Yonsei Medical Journal
Vol. 31, No. 4, 1990

Anti-retroviral Effect of Chlorophyll Derivatives (CpD-D) by Photosensitization

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A new photosensitizer, CpD(chlorophyll derivatives), previously reported as a promising agent for tumor therapy, was studied to determine its inhibitory effects on Gross leukemia virus(GLV), a mouse retrovirus isolated from the GLV-producing TGV cell line, and the cytocidal effect on the GLV infected cells in vitro. following photodynamic treatment with CpD-D and red light, the viral inactivation and infectivity were examined by measuring the reverse transcriptase(RT) activity of the virus itself and that in cell-free culture supernatnat of freshly GLV-infected secondary mouse embryo cells respectively. The cytocidal activity was measured by trypan blue exclusion test. Inhibition of GLV associated RT activity resulted from CpD-D and red light treatment. The RT inhibition effect was immediate and the infectivity of these photodynamically treated GLV to mouse embryo cells was also inhibited. However, specific cytotoxicity of GLV infected cells was not found. Thus, it is concluded that CpD-D may be used as an effective antiviral agent.

Key Words: Photosensitizer, anti-retroviral, chlorophyll derivatives (CpD-D), photodynamic, viral

The AIDS epidemic and the growing demand for virus-free blood products for the treatment of immunosuppressed patients have renewed the interest in blood sterilization procedures (Gomperts 1986; Prodouz 1987). Use of blood components involves significant risk to recipients because of the potential of transmission of infectious agent such as hepatitis B virus (HBV), cytomegalovirus (CMV), herpes simplex virus (HSV), human immunodeficiency virus (HIV) and non-A non-B (NANB) hepatitis virus. Screening of donors by medical history and serologic testing for antibodies to HIV and for circulating antigens of HBV reduces but does not eliminate the risk

One potential approach to prophylactic treatment of blood is photodynamic treatment (PDT). Photodynamic inactivation, the use of a photosensitizer and light energy to inactivate living organisms, has been known since 1900 (Jesionek and Tappeiner 1903). The antiviral potential of photosensitizing dyes was recognized in 1933 (Perdrau and Todd). Photodynamic sensitizers include naturally occurring pigments such as porphyrins, chlorins (chlorophyll, bonellin), flavin, bilirubin, and synthetic dyes (Spikes 1984). Tricyclic dyes such as neutral red and methylene blue were believed to intercalate in viral DNA and undergo oxidation reactions upon irradiation with visible light. The tricyclic dyes were intensely studied with HSV (Melnick and Wallis 1975; Swartz *et al.* 1979) and have found limited use in the treatment of herpetic skin and eye lesions (Wallis and Melick 1965; Felber *et al.* 1973; Chang and Weinstein 1975; O'Day *et al.* 1975). However, the efficacy of the treatment modality has been questioned (Myer 1973) and some authors have expressed concern that photoinactivated viruses are still mutagenic (Rapp *et al.*1973; Khan *et al.* 1977).

Porphyrins are a group of photoactive substances that are structurally different from heterotricyclic dyes and whose photochemical properties are responsible for the skin photosensitivity reaction observed in the porphyrias. Hematoporphyrin derivative (HpD), a mixture of naturally occurring tetrapyrroles, has been used to detect and treat tumors (Dougherty *et al.* 1975; Dougherty *et al.* 1978; Dougherty 1984; Spikes 1984). Killing of tumor cells by HpD is believed to be the result from photooxidation of both tumor and adjacent cell structures (Kessel 1984). Enveloped viruses such as HSV and HIV were effectively inactivated by HpD and light (Lewin *et al.* 1980; Schnipper *et al.* 1980; Matthews *et al* 1988).

Received March 28, 1990
Accepted June 13, 1990
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Previously we reported a new photosensitizer, chlorophyll derivatives (CpD), which is easily obtained from the extract of excreta of silkworms (Bombyx mori) (Park 1988; Choi 1989). The basic chemical structures of CpD, CpD-A, -B, -C and -D, belong to the porphyrin family (Choi *et al.* 1989) and have similar photochemical properties. We have observed singlet oxygen formation and red fluorescence upon light exposure (630-670nm) to CpD-A, and compared to HpD more intracellular uptake and greater photocytotoxicity (Park 1988). *In vivo* experiments indicate that CpD-D is effective in the treatment of murine subcutaneous and ascites tumors (Park *et al.* 1989a; Park *et al.* 1989b).

In this paper, we report the photodynamic effect of CpD-D on the Gross leukemia virus (GLV) and GLV infected mouse embryo cells.

MATERIALS AND METHODS

CpD-D

CpD-D was originally prepared and supplied by the department of Chemistry and Chemical Engineering, Ajou University, Suwon, Korea. CpD-D was extracted from fecal specimen of silkworm as a source of chlorophyll and was found to be a mixture of tetrapyrroles. CpD-D was dissolved in normal saline to 2mg/ml as a stock solution and was kept in the dark at −20°C until used.

The UV-visible absorption spectrum of CpD-D in normal saline was measured with a U-240 spectrophotometer (Shimazu, Japan) and its excitation spectrum was detected by a luminescence spectrophotometer (LS-5, Perkin-Elmer Ltd., USA)

Cell cultures

TGV (Machala et al. 1974) cell line which is established from leukemic C3HeB/Fe mouse and releases infectious GLV particles into the culture medium, was used for the source of GLV. Virus productivity of TGV was recognized by giant cell formation when TGV and XC cells were cocultured (Klement *et al.* 1969). Secondary cell cultures of 15- to 17-day C3H mouse embryo were stored in a nitrogen tank until used.

All cell cultures were maintained in Eagle's minimal essential medium (MEM, CSL, Australia) supplemented with 10% fetal calf serum (FCS, CSL, Australia), penicillin (100units/ml) and streptomycin (100ug/ml).

GLV

GLV (Gross 1957), type C murine leukemia virus (MuLV), was prepared from TGV cultures. To prepare cell-free viruses, TGV cells were cultivated until the monolayer was formed, then the cell-free supernatant containing viruses was collected twice whenever the medium was changed. The viral suspension of culture superantants was ultracentrifuged at 56,000g for 2 hours (Beckman, USA), and the pellet was resuspended in TNE buffer solution (10mM TrisHCl, 0.1M NaCl, 1mM EDTA). To prepare the virus stock, the suspensions were centrifuged (10,000g, 2 minutes) and the supernatant was passed through a 0.2μm filter (Acrodisc, Gelman Sciences Inc., Ann Arbor, MI). The concentrated virus stock was stored at −70°C.

Photodynamic treatment of GLV

GLV stock preparation was diluted fivefold in normal saline and placed into clear 35mm polystyrene tissue culture dishes (Corning, NY). CpD-D was added from 100μg/ml to a final concentration of 2.5μg/ml. GLV suspension with CpD-D was incubated in the dark for 10 minutes and subsequently illuminated for various time intervals. The light source was provided by commercial day light bulb (100V, PRS, 500WD, for color day light, Korea) at a fixed distance of 45cm from the petri dish, and the light was passed through the clear water chamber and red cellophane filter. This red light has been reported to provide a wavelength of 630-750nm according to Maxwell's color matching experiments (Linksz 1964). The GLV suspension was kept in the dark except when samples were intentionally irradiated.

For the measurement of infectivity, the photodynamically inactivated GLV was inoculated onto the normal mouse embryo cells and supernatants were obtained from the infected cell cultures at regular intervals and assessed for RT activity.

Photodynamic cytotoxicity

GLV infected mouse embryo cells and normal mouse embryo cells were seeded in 24 well culture plates (1×10^5 cells/well) and incubated for 24 hours in a CO_2 incubator. After the addition of CpD-D, cells were placed in the dark for 10 minutes and subsequently exposed to red light. Following irradiation, treated cells were immediately trypsinized and washed twice, then stained with trypan blue and counted for determination of cell viability.

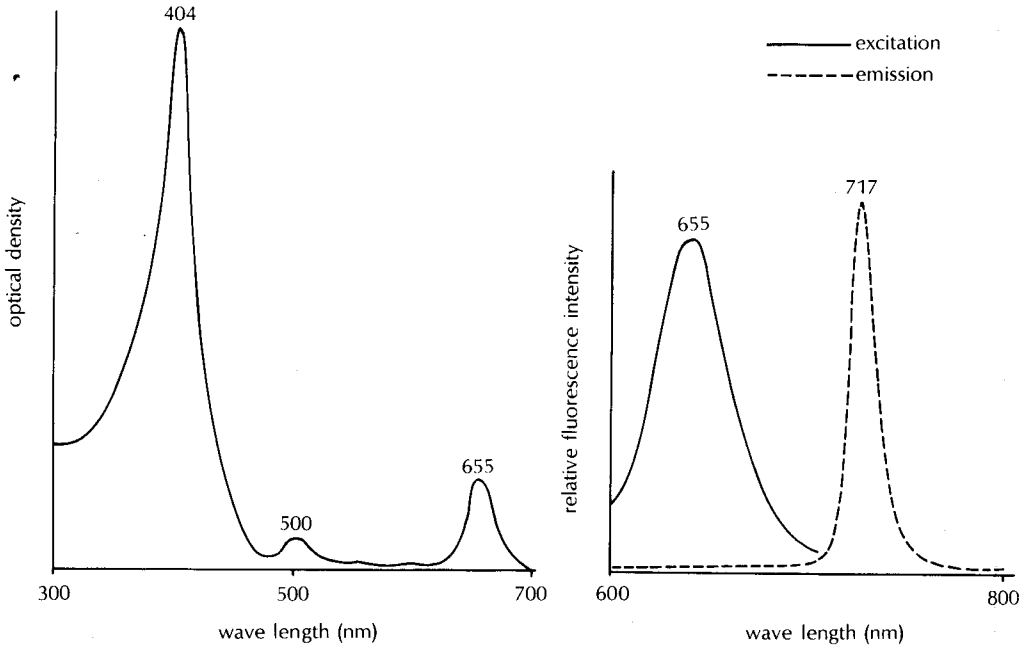

Fig. 1. *Absorption spectrum (left) and excitation and emission spectra (right) of CpD-D.*

Reverse transcriptase (RT) assay

The amount of GLV present in the culture medium was determined as follows. PEG 6000 was added to 2ml of cell-free culture medium to a final concentration of 10% and placed at 4°C for more than 2 hours and centrifuged at 1000g, 4°C for 45 minutes. The pellet was resuspended in a solution of 100mM TrisHCl (pH8.0), 0.1M NaCl, 1mM EDTA, and 0.01% Triton X-100 (v/v). Ten μl of samples was added to 90μl of reaction mixture containing (final concentration) 50mM TrisHCl (pH 8.0), 2mM MnCl2, 0.02M dithiothreitol, 50mM KCl, 0.1% Triton X-100, 10μCi/ml [^{3}H]dTTP (50-90Ci/mmol, Du Pont Co., Willmington, DE), and 12μg/ml poly (rA)·p (dT) (Pharmacia, Piscataway, NJ). The mixture was incubated at 37°C for 45 minutes, and then placed on ice for more than 30 minutes. Samples were spotted on glass fiber filters (Gelman, Ann Arbor, Michigan) and washed three times with 5% trichloroacetic acid and 0.02M sodium pyrophosphate and once with distilled water. The filters were dried and the radioactivity was determined in a toluene-based scintillation liquid.

In the case of photoinactivated GLV, 10μl of the treated GLV suspension was added to 90μl of reaction mixture.

RESULTS

Absorption and fluorescence spectrum of CpD-D

CpD-D dissolved in normal saline yielded absorption bands peaking at 404, 500 and 655nm and a similar band pattern of the excitation spectrum was detected. Excitaion and emission spectra of CpD-D are shown in Fig. 1. In this study, we used red light ranging from 630 to 710nm.

Effects of CpD-D on GLV

GLV can be monitored by the reverse transcriptase (RT) assay. Fig. 2 shows the relationship between the concentration of GLV and RT activity. There was an exponential reduction in RT activity as the concentration of GLV decreased. Fivefold diluted GLV suspensions were mixed with CpD-D at a final concentration of 50μg/ml and irradiated for 5 minutes. Immediately thereafter, the RT activity was determined. The results presented in Fig. 3 indicate that CpD-D does not inhibit the RT activity of GLV if the mixture is continuously shielded form light and a short period of photoirradiation results in loss of RT activity of the

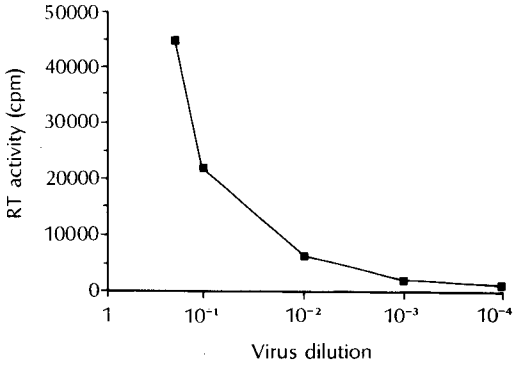

Fig. 2. Relationship between concentration of GLV and reverse transcriptase (RT) activtity.

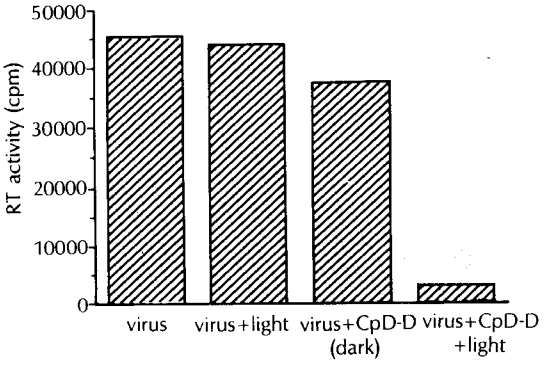

Fig. 3. Photodynamic inactivation of GLV by CpD-D and light. GLV was mixed with CpD-D (50μg/ml, final concentration)and irradiated with red light for 5 minutes. The treated virus was assayed in the dark for RT activity.

virus-CpD-D mixture.

To establish a dose-inactivation relationship for CpD-D, increasing concentrations of CpD-D were mixed with GLV, and irradiated for 5 minutes and 10 minutes respectively. As shown in Fig.4, as the concentration of CpD-D increased there was a decline in the RT activity. Exposure of GLV for a longer period to CpD-D enhances the susceptibility of GLV to photoinactivation by CpD-D, with no significant difference observed between exposure for 5 and 10 minutes.

To test the photodynamic effect of CpD-D on the infectivity of GLV, GLV was treated with 25μg/ml and 50μg/ml of CpD-D and was irradiated for 5 minutes, and inoculated onto the secondary mouse embryo cells. The amount of GLV released from the cells to the culture medium was accessed by RT activity in the

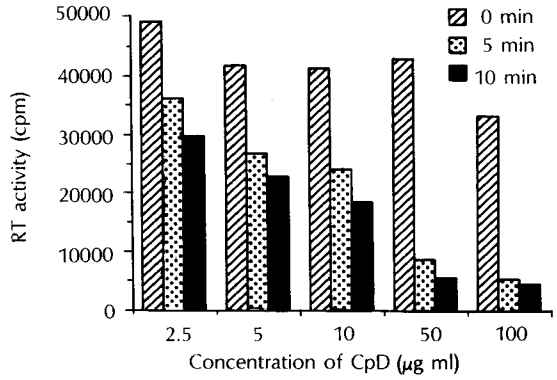

Fig. 4. Effect of CpD-D concentration and irradiation time on photodynamic inacitivation of GLV.GlV was mixed with increasing concentration of CpD-D and exposed to red light. Treated virus was then assessed for RT activity. (The RT activity observed without virus was about 1,000cpm).

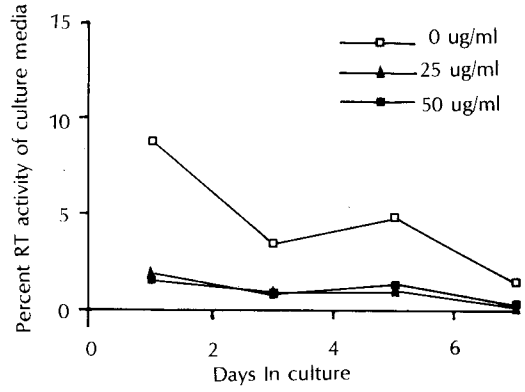

Fig. 5. Infectivity of photoinactivated GLV. GLV was mixed with CpD-D and exposed to red light for 5minutes. Photoinactivated GLV was inoculated into normal mouse embryo cells. Supernatants were obtained from the infected cell cultures at days 1, 3, 5 and 7 in culture, and assessed for RT activity. The RT activity of supernatants of uninfected cell cultures was 0.5-1.0%

infected cell culture supernatants. Supernatants were obtained from 1,3,5, and 7 days culture. It was observed that control samples yielded about 5-10% RT activities but the RT activity of supernatants infected with photoinactivated GLV was markedly reduced (Fig. 5).

Effects of CpD-D on GLV infected mouse embryo cells

To examine the *in vitro* cytotoxicity of CpD-D to

virus infected cells, normal and virus infected cells were treated with various concentrations of CpD-D. The normal mouse embryo cells which were treated with 10μg/ml and 25μg/ml of CpD-D and irradiated for 5 minutes showed percent survivals of 94.4% and 36% respectively at 3 hours after treatment (Fig. 6).

To determine the conditions that may induce significantly different cytotoxicity between the normal and virus infected cells, cells were treated with 10μg/ml of CpD-D and irradiated for various periods of time. The survival curves of boh normal and virus infected cells are illustrated in Fig. 7 and cell survivals at 4 hours after treatment are shown in Fig. 8. In the samples irradiated for 20 minutes and 30 minutes (Fig. 8), the percentages of viable cells appeared to be somewhat higher for the normal cells than virus infected cells, without significant difference. Thus, we concluded that there was no selectivie cytotoxicity of CpD-D for GLV infected cells.

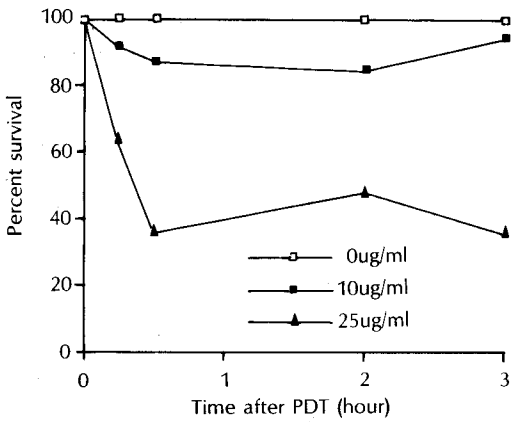

Fig. 6. *Survival curves of normal embryo cells treated with CpD-D and light. Cells were cultivated in a 24-well culture plate and were mixed with CpD-D and exposed to red light for 5minutes. Treated cells were trypsinized and washed. Percent survivals were measured by trypan blue exclusion test.*

DISCUSSION

Antiviral effects of hematoporphyrin derivative

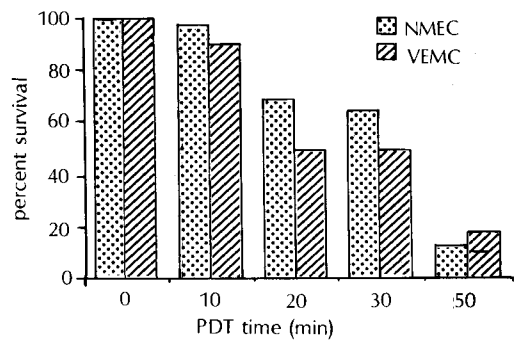

Fig. 8. *Percent survivals at 4 hours after PDT of normal mouse embryo cells (NMEC) and GLV infected mouse embryo cells (VMEC) treated with 10μg/ml of CpD-D and irradiated for various intervals.*

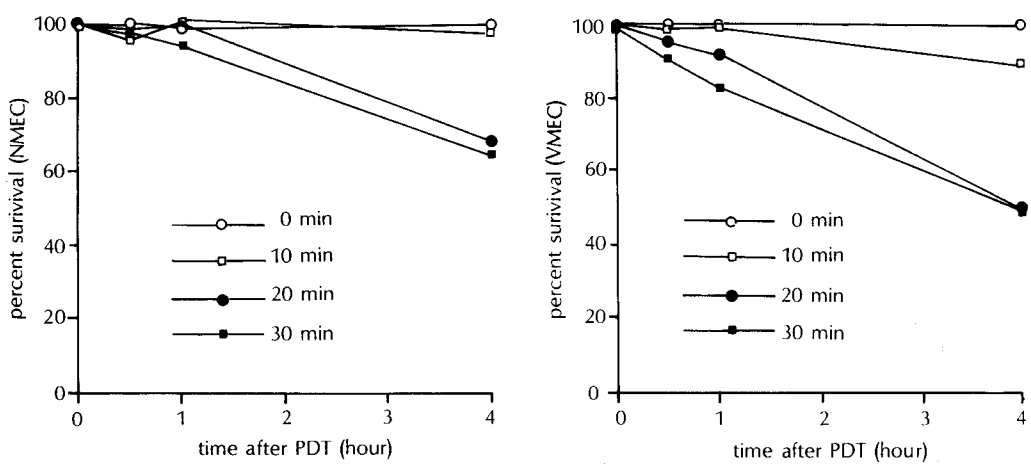

Fig. 7. *Survival curves of normal mouse embryo cells (NMEC) and GLV infected mouse embryo cells (VMEC) treated with 10μg/ml of CpD-D and irradiated for various intervals.*

(HpD) (Skiles *et al.* 1985) and merocyanine 540 (MC 540) (Sieber *et al.* 1987, Sieber *et al.* 1989), have indicated that photodynamic treatment would be useful in tumor destruction and selective killing of leukemic cells in autologous bone marrow grafts (Sieber 1983) but also in the effective removal of virus and virus infected cells contaminating in the blood.

When a photosensitizer is activated in the presence of molecular oxygen, two initial types of competing processes may occur. The type I process is initiated by the interaction of photosensitization with biomolecules and results in the formation of free radicals that can degrade biomolecules. Many photosensitizers interact with molecular oxygen and form the very reactive singlet oxygen which reacts efficiently with many biomolecules, resulting in their degradation, and this is called the type II process. In the case of porphyrins, singlet oxygen formation is assumed to be the predominant pathway (Moan and Sommer 1985).

In this work, we used a new photosensitizer, CpD-D, which is extracted from the excreta of silkworms, the source of chlorophyll, and processed to be water soluble. CpD-D is composed of more than 4 different compounds that belong to the porphyrin family (Choi 1989).

Porphyrins and other photosensitizers are activated by the absorption of photons throughout the absorption spectra. Porphyrins with absorption band near 630nm are usually used for photodynamic treatment of tumors (Gomer *et al.* 1984). In this study CpD-D, whose absorption peak is near 655nm, was used.

Photooxidative damage by porphyrins has been described in a variety of non-viral biologic systems, but the precise molecular targets are unknown. However, the cytoplasmic membrane is believed to be the main target. Photooxidation of biologic molecules causes oxidation of lipids and cross-linkages of proteins, resulting in the dysfunction of the transport system and changes in the membrane permeability (Kessel *et al.* 1977), cell swelling and cell lysis (Bellier and Dougherty 1982; Moan *et al.* 1982). DNA single-strand breaks and sister chromatid exchange have also been reported (Moan *et al.* 1980; Dubbleman *et al.* 1982).

There is very little information available regarding the mechanisms of photodynamic inactivation of viruses. Enveloped viruses such as HSV and HIV were effectively inactivated by the photodynamic effect of HpD (Lewin *et al.*: Schnipper 1980), but naked viruses were resistant to photoinactivation (matthew *et al.* 1987). HpD reacts primarily with the lipid portion of cytoplasmic membranes (Kessel 1977; Berenbaum *et al.* 1982) and, presumably, the lipid portion of virus envelopes which originate from various sites of cell membranes such as the cytoplasmic membrane, nuclear membrane, etc. The lipid composition and viscosity of the membrane may affect the adsorption, therefore, the susceptibility of the various kinds of enveloped viruses to photosensitization with porphyrins may depend on the lipid composition of the envelope. In studies of the effects of HpD on enveloped viruses, single stranded DNA breaks were observed and the treated viruses were unable to initiate the early stage of infection (Schnipper *et al.* 1980; Matthew *et al.* 1987), suggesting an HpD-associated alteration in the viral surface. And inactivation of reverse transcriptase has also been reported (Munson and Fiel 1977; Musser *et al.* 1980).

In this work, CpD-D remarkably inhibited the RT activities of the GLV treated with 50μg/ml of CpD-D and light. There were no such effects in the case of CpD-D or light alone samples. This suggests that the inhibitory effect is due to the photodynamic action of CpD-D. And this inhibitory effect was proportional to the concentration of CpD-D and the duration of irradiation. Inhibition of RT and defects in the envelope would be a possible mechanism of the suppression of GLV multiplication.

The low cholesterol content of leukemic cells determines a higher membrane fluidity compared to normal cells. For this reason, leukemic cells bound more porphyrins and were sensitive to the cytotoxic effect of porphyrins (Ben-Bassat *et al.* 1977; Kessel *et al.* 1981). Also cells transformed by Ki-MuLV were reported to be more sensitive to porphyrins than normal control cells. The increased sensitivity of these cells may be due to the changes in cell membrane and alterations of various intracellular metabolic processes. In this work, however, we could not find the differential cytotoxicity between normal and virus infected cells.

CpD-D mediated photosensitization inactivated GLV, inhibited the GLV associated RT activity and reduced infectivity of GLV. Thus, CpD-D appears to be a potentially attractive anti-retroviral agent. However, to eliminate viruses in the blood by using CpD-D, further studies are required. CpD-D should be more toxic to virus-infected cells than other normal cells. And the effectiveness against intracellular viruses and precise anti-retroviral mechanisms should be elucidated.

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