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OPTICAL IRRADIATION OF CYTOSTATICS: PHOTOPRODUCTS CHARACTERIZATION AND TESTS ON RABBIT EYE PSEUDOTUMORS

Adriana SMARANDACHE[1], Angela STAICU[1], Andra DINACHE[1], Ionut Relu ANDREI[1], Ruxandra Angela PIRVULESCU[2], Mihail Lucian PASCU[1,3]

[1] National Institute for Laser, Plasma and Radiation Physics, Magurele, Ilfov, Romania
[2] University of Medicine and Pharmacy "Carol Davila", Emergency University Hospital, Ophthalmology Clinic, Bucharest, Romania
[3] University of Bucharest, Faculty of Physics, Bucharest, Romania
Corresponding author: Mihail Lucian PASCU, E-mail: mihai.pascu@inflpr.ro

Abstract. This is a review about exposure of drugs solutions to uncoherent and laser radiation in order to induce changes in drugs' molecules and to generate photoproducts that alone or in mixtures have photo-enhanced activity. This could be a way to combat the failure of treatments when multidrug resistance is acquired by microorganisms or tumors. The structural modifications induced by optical radiation on cytostatic compounds were explored by optical absorption, fluorescence, and Fourier transform infrared spectroscopy. Results on 5-flurouracil, alongside with a new synthesized pyridine derivative (BG1120) are detailed. The effects of photoproducts mixtures on induced pseudotumors in rabbit eyes were estimated with respect to the effects on unirradiated compounds. The irradiation of the eye conjunctiva in which was injected unirradiated parent compound appeared to be more efficient in tumor's remission than the simple treatment of the eye with unirradiated compound, only. The increase of tumor cells membrane permeability may be responsible for the obtained practical results.

Key words: cytostatics, fluorescence, FTIR, multidrug resistance, optical irradiation, pyridine derivative, UV/VIS absorption spectroscopy, eye pseudotumor.

1. INTRODUCTION

Multidrug resistance (MDR) to chemotherapy is the main circumstance that causes failure of many cancer treatment schemes. Commonly, tumors are composed of mixed cells; some of them are drug-sensitive while others are resistant. Whereas in the first instance chemotherapy destroys the drug-sensitive cells, an important part out of resistant ones can remain active and residual tumors will withstand to treatment [1]. Under these circumstances, it is necessary to further find alternatives to existing drugs or treatment schemes. One of the methods that seems to be efficient in the enhancement of antitumor compounds effects is its use in combination with optical radiation [2, 3]. Once excited by absorbing optical radiation of suitable wavelength, photosensitive molecules can be deactivated *via* fluorescence emission, chemical photoreactions, generation of reactive oxygen species (ROS) – singlet oxygen, and free radicals. These processes can be used in development of alternative therapies for cancer [4–6]. Other phenomena that result from laser radiation interaction with tissues determining an antitumor effect are related to interstitial hyperthermia [7]. Photothermal therapy associated with chemotherapy might be another way to increase efficacy in tumor treatment. Furthermore, chemotherapeutics can be delivered to targets by functionalized nanoparticles, such as gold nanoparticles, which can induce photothermal effects in cancer cells [8].

The pulsed laser radiation mechanism of action to assist the chemotherapeutics administration in cancer treatment was lately explained by the effect of lasers on the microfluidic properties of tumor cells. There are theories showing that pulsed laser radiation induces changes of cell membrane and forces it to admit the antitumor drug by convection. If sent directly on the tissue, a laser beam is supposed to produce water nanolayers in the cells, which favor the drug convection through the membranes [9–12].

In order to overcome MDR, the modern drug discovery process aims to develop new pharmacologically active molecules and to increase their affinity, selectivity, efficacy, metabolic stability, and oral bioavailability. An important role in fighting MDR is played by the synthesis, characterization, and evaluation of novel formulation of compounds in order to find more effective, biologically active drug candidates [13–18].

In the context of using light in combination with new or existing drugs for enhancing their therapeutic effects, this report is a synthesis of authors' experience on the usage of optical radiation to induce photoreactions in medicines and to generate new photoproducts with photo-enhanced activity. Out of the investigated drugs, antibiotics, and cytostatics were often considered, since they are already widely used in the treatment of proliferative processes [19–21]. Other classes of new synthesized compounds like anthracycline, pyridine, quinazoline, phenothiazine and hydantoin derivatives were investigated. They do not have necessarily anticancer or antibacterial effects by themselves, but may generate photoproducts during exposure to incoherent or coherent optical radiation, which may be active compounds against (pseudo)tumors [13, 14, 22–31].

In this paper is considered 5-flurouracil (5-FU), one of the most used cytostatics, and a new synthesized molecule that belongs to pyridine series, named as BG1120, or 4,5 bis [thio(2-N-N-diethylaminoethyl)], 9methyl 1,8-diazantracene ($C_{21}H_{28}N_4S_2$). 5-FU belongs to the pyrimidine class of antimetabolites that is used in treatment of tumors [32, 33]. On the other side, due to its azaheterocyclic component, BG1120 is considered to have important benefits as antitumor, antifungal, and antibacterial drug candidate [34, 35].

The modifications induced by optical radiation in the studied compounds were investigated by analytical methods: optical absorption, emission (fluorescence), and Fourier Transformed InfraRed (FTIR) spectroscopy. The effects of the optical beams irradiation on pseudotumors induced on rabbit eyes injected with 5-FU/BG1120 were estimated relative to unirradiated rabbit eyes pseudotumors treated with the same drugs. Preclinical tests have shown that exposure to UV beams of "infected" rabbit eyes treated with aqueous solutions of selected compounds diminished the inflammation and the neovascularization in the conjunctival tissue.

2. RESULTS AND DISCUSSIONS

2.1. ABSORPTION SPECTROSCOPY

For 5-FU (Sindan, ROU), 10^{-4} M solutions in natural saline (0.9% NaCl) adjusted to pH = 8.4 were prepared for investigations. The effect of optical radiation on these solutions was estimated by exposing them to laser radiation provided by a N_2 pulsed laser with emission at 337.1 nm, energy per pulse 350 μJ, pulse time duration 1 ns, and 10 Hz pulse repetition rate [36, 37]. In Fig. 1, the absorption spectra evolution for irradiation times between 0 and 5 min is shown. Two absorption bands with peaks at 267 nm and 300 nm are characteristic to 5-FU molecule. It can be noticed that no significant changes are observed with irradiation time except a slight decrease of the absorption maxima.

BG 1120 was supplied by Faculty of Pharmacy, Université de la Mediterranée, Marseille, France. Solution sample of BG 1120, 5×10^{-5} M in distilled water was exposed to UV/VIS radiation emitted by a cw Xe lamp, up to 30 min. Molecular changes are put forward by the evolution of absorption spectra during irradiation that can be observed in Fig. 2. The absorption spectra of BG 1120 have a narrow band centered at 260 nm and a second broad band with maximum absorption at 375 nm. Another band with maximum intensity at 211 nm may be identified.

The peak at 211 nm disappears over cw UV/VIS exposure and the absorption magnitude of the two other bands decreases with time exposure increase. The presence of different photo-absorbing molecular species is suggested also by the two isosbestic points at 273 nm and 293 nm, respectively.

A stability assay of unirradiated BG 1120 solution samples for different storage environments was made in order to decide the time in which there are no changes of solutions properties. This helps to schedule further experiments, including those related to biomedical applications. The test proved that BG 1120 solution remains stable during 10 days, when it is kept in dark at 4°C.

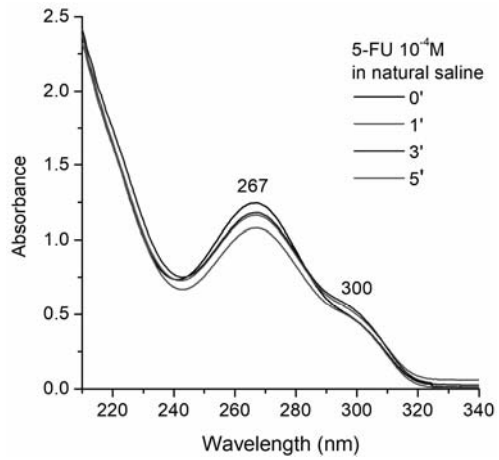

Fig. 1 – Absorption spectra of 5-FU at 10^{-4} M in natural saline; pH 8.4; exposed to N_2 laser radiation several irradiation times.

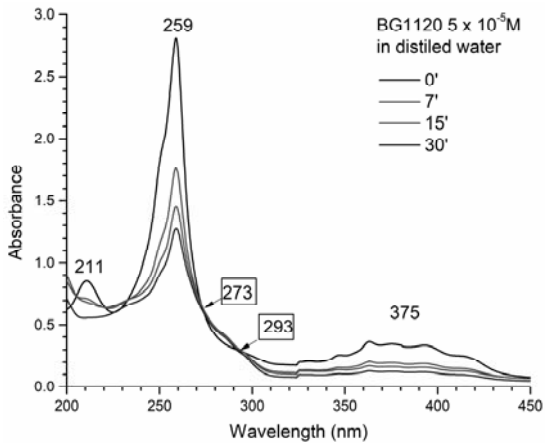

Fig. 2 – Absorption spectra of BG 1120, 5×10^{-5} M solutions in distilled water for several irradiation times, using cw Xe lamp.

As for solution kept in dark at room temperature (at about 20°C) for 10 days, the absorption spectra showed a slight decrease of the main absorption peak at 259 nm and a slight increase of the peak at 211 nm (figure not showed). These measurements recommend that unirradiated BG 1120 solutions in distilled water should be preserved in dark at 4°C and be used in maximum 10 days after preparation.

2.2. FLUORESCENCE SPECTROSCOPY

For 5-FU samples, studies were dedicated to the effect of exposure to N_2 laser radiation on excitation and emission fluorescence spectra. The irradiation was made 1 min, 3 min, and 5 min, respectively.

In Fig. 3, fluorescence excitation spectra are shown with emission wavelength set at 440 nm. One main excitation band is observed whose peak is shifted between 362 nm and 368 nm as function of irradiation time; an increase in intensity up to 3 min of exposure and then a decrease is noted. The same behavior has the emission spectra of 5-FU solutions as can be seen in Fig. 4. One emission band is noticed with the peak at 450 nm and its intensity increasing up to 3 min irradiation, followed by a decrease.

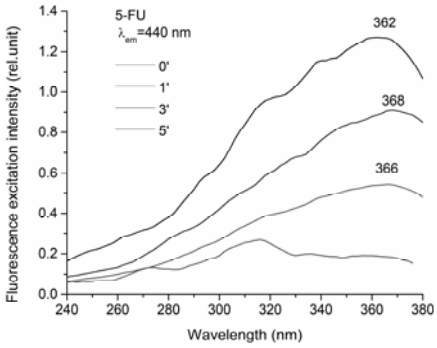

Fig. 3 – Fluorescence excitation spectra of 5-FU solution at 10^{-4}M in natural saline unirradiated and exposed 1 to 5 min to N_2 laser radiation.

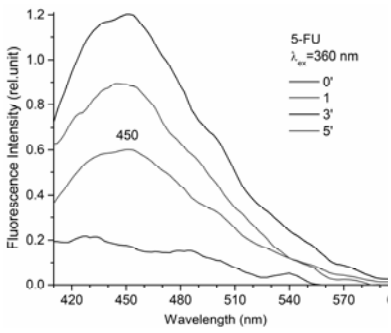

Fig. 4 – Fluorescence emission spectra of 5-FU solutions exposed to N_2 laser radiation.

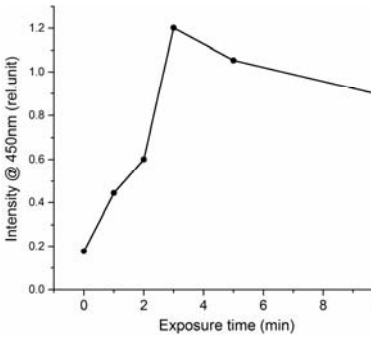

Fig. 5 – Intensity of fluorescence signal at 450 nm function of N_2 laser irradiation time.

This behavior is depicted in Fig. 5 where the intensity of the fluorescence peak at 450 nm is represented as a function of the irradiation time. The explanation of this trend could originate in the fact that 5-FU belongs to pyrimidines' class. This type of compounds have more tautomeric forms and differences between absorption and excitation spectra can be observed when distinct tautomer forms are present in the investigated solutions [38]. In this respect, the lactam form of 5-FU present in unirradiated solutions and weakly fluorescent is transformed under UV laser irradiation in the lactim tautomer that has enhanced emission.

The fluorescence emission of BG 1120 was excited at 300 nm wavelength (in the range where the absorption spectra remain unaltered during irradiation with cw Xe lamp) up to 30 min. The registered spectra reveal the evolution of fluorescence intensity with increasing exposure times as shown in Fig. 6. The fluorescence emission shows-up as a broad band with the main peak at 476 nm. It suffers a hypsochromic shift of 10 nm and a hypochromic one during irradiation, as follows: fluorescence intensity decreases with 46% after 7 min exposure, 54% after 15 min, and 70% after 30 min, compared to unirradiated sample.

Fluorescence excitation spectra measured at 460 nm for BG 1120 aqueous solution are presented in Fig. 7. Three bands with peaks at 310 nm, 377 nm, and 406 nm, respectively, appeared. The first peak shows a bathochromic shift of 4 nm along with a hypochromic one during exposure.

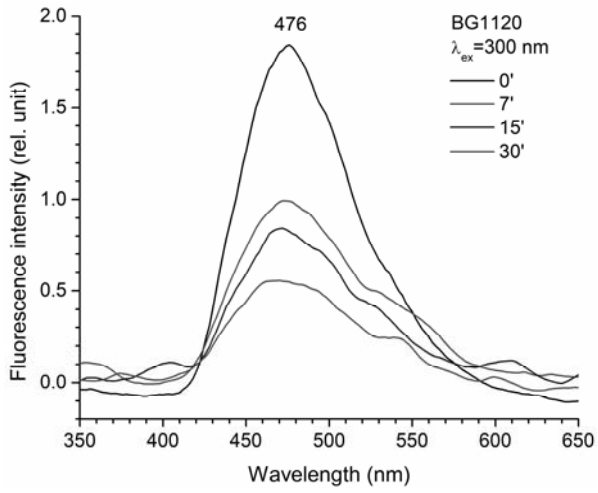

Fig. 6 – Fluorescence emission spectra of BG 1120 solutions, 5×10^{-5} M in distilled water for several irradiation times with cw Xe lamp; λ_{ex} = 300 nm.

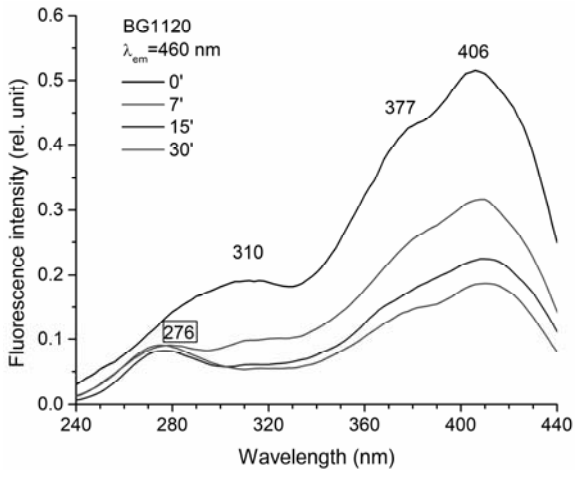

Fig.7 – Fluorescence excitation spectra of BG 1120 solutions 5×10^{-5} M in distilled water for several irradiation times with cw Xe lamp; λ_{em} = 460 nm.

The broader band with maximum at 310 nm suffers hypsochromic shift of 3 nm in the first 7 min and completely disappears after 15 min of exposure to UV/VIS radiation. A supplementary band at 276 nm may be also observed after 15 min irradiation without any change up to 30 min exposure. The decreasing of fluorescence excitation is produced as follows: 39% for solution irradiated 7 min, 57% for 15 min, and 65% for 30 min, compared to the evolution of unirradiated solution. The behavior of fluorescence spectra indicates that BG 1120 molecules suffer conformational changes, and a mixture of photoproducts in solution may occur.

2.3. FTIR SPECTROSCOPY

FTIR spectroscopic studies were approached to analyze the irradiated samples in order to identify the molecular modifications induced by exposure to UV [39]. The pyrimidines with OH group in orto or para position relative to N atom can be transformed into a keto tautomer form, which has usually a C=O vibration band near 1,700 cm^{-1}. FTIR spectra of unirradiated and irradiated 5-FU, as are exhibited in Fig. 8**,** show this specific vibration band assigned to C=O stretch at 1,655 cm^{-1}. The spectrum of unirradiated sample is characteristic to the diketo (lactam) tautomer. Following UV exposure of 5-FU during 3 min, the specific C=O vibrations for the keto form decrease in intensity together with the quadrant (1,555 – 590 cm^{-1} and 1,565 cm^{-1}) and semicircle (1,480 – 1,400 cm^{-1} and 1,410 – 1,375 cm^{-1}) stretch bands, assigned also to keto compound. A characteristic corresponding to N-H vibration at 3,500 – 3,300 cm^{-1} together with a wide band (3,500 cm^{-1}) due to OH bonded to pyrimidine ring could imply the change of the tautomeric forms equilibrium by shifting to the fluorescent form, i.e. the enol-keto tautomer (lactim form). All the spectroscopic investigations performed on 5-FU suggest that it is subject of tautomerization when exposed to certain doses of UV radiation.

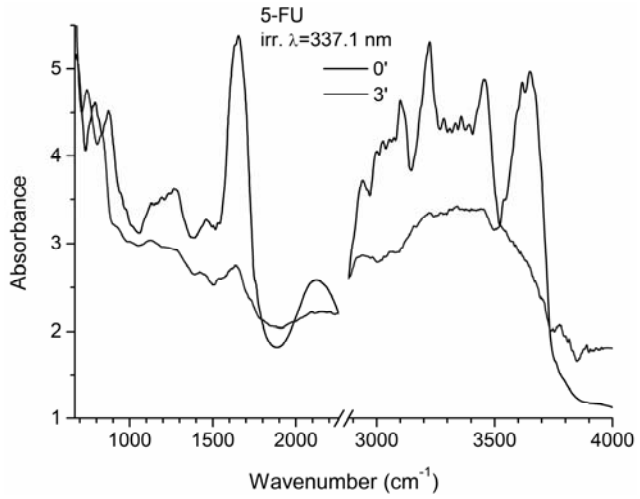

Fig. 8 – FTIR spectrum of 5-FU for unirradiated compared to 3 min laser irradiated solution at 337.1 nm.

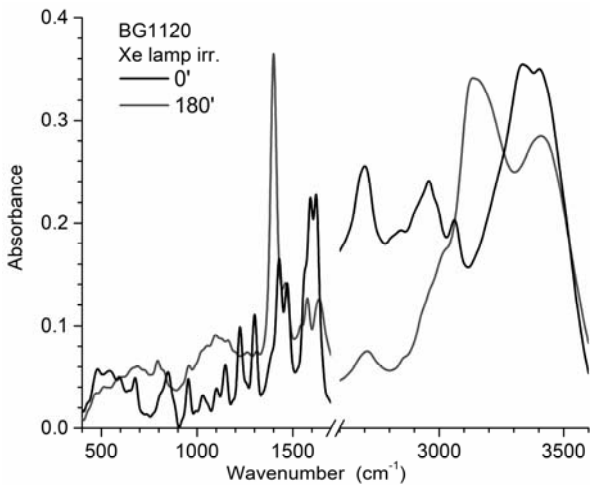

Fig. 9 – FTIR spectra of unirradiated and 180 min irradiated BG 1120 solution in distilled water with a cw Xe lamp.

After 3 min irradiation with laser beam at 337.1 nm, 0.3 mJ and 10 Hz pulse repetition rate, 5-FU transforms in the lactim form (keto-enol), which had an enhanced fluorescence [31].

As for FTIR spectrum of BG 1120 (Fig. 9), the unirradiated sample compared to the one exposed 3 h to cw Xe lamp reveals vibration changes, as follows: at 680 cm^{-1} it was identified the in-plane bending vibration of –C–S–H bonds, at 1,400 cm^{-1} could be assigned the stretching vibration of thiol, while vibrations at 1,098 cm^{-1}, 1,638 cm^{-1}, and 1,579 cm^{-1} might be contribution of –C=S bonds in lactam form. The ring stretching vibration of –C=C– can be responsible for vibrations appearing between 1,750–1,500 cm^{-1} and N–H (from NH^{4+}) stretching vibration may be assigned to peaks arising in the 3,500–3,100 cm^{-1} range [40].

These observations allow us to conclude that BG 1120 molecule undergoes transformations that yield to new photoproducts. As a consequence of the interaction of UV/VIS radiation with the considered molecules, possible photoinduced reactions may occur, which include the emergence of dithiol form (bis-lactim) at the end of irradiation sequence [15].

The changes that occur in BG 1120 molecules are present within minutes after exposure; a convenient time for exposure applicable to the subsequent experiments appears to be about 15 min, according to absorption and fluorescence data.

3. LABORATORY ANIMAL STUDIES

Laboratory tests were performed in order to establish the effect of studied cytostatic drugs, in combination with optical radiation, on corneal pseudotumors. The anatomopathological analysis of neovascularized tissue submitted to treatment can give information about the role that the studied medicines have in photodynamic anti-tumor therapy. These studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the University of Medicine and Pharmacy Carol Davila, Bucharest.

The tests were performed on rabbit eyes using the model developed by Schmidt-Erfurth et al. Details on experimental procedure are given elsewhere [6, 20, 21, 41]. In short, pseudotumors where induced at the sclero-corneal limbus of several rabbit eyes, after which these where subject to drug administration and further optical radiation was administered for several time intervals. Control eyes were used, on which the unirradiated medicines were injected.

The rabbit eyes were previously inoculated with 10^{-4} M 5-FU solutions in natural saline (0.9% NaCl). Three rabbit eyes injected with non-irradiated 5-FU were used as control. The next three eyes were irradiated with nitrogen pulsed laser, for 1 minute, other three were irradiated 3 minutes, and the last three 5 minutes.

All eyes were exposed to radiation 3 times a week. The duration of treatment was 4 weeks and then a pathological examination of conjunctive tissue was made with a microscope Nikon 6, with different maximization factors.

Results of the experiments are shown in Fig. 10 and Fig. 11. The eye treated with 5-FU and unexposed to nitrogen laser radiation shows a small inflammatory part (Fig. 10). One minute irradiation proved to be not enough for neovessels regression. In contrast, the increase of irradiation time intervals to 3 min seems to be curative, the inflammation area having a clear regression.

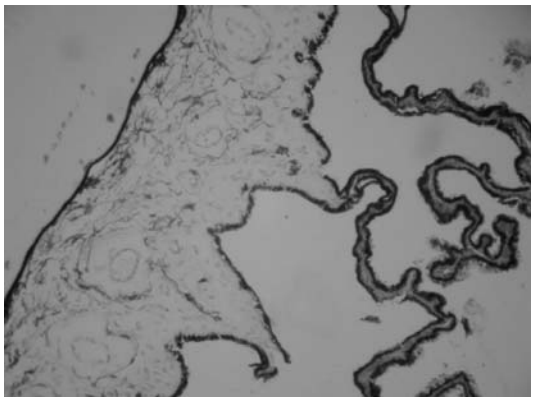

Fig. 10 – Images of the rabbit eye conjunctive tissue injected with unexposed 5-FU (100× magnification factor).

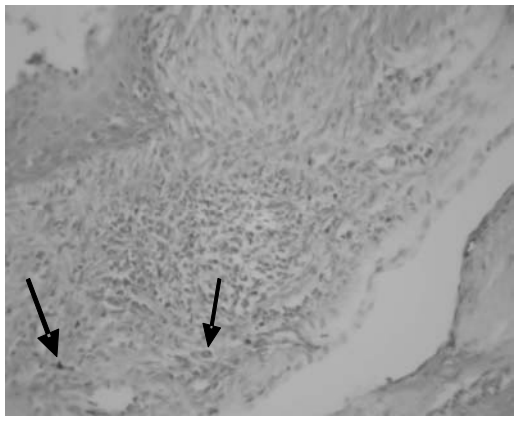

Fig. 11 – Image of conjunctive tissue treated with 5-FU and irradiated after drug administration, with N_2 pulsed laser radiation for sessions of 3 minutes (100× magnification factor).

As for the effect of BG1120 pyridine compound on rabbit eye pseudotumor, the same Schmidt-Erfurth experimental model was followed. One eye was kept for control. The second was treated with 0.1 ml BG 1120 at the concentration 5×10^{-5} M in distilled water. A third sample was exposed to cw radiation provided by a Xe lamp (11 mW) after drug administration.

In Fig. 12, the image of the pseudotumor tissue of the eye treated with BG 1120 solution is shown. This still presents inflammatory cells, neovessels, and a small fibrosis.

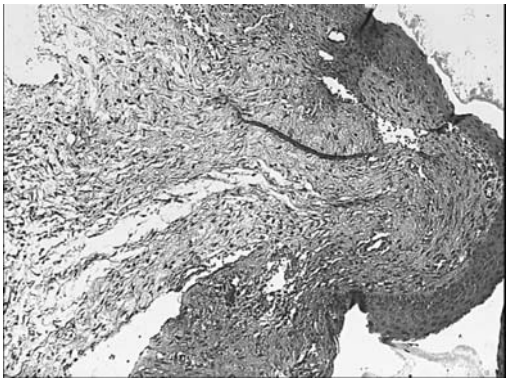

Fig. 12 – Image of rabbit eye tissue impregnated with BG 1120 solution in distilled water (100× magnification factor).

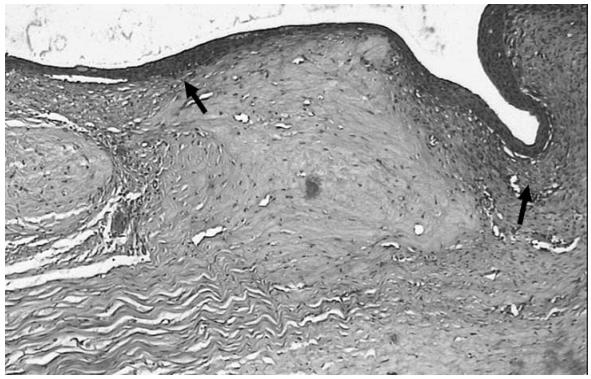

Fig. 13 – Image of eye tissue impregnated with BG 1120 solution, irradiated with Xe lamp beam (40× magnification factor).

Under exposure for 15 min to UV/VIS radiation emitted by Xe lamp, neovascularizations and inflammation are clearly reduced (Fig. 13). A fibrosis is still present which might be due to optical irradiation of healthy area surrounding tumor. This can lead to the conclusion that although optical irradiation can enhance the effect of antitumor compound, caution should be taken for exposing to radiation only the tumor area.

4. CONCLUSIONS

In this paper are reviewed data showing that optical irradiation in combination with antitumor compounds can be a possible method to enhance the effect of drugs and to further overcome MDR developed

by tumors. There are evidenced spectroscopic characteristics of compounds and of resulting photoproducts originating from them following exposure to UV/VIS optical radiation.

The same irradiation doses that induced structural modifications of drug photosensitive molecules also showed higher efficiency on eye pseudotumors impregnated with the compound. Eye conjunctiva neovascularizations are clearly reduced after treatment. The results could be explained by (i) the possible mechanisms of action of drug under UV/VIS exposure: increase of tumor cells membrane permeability and better compound penetration within cell and (ii) the drug photo-sensitising properties, which under excitation can transfer its energy to ROS species (singlet oxygen, free radicals), which can destroy the necrosis tissue.

Data presented here synthesize results regarding photosensitive properties of some antitumor compounds that can be used to develop therapeutic protocols for conjunctive neovascularization and tumors. It remains that furtherer detailed studies dig into the interaction mechanisms between the tumor structures and the medicines exposed to UV (uncoherent or coherent) optical beams.

ACKNOWLEDGEMENTS

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