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GROWTH INHIBITION OF *Acanthamoeba* BY HYDROPEROXYNAPHTALIMIDES UPON PHOTOIRRADIATION

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Abstract – *Acanthamoeba* strains were photoirradiated in the presence of light-sensitive organic peroxides (hydroperoxynaphthalimide derivatives) including a Photo-Fenton reagent at 366 nm. The survival rates of *Acanthamoeba* strains determined after 20 h culture showed a significant decrease only upon photoirradiated conditions. The most effective hydroperoxynaphthalimides among these compounds was the bromo-hydroperoxynaphthalimide (Br-HPO II). The minimum inhibitory concentration (MIC) of Br-HPO II is 100 times lower than that of hydrogen peroxide.

INTRODUCTION

Recently, there have been several reports of various diseases such as meningitis[1-3] induced by pathogenic *Acanthamoeba*. Much attention has been paid to the lifecycle of *Acanthamoeba* and *Negrelia*. In addition, there are more than 200 clinical reports of *Acanthamoeba* keratitis among contact lens users in USA[4] and also similar clinical reports in Japan.[5]

However, there is no report concerning drugs to cure these diseases and serious and fatal results might be anticipated in the case of meningitis. Meningitis can be contracted through nasal infection. For example, if the water in a swimming pool contains pathogenic *Acanthamoeba*, infection will take place through the nasal membrane. The usage of chloride gas is the general way to clean up water. In connection with this, several studies have been carried out to study the inhibitory effect of chloride gas on the growth of *Acanthamoeba*,[6,7]. However, due to the strong resistance of *Acanthamoeba*, satisfactory results were not obtained. In addition, there are also several reports on the growth inhibition of *Acanthamoeba* strains by using various drugs including anti-viral drugs[8-10] and the application of cryotherapy.[11] All these trials, however, did not produce any good results due to the high resistance of the cyst form of *Acanthamoeba* strains. It is, therefore, highly desirable to develop a system or a drug to cause the cell death of *Acanthamoeba*. In this paper, we examined the possibility to use the active oxygen radicals to induce *Acanthamoeba* growth inhibition because these species are quite toxic to induce the cell death.

We have already developed a series of compounds which generate a hydroxyl radical upon longer wavelength irradiation (366 nm, UVA region), and by using HPO III (referred to as the "Photo-Fenton reagent"), we examined the oxidative damage of DNA,[12,13,14] microsomes,[15] crystalline,[16] human low density lipoprotein,[17,18,19] chymotrypsin,[20] and lysozyme.[21] The major advantage to use of this compound lies in the longer wavelength (> 350 nm) irradiation and the generation of a clean and pure hydroxyl radical, which is strong in contrast to the commonly used metal-hydrogen peroxide system.[22,23] The application of the "Photo-Fenton reagent" as a new kind of drug for the growth inhibition of eucaryotic cells was examined using *Acanthamoeba polyphaga* and *Acanthamoeba astronyxis*. As a result, it was revealed the hydroperoxynaphthalimide derivatives including the "Photo-Fenton reagent" showed remarkable inhibitory effects on the growth of both *Acanthamoeba* strains.

MATERIALS AND METHODS

Proteose-peptone and yeast extract were purchased from Difco. Glucose and hydrogen peroxide (30%) were purchased from Wako Chemicals and t-butyl hydroperoxide (80%) was purchased from Nakarai Tesque Japan. All other reagents were purchased from Tokyo Kasei Organic Chemicals (TCI). The transilluminator (366 nm) used in these experiments was purchased from Funakoshi Co., Ltd., and the light intensity of the transilluminator at 10 cm was 1700 μW/cm^2.

Cell culture. *Achanthamoeba* used in these experiments are

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†*Abbreviations:* HPO II, 2-(2-Hydroperoxy-2-methoxyethyl)-1H-benz[de]isoquinoline-1,3-(2H)-dione; Br-HPO II, 2-(2-Hydroperoxy-2-methoxyethyl)-6-bromo-1H-benz[de]isoquinoline-1,3-(2H)-dione; HPO III, N,N'-bis(2-Hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetracarboxylic diimide, Photo-Fenton reagent; PYG culture medium, The solution contains 7.5 g proteose-peptone, 7.5 g yeast-extract and 1.5g glucose in 1000 ml distilled water.

A. polyphaga (CCAP, 1501/3A) and *A. astronyxis* (CCAP. 1501/9). As these strains did not require bacterial food sources, *Acanthamoeba* were grown axenically on shakers without rotating at 30 °C in a PYG culture medium [7.5 g proteose-peptone (Difco), 7.5 g yeast extract (Difco), 15 g glucose (Wako)/L)].[24] Stock cultures were restarted from aliquots of spores every 6 weeks. Experiments were carried out using *amoebas* harvested during the logarithmic phase of their growth at cell densities between 5×10^6 to 1×10^7 cells/mL.

The growth Inhibition of acanthamoeba by hydroperoxynaphthalimides derivatives. All hydroperoxynaphthalimide derivatives at 5 m*M* concentration were dissolved in t-BuOH, which was then diluted with NKMCT solution (12.0 g NaCl, 0.35 g KCl, 0.3 g $CaCl_2$, 0.4 g $MgSO_4$-$7H_2O$, in 100 m*M* Tris-HCl buffer solution, pH 6.8)[24] to the concentration required for the experiments. The concentration of *Acanthamoeba* solution was adjusted to 3.3×10^5 cells/mL by diluting the culture solution by NKCMT solution. The solution containing 0.9 mL of *Acanthamoeba* and 0.1 mL of hydroperoxynaphthalimide derivatives at defined concentrations was stood for 30 min at 30 °C, which was then photoirradiated from the transilluminator (366 nm) at a distance of 10 cm from the top of the sample solution for 20 min. The intensity of the transilluminator was 1700 $\mu W/cm^2$ at this distance. The non-photoirradiated samples were also kept at 30 °C for 20 min. After the reaction, 3 μL of the sample solution was taken out from the reaction mixture and was spread over the plates at three spots. The non-photoirradiated samples at the same concentrations of hydroperoxynaphthalimides were also treated in the same way.

The plates were prepared by dissolving 1.5 g of bacto agar in 100 mL of NKCMT solution, which was then autoclaved. The autoclaved agar solution was poured into the autoclaved plate and stood till the agar was completely fixed. The 0.3 g yeast extract in 4 mL of NKCMT solution was spread over the plate. After culturing for 20 h and staining using trypan blue, the survival rates of *Acanthamoeba* were determined by putting the number of surviving cells into the following equation†.

Survival Rate (%) = A × 100 / B × 10^{-3} × 9
A = The number of surviving cells
B = Concentration of the *Amoebas* used in the experiments

Similarly, control experiments were also carried out at the same time.

Growth inhibition by hydrogen peroxide and t-butyl hydroperoxide. The hydrogen peroxide (30%) and t-butyl hydroperoxide (80%) were diluted to the defined concentration by double distilled water. One portion of the hydrogen peroxide solution and 9 portions of the *Acanthamoeba* solution were mixed at 30 °C for 30 min without photoirradiation and 3 μL of the reaction mixture was spread onto the agar plate at three spots. The reading of the survival rates was also carried out in the same reaction conditions as those of hydroperoxynaphthalimide derivatives.

† This equation doesn't show the exact survival rate because within 20 hours culture, the active *Acanathamoeba* will carry out the cell division, which results in the increase of the cell number (A). So, the exact survival rate is anticipated same or a little bit lower than the values shown in Table 1.

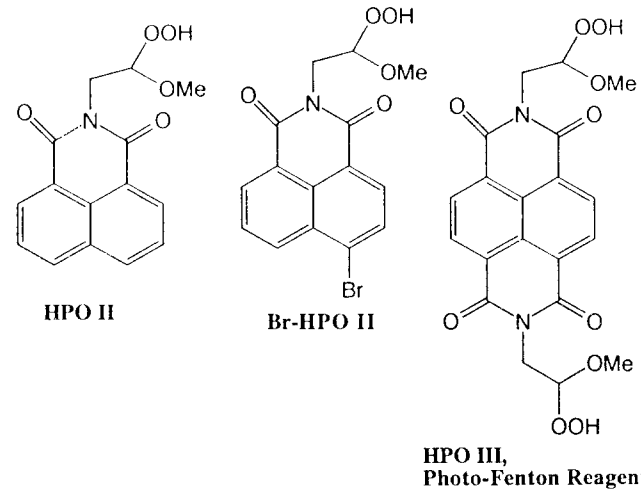

Figure 1. Chemical Structure of Hydroperoxynaphtahlimides and Photo-Fenton Reagent

RESULTS AND DISCUSSIONS

At first, we examined the morphorgic change of *Acanthamoeba* cells exposed to hydroperoxynaphtalimides using HPO II and *A. polyphaga*. When *A. polyphaga* was photoirradiated from the transilluminator for 20 min in the absence of HPO II, no obvious morphorgic change of the cell was observed; however, in the presence of HPO II, the significant cell membrane damage was observed in a concentration-dependent fashion. These damages are especially clear at 100 μM concentration of HPO II (Fig. 2). Without photoirradiation, no significant cell damage was observed in every case examined. So the cell damage was strongly connected with the hydroxyl radicals generated from HPO II upon photoirradiation. Next, we examined the viability of *Acanthamoeba* cells. Namely, 3 μL of the sample solution photoirradiated with the defined concentration of HPO II was dropped on the agar plate and microscopic photographs were taken after 10 days incubation (Fig. 3). Even if only 1 or 2 cells of *Acanthamoeba* survived in the reaction conditions employed, they will increase their cell numbers in 10 days spreading all over the plate within that period. As a result, at 1 μM concentration of HPO II, we observed a lot of *Acanthamoeba* spread all over the plate in both photoirradiated and non-photoirradiated cases. At 10 μM concentration of HPO II, a significant inhibition of the cell growth was observed in the photoirradiated case and no inhibition was observed in the non-photoirradiated case. At 100 μM concentration of HPO II, no *Acanthamoeba* survived in the photoirradiated conditions, however, a lot of amoebas was observed in the non-photoirradiated cases.

The photoirradiation without HPO II does not have any effect on the cell growth (Fig. 3, control). These results clearly demonstrate the importance of the photoirradiation for the cell death.

Next, the survival rates of *A. polyphaga* and *A. astronyxis* were determined after 20 h incubation of the 3 μL of the photoirradiated samples pasted on the agar plates. Table 1 shows a summary of the survival rate of *A.*

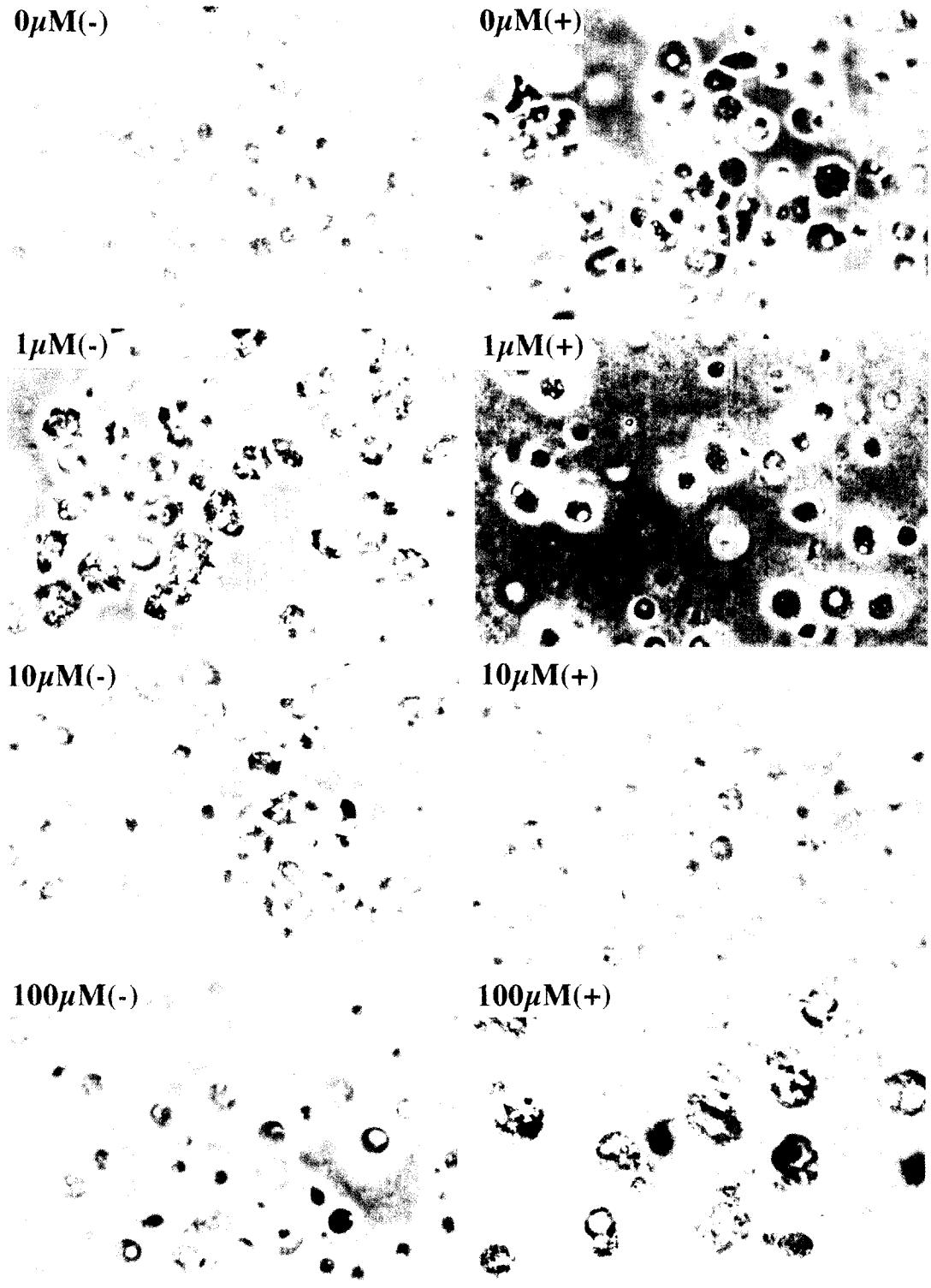

Figure 2. Effects on Acanthamoeba polyphaga with HPO II upon Photoirradiation (1 hour after treatment)

polyphaga exposed to the oxidative stress. Upon photoirradiated conditions, all these hydroperoxynaphthalimide derivatives showed the remarkable inhibitory effects on the growth of *A. polyphaga*; however, without photoirradiation, they did not show any clear inhibitory effects on the growth of *A. polyphaga*. The strongest inhibitory effect was observed in the case of Br-HPO II. This compound completely suppressed the growth of *A.*

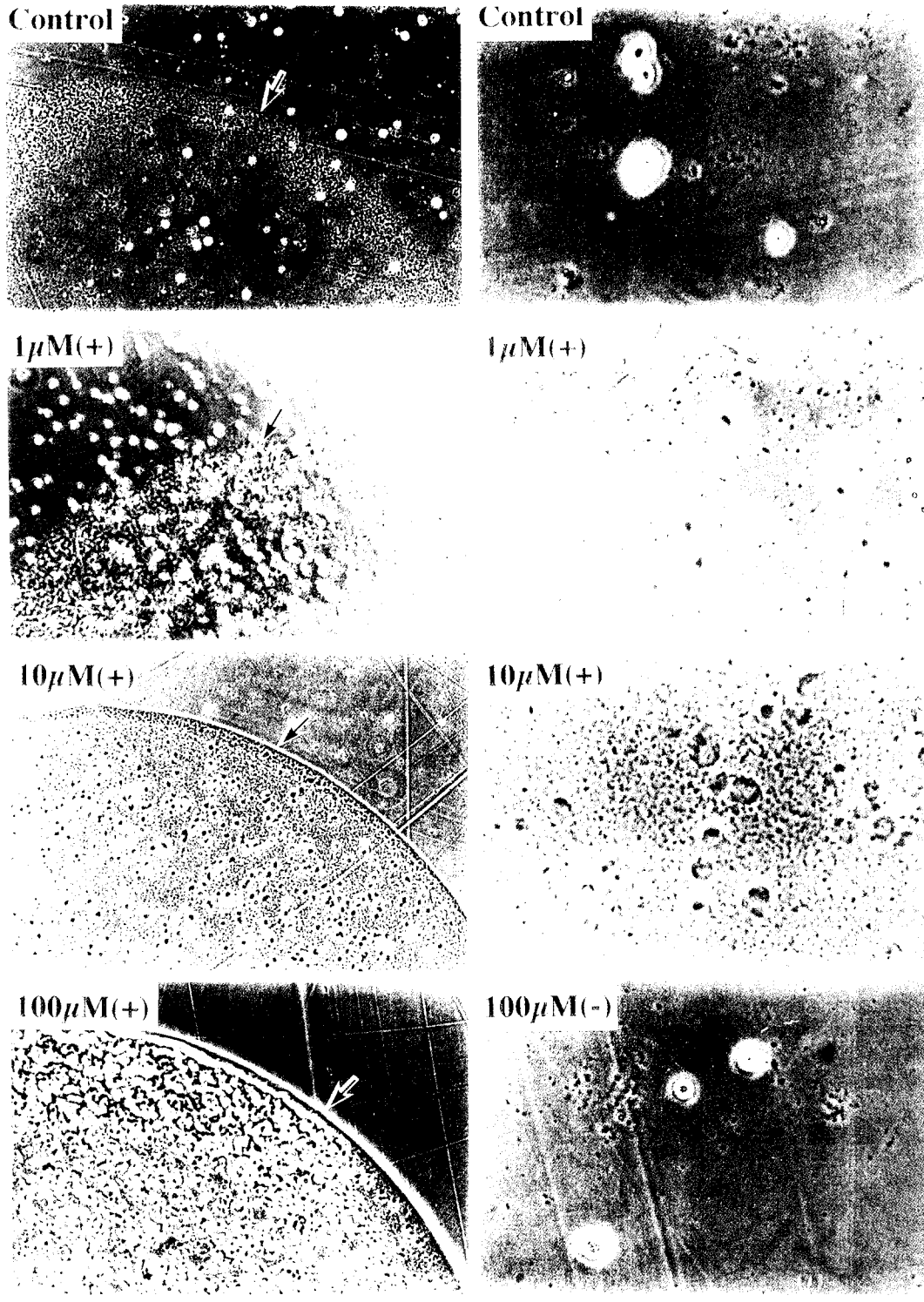

Figure 3. Effects on Acanthamoeba polyphaga by HPO II upon Photoirradiation (10 days after treatment)

Table 1. Survival Rates (%) of *Acanthamoeba polyphaga* (CCAP) with hydroperoxynaphthalimides, hydrogen peroxide and t-butyl hydroperoxide

Compound	Concentration (μM)	Irradiation	Survival Rate (%)
HPO I	25	+	5.728 ± 0.8
	6.25	+	a
	100	−	a
	50	−	a
HPO II	50	+	0
	25	+	0
	6.25	+	a
	100	−	a
	50	−	a
Br-HPO II	25	+	0
	6.25	+	0.397 ± 0.07
	3.125	+	a
	100	−	a
	50	−	a
HPO III	50	+	0.25 ± 0.06
	25	+	a
	100	−	a
	50	−	a
H_2O_2	1470	−	2.3 ± 0.19
	750	−	a
t-BuOOH	900	−	5.0 ± 0.43
	450	−	a

[a]The cell number is too big to count significant at the 0.1 level by Student's *t* test.

Table 2. Survival Rates (%) of *Acanthamoeba astronyxis* with hydroperoxynaphthalimides, hydrogen peroxide and t-butyl hydroperoxide

Compound	Concentration (μM)	Irradiation	Survival Rate (%)
HPO II	50	+	0
	25	+	0.7 ± 0.09
	6.25	+	a
	100	−	a
	50	−	a
Br-HPO II	25	+	0
	10	+	3.0 ± 0.43
	5	+	a
	100	−	a
	50	−	a
HPO III	50	+	2.8 ± 0.18
	25	+	a
	100	−	a
	50	−	a
H_2O_2	30000	−	5.2 ± 1.2
	15000	−	7.5 ± 2.36
	5000	−	a
t-BuOOH	9000	−	0.3 ± 0.08
	4500	−	7.5 ± 1.76
	900	−	a

[a]The cell number is too big to count significant at the 0.1 level by Student's *t* test.

polyphaga at 25 μM concentration and showed partial inhibitory effect at 6.25 μM concentration (0.4 % survival rate). Under similar reaction conditions, hydrogen peroxide and *t*-butyl hydroperoxide showed weaker inhibitory effects than at 1 mM concentration of the dose (Table 1). These results clearly demonstrate the efficacy of the photoirradiated hydroperoxynaphthalimide derivatives. Similarly, we examined the effects of these compounds on the growth of *A. astronyxis*. Almost similar effects were observed as observed in the case of *A. polyphaga*; however, the concentration needed to inhibit the cell growth is much higher than in the case of *A. polyphaga*. This is remarkable in the inhibitory effects of hydrogen peroxide on the growth of *A. astronyxis*. The concentration to inhibit the cell growth of *A. astronyxis* was more than 44 mM. This will be partially due to the difference in the catalase activity of the two strains; however, further studies are necessary to clarify this point (Table 2).

All these results clearly demonstrate the strong correlation between the cell death of *Acanthamoeba* strains and the photoirradiation of hydroperoxynaphthalimides. We have already established that the photoirradiation of hydroperoxynaphthalimide derivatives induces the specific generation of a hydroxyl radical.[12-21] So it is quite reasonable to consider that all these observations we found here are due to the hydroxyl radicals generated from the photoirradiation of hydroperoxynaphthalimide derivatives.

All these results examined in this paper showed the possible usage of hydroxyl radicals (active oxygen radicals) generating compounds in the cell death of pathogenic *Acanthamoeba*. Although the dose required for the necrosis of the cell is not so low, the specific application of this method by using some kind of laser light opens up the potentiality of this kind of drug as a light-activated drug.[25] The difference in the sensitivity of the doses may partially be attributed to the ability of the penetration of the compound through cell membranes. In this respect, the improvement of the drug will make it possible to obtain a superior light-activated drug. Further modification of this kind of drugs is now in progress in our laboratory.

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