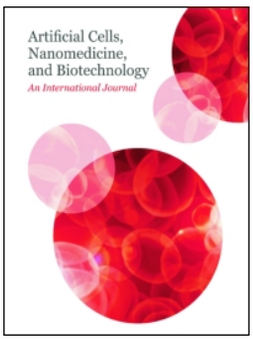

Artificial Cells, Blood Substitutes, and Biotechnology

ISSN: 1073-1199 (Print) 1532-4184 (Online) Journal homepage: http://www.tandfonline.com/loi/ianb19

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Andrei P. Sommer, Dan Zhu, Adam R. Mester & Horst-Dieter Försterling

**To cite this article:** Andrei P. Sommer, Dan Zhu, Adam R. Mester & Horst-Dieter Försterling (2011) Pulsed Laser Light Forces Cancer Cells to Absorb Anticancer Drugs – The Role of Water in Nanomedicine, Artificial Cells, Blood Substitutes, and Biotechnology, 39:3, 169-173

**To link to this article:** http://dx.doi.org/10.3109/10731199.2010.516262

Published online: 17 Sep 2010.

*Artificial Cells, Blood Substitutes, and Biotechnology,* 39: 169–173

ISSN: 1073-1199 print / 1532-4184 online
DOI: 10.3109/10731199.2010.516262

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# Pulsed Laser Light Forces Cancer Cells to Absorb Anticancer Drugs – The Role of Water in Nanomedicine

**Andrei P. Sommer and Dan Zhu**
Institute of Micro and Nanomaterials, University of Ulm, Ulm, Germany

**Adam R. Mester**
National Laser Centre, Peterfy Sandor Teaching Hospital, Budapest, Hungary

**Horst-Dieter Försterling**
Department of Chemistry, Philipps University of Marburg, Marburg, Germany





**Abstract:** Anticancer drugs executing their function intracellularly enter cancer cells via diffusive processes. Complementary to these slow processes, cells can be forced to incorporate drugs by convection – a more efficient transport process. Transmembrane convection is induced by moderately intense pulsed laser light (or light emitting diodes) changing the structure of nanoscopic water layers in cells. This is a fundamental difference with the method of photodynamic therapy. In a model system we demonstrate that a total irradiation time of one minute is sufficient to completely inhibit proliferation of cancer cells. Transmembrane convection protects healthy cells from extended chemotherapy exposure, could be exploited to overcome multidrug resistance, and is a promising new tool in a variety of therapies as well as in skin rejuvenation.

**Keywords:** transmembrane convection, intracellular interfaces, laser, light emitting diodes, cells, cancer, cytostatic, cytotoxic

## INTRODUCTION

The space in the interior of cells is densely crowded with a variety of hydrophilic surfaces: macromolecules and organelles [1]. Necessarily, a substantial fraction of the intracellular water prevails in the form of interfacial water layers (IWL), confined between proximal surfaces. Experiments performed on hydrophilic model surfaces at room temperature provided insight into the organization of the molecules constituting IWL, which is different from bulk water [2]. The difference manifests itself qualitatively and quantitatively in the absorption of 670 nm laser light – a wavelength virtually not absorbed by bulk water – by the rigid IWL, and is reflected in significantly elevated viscosities and densities compared to bulk water [3,4]. These parameters decreased instantly in response to moderately intense ($\leq$1000 $Wm^{-2}$) 670 nm laser light [5]. Here we demonstrate that intracellular IWL can be modulated by exposure of cells to 670 nm laser light at intensities as low as 1000 $Wm^{-2}$. Moderately intense red to near-IR radiation, applied in a continuous mode at fluences in the range of $1 - 4 \times 10^4$ $Jm^{-2}$, was shown to restore the vision of methanol-blinded rats [6], to promote cell survival in vitro, and has been used for more than 40 years in clinical wound healing [7]. There is evidence in vitro that moderately intense 670 nm light delivered in a pulsed mode provokes cellular responses superior to those stemming from continuous mode irradiation. The efficacy of pulsed light is documented in vitro [8] and in vivo [9], but the intrinsic cause for the effect of pulsed light is not clear. Indeed, on a biological basis it is not explicable. However, a coherent interpretation of the reported effects of moderately intense laser light on IWL offers a compelling explanation. From the instant decrease in density and viscosity of the IWL on hydrophilic model surfaces in response to the light we are led to expect analogous processes in cells, i.e., a simultaneous reduction of the density and viscosity

Address correspondence to Andrei P. Sommer, Institute of Micro and Nanomaterials, University of Ulm, 89081 Ulm, Germany. E-mail: andrei.sommer@uni-ulm.de

of the intracellular water prevalent as IWL. Because of the reciprocal behavior between volume and density, this can only mean that upon exposure to moderately intense 670 nm laser light, cells are forced to push out a fraction of their fluid content. Importantly, once the laser is turned off the intracellular IWL consolidates practically instantly [10], inducing a reflux of molecules into the cell. Concomitant with a transient inertia of the cell membrane, alternating expansion and contraction of the cytosol is equivalent with an externally triggered cell metabolism. The principle of the light modulated pumping process was put forward by us in an earlier paper [11] and is validated in this work. It is instructive to relate the mechanisms of the light-cell pump to the definition of density.

## MATERIALS AND METHODS

In vitro verification of the validity of the light-cell pump mechanism is simple and consists of supplementation of the cell culture medium with a cytostatic drug and application of pulsed light. We used HeLa cells as a model system and green tea-extracted polyphenols as a cytostatic (cytotoxic) drug. Green tea is rich in epigallocatechin gallate (EGCG). Both the tea and pure polyphenol are potent tumor inhibitors [12]. HeLa cells were seeded in 24-well plates, 100.000 cells in 1 mL culture medium per well, and supplemented with 10, 50 and 100 µL filter-sterilized (pore Ø 0.45 µm) green tea solution, respectively. The solution was prepared from 3 g of dry leaf mass per 250 mL of ultrapure water (brewing temperature 100° C, cooling time 30 min) [13]. Subsequent to supplementation of the culture medium with the drug the cells were irradiated for 1 min by scanning the line-shaped beam of a 670 nm laser over the wells containing the cells. Power, intensity, fluence, scanning frequency and beam geometry were 33 mW, 1000 $Wm^{-2}$, $1 \times 10^4$ $Jm^{-2}$, 1 Hz and $2 \times 15$ mm, respectively. Irradiated cells were incubated with non-irradiated controls for 52 h and counted using a CASY Cell Counter (Innovatis, Reutlingen, Germany).

## RESULTS

Calculated maximum EGCG concentrations per well were 5, 25 and 50 µM, respectively [14]. In HeLa cells EGCG exhibits its full cytotoxic potency at approximately 50 µM. In most in vitro studies EGCG is reported to show a pronounced cytostatic activity at 50 µM [15]. For the HeLa cells grown in this concentration exposure to the pulsed light resulted in complete proliferation arrest. Explicitly, the initial and final cell numbers were virtually equal (Figure 1). In the non-irradiated group exposed to 50 µM EGCG cell numbers

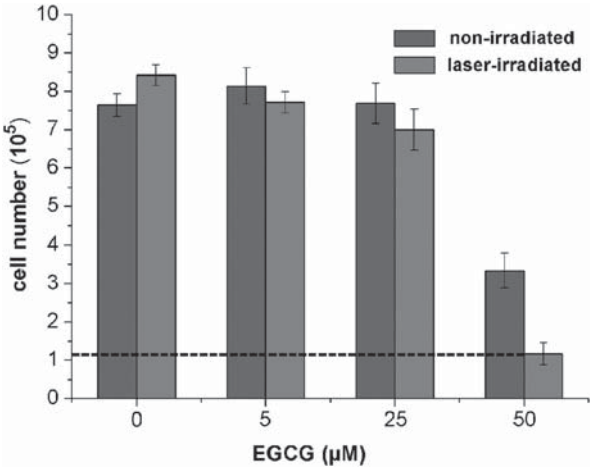


***Figure 1.*** Effect of EGCG and periodic laser irradiation on proliferation of HeLa cells. Complete proliferation arrest at 50 µM EGCG and 1 min periodic irradiation [13].

increased to 330.000, and in the non-irradiated control group without cytostatic drug they increased to 760.000.

### Theory of the Light-Cell Pump

Normally, the mean density $\rho_c$ of a cell in a state of equilibrium is expressed as the ratio between cell mass $m_c$ and volume $V_c$ (equation 1 in Figure 2), where $V_c$ comprises the space within the cell membrane. Equation 2 (Figure 2) provides a more accurate description of the physical situation within the cell membrane. Explicitly, it accounts for both the volume of the water $V_w$, and that of the solid granular material $V_s$ within the cell membrane, i.e., macromolecules and organelles. On this basis, experiments probing the softness of living cells by recording their response to shear indicated a resemblance to soft materials, including colloidal glasses and toothpaste [16]. One deficiency of equation 2 (Figure 2) is that it does not discriminate between bulk water and IWL, co-present in

$$\bar{\rho}_c = \frac{m_c}{V_c} \quad (1)$$

$$\bar{\rho}_c = \frac{m_c}{V_s + V_w} \quad (2)$$

$$\bar{\rho}_c = \frac{m_c}{V_s + V_{bw} + V_{iw}} \quad (3)$$

***Figure 2.*** The molecular organization in interfacial water layers (*iw*) on hydrophilic surfaces is different from that in bulk water (*bw*). Whereas equation (1) describing the density is simple, its extension for implementation of interfacial water layers is by no means trivial. Equations 1-3 provide a mathematical explanation of the relationship between the structural variations in intracellular water in response to the irradiation of cells with moderately intense laser light – the key for understanding the mechanism of the light-cell pump.

cells. More close to reality is therefore the picture reflected by equation 3 (Figure 2), where the terms $V_{bw}$ and $V_{iw}$ stand for the volume of the intracellular bulk water and IWL, respectively. On the basis of equation 3 (Figure 2), the conversion of the intracellular IWL to virtually bulk water, in response to irradiation with moderately intense 670 nm light, clearly predicts that the aqueous fraction of the cellular volume will instantly expand. This expansion is primarily ascribed to the decrease in the density of the intracellular IWL, associated with an increase in the term $V_{iw}$. From the intuitive picture of rigid IWL confined between solid granular material, and irradiation-induced sublimation of the water constituting the IWL [10], it is expected that the density of the irradiated confined phase could temporarily even undershoot normal bulk water densities. In accord with the simultaneous decrease in the viscosity (increase in fluidity) of the IWL, the cell will either expand its total volume or keep it constant and push out a fraction of its aqueous content. However, expansion in total volume involves stretching the cell membrane – an energy-costly process. Thus, it is reasonable to expect that cells with a certain permeability to water will favor the second possibility, and regulate the variation in $\rho_c$ by pushing out a fraction of their aqueous content. Conversely, upon interrupting the irradiation, the intracellular IWL will instantly consolidate, thereby diminishing the intracellular bulk water content. The only possibility left to the cell to compensate for the instant drop in $\rho_c$ is to absorb water from the environment.

Interestingly, the usefulness of the pulsed delivery mode in photobiomodulation has been challenged [17,18]. However, full insight into the effect of 670 nm laser light on IWL has been provided only recently; otherwise the impact of the pulsed delivery mode on IWL and its extrapolation to the intracellular water would have been ascertain earlier. The recent finding that pulsed, moderately intense 670 nm light not only significantly increased the proliferation of HEp-2 cells, as compared to continuous mode irradiation, but also their metabolic activity (greater oxidative burst) [8] is now fully explained. From the dynamic character of the pumping process, it is plausible that the transport across the cell membrane is not limited to water molecules. Under the influence of the convective flow, other molecules within the range of the flow will cross the membrane as well. By facilitating the transmembrane transport, pulsed light force the cells to incorporate additional amounts of nutrients from the culture medium.

The explanation of two photobiological effects as different as the enhanced proliferation and metabolic activity of HEp-2 cells, on the one hand, and the inhibition of HeLa cells, on the other hand, validates the light-cell pump mechanism. From considering the instrumentality of the light-cell pump in enhancing the potency of anticancer drugs, it is clear that it will gain importance in the therapy of cancer, in particular melanoma [19]. On the same grounds, the light-cell pump is expected to become a tool for the transport of topically applied nutrients and/or antioxidants along successive cell layers deep into the skin. These applications are visually summarized in Figure 3.

## DISCUSSION

In photobiomodulation experiments performed with devices operating in a continuous mode and delivering fluences and intensities virtually equal to those used in our study, the standard action spectrum of the light involves the increase in cellular ATP by activation of the mitochondrial respiratory chain, and/or release of stimulating levels of reactive oxygen species generated by mitochondria. In both cases the normal cellular response is proliferation, even in the presence of cytostatic drugs. When the irradiation is performed in a continuous mode – with or without a cytostatic drug – inhibitory effects require fluences surpassing the upper limit described by the classical biostimulatory windows [20]. The validity of this important aspect was illustrated in a recent pilot study designed to explore synergistic effects of moderately intense 660 nm laser light and cytostatic drugs on murine mesenchymal stem cells [21]. In other words, the efficacy of the light-cell pump could be systematically improved by extending the total time of irradiation. For applications in vivo this possibility necessitates consideration of higher light fluences on healthy cells [20]. A further possibility to improve the efficacy of the light-cell pump consists in finding the optimal pulse frequency profile. Optimizing drug uptake in these ways represents an unexplored field. There are uncertainties in our straightforward approach, which should be clearly mentioned. The first concerns the cell itself (a reasonable permeability to water is pivotal for the susceptibility of cells to the pulsed light), the second, its direct environment. Cells studied in vitro are surrounded

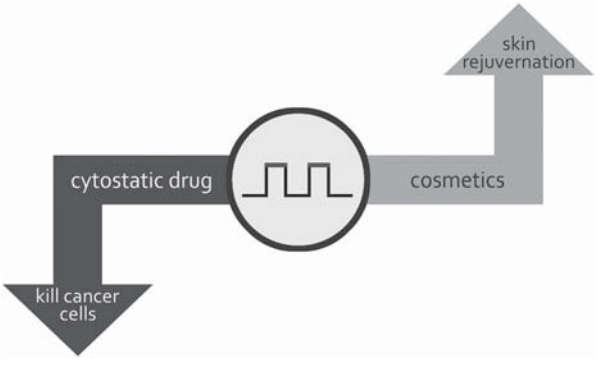


***Figure 3.*** Self-explanatory representation of two sides of the light-cell pump: In oncological applications it can transport chemotherapy drugs into cancer cells, whereas in dermatological applications it can serve as a tool to bring nutrients and/or antioxidants deep into the skin.

by copious amounts of culture liquid, including water. Corresponding cells in an in vivo system are surrounded by less water. However, in order to operate in vivo the light-cell pump needs a water-rich milieu. An extracellular matrix, which is poor in water, could limit the functionality of the light-cell pump in vivo. Because of the limited range of the convective flow, the efficacy of the light-cell pump should depend on the local concentration of the substance designated for cellular import. Thus, the transfer of results ascertained in an in vitro model into an in vivo system is by no means trivial. Solutions to these fundamental problems are an attractive challenge to computer simulations. Interestingly, the light-cell pump recommends itself to be utilized complementary to chemical and biochemical methods, and there, even in combination with EGCG. The motivation for the combinational strategy roots in increasing observational evidence that EGCG is suppressing multidrug resistance in various cancer cells. Thus, even in cancer cells where the light-cell pump fails to carry anticancer drugs into the cells, administration of EGCG (or green tea) and suitable light modulation promise to enhance the Trojan horse function of EGCG, thereby eventually helping to destroy cancer. Prospective in vitro experiments involving the evaluation of the efficacy of the light-cell pump in selected cell-drug pairings will also have to consider the effect of the substrate and that of interfacial water layers on the substrate on cell performance [22], as well as the possible effect of the irradiation on the cell-substrate interaction. Preferably, ideal tests will be carried out on substrates preserving their chemical and biological inertness, thereby accounting for the potential of the light-cell pump to carry both ions and molecules from the environment into the cells. Presumably, the light-cell pump is even applicable to nanoparticles and drugs with low water solubility. Experiments using modern anticancer drugs are in the course of preparation.

## CONCLUSIONS

Enhanced cell proliferation in response to moderately intense laser irradiation is widely exploited in clinical practice. Despite the reproducibility of the effect, there was no immediate explanation of the biological mechanism, at least not in the early years of photobiomodulation. Today, the cause for the proliferative effect is understood and traced back to the activation of chemical and biochemical cellular processes. Recently, several groups reported that the biological effect of light delivered by lasers and light emitting diodes is more pronounced when it is delivered in a pulsed mode, as opposed to continuous mode. Here we provide an explanation of the difference, and show that pulsed light is involved in the activation of physicochemical cellular processes. We arrive to their formulation by modelling the results of current laboratory experiments focusing on the impact of moderately intense laser light on interfacial water layers. Their systematic analysis leads to the discovery of the principles of light-induced transmembrane convection – a powerful biomedical approach and basis of the light-cell pump. Potential applications include but are not limited to novel anticancer therapies and deep facial rejuvenation. In the latter, pulsed irradiation is supposed to transport nutrients and antioxidants from cell to cell into deeper skin layers.

***Declaration of interest:*** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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