

IN VIVO STUDIES OF THE EFFECTS OF ALKYL SUBSTITUTED BENZO[B]PYRIDINIUM COMPOUNDS EXPOSED TO OPTICAL RADIATION

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Abstract. The paper reports about the effects of selected alkyl substituted benzo[b]pyridinium compounds which are potential or effective cytostatics, on the pseudotumoural tissues after their exposure to ultraviolet visible optical beams. The study of the modifications of the absorption, fluorescence and FTIR spectra of the investigated compounds was performed to evidence the molecular modifications after exposure to uncoherent optical radiation emitted in ultraviolet and visible. Based on these data the selection of compounds which were most sensitive to the exposure to optical beams was made and their action on the pseudotumoural issues was studied. In this paper are reported the results obtained for “in vivo” application of the BG 1120 on pseudotumoural tissues; the conclusions were that BG 1120 is able to diminish faster, when irradiated, the inflammations and the neovascularisations in the conjunctival tissue; possible mechanisms of its action are proposed.

Key words: benzo[b]pyridinium compounds, cytostatics, pseudotumoural tissues, UV-VIS radiation.

1. INTRODUCTION

Since the early 90's it was demonstrated that azaheterocycles are efficient chemosensitizers [1]. With respect to this, thioacridines [2], benzo[b]-1,8-naphthyridines [3] and pyridoquinoline derivatives [4, 5] have been successfully tested. In addition, some of them exhibit anticancer properties [6, 7] due to their capability to intercalate into DNA [8].

Other reports show that cytostatics such as Methotrexate and 5-Fluorouracil (5-FU) may become more efficient in acting on pseudotumour tissues when exposed to ultraviolet – visible (UV-VIS) optical radiation [9, 10].

2. MATERIALS AND METHODS

Four test samples compounds were studied with a common benzo[b]pyridine nucleus. They belong to the acridine, the naphthyridine and the pyridoquinoline series (Fig. 1). Chemical data of these compounds, quoted as, respectively, BG 186, BG 204, BG 558 and BG 1120 are listed in Table 1.

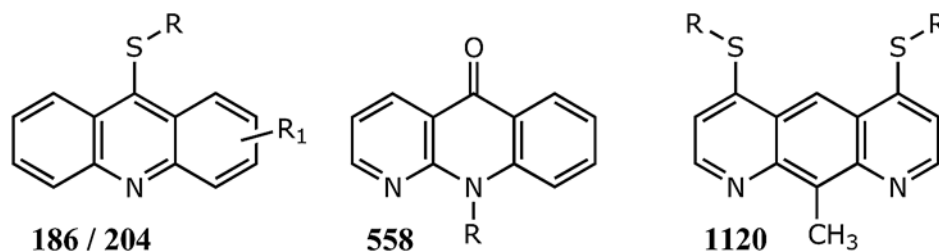


Fig. 1 – Chemical series selected for investigation.

Table 1

Chemical data

Code number	Series	R (Fig. 1)	R ₁ (Fig. 1)	Bp (°C)	¹ H NMR data, δ (ppm)
BG 186	Acridine	CH ₂ CH ₂ -N(Et) ₂	4-OMe	64	0.9(t, 6H); 2.2 q, 4H); 2.8(t, 2H); 3.0(t, 2H); 4.1(s, 3H); 7.0(m, 1H); 7.6(m, 3H); 8.2(m, 2H); 8.8(m, 1H)
BG 204	Acridine	CH ₂ CH ₂ -N(Et) ₂	3-NH ₂	128	1.40(t, 6H); 3.40(d, 4H); 3.60(d, 2H); 3.80(d, 2H); 8.10(m, 3H); 8.30(m, 2H); 9.10(m, 2H)
BG 558	naphthyridine	CH ₂ CH ₂ -N(Et) ₂		130	1.60(t, 6H); 3.60(q, 4H); 4.00(t, 2H); 5.40(t, 2H); 7.80(m, 3H); 8.20(t, 1H); 8.80(d, 1H); 9.30(d, 1H); 9.50(d, 1H)
BG 1120	pyridoquinoline	CH ₂ CH ₂ -N(Et) ₂		115–117	1.08(t, 12H); 2.66(q, 8H); 2.90(t, 4H); 3.22(t, 4H); 3.36(s, 3H); 7.22(d, 2H); 8.84(d, 2H); 8.88(s, 1H)

The samples were diluted in distilled water to $5 \times 10^{-5} \text{M}$ and exposed to optical radiation emitted by a Xe lamp in the spectral range 200nm – 900 nm; the beam power density was 11mW/cm^2 [11]. The Xe lamp measured emission spectrum in the near ultraviolet is given in Fig. 2.

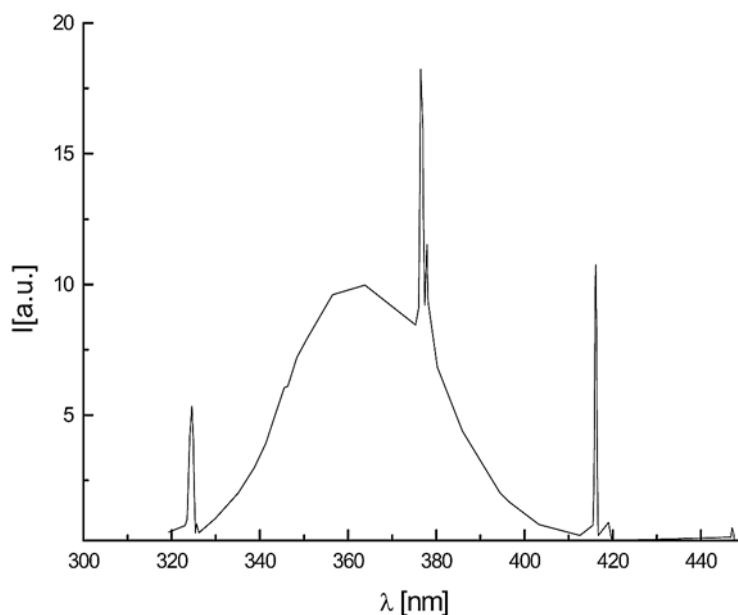


Fig. 2 – The Xe lamp emission spectrum.

The absorption, fluorescence and FTIR spectra of all the compounds were measured using standard equipment, respectively UV-VIS absorption spectrophotometer, spectrofluorimeter and FTIR photometer.

For the FTIR measurements the BG 1120 sample solutions were prepared at $5 \times 10^{-5} \text{M}$ concentration in distilled water; they were exposed to the Xe-lamp radiation for up to 3 hours and then were poured on KBr crystals and let to become dry for one day. The resulted solid samples were grounded and pressed at $1.575 \times 10^5 \text{ kg cm}^{-2}$ obtaining pellets which were then used to be measured by FTIR.

The modifications of the absorption and fluorescence spectra were measured for both irradiated and un-irradiated samples to obtain information about the changes in electronic structure of the molecules and the vibrational levels associated with it; the complementary FTIR spectra measurements were made to detect the molecules conformational changes for the respective substances using the schemes reported in [12, 13].

For the “in vivo” study it was used the experimental model of the rabbit eye (Schmidt-Erfurth) featuring conjunctive tissue inflammation and neovascularization. Pseudo-tumours with new vascularisation were induced by sewing a catgut stitch

2.0 at the sclero-corneal limbus and by injecting a prostaglandinic-like substance (Travatan 0.1 ml – which has a slightly proinflammatory action) nearby, under the conjunctiva.

Four rabbits were used, for each of them keeping one unaltered eye for reference.

3. RESULTS AND DISCUSSIONS

After the exposure to optical radiation in UV – VIS only two of the studied compounds exhibited modifications of the absorption, fluorescence and FTIR spectra: BG 204 and BG 1120. In this paper the results obtained on BG1120 are reported.

As it is shown in Fig. 3, there is an obvious modification of the absorption spectrum of BG 1120 as the irradiation/exposure time increases.

All the absorption spectra have a maximum at around 260 nm which is decreasing with the increasing of the exposure time; the absorption spectra remain unchanged in intensity at about 290 nm – 300 nm; they exhibit a second broad peak at about 375 nm which has the same evolution with increasing of the exposure time.

The comparison of the Xe lamp emission spectrum in the near ultraviolet with the absorption spectrum of the sample shows the following:

- the emission of the Xe lamp has a peak at about 320 nm followed by a continuum between 330 and 420 nm with two strong peaks superposed on it at respectively 380 nm and 418 nm;
- the absorption spectrum has a peak at about 210 nm at the limit with the vacuum ultraviolet, another one very intense at 260 nm and a continuous absorption band between 325 nm and 440 nm.

The superposition of the two spectra shows that the absorption maxima at 210 nm and 260 nm may not be excited by the Xe lamp radiation and consequently the implied molecular electronic states may not be directly responsible for the modifications of the absorption spectrum after exposure of the molecules to the radiation. On the other hand, the continuous absorption between 325 nm and 440 nm coincides with the emission continuum of the lamp between 330 nm and 420 nm and the absorption of the optical radiation takes place mainly in these spectral ranges; the evolution of the absorption maxima suggests that modifications in the electronic structure of the BG1120 molecule take place following the absorption in the 325 nm – 440 nm band, but are reflected in the decreasing of all the absorption bands, out of which the peak at 210 nm vanishes.

This evolution recommends the measurement of the BG 1120 fluorescence emission spectrum at 300 nm (Fig. 4) where the absorption signal remains unchanged in intensity while the exposure time to optical radiation is increased.

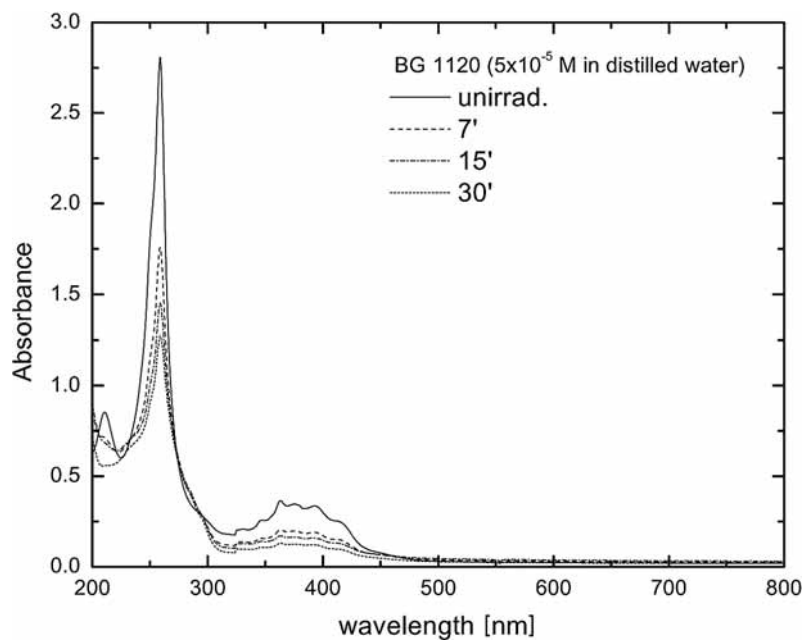


Fig. 3 – The absorption spectra of BG 1120 unirrad – not exposed to the optical beam; 7', 15', 30' – exposure times in minutes.

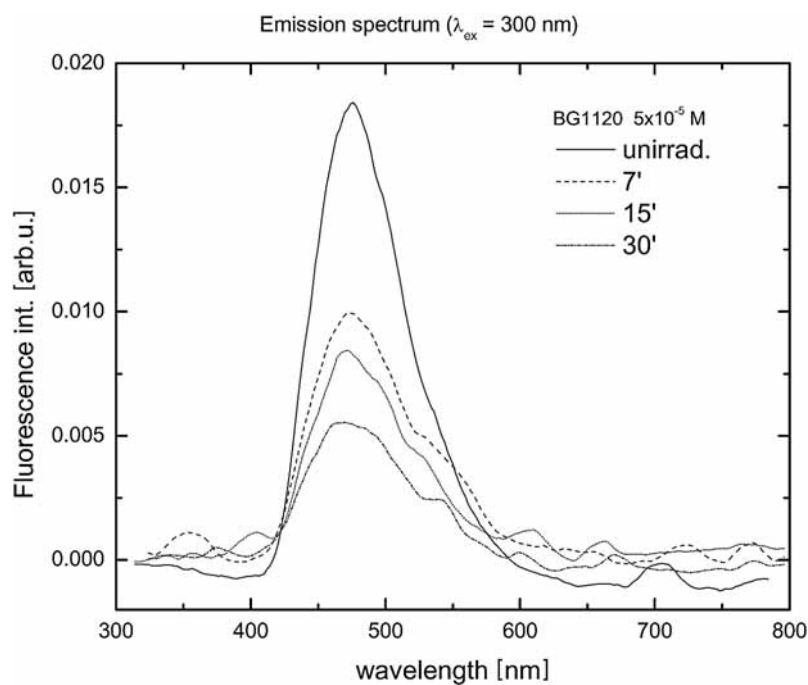


Fig. 4 – The fluorescence spectrum of BG 1120.

The fluorescence emission spectrum has a peak at 460 nm which also decreases with the increasing of the exposure time. Measurement of the excitation spectrum at 460 nm (Fig. 5) has shown four emission bands (at 275 nm, 310 nm, 375 nm, 408 nm, respectively) partially superposed, which decrease in intensity with the exposure time so that one of them (310 nm) even disappears after 7 min.

The modifications of the absorption and fluorescence spectra show that the electronic structure of the BG 1120 molecules is changed so that it is reflected both in decreasing of the absorption intensities in near ultraviolet and visible as well as the decreasing of the emission fluorescence in the visible when the exposure time of the solutions to near ultraviolet and visible radiation is produced.

The decreasing process is not saturated with the increase of the exposure time up to 30 min, although the most important drop takes place in the first 7 min of exposure after which the signals decrease to 60% from their initial values.

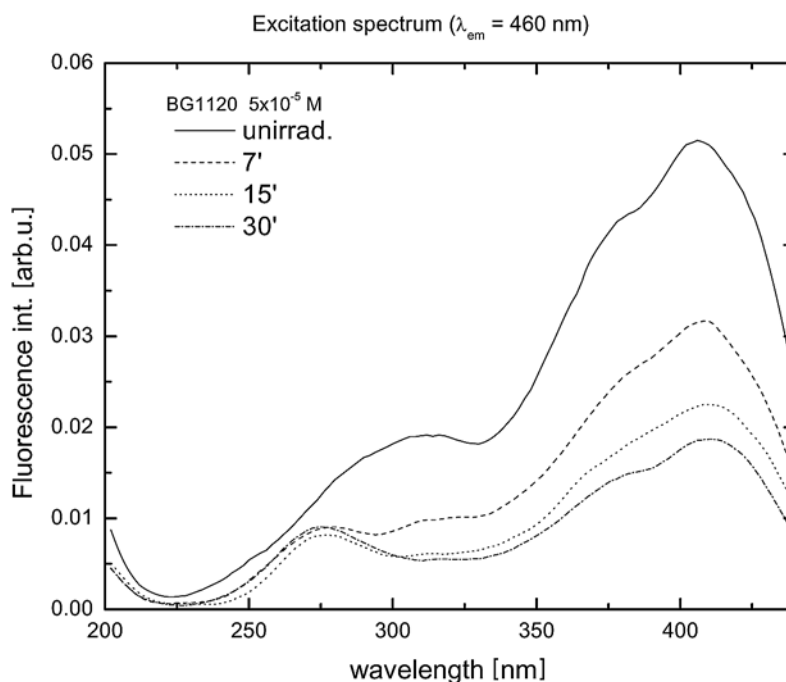
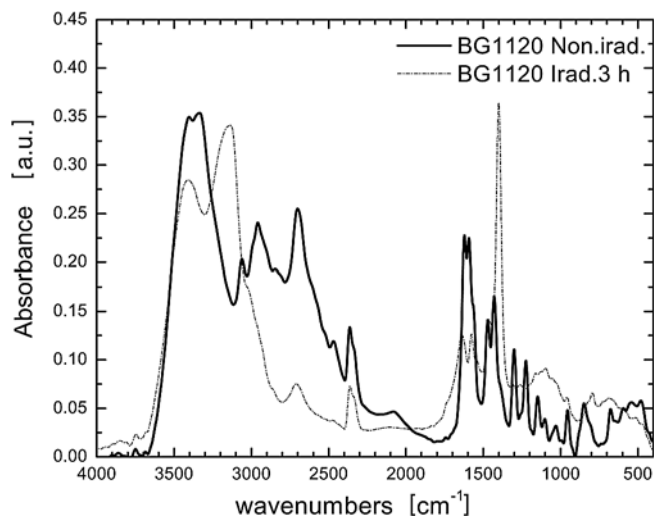


Fig. 5 – The excitation spectrum of BG 1120.

To obtain more information about these modifications, the FTIR spectra were measured in the spectral range $4000\text{ cm}^{-1} - 400\text{ cm}^{-1}$. In Fig. 6 are shown two superposed spectra, one for a solution unexposed to radiation and the second for the same solution after 3 hours irradiation time. The spectrum exhibits a strong and sharp line at 1400 cm^{-1} attributed to the ring stretching mode in thiols and a weak and broad signal at 680 cm^{-1} which corresponds to the in-plane bending C-S-H [14].

Fig. 6 – The FTIR spectrum of BG 1120.



The spectrum suggests that the bond S-C with tertiary amine is broken and dithiols and tertiary amine are formed (Fig. 7) [14].

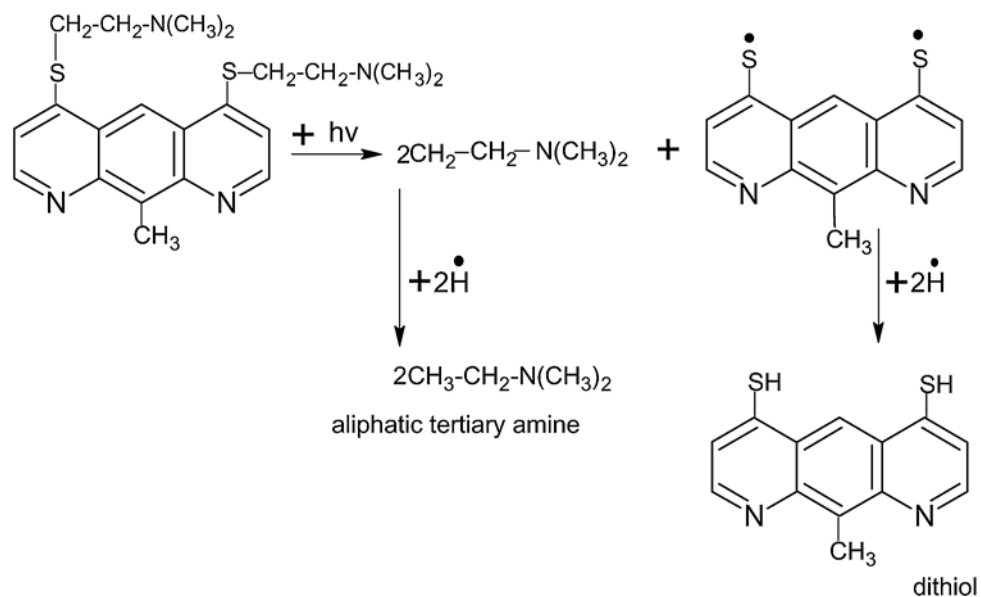


Fig. 7 – The transformation of the BG 1120 molecule under optical radiation.

The FTIR spectrum also exhibits a broad band at 1098 cm^{-1} which corresponds to compounds that contain a thiocarbonyl group ($\text{C}=\text{S}$) and two sharp, medium intensity signals at 1638 cm^{-1} , respectively 1579 cm^{-1} that correspond to vibrations of the $\text{C}=\text{S}$ bond met in the lactam form of the aromatic rest. The two S

atoms which bond the azaantracenic rest unstabilize the molecule and a complex process of tautomerization may take place. The rest of the molecule transforms in one of the three tautomeric form: bis-lactim, lactim-lactam or bis-lactam [14].

The band at 1400 cm^{-1} can be assigned to the C-C ring stretch in thiols (SH) and to N-H bending vibration in the NH_4^+ ion and the broad band at 2710 cm^{-1} may be attributed to $-\text{CH}_2$ stretching mode in $\text{CH}_2\text{-N}$ group from the tertiary amine.

Considering the data obtained about the modifications induced in the absorption, fluorescence and FTIR spectra following the interaction with the optical radiation, one may conclude that the initial BG 1120 molecules are transformed (Fig. 7) so that the resulting compounds have new properties which may be responsible for the more efficient action on pseudotumoural tissues in eyes.

The modifications of the BG 1120 molecules are produced after some minutes of exposure; a convenient exposure time for further experiments is 15 min since the absorption and fluorescence signals which express the molecules modifications show a decrease to about 40% from the initial values after 15 min exposure.

On the other hand, the pH of the solutions, which are made in distilled water, remains neutral even if modifications of the BG 1120 molecules are produced. One considers also that the pH of the pseudotumours produced on the rabbit eyes is not changed during the experiments.

Fig. 8 shows one of the pseudotumours which was produced (the obtained injuries looked approximately the same in all four studied eyes) using the procedure mentioned above at materials and methods chapter. At 12 days after this procedure the BG 1120 solution was injected in the eye under the conjunctiva, near the affected area, as follows: the 1st eye was not treated; the 2nd eye was injected with 0.1ml BG 1120 $5 \times 10^{-5}\text{ M}$ in distilled water, the solution being not irradiated; the 3rd eye was injected with the same amount of the irradiated substance (15 min exposure time); the 4th eye was injected with not-irradiated substance, then irradiated *in vivo* with the Xe lamp, for 15 minutes.

After two days the eyes were submitted to the anatomopathological examination using a Nikon 6 microscope. The tissues were coloured by hematoxiline- eosine.

As Fig. 9 shows, the 1st eye(not-treated) presents around the necrotic stitch a large quantity of inflammatory cells (A) as well as neovascularisations (B) and a small amount of fibrosis (C).

The 2nd eye (in which not irradiated substance was introduced) has the same tissue modifications, however much more reduced (Fig. 10).

In the 3rd eye (treated with irradiated substance) the quantity of the inflammatory infiltrate and oedema is significantly decreased comparing to the 2nd eye (Fig. 11). This shows that the substance exposed to optical radiation is more efficient (by a factor 1.5 to 2) in the reduction of the pseudo-tumour tissue dimensions than the non-exposed one.

In the 4th eye (Fig. 12) while the neovascularisations and the inflammatory cells are diminished, there is a large quantity of fibrosis; this might be due to the effects of the optical radiation on the tissue following the interaction of it with the constituents of the successive layers of the cornea. This fact leads to the conclusion that if the optical radiation is used to interact with the ocular tissues, it should be directed on the treated area only, to avoid any damage on the healthy tissue. On the other hand, this suggests that direct exposure of the impregnated eyes to optical radiation should be avoided due to the fibrosis of the tissues which may be produced.

4. CONCLUSIONS

While some of the analyzed chemical compounds present stability at the near ultraviolet – visible radiation, others such as BG 1120, show a decrease of the absorption, excitation and emission band intensities with the increase of the irradiation time; this suggests significant molecular transformations which might make the solutions more efficient for therapeutic effects; these could be improved by exposure to optical radiation (in the near ultraviolet and visible), as it is suggested by the data reported in this paper.

The modifications are stable at short time ranges (typically days); this can be understood according to a rough calculation if one takes into account that the average cross section of the absorption of radiation by BG 1120 molecules is about 10^{-17}cm^{-2} , the number of molecules in 2ml of solution at $5 \times 10^{-5}\text{M}$ concentration is around 10^{16} and the total number of photons sent by the Xe-lamp in 15 min to the exposed volume (2ml) of solution is around 10^{18} . At 15 min exposure time the most part of the molecules absorb radiation which produces irreversible molecular modifications at short time, even if the process is not saturated.

BG 1120 was able to decrease the inflammation and the neovascularisations in the conjunctival tissue, after being exposed to Xe-lamp radiation by a factor of 1.5–2 with respect to the unexposed substance.

Two mechanisms could be involved in the process: the cytostatic mechanism which implies the interference with the synthesis of the DNA and the cellular replication in the tissues with active proliferation, and the photosensitising mechanism that leads to the singlet oxygen and free radicals and finally to necrosis of the tissue involved.

These experiments point out the photosensitizing properties of some yet to be studied substances and their possible use in the treatment of some important affections such as conjunctive neovascularisation and cancer.

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