0.008$, $p=0.039$ and $p=0.001$, respectively). Similar effects were observed after ZA exposure. Here, LLLT significantly reduced cell viability at the two highest concentrations of 100 and 200 μM of ZA ($p=0.039$ and $p<0.001$) (Figure 2B). Although showing a reduction, no significant difference in viability was found after LLLT in the presence of 50 μM ZA ($p=0.345$).

Effect of cisplatin and ZA on HeLa cell metabolism. Extracellular flux analysis was performed on HeLa cells exposed to 13 μM cisplatin (Figure 3) or 100 μM ZA (Figure 4). Cisplatin significantly inhibited oxidative phosphorylation ($p<0.001$) (Figure 3A), glycolysis ($p=0.00539$) (Figure 3B) and glycolytic reserve ($p<0.001$) (Figure 3C). The Warburg effect was significantly enhanced in comparison to untreated control groups ($p<0.001$) (Figure 3D).

Similar results were seen after incubation with 100 μM ZA (Figure 4). Oxidative phosphorylation ($p<0.001$) (Figure 4A), glycolysis ($p=0.015$) (Figure 4B) and glycolytic reserve ($p=0.009$) (Figure 4C) were significantly impaired, with a concomitant increase in the Warburg effect ($p=0.039$) (Figure 4D).

Laser therapy. LLLT alone significantly increased glycolysis ($p=0.0026$) (Figure 3B and 4B) and reduced glycolytic reserve ($p=0.0025$) (Figure 3C and 4C). Although oxidative phosphorylation was diminished ($p=0.0039$) (Figure 3A and 4A), the Warburg effect, defined as the ratio between ECAR and OCR, revealed significantly higher values in comparison to non-irradiated control cells ($p<0.001$) (Figure 3D and 4D).

In the presence of 13 μM cisplatin, supplemental LLLT significantly reduced oxidative phosphorylation ($p<0.029$) (Figure 3A), glycolysis ($p=0.042$) (Figure 3B) and glycolytic reserve ($p=0.037$) (Figure 3C), whereas the Warburg effect was elevated in comparison to cisplatin treatment alone ($p<0.001$) (Figure 3D). In the presence of 100 μM ZA, additional LLLT generated a significant reduction of oxidative phosphorylation ($p=0.0019$) (Figure 4A), glycolysis ($p=0.041$) (Figure 4B) and glycolytic reserve ($p=0.028$) (Figure 4C), whereas the Warburg effect was enhanced in comparison to ZA treatment alone ($p<0.001$) (Figure 4D).

Influence of laser-mediated heat. To evaluate the presence of thermal effects due to LLLT, we investigated the expression of HSP70, as well as that of the cellular proliferation marker PCNA in HeLa cells. Immunocytochemistry showed HeLa cells without heat exposure exhibited basal expression levels of HSP70, which appeared markedly induced after heat exposure (42.5˚C) for 6 h but not after short (15 min) heat (42.5˚C) exposure or LLLT for 15 min (Figure 5A). Western blot analysis supported these observations (Figure 5B). Here,

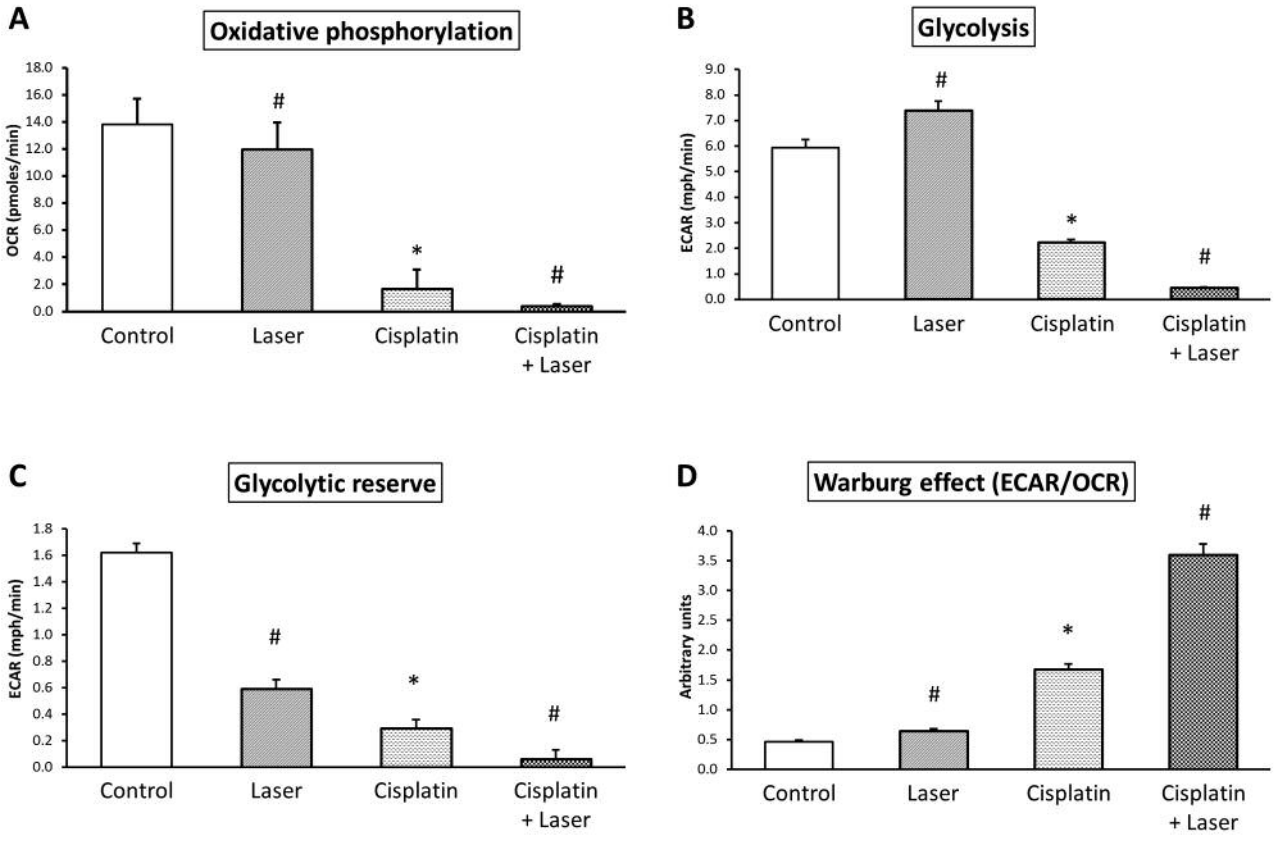

Figure 3. *Multiparameter metabolic analysis of measuring oxygen consumption rate (OCR) for oxidative phosphorylation (A) and extracellular acidification rate (ECAR) for glycolysis (B), glycolytic reserve (C) and for the Warburg effect (D) in the presence of 13 μM cisplatin (CDDP) with and without low-level laser therapy (LLLT). Significantly different at *p<0.05 (chemotherapy vs. untreated), #p<0.05 (LLLT vs. untreated). Data are means±SEM.*

a reduction of PCNA, reflecting the proliferative state of the cell, was also seen (Figure 5B). Induction of HSP70 and reduction of PCNA expression in long-term (6 h) heat-treated (42.5˚C) cells was statistically significant (p=0.0305 and p=0.0254) (Figure 5C).

Influence of treatment on PCNA expression. Treatment of HeLa cells with cisplatin or a combination of cisplatin and LLLT led to an average reduction (not significant) of PCNA protein expression (Figure 6A). Treatment of cells with ZA or a combination of cisplatin and LLLT significantly lowered the PCNA expression level (p=0.035 and p=0.0178, respectively) (Figure 6B).

Discussion

Cancer cell metabolism is characterized by high rates of glycolysis, which makes tumor cells highly dependent on glucose for survival (28). The potential of changing tumor cell metabolism for therapeutic benefit using LLLT was investigated, based on the assumption that manipulation of cellular metabolism by laser light may chemosensitize cancer cells.

Cisplatin and ZA are widely used chemotherapeutic agents in modern oncology. Cisplatin is typically deployed in the treatment of solid tumors (8). Clinical indications for use of the *bis*phosphonate ZA are inhibition of bone loss associated with several bone diseases, as well as tumor metastasis to bone (5). In which way the cellular metabolism of bone metastatic human cervical cancer is influenced by LLLT alone or in combination with cisplatin or ZA is incompletely understood.

Cisplatin lowered the viability of HeLa cells at all tested concentrations. Such a dose-dependent cytotoxicity is well established (23, 29). Similar results were also reported for ZA by other study groups (24).

Focusing on tumor cell metabolism, both chemotherapeutic agents reduced oxidative phosphorylation, glycolysis and glycolytic reserve, while enhancing the Warburg effect. The described effects were more distinct for cisplatin than for ZA. There is growing evidence that cisplatin, as well as its action

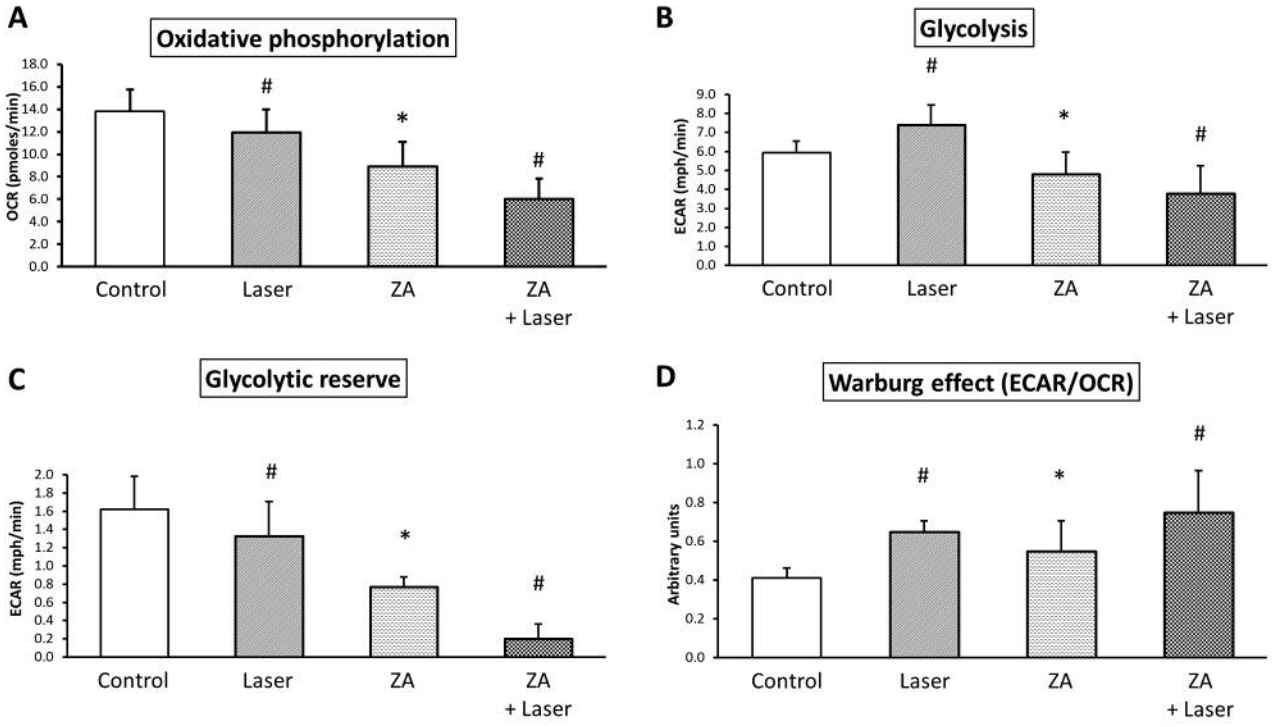

Figure 4. *Multiparameter metabolic analysis of oxygen consumption rate (OCR) for oxidative phosphorylation (A) and extracellular acidification rate (ECAR) for glycolysis (B), glycolytic reserve (C) and for the Warburg effect (D) in the presence of 100 μM zoledronic acid (ZA) with and without low-level laser therapy (LLLT). Significantly different at *p<0.05 (chemotherapy vs. untreated), #p<0.05 (LLLT vs. untreated). Data are means±SEM.*

on genomic DNA, has anti-metabolic activities resulting in reduced glycolysis and oxidative phosphorylation, as was seen in the present study. Wang *et al.* reported for the human uterine cervical cancer cell line SiHa that cisplatin reduced glycolysis and suppressed glycolysis-related protein expression, including that of glucose transporter 1 and 4 (30). Furthermore, mitochondrial accumulation of cisplatin was reported to damage mitochondrial structure and function (31). This could be one possible explanation for the impaired cellular respiration observed in our study for HeLa cells and by Alborzinia *et al.* for the colon carcinoma cells HT-29 and HCT-116 (32).

The influence of *bis*phosphonates such as ZA on the metabolism of tumor cells remains unclear. Kopecka *et al.* reported the decrease of glycolysis and the inhibition of oxidative phosphorylation, as observed in our study, for multidrug-resistant cancer cells (33). The distinct molecular effects of *bis*phosphonates on mitochondria are yet to be clarified. It was suggested that their observed pro-apoptotic effect on tumor cells, resulting in reduced invasion, adhesion, proliferation, and angiogenesis, could directly be induced *via* a mitochondria-dependent pathway (34), possibly being associated with indirect inhibition of protein isoprenylation or direct inhibition of the mitochondrial enzyme adenine nucleotide translocase through the ATP analog 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester (35, 36).

LLLT of ZA- or cisplatin-treated HeLa cells reduced cell viability even more than did the single agents. Lower levels of oxidative phosphorylation, glycolysis and glycolytic capacity accompanied this treatment, whereas the Warburg effect appeared to be induced. The increasing cellular effect on cisplatin-induced toxicity after additional irradiation is in accordance with findings of other research groups using different laser-based technologies.

Photodynamic therapy, in which a photosensitizer is activated by laser light of a specific wavelength, enhanced the anticancer effect of cisplatin as reported for colon cancer cells *in vitro* and *in vivo* by Ge and co-workers (11), as well as for squamous cell carcinoma *in vivo* by Uehara *et al.* (37). Further *in vivo* research demonstrated that the antitumor effect of a local cisplatin application can be boosted by laser thermal therapy (9) or by interstitial laser therapy (38).

Taken together, LLLT was able to raise the cytotoxicity of the applied drug at nearly every concentration tested, which is known as laser photochemotherapy (39). There is evidence

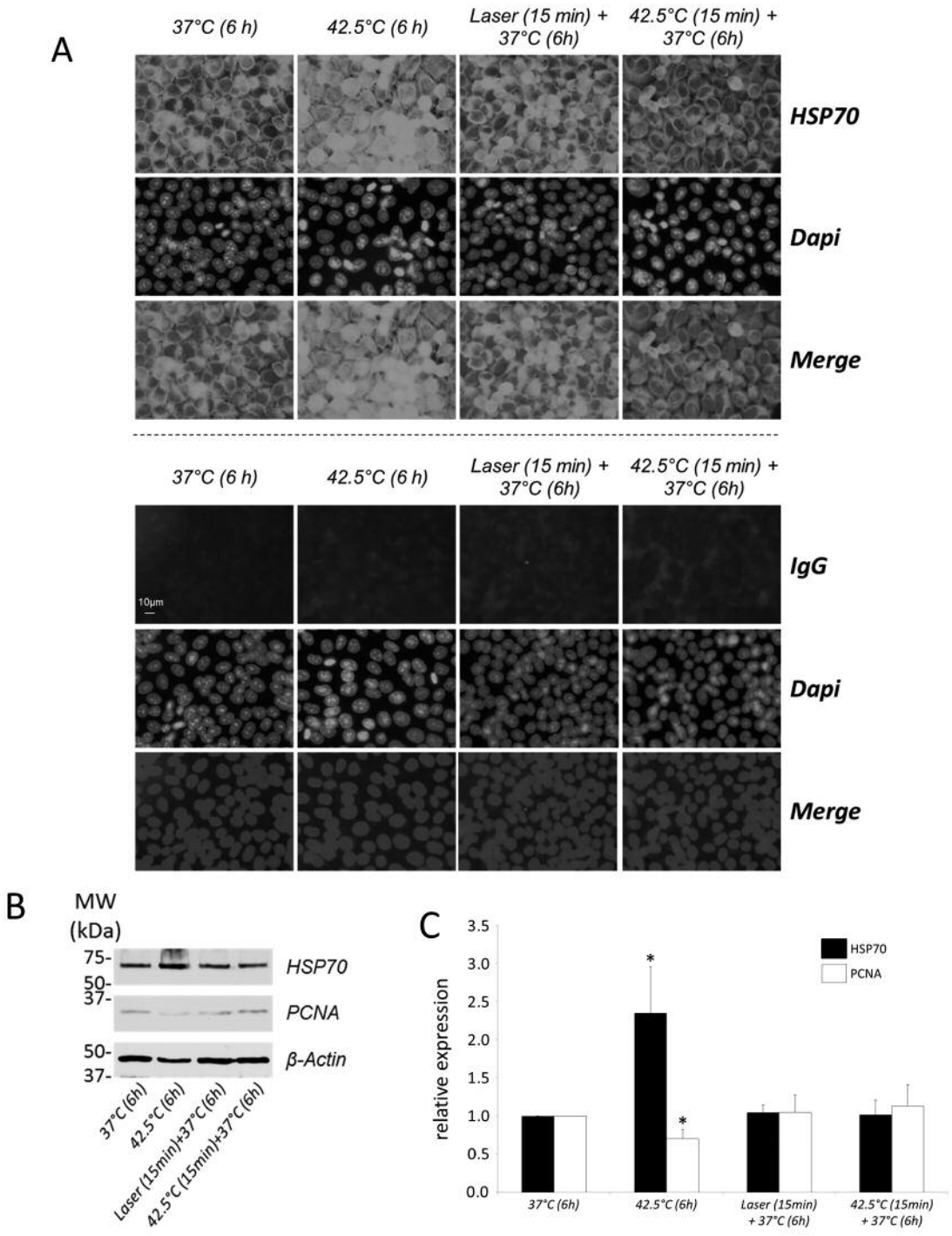

Figure 5. *Laser exposure of cultured HeLa cells does not induce expression of heat-shock protein 70 (HSP70). A: Basal expression of HSP70 was detected in non-stressed (37˚C, 6 h) as well as all treated cells by immunocytochemistry (upper panel). Only long (6 h) exposure to 42.5˚C visibly induced HSP70 expression in HeLa cells, whereas treating cells for 15 min with laser (typical therapeutic duration) or at 42.5˚C with subsequent incubation for 6 h at 37˚C did not appear to affect HSP70 expression levels. The lower panel shows control staining of HeLa cells using normal IgG to document the specificity of the HSP70 antibody. Note that all HSP70 and IgG images [fluorescein isothiocyanate channel (FITC)] were photographed at identical microscope settings (exposure times) to make images comparable with each other. The size bar shown in the upper left photograph of the lower panel is representative of all images. B: Western blot analysis confirms the observation made from (A) that only long (6 h) exposure at 42.5˚C but not short (15 min) laser or high temperature (42.5˚C) treatment was capable of significantly inducing HSP70 expression over basal levels. Similarly, proliferating cell nuclear antigen (PCNA) expression, as an indicator of cellular proliferation, was significantly reduced only in cells incubated for 6 h at 42.5˚C. Bands were quantified as described in the Materials and Methods section. Data are means±SEM (n=3). *Significantly different at p<0.05. 4',6-diamidino-2-phenylindole (Dapi).*

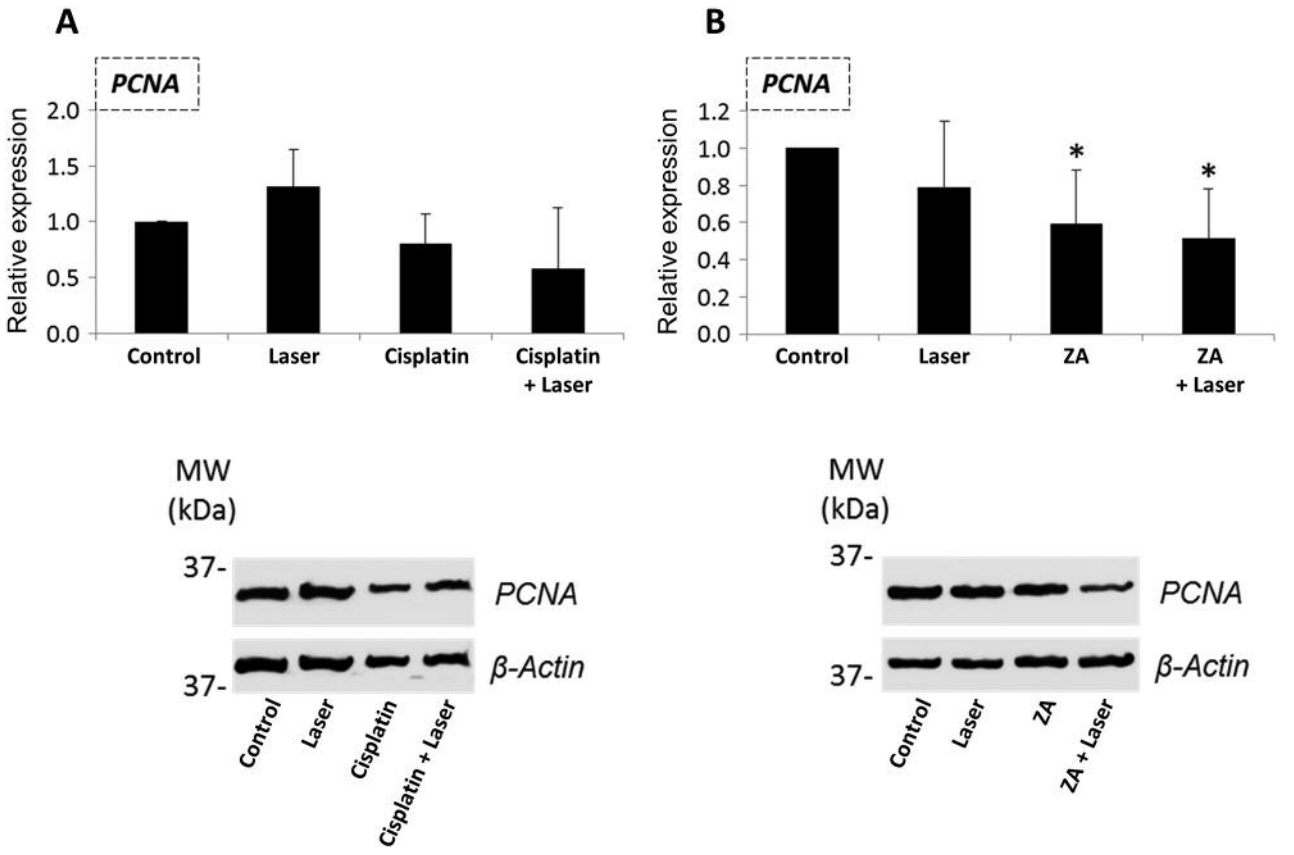

Figure 6. *Treatment of HeLa cells with laser or in combination with cisplatin or zoledronic acid (ZA) affects expression of proliferating cell nuclear antigen (PCNA). A: Western blot analysis revealed an average reduction (not statistically significant) in the expression of the proliferation marker PCNA in HeLa cells treated with CDDP or a combination of laser and CDDP (n=3, t-test). B: Similarly, PCNA expression was also reduced ($p<0.05$) in HeLa cells treated with ZA or a combination of laser and ZA (n=4, t-test). Bands were quantified as described in the Materials and Methods section. *Significantly different at $p<0.05$.*

that chemotherapeutic agents such as cisplatin are potential candidates for light or heat activation in tumor cells (40).

Furthermore, it has been demonstrated that tumor cells are able to switch between glycolysis and mitochondrial metabolism (oxidative phosphorylation) depending on environmental conditions (41, 42). Since cisplatin and ZA both have anti-glycolytic activities, glycolysis of HeLa cells will be disturbed after exposure of cells to either of the two compounds. Compensation of this anti-glycolytic activity can result in a higher mitochondrial respiration rate, in the sense of metabolic escape (43). As single LLLT without the presence of cytostatic drugs was able to lower mitochondrial respiration, LLLT in the context of laser photochemotherapy could help to disable such a metabolic escape of glycolysis-impaired cells by inhibiting mitochondrial function (Figure 7). This may also explain the observations that LLLT increased cytotoxicity of cisplatin or ZA by additional reduction of cellular respiration, thereby avoiding bioenergetic adaptation, resulting in cell growth inhibition. However, the impact of laser irradiation on HeLa cells appears to be ambivalent. Without chemotherapy, LLLT increased viability and metabolism of HeLa cells, which has also been reported in recent literature for other cell lines (44). Interestingly, LLLT was able to enhance cytotoxicity of chemotherapeutic agents, possibly by direct impact on glycolysis and mitochondrial function.

As a proof of principle, the present study evaluated the effect of laser photochemotherapy in conjunction with cisplatin or ZA on the human cervical cancer cell line HeLa. Cervical cancer is one of the most frequent malignancies affecting women, with a 5-year survival rate of only 16.5% for patients with metastatic disease (45). There is growing evidence that cisplatin-based chemotherapy has a positive impact on survival (46). As bone metastases are present in up to 22.9% of patients (21), bisphosphonates such as ZA are clinically effective (47), even though no positive effects on survival rates were observed (48).

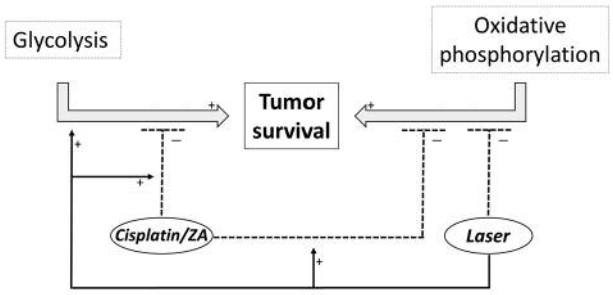

Figure 7. *Scheme depicting the possible influence of laser photochemotherapy on tumor cell metabolism. Preventing metabolic escape of glycolysis (–) arrested HeLa cells due to low-level laser therapy (LLLT)-mediated inhibition of mitochondrial function.*

In a clinical setting, laser photochemotherapy comprising cisplatin or ZA together with additional LLLT could help to improve treatment of locally advanced cancer. Another therapeutic concept, *e.g.* intraoperative brachytherapy after neo-adjuvant chemotherapy with cisplatin, could be used to kill remaining cancer cells or control bone metastases with the addition of ZA. The observation of anti-metabolic properties of cisplatin and ZA in a cell line sensitive to both chemotherapeutic compounds will also be interesting to test in other types of solid tumors such as head and neck cancer.

In conclusion, LLLT can modulate cell viability and metabolism of human cervical cancer cells. In combination with cisplatin and ZA, LLLT increases cellular toxicity, which might be explained by additional LLLT preventing metabolic mitochondrial escape of tumor cells in the presence of cytostatic drugs with anti-glycolytic effects.

The presented approach could be of interest in the evaluation of new therapeutic strategies against locally advanced tumors or when bone metastases are present.

Conflicts of Interest

The Authors report no conflicts of interest in regard to this study.

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