

Photodynamic inactivation of *Escherichia coli* by methylene blue incorporated in ZSM-5 zeolite channels under red LED light

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Abstract

Photodynamic therapy (PDT) is a method that combines the use of nontoxic components, namely photosensitizer, light and oxygen to cause localised oxidative photo-induced damage. The aim of this study was to contribute to PDT development by studying an alternative light sources using red light-emitting diode (LED) light and evaluated its effect on the photodynamic activity of methylene blue (MB) in biological experiments. To prepare a new heterogeneous photocatalyst by incorporating the MB into ZSM-5 zeolite structures, which effectively produces ROS by photoactivation with visible light. The investigations of the effectiveness of PDT with red LED light and MB, afterwards with ZSM-5-MB were carried out on Gram-negative bacteria *Escherichia coli* CCM 3988.

Keywords: methylene blue, zeolite, *Escherichia coli*, light-emitting diode, inhibition

Introduction

Photodynamic therapy (PDT) is a method that utilizes the harmless visible or ultraviolet light in combination with a photosensitizing agent. It induces several phototoxic reactions which results in cell damage (photodamage). The photodynamic reaction involves a light absorption by a photosensitizer, non-toxic photoactive dye, to excite the molecule into the excited singlet state. This state undergoes a transition leading to the long-living triplet state, which can react with molecular oxygen inducing the formation of reactive oxygen species (ROS), such as singlet oxygen, superoxide, and radicals. These ROS can oxidize the surrounding bioorganic

molecules, such as proteins, lipids, nucleic acids, leading to cell death (Mantareva et al. 2007, So et al. 2009, Hamblin et al. 2002).

The laser sources and their high-potency are preferable for PDT, but their high costs make it inaccessible in most countries. Nowadays, various alternative light sources have been appeared. One of the most interesting of these is the light-emitting diode (LED) because of its low price. It is possible to find different colours of LED light in the market, with radiations covering almost all of the visible electromagnetic spectrum (Machado 2000, Mang 2004). The commercial phenothiazine dyes are a class of aromatic photosensitizing molecules and several compounds based on the phenothiazinium chromophore possess antibacterial properties (Phoenix et al. 2003). Methylene blue (MB, Figure 1) with high light absorption at 665 nm is an effective photosensitizing agent for the inactivation several types of microorganisms (Peloi et al. 2008, Usacheva et al. 2001). In recent years, several approaches have been proposed for applying MB and other photosensitizers in medicine, namely for the disinfection of blood and photodynamic antimicrobial chemotherapy (Wainwright 2002, Ulatowska-Jarza et al. 2006).

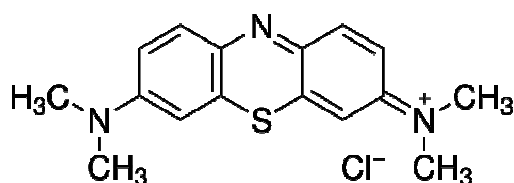


Figure 1. Structure of methylene blue

PDT appears as a promising technique against viruses, bacteria, and fungi. Therefore it used as a therapy for the local treatment of infections (Demidova and Hamblin 2005). This therapy has been successfully used for the treatment of several diseases involving abnormal cell growth (cancer), rheumatoid arthritis, vitiligo, arteriosclerosis and other diseases. The broad spectrum of antimicrobial activity makes photodynamic techniques quite promising also for the disinfection of microbiologically contaminated water (Jori and Brown 2004). PDT is considered to be safe method and all efforts are being made to minimize its cost, which returns to the photosensitizers used (Peloi et al. 2008, Solouma and Fekry 2005).

The aim of this study is to contribute to PDT development by studying alternative light sources using red LED light and its effect on the photodynamic activity of MB in biological experiments. The further aims of this work are to prepare a stabile heterogeneous photo catalyst by incorporating the MB into ZSM-5 zeolite structures and to evaluate the effect of

this photo catalyst. The investigations of the effectiveness of PDT with LED light and MB including ZSM-5-MB were carried out on Gram-negative bacteria *Escherichia coli*.

Materials and Methods

Chemicals and light

MB (SIGMA-ALDRICH, USA, $C_{16}H_{18}ClN_3S \cdot 3H_2O$, MW 373.9 g.mol⁻¹), ZSM-5 (VÚRUP, Bratislava, Slovak Republic) and all other reagents and solvents used were of analytical grade and used without purification. The LED light system (PowerR export-import, Slovak Republic) was constructed using 4 units (every of units consist of 16 LED lamps with the power of 1 W) that emitted red light.

Bacterial strain

Escherichia coli CCM 3988 from the Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic was used in experiments.

Preparation of the catalyst

To prepare the impregnating solution 0.4 g of MB was suspended in 100 cm³ of demineralised water. 5 g of zeolite ZSM-5 was added to solution and the suspension was stirred by magnetic mixer at 15 °C. After 15 h of stirring, the product was filtrated through a paper filter and rinsed with distilled water to gain a colourless filtrate. Furthermore, it was washed in 100 cm³ of demineralised water, filtrated and dried in a desiccator.

Spectral characterization of the catalyst and irradiation lamp

The diffuse reflectance UV/VIS spectrum was measured with Specord M-40 apparatus (Zeiss, Germany) and measured data was transformed to Kubelka-Munk parameters. For measuring the spectrum, BaSO₄ was used as standard. Spectrum of used lamp was measured with UV/VIS spectrometer Ocean Opt. HR4000 from a distance of 5 cm.

Determination of the effect of MB on E. coli

The effect of MB on *E. coli* growth was determined by microdilution method, carried out in micro-plates type P (GAMA, České Budějovice, Czech Republic). Mueller-Hinton broth (MHB) was inoculated with overnight grown culture of *E. coli* (final cells density in MHB was $10^4 \cdot \text{ml}^{-1}$). All experiments were carried out in two groups. One group was exposed to the light and the other group was kept in the dark. Two control groups were prepared: one by adding 200 μl MHB with cells of *E. coli* (positive control) and the other by mixing 150 μl of MHB without bacteria + 50 μl of MB solution - final concentrations in MHB were 7, 13, 20, 35 and 50 $\mu\text{mol} \cdot \text{l}^{-1}$ (as a background). 150 μl MHB inoculated by *E. coli* was added to 50 μl of MB solution at final concentrations ranging from 7-50 $\mu\text{mol} \cdot \text{l}^{-1}$ (Jantová et al. 1995, Peli et al. 2008). The light source was placed vertically at a distance of 12 cm above the micro-plates. These micro-plates were irradiated under shaking at 37 °C for 8 h. The growth of *E. coli* was quantified spectrophotometrically by Humareader (Lamda Life, Bratislava, Slovakia) at 630 nm, measured until confluent growth.

The antimicrobial effect of MB was characterized by the IC_{50} values (concentration of MB, which in comparison to the control inhibits the growth of bacteria to 50 %) and MIC values (minimal inhibitory concentration of MB, which inhibits bacterial growth by 100 %). The IC_{50} and MIC values were read from toxicity curves (Dudová et al. 2002).

Effect of ZSM-5-MB on E. coli

MHB was inoculated with overnight grown culture of *E. coli* (final cells density in MHB was $10^4 \cdot \text{ml}^{-1}$). The experiments were carried out in 5 ml in glass bacterial tubes (MHB with cells of *E. coli* and particular amount of ZSM-5-MB). The amounts of ZSM-5-MB were ranging from 500 to 2000 mg. The control samples were cultivated without the presence of photocatalyst and every sample in experiments was prepared in three parallels. The light sources were placed vertically at a distance of 12 cm above the glass bacterial tubes. These tubes were irradiated under shaking at 37 °C for 10 h. The standard plate count method was used to determine the total number of viable cells of *E. coli* as the colony forming units (CFU) (Houghtby et al. 1992). The initial sample was diluted through serial dilution in saline (10^{-1} - 10^{-8}). Diluted sample (100 μl) was placed on a sterile Petri agar plate with the Mueller-Hinton agar (MHA) in triplicate sets. After incubation (24 h at 30 °C) the number of colonies

was counted (mean of 3 experiments). Each experiment was followed by an experiment carried out in the dark. The number of viable cells as CFU was calculated as a percentage in comparison to the control without zeolite system (100 % growth).

Results and Discussion

Differential reflectance UV/VIS spectrum was achieved by measuring the diffuse reflectance spectra of ZSM-5-MB and raw ZSM-5 and collected Kubelka-Munk's parameters were subtracted to obtain the differential spectra (Figure 2).

It can be seen that the prepared heterogeneous photocatalyst ZSM-5-MB effectively absorbs light with $\lambda_{\max} \approx 645$ nm (Figure 2). Therefore, we may assume that the photocatalyst may be effectively excited by irradiated with red LED light (λ ranging from 590 to 670 nm) and following photophysical processes of energy transfer to the present molecular oxygen will lead to formation of various ROS ($^1\text{O}_2$, O_2^- , $\cdot\text{OH}$, H_2O_2) (Konovalova et al. 2004). The red LED is emitting light in the visible region of the spectrum. Therefore, it was considered as suitable for our experiments.

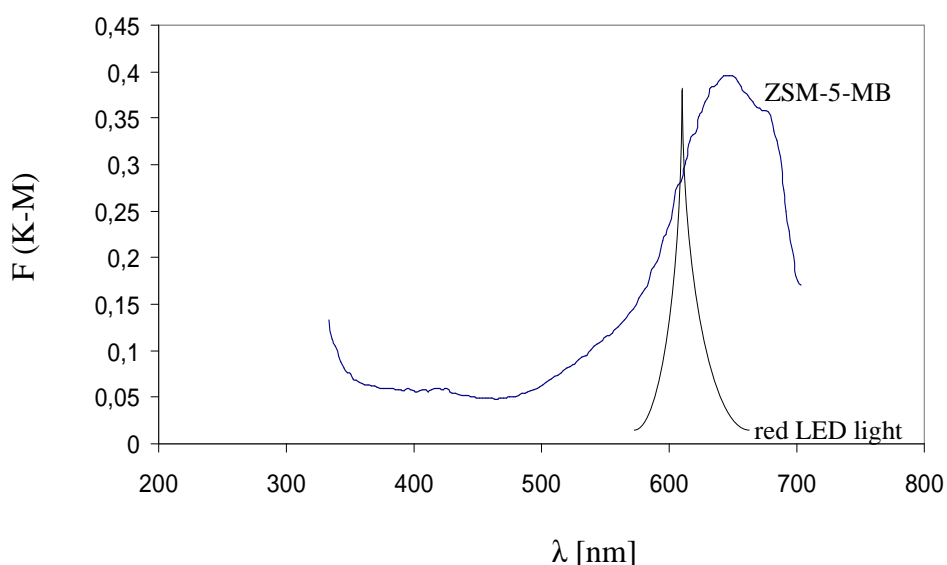


Figure 2. Differential reflectance UV/VIS spectrum of ZSM-5-MB photocatalyst and red LED light emission spectrum.

In our study the effect of photodynamic activity of MB using red LED light system was determined. It was found that irradiated cells of *E. coli* were eradicated to some extent by red LED light in the presence of MB. Though, the degree of photo-induced damage was

dependent upon the concentration of dye. The confluent growth of *E. coli* was reached after 8 h cultivation in MHB (positive control group). The red LED light had no effect on the growth of *E. coli*. After addition of MB at the different concentrations, the percentage of growth (*E. coli*) decreased proportionally to the concentration of MB increased (Figure 3). In Table 1, it is shown that the increasing concentration of MB under the red LED light resulted in greater by growth inhibition of *E. coli*, after 8 h. By comparing the percentage of growth inhibition, the light has significant effect on cells growth.

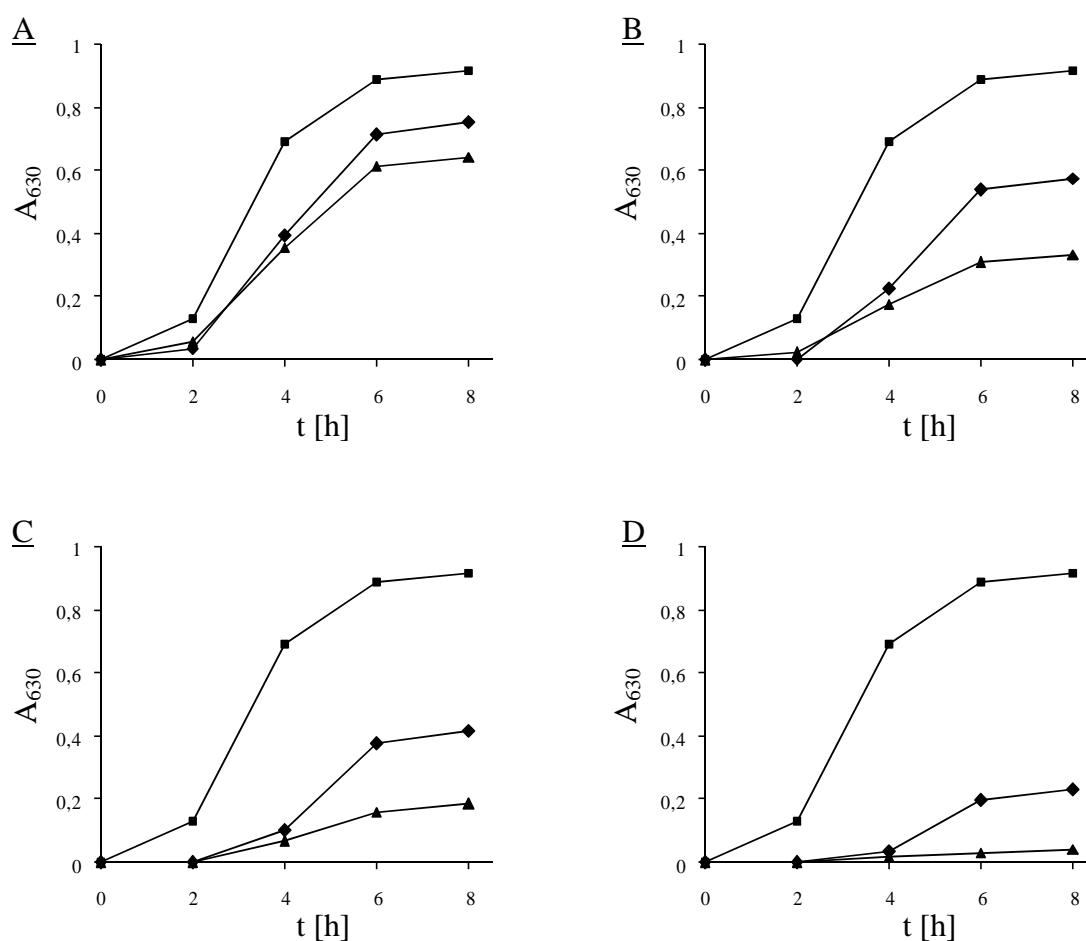


Figure 3. The effect of red LED light at the different concentrations of MB on *E. coli*. (A) 7 $\mu\text{mol.l}^{-1}$ MB, (B) 13 $\mu\text{mol.l}^{-1}$ MB (C) 20 $\mu\text{mol.l}^{-1}$ MB (D) 35 $\mu\text{mol.l}^{-1}$ MB; (■) control - *E. coli* (100 % growth), (▲) MB irradiated with red LED light, (◆) MB in dark conditions.

Table 1. The growth of *E. coli* at various concentrations of MB.

MB [$\mu\text{mol.l}^{-1}$]	Inhibition [%]	
	Light	Dark
7	31.0	23.9
13	65.2	42.8
20	81.9	59.7
35	100	78.9
50	100	100

The 50 % inhibition (IC_{50}) of *E. coli* was observed at $10.4 \mu\text{mol.l}^{-1}$ for irradiated samples and $16.1 \mu\text{mol.l}^{-1}$ for non-irradiated samples. The MIC values were observed at $35 \mu\text{mol.l}^{-1}$ for irradiated samples and $50 \mu\text{mol.l}^{-1}$ for non-irradiated samples. Preparing of zeolite system (ZSM-5-MB) could significantly enhance the production of $^1\text{O}_2$ on the surface and the antibacterial efficiency of MB too (Bujdák et al. 2009).

In our experiments, the effect of photocatalyst ZSM-5-MB on the cells growth of *E. coli* was evaluated. Figure 4 demonstrates that the number of viable cells of *E. coli* decreased in dependence on the amount of photocatalyst ZSM-5-MB after irradiation by red LED light ($t_{\text{exp}}=10 \text{ h}$). The control experiments carried out in the dark conditions indicated no growth inhibition (100 % growth for ZSM-5-MB at any amount). From the results listed in Table 2, it is clear that the amount of photocatalyst affects the growth of *E. coli*.

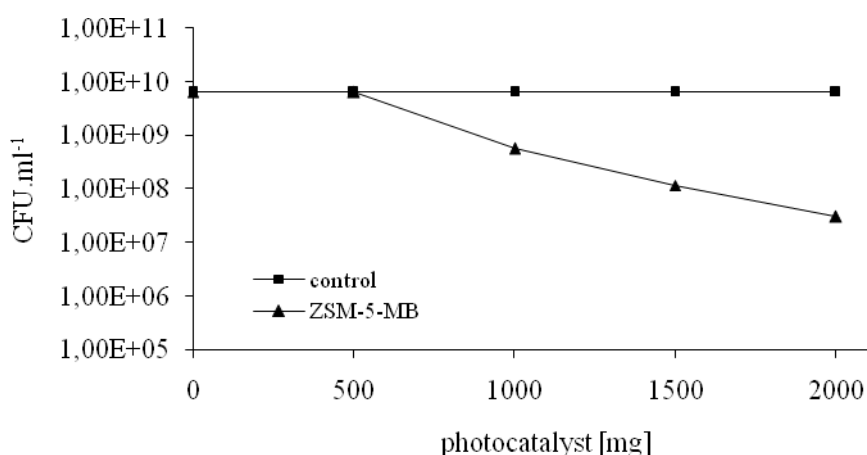


Figure 4. The effect of irradiated photocatalyst ZSM-5-MB on the *E. coli* growth after 10 h cultivation.

Table 2. The growth of *E. coli* at various amounts of ZSM-5-MB.

ZSM-5-MB [mg]	Number of viable cells as CFU.ml ⁻¹	Inhibition [%]
500	6.42×10^9	0
1000	5.60×10^8	11.0
1500	1.18×10^8	18.2
2000	3.05×10^7	45.5

It can be seen from Figures 5 and 6 that the number of viable cells of the bacteria *E. coli* decreased inverse proportionally with the amount of ZSM-5-MB.

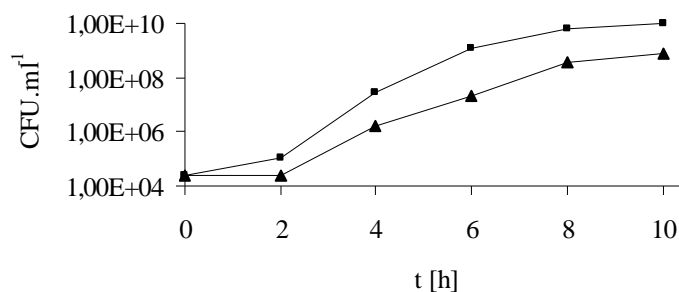


Figure 5. The effect of red LED light on the *E. coli* cell growth in the presence of photocatalyst ZSM-5-MB (1500 mg). (■) control - *E. coli* (100 % growth), (▲) ZSM-5-MB irradiated with red LED light (18 % inhibition).

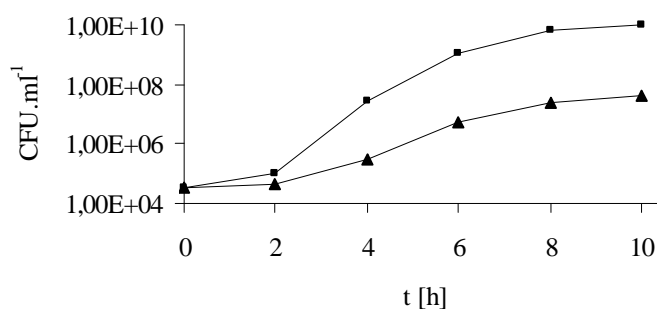


Figure 6. The effect of red LED light on the *E. coli* cell growth in the presence of photocatalyst ZSM-5-MB (2000 mg). (■) control - *E. coli* (100 % growth), (▲) ZSM-5-MB irradiated with red LED light (46 % inhibition).

While MB inhibited the growth of *E. coli* in the dark and in the light, the photocatalyst ZSM-5-MB inhibited the growth of *E. coli* only by the effect of red LED light. The increased inhibition activity of MB and ZSM-5-MB in light may be explained by the generation of ROS. In the studies carried out by Ploi et al (2008) a concentration of $35.2 \mu\text{mol.l}^{-1}$ MB exposed to LED light at intensities of 2 and 4 J.cm^{-2} led to 89 % and 94 % inhibition, respectively, and at a concentration of $70.4 \mu\text{mol.l}^{-1}$ MB with 6 J.cm^{-2} , induced 96 % inhibition. In this study, the best PDT response to eliminate *E. coli* was achieved with exposure to red LED light in combination with the photosensitizer at concentration of $35 \mu\text{mol.l}^{-1}$, induced 96 % inhibition and in combination with the photocatalyst ZSM-5-MB at amount of 2000 mg, led to 46 % inhibition.

Conclusion

The aim of this work was to contribute to PDT development using red LED light and evaluated its effect on the photodynamic activity of MB in biological experiments, to prepare a new stable heterogeneous photocatalyst, which by photoactivation with visible light effectively produces ROS and is utilizable in photoinactivation of pathogen microorganisms. The investigations of the effectiveness of PDT with red LED device and MB, and with ZSM-5-MB were carried out on Gram-negative bacteria *Escherichia coli*. The prepared system effectively absorbs light in the visible region of the spectra with $\lambda_{\text{max}} \approx 645 \text{ nm}$. While MB inhibited the growth of *E. coli* in the dark and in the light, the photocatalyst ZSM-5-MB inhibited the growth of *E. coli* only by the effect of red LED light. The number of dead cells was increased with the increase of MB concentration as well as of ZSM-5-MB amount present in the growth medium. The red LED light appears to be a very good option for PDT because of its low cost and efficacy.

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References

- Bujdák J, Jurečková J, Bujdáková H, Lang K, Šeršeň F (2009) Environmental Science & Technology 43: 6202-6207
- Demidova TN, Hamblin MR (2005) Antimicrobial Agents and Chemotherapy 49: 2329-2335.
- Dudová B, Hudecová D, Pokorný R, Mičková M, Palicová M, Segl'a P, Melník M (2002) Folia Microbiol. 47: 225-229
- Hamblin MR, O'Donnell DA, Murthy N, Rajagopalan K, Michaud N, Sherwood ME, Hasan T (2002) Journal of Antimicrobial Chemotherapy 49: 941-951
- Houghtby GA, Maturin LJ, Koenig EK, Wesser JW (1992). In: Marshall RT (Ed) Standard Methods for the Examination of Dairy Products, 16th Edition (pp 213-246). American Public Health Association, Washington (DC)
- Jantová S, Hudecová D, Stankovský Š, Špirková K, Ružeková L (1995) Folia Microbiol. 40: 611-614
- Jori G, Brown SB (2004) Photochem. Photobiol. Sci. 3: 403-405
- Konovalova TA, Lawrence J, Kispert LD (2004) J. Photochem. Photobiol. A 162: 1-8
- Machado AEH (2000) Química Nova 23: 237-243
- Mang TS (2004) Photodiagnosis and Photodynamic Therapy 1: 43-48
- Mantareva V, Kussovski V, Angelov I, Borisova E, Avramov L, Schnurpfeil G, Wöhrle D (2007) Bioorganic & Medicinal Chemistry 15: 4829-4835
- Peloi LS, Soares RRS, Biondo CEG, Souza VR, Hioka N, Kimura E (2008) J. Biosci. 33: 231-237
- Phoenix DA, Sayed Z, Hussain S, Harris F, Wainwright M (2003) FEMS Immunology and Medical Microbiology 39: 17-22
- So CW, Tsang PWK, Lo P-C, Seneviratne CJ, Samaranayake LP, Fong W-P (2009) Photodynamic inactivation of *Candida albicans* by BAM-SiPc, Hong Kong, China: 1-6
- Solouma NH, Fekry O (2005) Assessment of the Validity of Photodynamic Therapy by Image Analysis and Interpretation, GVIP 05 Conference, Cairo, Egypt
- Ulatowska-Jarza A, Holowacz I, Razik J, Wieliczko A, Nowak K, Streck W, Czernielewski L, Podbielska H (2006) Proceedings of the Symposium on Photonics Technologies for 7th Framework Program, Wroclaw, Poland: 546-549
- Usacheva MN, Teichert MC, Biel MA (2001) Lasers in Surgery and Medicine 29: 165-173
- Wainwright M (2002) Current Medicinal Chemistry 9: 127-143