RESEARCH COMMUNICATION

# Chemopreventive Activity of Porphyrin Derivatives Against 6-Sulfooxymethylbenzo[a]pyrene Mutagenicity

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## Abstract

**Porphyrins exhibit potent antimutagenic activity in the range of assays *in vitro* and *in vivo*. The antimutagenic activities of porphyrin derivatives including phthalocyanines (Pcs) were investigated using 6-sulfooxymethylbenzo[a]pyrene (SMBP) and its proximate metabolite 6-hydroxymethylbenzo[a]pyrene (HMBP) in the Ames assay and hypoxanthine:guanine phosphoribosyl transferase (*hprt*) point mutation assay in V79 cells. Pcs, irrespective of coordinated metal, showed highly antimutagenic activity against both HMBP and SMBP in the Ames assay. However, their inhibitory effects against HMBP were in general less dramatic than against SMBP. Treatment with chlorophyllin (CHL) and protoporphyrin reduced the mutation frequency to 24.8% and 19.1% of that with SMBP, and 56.5% and 40.7% of that with HMBP, respectively. Hemin, biliverdin and chlorophylls had a lower antimutagenic activity compared with other porphyrin derivatives although they still retained inhibitory capacity against SMBP. The results of the Ames test for SMBP were extended to the mammalian system to confirm the antimutagenicity of porphyrin derivatives. The antimutagenic strength of porphyrin derivatives was in order of hemin, Pcs, CHL and protoporphyrin. Biliverdin, chlorophyll a and chlorophyll b had a little effect against HMBP and SMBP. Among the compounds tested here, hemin had a strong inhibitory effect against SMBP-induced mutation in V79 cells while Pcs were most effective in the microbial system. It is assumed that these discrepancies are partly due to differences in membrane permeability to the chemicals, their metabolisms and chromosomal organization. Furthermore, carcinogen-DNA binding in calf thymus DNA and plasmid was carried out with a series of porphyrin derivatives to understand their protective mechanism. Most of the porphyrin derivatives exhibited inhibitory activity against SMBP with a variety of degrees. A significant reduction of SMBP-mediated DNA damage by Pcs was confirmed on agarose electrophoresis. There was little correlation between the levels of SMBP-DNA adducts and their modulation in mutation frequencies, indicating that porphyrin compounds somehow affect the antioxidant function and the reactivity of SMBP to cellular components.**

**Keywords**: Prophyrin - 6-Sulfooxymethylbenzo[a]pyrene - Antimutagenicity

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## Introduction

Chemoprevention is the process of inhibiting or reversing carcinogenesis, resulting in providing enormous benefits to public health by lowering the incidence of human cancer. A large number of potential chemopreventive agents have been identified from epidemiological surveys, experimental, preclinical and clinical observations, and structural homology with known chemopreventive agents. Some pharmaceuticals, dietary constituents, micronutrients and trace elements are included (Helzsouer *et al*., 1994; Morse and Stoner, 1993) and have been categorized using a range of end points, from short-term genotoxicity assays to biochemical marker assays such as the reduction of carcinogen-DNA binding (Sharma *et al*., 1994; Wattenberg,

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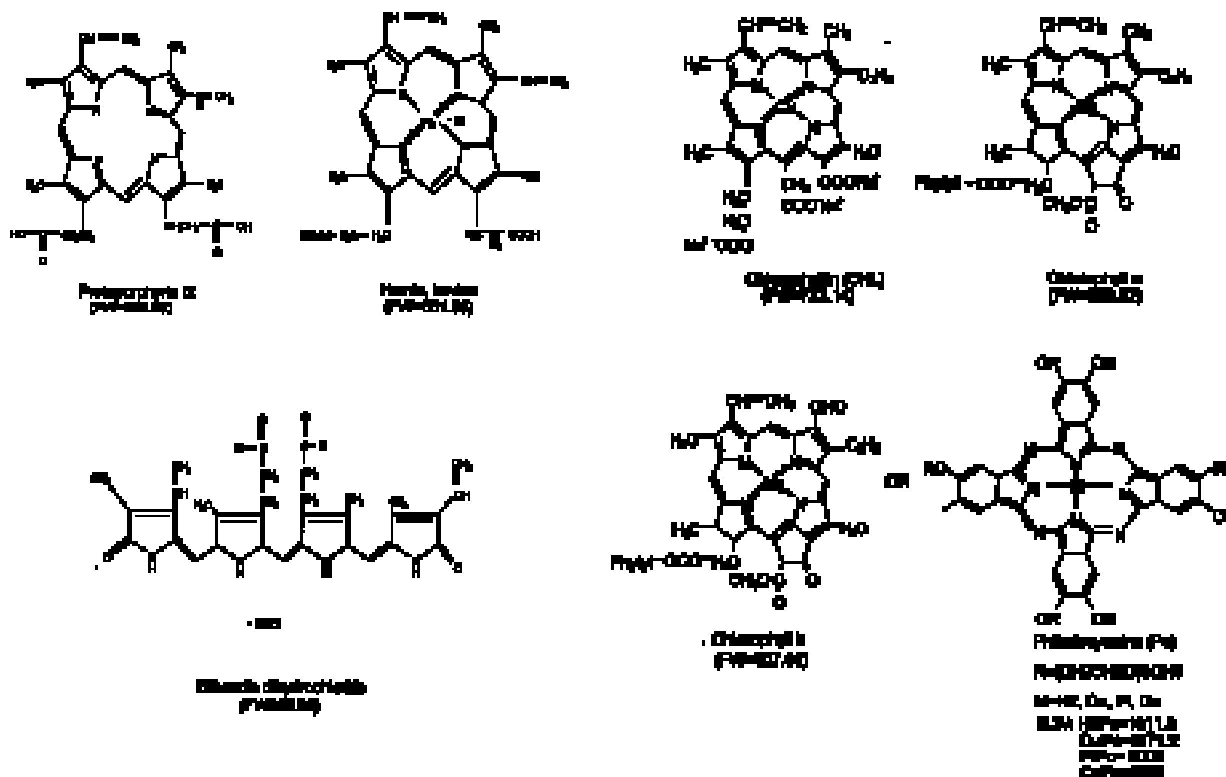

**Figure 1. Structures of Pcs and Related Porphyrins Investigated**

1985). Among the natural products, the potent effects of porphyrins on chemicals with different mechanisms of action have been investigated in the Ames assay and the V79 cell assay (Romert *et al*., 1992). Porphyrins have primarily been known for their use as photosensitizers in photodynamic therapy (Moan and Berg, 1992). The oxidation of cellular constituents, such as lipids and proteins, by activated oxygen species mediated through photodynamic reactions has been extensively studied. In contrast, porphyrins inhibit lipid peroxidation by scavenging of peroxyl radicals (Williams *et al*., 1994). A porphyrin-like molecule copper Pc trisulfonate has been shown to have strong affinity to polycyclic compounds and to be potential inhibitors against exposure to carcinogens and mutagens . In this study, we investigated the antimutagenic activities of Pcs and their related porphyrins on 6-sulfooxymethylbenzo[a]pyrene (SMBP), an ultimate metabolite of substituted methylbenzo[a]pyrene, using a screening procedure of the Ames assay and the mutagenicity assay in V79 cells. SMBP itself exhibits stronger mutagenicity in bacterial and mammalian systems without further activation than its corresponding proximate compound 6-hydroxymethylbenzo[a]pyrene (HMBP) (Cho *et al*., 1998). Most of porphyrins exerted antimutagenicity against SMBP, synthesized either chemically or metabolically from HMBP, and their inhibitory profiles were similar results in bacterial and mammalian systems. In addition, a reactive SMBP bears a good leaving group, e.g., a sulfate group, which generates a highly reactive carbonium ion to react with DNA. Therefore, Pcs including related compounds hemin, chorophyllin, chlorophyll and biliverdin were also tested for their inhibitory activity with respect to reactivity of SMBP to DNA. Pcs were quite effective in reducing the carcinogen-DNA adducts, but other porphyrin derivatives were slightly effective.

## Materials and Methods

*2. 1. Chemicals*

CHL, hemin, protoporphyrin, chlorophyll a and chlorophyll b were purchased from Aldrich Chemical Co. (Milwaukee, WI). Phthalocyanine (Pc) and metallophthalocyanines (copper, cobalt and platinum phthalocyanines) were synthesized as described previously (Ford *et al*., 1994). The structures of Pcs and related porphyrins under investigation in this study are illustrated in Fig. 1. Fetal bovine serum was purchased from Gibco-BRL (Grand Island, NY). Dulbecco's modified Eagle's medium (DMEM), calf thymus DNA, dimethylsulfoxide (DMSO), 6-thioguanine (6-TG) was obtained from Sigma Chemical Co. (St Louis, MO). HMBP and SMBP were synthesized as described previously (Natarajan and Flesher, 1973). Other reagents were of analytical grade.

*2. 2. Preparation of rat liver cytosolic fraction*

Cytosols from the liver of 4-week old female Sprague-

Dawley rats were prepared as described previously (Surh *et al.*, 1989) and stored at -70°Cuntil use. The female liver cytosol was used because there is a large amount of hydroxysteroid sulfotransferase involved in activation of HMBP in the presence of sulfo-group donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS). Otherwise indicated, HMBP is activated in the presence of PAPS and female liver cytosol as prepared above. The protein contents of cytosol prepared were determined by the Lowry assay with bovine serum albumin as standard (Lowry *et al.*, 1951).

*2. 3. Bacterial mutagenicity assay*

HMBP (7.2 nmol) was incubated for 60 min at 37°C together with the test agents (10 nmol) under investigation in a final volume of 1.1 ml of 0.1 M $KH_2PO_4$-$Na_2HPO_4$ buffer (pH 7.4) including the liver cytosol (S105) prepared before and the PAPS-generating system (5 mM ATP, 0.1 mM EDTA, 5 mM $Na_2SO_4$, 3 mM $MgCl_2$). The cytosolic fraction and the PAPS-generating system were omitted from the mixture when SMBP (1 nmol) dissolved in DMSO was used instead of HMBP. The incubation condition for SMBP was 30 min at 37°C. After incubation, the mixtures were diluted with soft agar, poured onto a hard agar plate and further incubated for 48 hrs to allow the growth of $His^+$ revertant colonies.

*2. 4. Cytotoxicity and mutagenicity in V79 cells*

Exponentially growing V79 cells were treated with SMBP (2.5μM) along with the individual porphyrin derivatives at a concentration of 12.5 μM for 1 hr, followed by reseeding 200-2000 cells on a 60-mm dish. Their inhibitory effect on cytotoxicity of SMBP was evaluated by counting the colonies 7 days after seeding. The direct mutagenicity assay in Chinese hamster V79 cells was carried out as described previously with some modifications (Cho *et al.*, 1996; Jenssen, 1984). Mutations at hypoxanthine:guanine phosphoribosyl-transferase (*hprt*) locus were measured by their resistance to 6-thioguanine (6-TG). Exponential phase cultures, preincubated with a series of porphyrin derivatives, were exposed to HMBP (90 μM) and SMBP (2.5 μM) for 60 min, followed by subculture. Cloning efficiency was determined by counting the number of colonies 7-8 days after seeding 200-2000 cells per petri dish while the cells were subcultured to select mutants. Mutation frequencies for 6-TG were expressed as mutants/$10^6$ survivors and were corrected for cloning efficiency.

*2. 5. Inhibition of covalent bindings of SMBP to calf thymus DNA by porphyrin derivatives*

To determine covalent binding of HMBP to DNA, incubation was done at 37°C for 90 min in a final volume of 500 μl, 0.1 M Tris-HCl buffer (pH 7.4) containing 500μg calf thymus DNA, 10 nmol HMBP, 50 μl of liver cytosol (~50 mg/ml) and the PAPS-generating system. As described previously, the cytosolic fraction and the PAPS-generating system were omitted from the mixture when SMBP (1 nmol), dissolved in 10 μl of DMSO, was used instead of HMBP. These incubation mixtures were then tested together with various porphyrin derivatives at a constant dose of 10 nmol. To compare the inhibitory activities of porphyrins on SMBP binding to DNA, calf thymus DNA was incubated as a nucleophilic acceptor to capture the labile reactive sulfuric acid ester of [$^3$H-$CH_2$]SMBP (sp. act. 12.0 mCi/mmol) in the incubation mixture containing the porphyrins under investigation.

*2. 6. Electrophoresis*

Standard reactions were carried out in 30 μl containing 0.1 M Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, SMBP and plasmid DNA as nucleophilic acceptor. SMBP (1 nmol) was incubated in a reaction mixture containing the pUC19 plasmid and antimutagens under investigation for 30 min at 37°C, followed by electrophoresis onto 0.8% agarose gel at 100 V for 50 min. SMBP-modified DNA was detected as a violet fluorescence band under UV light due to BP moiety of SMBP, and DNA itself was also visualized under UV light after staining with ethidium bromide (EtBr).

## Results

Ames tests were performed to assess the antimutagenic activities of porphyrin derivatives against SMBP and HMBP using *S. typhimurium* TA98. The inhibitory effects of Pcs and related compounds on the HMBP- or SMBP-induced mutagenesis are shown in Fig. 2. $H_2$Pc inhibited the mutagenic activity of SMBP in a dose-dependent manner up to 10 nmol, where mutation frequency was significantly reduced to 15% (Table 1). The antimutagenic strengths of the porphyrin derivatives were in the following order: Pcs, CHL, protoporphyrin, chlorophyll a, chlorophyll b, hemin and biliverdin. The antimutagenic effects of porphyrin derivatives against HMBP were in the large less remarkable than those against SMBP. All the Pcs showed more

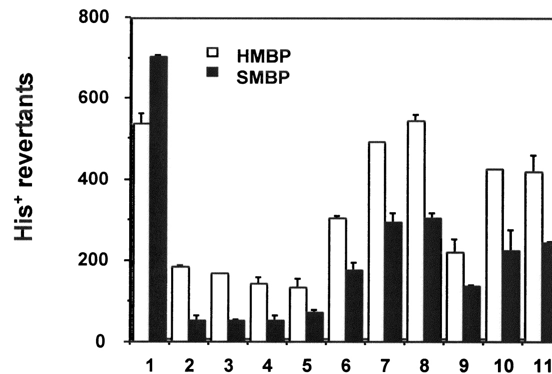


**Figure 2. Comparative Inhibition of HMBP- or SMBP-induced Mutagenesis in *S. typhimurium* TA98 by Porphyrin Derivatives.**

Porphyrin derivatives (10 nmol) were incubated with 7.2 nmol HMBP or 1 nmol SMBP at 37°C 1, No porphyrin; 2, $H_2$Pc; 3, CuPc; 4, PtPc; 5, CoPc; 6, CHL; 7, Hemin; 8, Biliverdin; 9, Protoporphyrin; 10, Chlorophyll a; 11, Chlorophyll b.

Table 1. Reduction of mutation frequency with SMBP by H2Pc in Salmonella typhimurium TA98.

| Concentration of phthalocyanine (mM) | $His^+$ revertants |
|---|---|
| 0.0 | 520.5 + 34.7 |
| 0.25 | 358.5 + 21.9 |
| 0.5 | 371.5 + 92.6 |
| 1.0 | 300.5 + 5.0 |
| 5.0 | 165.0 + 7,1 |
| 10.0 | 99.0 + 26.9 |

SMPB (1nmol) dissolved in DMSO was used without activation and the incubation condition was 30 min at 37°C

antimutagenic activity against both SMBP and HMBP than other related compounds. The inhibitory effect of $H_2Pc$ on the mutation frequency was comparable to or slightly less than that of the metal coordinated Pcs such as CuPc, PtPc and CoPc, suggesting that the coordinated metals do not significantly affect mutagenecity induced by SMBP. In contrast, hemin, biliverdin, chlorophyll a and chlorophyll b exerted weak antimutagenic activities.

The inhibitory effects of Pcs and related compounds on the cytotoxicity and mutagenicity induced by SMBP in V79 cells are presented in Fig. 3A and 3B. The porphyrin

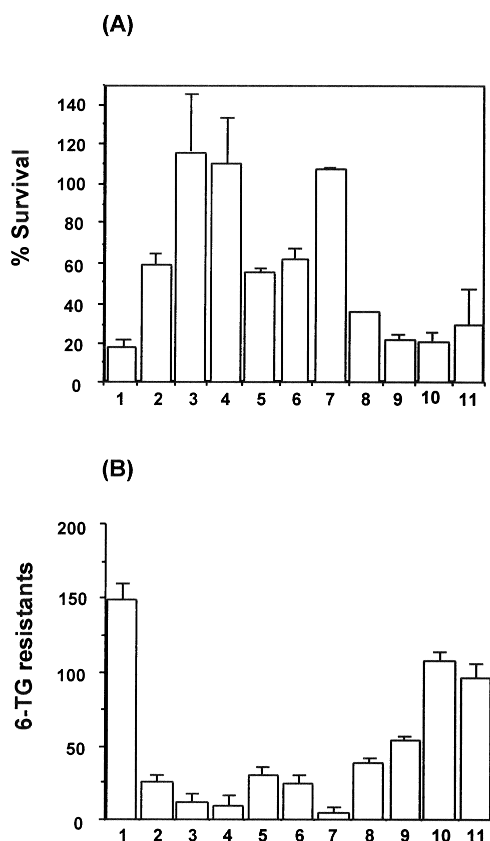


**Figure 3. Effects of Porphyrin Derivatives on SMBP-induced Cytotoxicity and Mutagenicity in V79 cells.**
Treatments of SMBP and porphyrins were of 1.5 and 12.5 _M concentration, respectively, and porphyrin derivatives added to the medium were as follows. 1, no porphyrin; 2, $H_2Pc$; 3, CuPc; 4, PtPc; 5, CoPc; 6, Chlorophyllin; 7, Hemin; 8, Biliverdin; 9, Protoporphyrin; 10, Chlorophyll a; 11, Chlorophyll b.

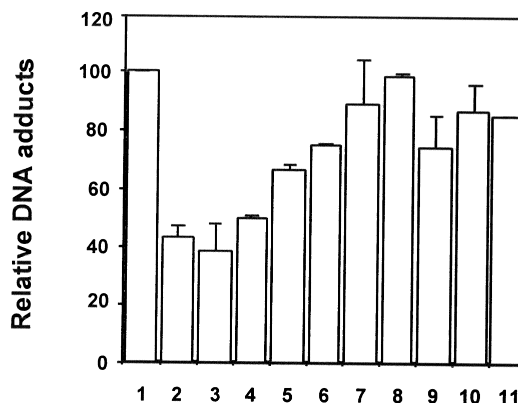


**Figure 4. Effects of Pc and Related Compounds on [$^3$H-$CH_2$]SMBP Binding to Calf Thymus DNA.**
The reaction was started by addition of 0.6 nmol SMBP to the incubation mixture containing 500 μg DNA and 10 nmol porphyrin derivatives and continued for 30 min at 37°C. Adduct formation was expressed in terms of DPM/mg calf thymus DNA for comparison. 1, no porphyrin; 2, $H_2Pc$; 3, CuPc; 4, PtPc; 5, CoPc; 6, CHL; 7, Hemin; 8, Biliverdin; 9, Protoporphyrin; 10, Chlorophyll a; 11, Chlorophyll b.

**Figure 5. Agarose Gel Eectrophoregram of pUC19 DNA treated with 2 nmol SMBP in Combination with 5 nmol Porphyrin Derivatives.**
Lane 1: control, lane 2: no porphyrin, lane 3: $H_2Pc$, lane 4: CuPc, lane 5: PtPc, lane 6: CoPc, lane 7: CHL, lane 8: Hemin, lane 9: Biliverdin, lane 10: Protoporphyrin, lane 11: Chlorophyll a, lane 12: Chlorophyll b. Upper and lower panel represent photographs of gel visualized by BP moiety of SMBP bound to DNA, and ethidium bromide (EtBr) intercalated into DNA under UV illumination, respectively.

derivatives alone did not show any cytotoxic and mutagenic activity at concentrations used in this experiment (data not shown). All the porphyrin derivatives reduced both cytotoxicity and mutagenicity caused by SMBP, but their inhibitory degrees were markedly variable with the porphyrin compounds. As shown in Fig. 3b, Pcs, hemin and CHL were quite effective in reducing the mutation frequency to a control level while biliverdin, protoporphyrin, chlorophyll a and chlorophyll b were slightly potent. The fluctuation of survival rate by the porphyrin derivatives was closely correlated with that of the mutagenicity. Among them, CuPc and PtPc showed the most effective antimutagenic activity against SMBP in V79 cells, with which an enhancement of the survival rate was accompanied. The reduction rate of SMBP-mediated cytotoxicity by $H_2Pc$ was weak as compared with CuPc and PtPc but comparable to that by CoPc. The reduction of cytotoxicity and mutagenicity caused by SMBP was less remarkable by the treatment with protoporphyrin, chlorophyll a and b but the effectiveness of biliverdin was higher than the above compounds. Thus, Pcs and the related compounds inhibited the mutagenesis induced by SMBP in the mammalian system as well as the microbial system, suggesting that inhibitory effects of porphyrin derivatives on the mutagenicity induced by SMBP are a general phenomenon in biological systems.

SMBP readily reacts with calf thymus DNA to produce DNA adducts, which is measured by the radioactivity of SMBP bound to DNA. The inhibitory effects of porphyrins on adduct formation are shown in Fig. 4. The Pcs were quite effective in reducing the adducts, but other porphyrin derivatives were slightly effective. A decrease of the adducts by treatment with porphyrins could also be confirmed by a decreased fluorescence intensity and the migration pattern of SMBP-modified plasmid on agarose gel (Fig.5). SMBP produced adduct formation in DNA, resulting in the diffused migration pattern of BP-bound DNA and its visualization without staining with EtBr under UV light. As would be expected from above results, the treatment with Pcs almost recovered the normal migration pattern and dramatically reduced the fluorescence intensity. Similar results were observed by other porphyrin derivatives except protoporphyrin. It was demostrated that $H_2Pc$ has activity equivalent to metal coordinated Pcs with respect to the reduction of the SMBP-DNA adduct and these properties extend to cytotoxicity and mutagenicity, suggesting that the inhibitory effects of Pcs are closely related to their structure and not to coordinated metal species.

## Discussion

Most of the porphyrins under investigation exhibited strong antimutagenic activity against both SMBP and HMBP in the Ames. SMBP was in general more effectively antagonized by the potent antimutagenic compounds than the HMBP. A large number of proteins present in the liver cytosol, which are supplemented for the activation of HMBP, may modulate antimutagenic activities of the porphyrin derivatives against HMBP. Since SMBP is also rapidly hydrolyzed to a HMBP in aqueous environment ($t_{1/2}$ ~7min), the mutagenicity or reactivity depends on its maintenance in an aqueous phase. The water-labile ultimate electrophiles are transported intracellularly by various components in plasma such as carrier proteins (Hanson-Painton *et al*., 1983) or lipids (Busbee *et al*., 1982; Chen *et al*., 1979; Shu and Nichols, 1979) to tissues and cellular target macromolecule DNA (Singer and Grunberger, 1983). The antimutagenic activity of porphyrins was also confirmed in a mammalian cell system, in which an enhancement of cell survival and a reduction of the mutagenicity were observed. Hemin was the most effective in reducing the mutagenicity of SMBP in V79 cells as similar to or even better than Pcs and CHL but less dramatic compared to antimutagenic strength of Pcs and CHL in Ames assay. The effective orders of porphyrins on the mutagenicity of SMBP in the V79 cells were different from that in the bacterial system. Bacterial cells differ from mammalian cells with respect to membrane permeability to chemicals, their metabolisms and chromosomal organization and others, and then their antimutagenic responses may be different from those in mammalian cells. Furthermore, there was not a strong correlation between the levels of DNA adducts and their mutation frequencies, suggesting that porphyrins in biological systems might offer other protective mechanisms in addition to the interference of adduct formation between SMBP and DNA.

Since porphyrins have a pyrrole ring structure with a large delocalized $\pi$ system, these molecules have been suggested to form reversible or noncovalent complexes with aromatic carcinogen bearing a plane ring structure through $\pi$-$\pi$ or face-face interactions (Arimoto *et al*., 1993; Breinholt *et al*., 1995). Hydrophobic and van der Waals interactions may also enhance the stability of the resulting complex. Interaction between porphyrins and DNA has been characterized by a variety of physical techniques. Specifically, porphyrins intercalate into GC-rich regions and bind in an outside manner at AT sites (Fiel, 1989). Their binding mechanisms depend on the nature of the metal ion, and the size and location of the substituent groups on the periphery of the porphyrins (Gibbs and Pasternack, 1989). Our results demonstrated that the coordinated metal ion did not affect the cytotoxicity and mutagenicity of SMBP, suggesting that complex formation is a major factor for relieving the adverse effect of SMBP. It has not been demonstrated whether intercalation of porphyrins modulates carcinogenic or mutagenic potential. However, it seems plausible that porphyrins exert their protective action through tight binding with mutagen or intercalating into DNA, which may alter the DNA structure or occupy the available site for mutagens to inhibit interaction between DNA and mutagen. Even though the molecular complex may be one of the most favorable candidates for the chemoprotective mechanism of porphyrins, it has also been suggested that scavenging of radicals and suppression of metabolic activation are other possible mechanisms (Kim *et al*., 1982; Lai *et al*., 1980; Ong *et al*., 1986). Porphyrins themselves, a chelated metal

in the center of the molecules, posses the structure being able to scavenge radicals or suppress metabolic activation. In our study, CHL is quite effective to scavenge hydroxyl radicals by measuring the degradation of deoxyribose and further by reducing lipid hydroperoxides (Kim and Chung, 1999). Our other study demonstrates that CHL can inhibit both tumor promotion and progression of papillomagenesis in the two stage mouse skin carcinogenesis (Chung *et al*., 1999). Further studies will clarify that the antioxidant function of CHL is related to the inhibition of the tumor promotion and progression. Recently, it has been reported that CHL has a radioprotective capacity in addition to an antimutagenic activity (Morales-Ramirez and Garcia-Rodriguez, 1994; Zimmering *et al*., 1990). Another possible chemoprotective mechanism of porphyrins is trapping reactive forms of carcinogens by nucleophilic attack (Park and Surh, 1996). In addition to this molecular trapping, porphyrins appear to inhibit conversion of procarcinogens to ultimate carcinogenic metabolites, which may be an alternative mechanism for their protection against chemically induced mutagenesis and carcinogenesis (Yun *et al*., 1995). Taken together, prophyrins appear to scavenge the reactive oxygen species and to prohibit the reactive carcinogen by a combination of diverse mechanisms mentioned previously rather than one mechanism.

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**Personal profile: An-Sik Chung**

Dr An-Sik Chung, Professor and Chairman of the Department of Biological Sciences, Korean Advanced Institute for Science and Technology, since 1989, has a long experience of research. He received his Ph.D. at the Universityof Wisconsin and then spent a post-doctoral period

in the University of Illinois in the USA. Since his return to Korea he has been very active, first in the Department pf Biochemistry, Kosin Medical College, Pusan, then in KIT and KAIST.

The main research interests of Dr Chung are in four areas:
1) Reactive oxygen species (ROS), cellular proliferation and tumor promotion
2) Inhibition of MMP-2 and MMP-9 expression and underlying mechanisms
3) Induction of apoptosis by selenium compounds
4) Mechanisms of action of bioflavonoids with regard to proinflammatory cytokines an UV protection