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5-Fluorouracil as a Phosensitiser

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Abstract. *5-FU exhibits a high fluorescence after irradiation with UV-vis light. An enhancement of the cytostatic activity of 5-FU under UV-vis irradiation was observed on an in vivo experimental model.*

The usefulness of 5-FU is limited because of the rapid development of acquired resistance (1). Techniques leading to an increase of its cytostatic activity are still an important objective. The photo-physical properties of 5-FU (*e.g.* fluorescence after UV-vis irradiation) may be of interest in this respect (2-6). It has been shown that incorporation of 5-FU into the RNA increases sensitivity to UV irradiation (7).

Materials and Methods

5-FU (Sindan, Romania) solutions in physiological saline (0.9% NaCl) at concentration of 10^{-4} M, adjusted to pH 8.4 by addition of NaOH, were studied (4, 8-10). They were exposed to UV-vis light for 1 to 10 minutes by using a Hg lamp (11). The Hg lamp emission was in continuous wave (cw) mode, in a spectral range of 300 to 600 nm, with low (337, 549 and 580 nm) and high (365, 406 and 435 nm) intensity lines.

A second light source was a nitrogen pulsed laser (N_2 laser), emitting pulses at 337.1 nm with the following characteristics: total pulse time width: 1 ns, peak power/pulse: 350 kW, pulse repetition rate: 10 pulses/s and a mean beam energy of 350 μJ. For optimal application of the radiation to the tumor cells, an optical fiber was used.

The absorption spectra of the irradiated and non-irradiated samples were recorded in the UV-vis range with a Perkin Elmer spectrometer. An Aminco-Bowman spectrophotofluorimeter was used to record the excitation and emission fluorescence spectra, while Raman and FTIR spectra were obtained by using an Ocean Optics Raman System 2000 and Nicolet Magna – IR™ 550 spectrometer, respectively.

Experiments on rabbit eyes with induced pseudo-tumors (6, 9, 11, 20) were performed using the nitrogen pulsed laser.

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Results and Discussion

Pyrimidine bases predominantly occur in one isomeric form; however, they can exist in other tautomeric forms depending on their environment, which could create base-pair mismatching and lead to mutations (12). It was shown that the excitation spectrum is different than that of absorption; this anomaly could be explained (13) by the existence of two distinct molecular species in the solution, namely: (i) the diketo (lactam) form, which is a major component, a stable and weakly fluorescing tautomer, and (ii) the enol-keto (lactim) form (Figure 1), as a minor component, but fluorescing tautomer. In solutions of pH 8, this phenomenon could be due to a deprotonation effect (12).

5-FU solutions irradiated with N_2 laser beam. The absorption spectra of the 5-FU solutions irradiated with N_2 laser beam, at exposure time between 1 and 5 minutes, are shown in Figure 2.

In Figures 2-4 the terms "lif X" and "nif" refer to irradiated 5-FU solutions (X=1-5 min) and non-irradiated 5-FU solution, respectively.

The absorption spectra exhibited bands in the range 250 nm – 350 nm. The main absorption band wavelength was 279 nm. No changes in these absorption spectra were observed, indicating that, after irradiation with N_2 laser beam, the structure of the 5-FU molecule does not change.

The excitation fluorescence spectra were recorded at 440 nm emission wavelength. They are shown in Figure 3, where the relative intensity of the fluorescence excitation is recorded as a function of irradiation time. The term I_{ex} represents the fluorescence intensity. The highest fluorescence excitation intensity was detected at 360 nm. This value was selected as the excitation wavelength for the emission fluorescence spectra.

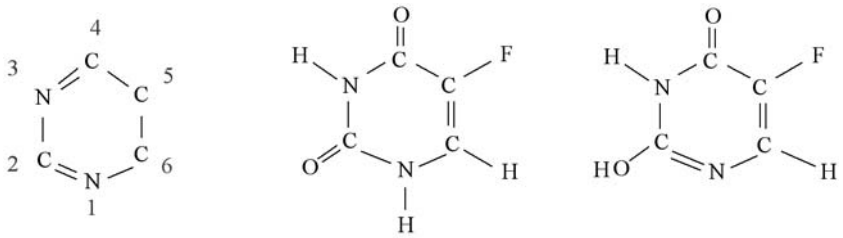

Figure 1. *The pyrimidine ring and the two 5-FU tautomers: lactam and lactim forms.*

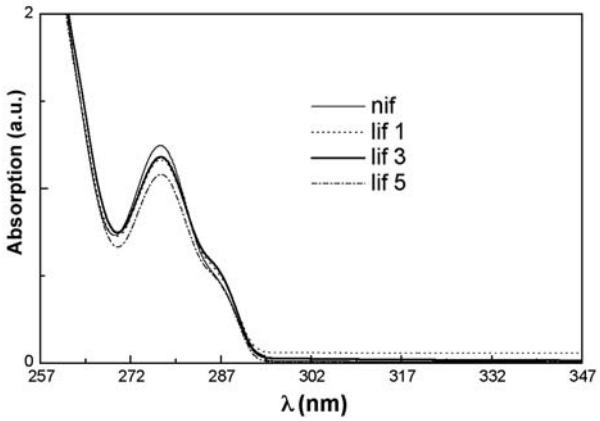

Figure 2. *The absorption spectra of the non-irradiated 5-FU solution and the irradiated 5-FU solutions with N_2 laser beam.*

The emission fluorescence spectra of the non-irradiated and irradiated 5-FU solutions, as a function of the exposure time to N_2 laser beam, are shown in Figure 4. This spectral analysis was performed in the 400 nm - 600 nm range. The term I_{em} represents the emission fluorescence intensity. Using an excitation wavelength of 360 nm, maximum emission fluorescence intensity was observed at 450 nm. 5-FU became strongly fluorescent under UV-vis irradiation.

As shown in Figure 5, after irradiation with N_2 laser beam, the emission fluorescence of the 5-FU solution increased more than six times.

5-FU solutions irradiated with Hg lamp. The absorption spectra of the 5-FU solutions irradiated under Hg lamp for 1 to 10 minutes are shown in Figure 6. In Figures 6 and 7 the terms "uif X" and "nif" refer to irradiated (X=1-5 min) and non-irradiated 5-FU solutions, respectively. The absorption spectra exhibited bands in the range 200 – 500 nm. The main absorption band was also located at about 279 nm. As in the case of laser irradiation, the absorption spectra presented no obvious modification, also suggesting that the structure of 5-FU does not change.

Figure 7 shows the emission fluorescence spectra of the non-irradiated and irradiated 5-FU solutions, as a function of the exposure time to Hg lamp. This analysis was performed in the 400 - 550 nm spectral range. The term I_{em} represents the emission fluorescence intensity. The excitation wavelength of 350 nm was used. The maximum emission fluorescence intensity was also observed at 450 nm. 5-FU became fluorescent under UV-vis irradiation.

The emission fluorescence of the 5-FU solution increased three times after Hg lamp irradiation (Figure 8).

In the present experimental conditions, it is suggested that ultraviolet light produces free radicals whose formation is influenced by the matrix in which they are irradiated. It has already been shown that pyrimidines can undergo a keto-enol tautomeric shift. The radicals are formed by the deprotonation of 5-FU and can give pyrimidine dimers, which cannot fit into the double helix, thus blocking the replication/transcription of the nucleic acids. The radical seen most clearly after irradiation at room temperature (Figure 9) was suggested to be a reduction product formed from 5-FU (14, 15).

A comparison of the emission fluorescence spectra obtained after laser and Hg lamp irradiation shows a remarkable increase of the band centered at about 450 nm, with about four times greater intensity in the case of laser irradiation. This could be attributed to the lactim-form presence (the fluorescing tautomer) as well as to the monochromatic and high intensity laser radiation, which allows a more effective and selective excitation.

It has been suggested that the mechanism of tumoricidal activity of light-excited sensitizers involves the production of free radicals (16, 17). This process could be amplified by the dimeric pyrimidine which blocks RNA replication/transcription.

FTIR spectra. Since no structural modifications were inferred from the UV-vis absorption spectra, FTIR and

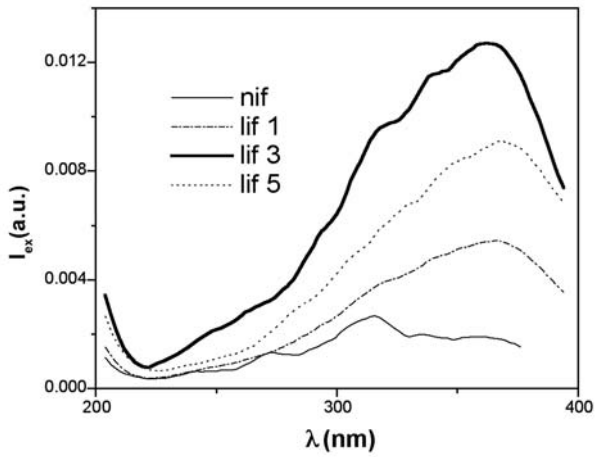

Figure 3. *The excitation fluorescence spectra of the non-irradiated 5-FU solution and the irradiated 5-FU solutions with N_2 laser beam.*

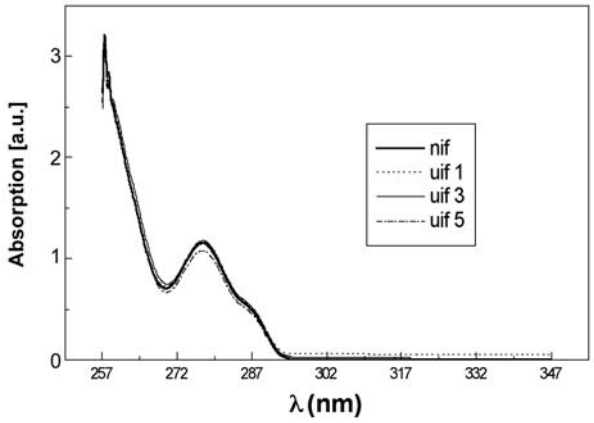

Figure 6. *The absorption spectra of the non-irradiated 5-FU solution and the irradiated 5-FU solutions with Hg lamp.*

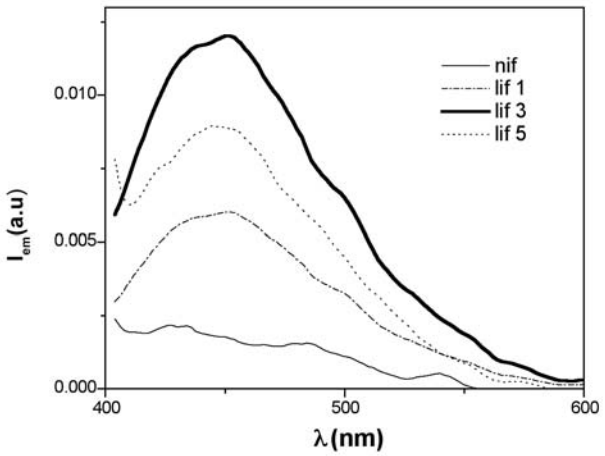

Figure 4. *The emission fluorescence spectra of the non-irradiated and irradiated 5-FU solutions with N_2 laser beam.*

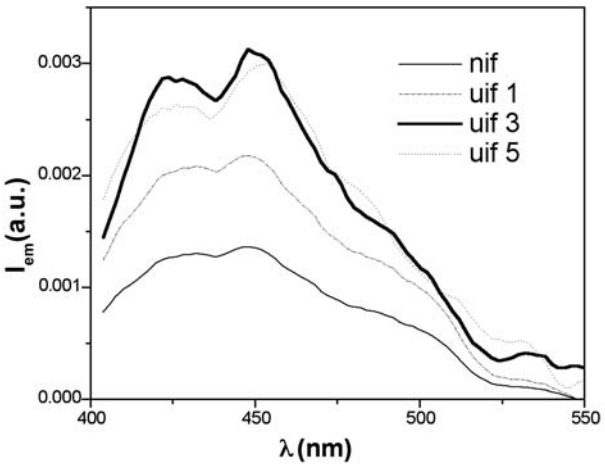

Figure 7. *The emission fluorescence spectra of the non-irradiated 5-FU solution and the irradiated 5-FU solutions with Hg lamp.*

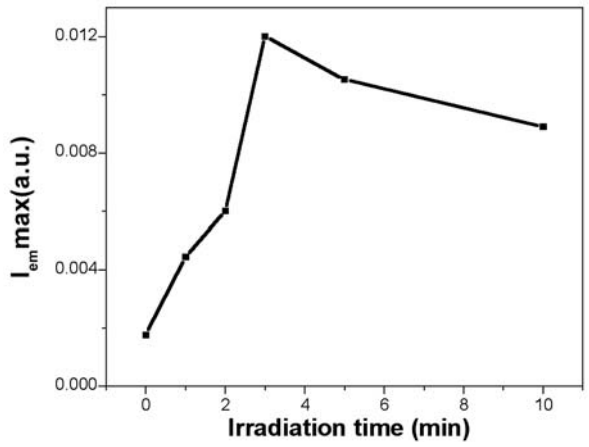

Figure 5. *The relative intensity of emission fluorescence maximum (at 450 nm) versus irradiation time with N_2 laser beam.*

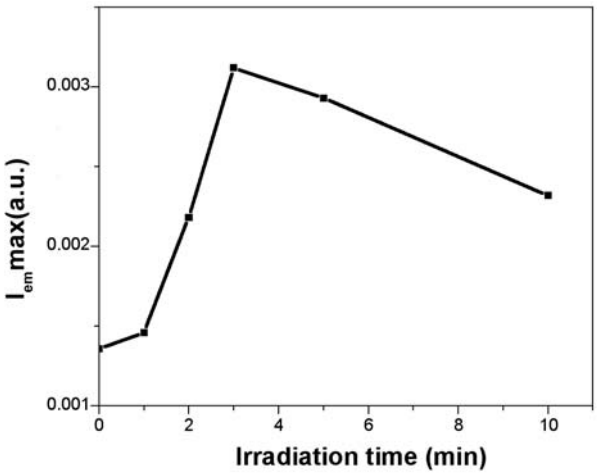

Figure 8. *The relative intensity of emission fluorescence maximum (at 450 nm) versus irradiation time with Hg lamp.*

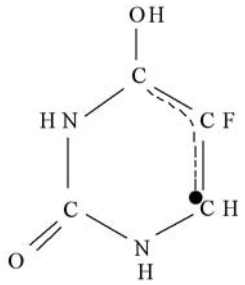

Figure 9. *The reduction product formed from 5-FU observed frequently during UV irradiation at room temperature.*

Raman investigations were used to identify any possible change of the molecule's geometry appearing as a result of UV irradiation (18). Pyrimidines with a hydroxy group at ortho or para position usually transform into the keto form, with a C=O band near 1700 cm^{-1}.

The FTIR spectrum of the non-irradiated 5-FU solution at a concentration of 5.10^{-4} M and pH = 8.6 - 9.4 is presented in Figure 10 and shows the typical bands in the range 1700 – 1650 cm^{-1}, assigned to conjugated (at 1655 – 1670 cm^{-1}) and non-conjugated (at 1695 cm^{-1}) C=O stretch. This spectrum seems to be characteristic of the more weakly fluorescing but largest group diketo (lactam form), in equilibrium with the enol-keto (lactim form). This equilibrium has been suggested by Raman spectra (not shown) of both non-irradiated and irradiated samples, which present the characteristic band of substituted pyrimidine (2,4-hydroxy, 5-fluorine) near 1005 cm^{-1}, caused by the 1, 3, 5 ring atoms moving radially in phase (19).

The FTIR spectrum of the 5-FU solution, irradiated for 3 minutes with pulsed nitrogen laser (Figure 11), shows the change of the 5-FU molecule's geometry after irradiation. Apart from the very strong peak at 2284 cm^{-1} due to ν_3 antisym stretch mode of carbon dioxide, the peaks attributed to conjugated and non-conjugated C=O stretch are strongly diminished, as well as the quadrant (1590 – 1555 cm^{-1} and 1565 – 1562 cm^{-1}) and semi-circle (1480 – 1400 cm^{-1} and 1410 – 1375 cm^{-1}) stretch bands, assigned to the keto form.

Also, a signal for only one N-H bond at 3300-3500 cm^{-1} and the superimposed wide band due to OH groups (~3500 cm^{-1}) bonded to pyrimidine ring could suggest that after irradiation the equilibrium was shifted to enol-keto tautomer (lactim form), which explains the enhancement of the fluorescence.

All these observations lead to the conclusion that UV irradiation causes tautomerization of 5-FU (12), with fluorescence increase after exposure to irradiation for 3 minutes. After irradiation, 5-FU is transformed to the

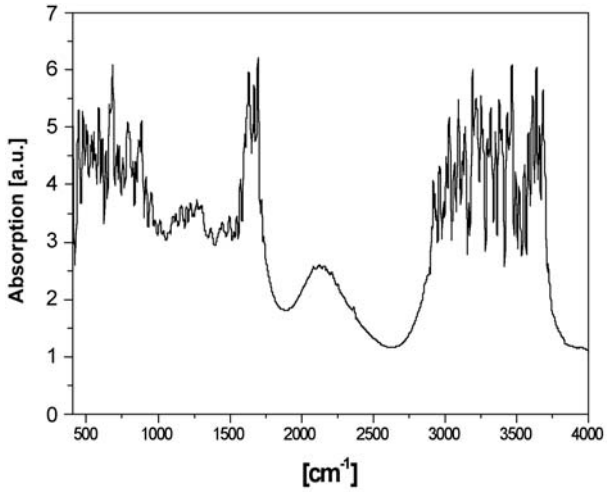

Figure 10. *The FTIR spectrum of the non-irradiated 5-FU solution.*

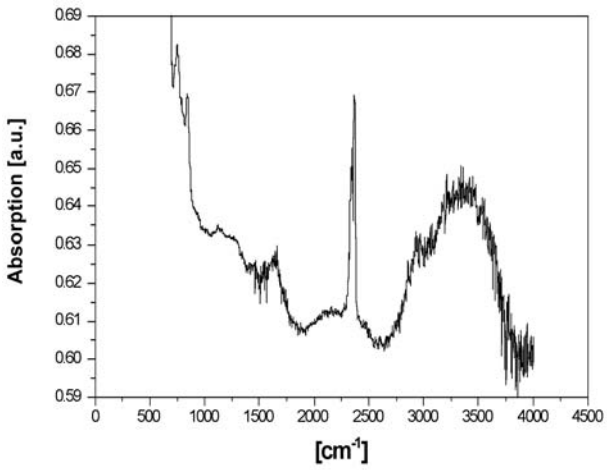

Figure 11. *The FTIR spectra of 5-FU irradiated for 3 minutes with nitrogen pulsed laser.*

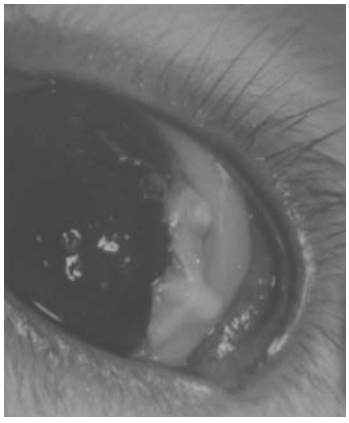

Figure 12. *The rabbit eye pseudo-tumor before 5-FU injection and exposure to radiation.*

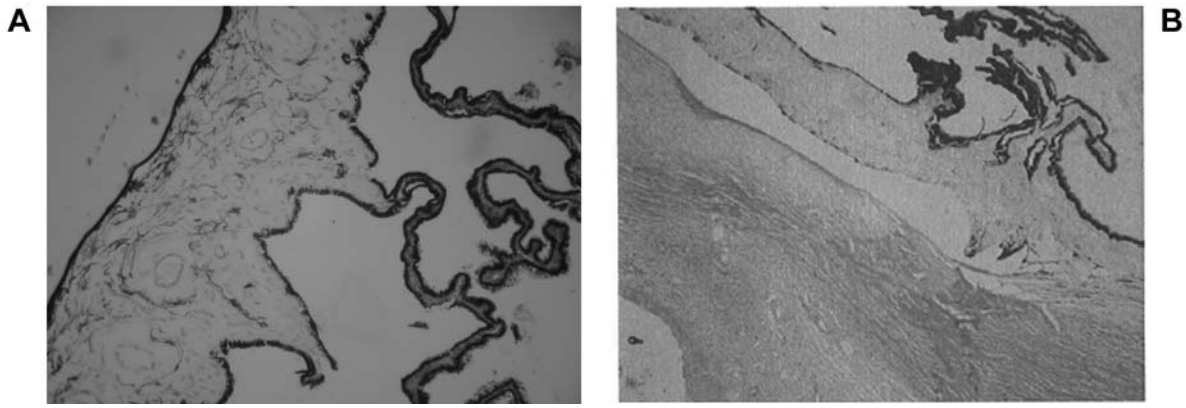

Figure 13. *The images of the rabbit eye treated with 5-FU: a. non-irradiated (witness); b. irradiated for 1 minute.*

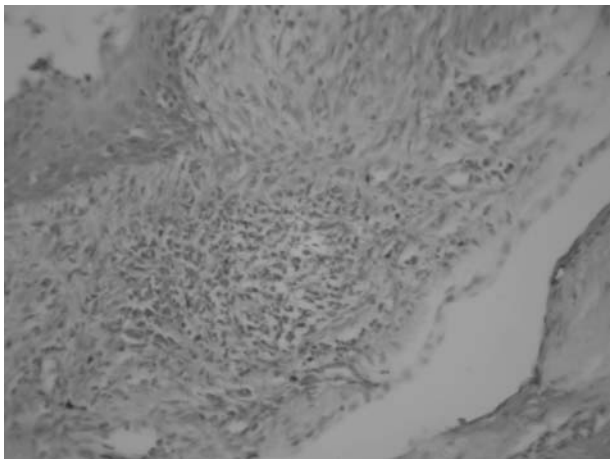

Figure 14. *The image of an eye which presents an inflammation regress after injection with 5-FU and 3-minute irradiation with the nitrogen pulsed laser.*

tautomeric keto-enol (lactim) form, which is the major component of the solution.

Experimental model. The experimental model of the rabbit eye pseudo-tumor, developed by Schmidt-Erfurth and co-workers (6, 20), features conjuctive tissue inflammation and neovascularization. Pseudo-tumors with new vascularization were induced by sewing a catgut stitch (diameter = 0.88 mm) at rabbit eye cornea. The experiment was performed on six rabbits. The rabbit eyes were treated with 10^{-4}M 5-FU solutions in physiological saline (0.9% NaCl). Three rabbit eyes treated with 5-FU and non-irradiated were used as controls. The next three eyes were irradiated with nitrogen pulsed laser for 1 minute. Another three eyes were irradiated for 3 minutes, and the last three for 5 minutes.

An image of the eye pseudo-tumor before 5-FU treatment and irradiation is shown in Figure 12. Irradiation of eyes was performed 3 times a week. The duration of the treatment was 4 weeks and then a pathological examination of the conjunctive tissue was made using an electron microscope Nikon 6, with a 10x zoom. The results of this experiment are shown in Figures 13 A, B and 14. In the first image (Figure 13 A), a non-irradiated rabbit eye treated with 5-FU is shown (control); a small inflammatory part could be observed. In Figure 13 B, the homogeneous dye blemishes represent newly-formed vessels without own walls. This eye was irradiated with the nitrogen pulsed laser for 1 minute after treatment with 5-FU.

Increasing the irradiation time to 3 minutes led to an inflammation regress, as shown in Figure 14.

Conclusion

5-FU is weakly fluorescent, but it presents a high fluorescence after exposure to UV-vis light. The UV-vis absorption spectra of 5-FU did not show any structural

modification, but the FTIR spectra showed that the molecular geometry of 5-FU changed after irradiation. The UV-vis irradiation transformed 5-FU into a tautomeric form. The increase of the fluorescence is attributed to a higher conversion to the keto-enol (lactim form) fluorescing tautomer due to monochromatic and high intensity laser radiation, which allows a more effective and selective excitation. The radicals formed by the deprotonation of 5-FU could initiate the tumoricidal effect. The light excited sensitizers directly interact with substrate and/or solvent molecules to yield radical or radical ions.

The present spectroscopic studies were coupled with preliminary tests on 5-FU photosensitizing properties measured on rabbit eyes conjunctive tissue. Comparative data were obtained about the evolution of neovascularisations in the conjunctive tissue between an untreated rabbit eye and an eye impregnated with 5-FU 10^{-4} M in physiological saline and exposed to a UV-light beam emitted by a nitrogen pulsed laser. This preliminary experiment yielded encouraging results showing that the eye conjunctive inflammation and neovascularisation disappeared after the treatment, and pointing out the photosensitizing properties of 5-FU and their possible use in the treatment of conjunctive neovascularisation and cancer (3,7,10).

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