*Short Communication***Anticancer Photodynamic and Non-photodynamic Effects of Pterin Derivatives on a Pancreatic Cancer Cell Line**

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Abstract. To evaluate the feasibility of two kinds of pterin derivatives, 2-(*N,N*-dimethylaminomethyleneamino)-6-formyl-3-pivaloylpteridin-4-one (DFP) and 2-(*N,N*-dimethylaminomethyleneamino)-3-pivaloylpteridin-4-one (DP), as anticancer drugs, their photodynamic and non-photodynamic effects on pancreatic cancer cell line Panc-1 cells were examined. For photodynamic effects, cell death 48 h after UV-A irradiation was more prominent in cells preloaded with DP than DFP. When cells were simply incubated for 96 h without irradiation, DFP induced cell death, while DP suppressed cell proliferation. Furthermore, DP was much more soluble in water than DFP. These findings collectively indicated that DP is more feasible as an anticancer drug than DFP.

Keywords: pterin derivative, anticancer drug, photodynamic effect

6-Formylpterin (6FP, Fig. 1A) is known as a potent xanthine oxidase inhibitor with an inhibition constant (K_i) of approximately 0.6 nM (1, 2). Although 6FP does not usually occur in vivo, it is produced from folic acid in vivo in some pathological conditions such as carcinoma (3). 6FP reacted with reducing agents in cells, such as NAD(P)H, and intracellularly generated reactive oxygen species (ROS), which induced apoptosis in human promyelocytic leukemia HL-60 cells and suppressed cell proliferation in human pancreatic cancer cell line Panc-1 cells (4). Furthermore, 6FP produces singlet oxygen (1O_2) under long-wavelength ultraviolet (UV-A) (5), which shows that 6FP may serve as a photosensitizer in anticancer photodynamic therapy (PDT) (6, 7). However, 6FP is insoluble both in water and in lipid, so it is poorly taken up by cells, which is a crucial drawback as a drug.

In the previous study, we developed a novel 6FP derivative, 2-(*N,N*-dimethylaminomethyleneamino)-6-

formyl-3-pivaloylpteridin-4-one (DFP, Fig. 1B), which is more soluble in water and lipid than 6FP (8). Furthermore, DFP was taken up by Panc-1 cells and photodynamically induced cell death, indicating that DFP may have applicability in anticancer PDT (9). However, DFP has drawbacks as a photosensitizer. First, the water solubility of DFP is still low, and the maximum concentration in phosphate-buffered saline (PBS) was estimated to be 4 – 5 mM at best. Second, DFP generates ROS through NADH oxidation at much higher rate than 6FP (10), which may induce undesirable cell death without UV-A irradiation. Third, the photodynamic effects of DFP on Panc-1 cells were not so potent, and high concentrations as much as 500 μ M were required to induce cell death (9).

Among the drawbacks of DFP as a photosensitizer, the insolubility in water and the intracellular ROS generation are conceivably caused by the formyl group on the 6-position of DFP. A compound in which the formyl group was removed from DFP was already developed in our laboratory as 2-(*N,N*-dimethylaminomethyleneamino)-3-pivaloylpteridin-4-one (DP, Fig. 1C) (10). So, in the present study, to develop more potent

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Fig. 1. Molecular structure of 6-formylpterin (6FP) (A), 2-(*N,N*-dimethylamino-methyleneamino)-6-formyl-3-pivalolpteridine-4-one (DFP) (B), and 2-(*N,N*-dimethyl-aminomethyleneamino)-3-pivalolpteridin-4-one (DP) (C).

photosensitizers than DFP, photodynamic and non-photodynamic effects of DP were examined and compared with those of DFP.

6FP was obtained from Sankyo Kasei Kogyo (Hiratsuka). DFP and DP were synthesized in our laboratory from 6FP according to the procedure reported previously (8). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and penicillin-streptomycin liquid were purchased from Invitrogen (Carlsbad, CA, USA); 2',7'-dichlorofluorescein diacetate (DCFH-DA) and tetramethylrhodamine methyl ester (TMRM) were purchased from Molecular Probes (Eugene, OR, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Nacalai Tesque (Kyoto). Panc-1 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD USA) and cultured in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified incubator with 5% CO₂.

First, the maximum solubility of DFP and DP in PBS solution was examined. PBS solutions containing oversaturated DFP or DP were prepared and stirred at room temperature for 2 h. After filtration, UV absorption spectra of these solutions were measured using a UV spectrophotometer (Jasco UV/VIS Spectrophotometer V-550; Japan Spectroscopic Co., Tokyo). The solubility of DFP or DP was calculated using a molar absorbance coefficient of $\epsilon_{316} = 27172 \text{ M}^{-1} \text{ cm}^{-1}$ for DFP or

$\epsilon_{309} = 24056 \text{ M}^{-1} \text{ cm}^{-1}$ for DP. The maximum solubility of DP was 61 mM and that of DFP was 4 mM. According to this result, DP and DFP were directly dissolved in PBS; 40 mM DP and 4 mM DFP solutions (pH 7.4, in each) were prepared and filtered for sterilization.

The photodynamic effects of DP and DFP on Panc-1 cells were examined as follows: Cells ($1 \times 10^5/\text{ml}$) were incubated in 12-well tissue culture plates (BD Biosciences, Bedford, MA, USA) with 1 ml medium in each well for 48 h, until they became confluent, and then incubated with 1–4 mM DP or 100–400 μ M DFP for 4 h. After washing the cells twice with PBS, the medium was replaced with phenol red-free medium; phenol red was omitted because of possible interference with the photodynamic action. UV-A irradiation was performed for 1 h at 2.5 mW/cm² using a UV-A radiation apparatus (XX-15BLB, 625 nm; UVP, Upland, CA, USA). After irradiation, the cells were washed once and incubated for another 48 h. Cell viability 48 h after UV-A irradiation was assessed by the mitochondrial-dependent reduction of MTT to formazan (MTT assay) (11).

Non-photodynamic effects of DP and DFP on Panc-1 cells were examined as follows: Cells ($5 \times 10^4/\text{ml}$) were incubated overnight in 96-well tissue culture plates (BD Biosciences) with 100 μ l medium in each well and then incubated with 1–4 mM DP or 100–400 μ M DFP for 0–96 h. After incubation with DP or DFP for 0, 24, 48,

Fig. 2. Effects of DP and DFP on cell viability of Panc-1 cells. Upper: Cells preloaded for 4 h with 1–4 mM DP (left) or 100–400 μ M DFP (right) were irradiated with UV-A for 1 h. Then, cell viability at 48 h after irradiation was assessed by MTT assay. Cell viability is shown as a percentage, and that obtained from Panc-1 cells incubated without DP or DFP was assigned as 100%. The open bar shows cell viability when cells were irradiated with UV-A, while the closed bar shows viability in the cells that were not irradiated. The values are shown as the means $\pm$ S.D. ($n = 6$). * $P < 0.05$ and ** $P < 0.01$: significantly different from the value obtained from the cells incubated without DP or DFP with irradiation. § $P < 0.05$: significantly different from the value obtained from cells incubated without DP and not irradiated. Lower: Cells were incubated with 1–4 mM DP (left) or 100–400 μ M DFP (right) for 0–96 h without UV-A irradiation. Cell viability after 0, 24, 48, 72, and 96 h after incubation was assessed by the MTT assay. Cell viability is shown as a percentage, and that obtained from Panc-1 cells incubated for 0 h with or without DP or DFP was assigned as 100%. The values are shown as the means $\pm$ S.D. ($n = 8$). NS: values obtained at 24, 48, 72, and 96 h incubation were not significantly different from values obtained at 0 h incubation. DP at 2 mM and DFP at 400 μ M are shown. In other cases, the values obtained at 24, 48, 72, or 96 h incubation were significantly different from the values obtained at 0 h incubation.

72, and 96 h, cell viability was assessed by MTT assay.

Values are shown as the means $\pm$ S.D. Statistical comparisons were made using one-way ANOVA followed by the Student's paired t -test with a Bonferroni correction. P values < 0.05 were regarded as significant.

Intracellular ROS generation, cell death, and proliferation in Panc-1 cells were observed using a time-lapse imaging system (BioStation IM; Nikon, Tokyo), which consists of an incubator incorporating fluorescent microscopy equipped with a digital CCD camera and a PC computer for data acquisition and analysis. Cells (1×10^5 cells/ml) were incubated overnight in a 35-mm glass base dish (IWAKI, Funabashi) with 2 ml medium. Cells were then incubated with 100 nM TMRM, a mitochondrial membrane potential (MMP)-sensitive dye (12) for 20 min. The medium was then changed, and cells were incubated with various concentrations of DP

or DFP and 2 μ M DCFH-DA, a ROS-sensitive dye (13) at 37°C and 5% CO₂ in the time-lapse imaging system; and the phase-contrast images and fluorescent images were acquired every 15 min for 48 h. Green fluorescence images for DCF and red fluorescence images for TMRM were acquired. In addition, a Vybrant apoptosis assay kit (Molecular Probes) was used to distinguish apoptotic and necrotic cells by YO-PRO-1 and propidium iodide (PI) staining. The apoptotic and necrotic cells exhibit green fluorescence of YO-PRO-1 and red fluorescence of PI, respectively (14).

In cells preloaded with DP for 4 h and irradiated, cell viability at 48 h after irradiation decreased significantly in a dose-dependent manner (Fig. 2, upper left, open bar). In cells preloaded with DP but not irradiated, cell viability decreased slightly but significantly only when 4 mM DP was applied (Fig. 2, upper left, closed

Fig. 3. Time-lapse cell imaging and cell cycle analysis. A: Cells were preloaded with 100 nM TMRM for 20 min. After changing the medium, cells were loaded with 2 μ M DCFH-DA and incubated without DP or DFP (Control) or with 2 mM DP, 4 mM DP, or 400 μ M DFP. To compare the cell growth, the cells (1×10^5 cells/ml) were incubated overnight in a 35-mm glass base dish with 2 ml medium (Control vs 2 mM DP). To compare mechanisms for the cell death, the cells were incubated for 48 h, until they became confluent (4 mM DP vs 400 μ M DFP). First, second, and third rows show the images of cells at 0, 24, and 48 h after incubation, respectively. Phase contrast images, green fluorescence images for DCF, and red fluorescence images for TMRM were acquired every 15 min for 48 h. Merged images are shown. B: Cells were incubated with 4 mM DP, YO-PRO-1 (1 μ M), and PI (5 μ g/ml), and then time-lapse imaging was performed and merged images was acquired every 5 min for 24 h. Note that necrotic cells exhibited red fluorescence of PI, whereas apoptotic cells exhibited green fluorescence of YO-PRO-1. C: DNA content in Panc-1 cells incubated with 2 mM DP or without DP (Control) was determined by propidium iodide staining. DP-treated cells tended to fall within the G2/M phase compared with the untreated cells, which indicated that G2/M arrest was induced by DP.

bar). On the other hand, in cells preloaded with DFP and irradiated, cell viability decreased significantly only when 400 μ M DFP was applied (Fig. 2, upper right, open bar), and in cells preloaded with DFP but not irradiated, cell viability did not decrease (Fig. 2, upper right, closed bar). The stronger effects were shown in another cancer cell line, HeLa cells (data not shown).

When cells were incubated for 96 h without UV-A irradiation, cell viability increased significantly in a time-dependent manner in cells incubated without DP or DFP, 1 mM DP, 100 μ M DFP, and 200 μ M DFP; however, when cells were incubated with 2 mM DP or 400 μ M DFP, cell viability did not change significantly during 96 h. Furthermore, when cells were incubated with 4 mM DP, cell viability significantly decreased during the first 24 h and did not recover (Fig. 2, lower left and right).

In time-lapse cell imaging, when Panc-1 cells were incubated without DP or DFP, cells with red fluorescence became confluent for 48 h (Fig. 3A, Control). Since red fluorescence reflects intact MMP (15), these images showed normal cell proliferation. When the cells were incubated with 2 mM DP, they did not increase in number while maintaining red fluorescence, which showed the suppression of cell proliferation (Fig. 3A, 2 mM DP). Cell cycle analysis by flow cytometry showed that DP-treated cells tended to fall within the G2/M phase compared with untreated cells (Fig. 3C), which indicated that G2/M arrest was induced by 2 mM DP. When cells at confluence were incubated with 4 mM DP, discoloration of red fluorescence was observed at 24 and 48 h (Fig. 3A, 4 mM DP). Since discoloration of red fluorescence reflects loss of MMP, these images showed cell death. In the Vybrant apoptosis assay, the cells incubated with 4 mM DP showed green fluorescence of YO-PRO-1 first and then red fluorescence of PI (Fig. 3B), which indicated that apoptotic cell death first occurred and then necrotic cell death occurred. When the cells at confluent state were incubated with 400 μ M DFP, appearance of green fluorescence and discoloration of red fluorescence were observed at 24 and 48 h, which showed ROS generation and cell death (Fig. 3A, DFP 400 μ M).

In conclusion, DP is superior to DFP as a photosensitizer. First, the effective concentration of DP for PDT was more than 1 mM and 1/60th of its maximum soluble concentration, while that of DFP was more than 400 μ M and 1/10th of its maximum soluble concentration. Second, unlike DFP, the effective concentration of DP for PDT did not induce cell death but suppressed cell proliferation without UV-A irradiation; however, to be

effective, a high concentration was still required. Cell permeability and water solubility should be further improved by structural modification.

References

- Lowry OH, Bessey OA, Crawford EJ. Pterine oxidase. *J Biol Chem.* 1949;180:399–410.
- Kalckar HM, Kjeldgaard NO, Klenow H. 2-Amino-4-hydroxy-6-formylpteridine, an inhibitor of purine and pterine oxidases. *Biochim Biophys Acta.* 1950;5:586–594.
- Halpern R, Halpern BC, Stea B, Dunlap A, Conklin K, Clark B, et al. Pterin-6-aldehyde, a cancer cell catabolite: identification and application in diagnosis and treatment of human cancer. *Proc Natl Acad Sci U S A.* 1977;74:587–591.
- Arai T, Endo N, Yamashita K, Sasada M, Mori H, Ishii H, et al. 6-formylpterin, a xanthine oxidase inhibitor, intracellularly generates reactive oxygen species involved in apoptosis and cell proliferation. *Free Radic Biol Med.* 2001;30:248–259.
- Thomas AH, Lorente C, Capparelli AL, Martinez CG, Braun AM, Oliveros E. Singlet oxygen ($^1\Delta$) production by pterin derivatives in aqueous solutions. *Photochem Photobiol Sci.* 2003;2:245–250.
- Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbek M, et al. Photodynamic therapy. *J Natl Cancer Inst.* 1998;90:889–905.
- Pervaiz S. Reactive oxygen-dependent production of novel photochemotherapeutic agents. *Faseb J.* 2001;15:612–617.
- Nonogawa M, Arai T, Endo N, Pack SP, Kodaki T, Makino K. Novel 6-formylpterin derivatives: chemical synthesis and O₂ to ROS conversion activities. *Org Biomol Chem.* 2006;4:1811–1816.
- Yamada H, Arai T, Endo N, Yamashita K, Nonogawa M, Makino K, et al. Photodynamic effects of a novel pterin derivative on a pancreatic cancer cell line. *Biochem Biophys Res Commun.* 2005;333:763–767.
- Nonogawa M, Pack SP, Arai T, Endo N, Sommani P, Kodaki T, et al. Reactive oxygen species generation through NADH oxidation by 6-formylpterin derivatives in the dark. *Biochem Biophys Res Commun.* 2007;353:1105–1110.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983;65:55–63.
- Petrat F, Pindiur S, Kirsch M, de Groot H. NAD(P)H, a primary target of $^1\text{O}_2$ in mitochondria of intact cells. *J Biol Chem.* 2003;278:3298–3307.
- Royall JA, Ischiropoulos H. Evaluation of 2',7'-dichlorofluorescein and dihydrorhodamine 123 as fluorescent probes for intracellular H₂O₂ in cultured endothelial cells. *Arch Biochem Biophys.* 1993;302:348–355.
- Bollimuntha S, Singh BB, Shavali S, Sharma SK, Ebadi M. TRPC1-mediated inhibition of 1-methyl-4-phenylpyridinium ion neurotoxicity in human SH-SY5Y neuroblastoma cells. *J Biol Chem.* 2005;280:2132–2140.
- Scaduto RC Jr, Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys J.* 1999;76:469–477.