The Long-lasting Antiproliferative Effect of 15-Deoxyspergualin through its Spermidine Moiety

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15-Deoxyspergualin (DSG) inhibited growth of mouse EL-4 lymphoma cells with an IC\textsubscript{50} 0.02 \mu g/ml. Even though the cells were treated with DSG for only 4 hours and then washed, the antiproliferative effect lasted long with an IC\textsubscript{50} 0.4 \mu g/ml. DSG has spermidine and guanidine moieties in its structure. One decomposed element containing guanidine moiety inhibited the growth at higher doses than DSG, but the effect did not last long unlike DSG. While another element containing spermidine moiety did not affect the growth, it diminished the long-lasting antiproliferative effect of DSG by pretreatment of the cells. Pretreatment with polyamines such as putrescine, spermidine, and spermine also diminished the effect of DSG. Furthermore, N-alkylation of spermidine moiety in DSG abolished the antiproliferative effect. These results suggested that DSG binds to the cells through its spermidine moiety and exerts its long-lasting antiproliferative effect.

Materials and Methods

General
Putrescine, spermidine, spermine, and aminoguanidine were from Sigma (St. Louis, MO). DL-\alpha-Difluoro-methylornithine (DFMO) was from Calbiochem (La Jolla, CA). IR spectra were recorded on a Hitachi 260-10 spectrometer. \(^1\)H NMR spectra were measured with a JEOL JNM A400 spectrometer. HRFAB-MS spectra were measured with a VG AutoSpec mass spectrometer.

Preparation of DSG Derivatives

DSG and decomposed elements of DSG (DSG-A and DSG-B) were provided from Takara Shuzou Co. Ltd. (Japan) and Nippon Kayaku Co. Ltd. (Japan), respectively. Methyldeoxyspergualin (MeDSG) was prepared by the method of Umeda et al.,\(^2\) as hydrochloride. To obtain N-cyclopropylmethylated DSG (3-CP-DSG), triethylamine (27 \mu l, 0.193 mmol), cyclopropanecarboxaldehyde (21.2 \mu l, 0.288 mmol) and sodium cyanoborohydride (19.2 mg, 0.288 mmol) were added to a solution of MeDSG (49.3 mg, 0.096 mmol) in MeOH (2 ml) under ice cooling. After stirring for 4 hours at room temperature, the reaction mixture was diluted with water and chromatographed on a CM-Sephadex C-25 (Na\textsuperscript{+}) column with a linear gradient elution using water and 0.5 M NaCl solution. Fractions containing 3-CP-DSG were collected and evaporated to
dryness. The residue was extracted with MeOH and the MeOH extract was chromatographed on a Sephadex LH-20 column using MeOH as an eluent. Fractions containing 3-CP-DSG were pooled and evaporated to give 22.0 mg (34%) as a syrup. HRFAB-MS m/z 564.4604 (M+H)+ calcd. for C_{30}H_{58}N_{7}O_{3} m/z 564.4601; IR (KBr) 3250, 2935, 1660, 1525, 1470, 1375, 1350, 1190, 1090 cm⁻¹; ¹H NMR (CD_{3}OD) δ: 0.3~0.5 (6H, m, cyclopropyl-CH₂), 0.6~0.8 (6H, m, cyclopropyl-CH₂), 1.0~1.2 (3H, m, cyclopropyl-CH), 1.2~2.0 (12H, m, CH₂), 2.0~2.4 (4H, m, CH₂), 2.7~3.4 (16H, m, CH₂), 3.39 (3H, s, OCH₃), 5.30 (1H, s, 11-CH).

Cells
Mouse EL-4 lymphoma cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 100 units/ml of penicillin G, and 100 μg/ml of streptomycin at 37°C with 5% CO₂.

Cell Growth
Cells were inoculated into 96-well plates at 3000 cells/well and incubated with or without test drugs for 4 days. The growth was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described. In case of 4h-treatment experiment, the cells were incubated with or without test drugs for 4 hours at 37°C, and then the cells were washed twice with the growth medium and further cultured for 4 days. For competitive assay of DSG, test drugs were added to cells 30 minutes before the addition of DSG.

Results and Discussion
15-Deoxyspergualin (DSG) has antiproliferative effect on various cancer cell lines. Among them, DSG showed a relatively strong effect on hematopoietic malignant cells. In this study we used mouse EL-4 lymphoma cells. As shown in Fig. 2, DSG inhibited the growth of EL-4 cells with an IC₅₀ 0.02 μg/ml. In contrast, when the cells were treated with DSG for only 4 hours and then washed out the drug, DSG inhibited the growth apparently with an IC₅₀ 0.4 μg/ml, even though its effect was weakened (Fig. 2). On the other hand, cycloheximide used as a negative control
inhibited the growth with an IC_{50} 0.1 \mu g/ml, but the antiproliferative effect was diminished by 4-hour treatment (Fig. 2). This result shows that the antiproliferative effect of DSG lasts long.

DSG has spermidine and guanidine moieties in its structure (Fig. 1). We next examined the effect of the decomposed elements of DSG. Although one decomposed element containing spermidine moiety (DSG-A) did not affect the growth of EL-4 cells, another element containing guanidine moiety (DSG-B) inhibited it at a high dose of 100 \mu g/ml (Fig. 3A). Unlike DSG, DSG-B failed to inhibit the growth by only 4-hour treatment (Fig. 3B). Addition of DSG-A along with DSG-B did not enhance the effect of DSG-B (Fig. 3C). We therefore hypothesized that DSG would bind to cells and internalize into the cells through a spermidine moiety and then the internalized DSG would exert its antiproliferative effect through a guanidine moiety.

To ascertain the hypothesis, we examined whether the effect of DSG should be abolished by pretreatment of the cells with DSG-A. The cells were preincubated with excess amount of DSG-A for 30 minutes, and then further incubated with DSG along with DSG-A for 4 hours. After washing out the drugs, the cell growth was determined. As a result, pretreatment with DSG-A expectedly diminished the antiproliferative effect of 4-hour treatment DSG dose-dependently (Fig. 4A). As well as DSG-A, pretreatment of the cells with excess amount of polyamines such as putrescine, spermidine, and spermine also abolished the antiproliferative effect of DSG rather strongly (Fig. 4B). The same results were also obtained using spergualin, an original compound of DSG (data not shown). These effects of polyamines were not thought to be due to prevention of polyamine depletion by DSG. It is reported that intracellular polyamines are decreased by DSG treatment.\(^\text{14}\) DL-\(\alpha\)-Difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, also decreases the intracellular polyamines.\(^\text{15}\) As shown in Fig. 5, DFMO inhibited the growth with an IC_{50} 50 \mu g/ml, but the continuous presence of polyamines along with DFMO throughout cultured days diminished the antiproliferative effect of DFMO. This result...
Fig. 4. Reversal of DSG effect by DSG-A and polyamines.

EL-4 cells were preincubated with DSG-A or polyamines for 30 minutes, and then further incubated with DSG at 1 µg/ml along with DSG-A or polyamines for 4 hours at 37°C. The cells were washed and cultured for 4 days. (A) DSG-A was added at 0 (○), 10 (□), 100 (■), and 1000 (▲) µg/ml. (B) Polyamines were added as follows, putrescine 32 µg/ml (200 µM, □), spermidine 51 µg/ml (200 µM, ■), spermine 70 µg/ml (200 µM, ▲), and DSG-A 58 µg/ml (200 µM, ▼).

Fig. 5. Effect of polyamines on the antiproliferative effect of DSG and DFMO.

EL-4 cells were preincubated with DSG for 4 hours, washed and then cultured for 4 days in the absence (○) or the presence of putrescine 100 µg/ml (●), aminoguanidine 100 µg/ml (■), spermidine 10 µg/ml (▲), or spermine 10 µg/ml (▲). Because of non-lasting effect of DFMO, DFMO was added along with polyamines without washing. Spermidine and spermine were added with aminoguanidine 100 µg/ml to inhibit oxidation.

indicated that depletion of the intracellular polyamines was prevented by the extra-addition of polyamines. On the other hand, when the cells were preincubated with DSG for 4 hours and then polyamines were added after washing out DSG, the antiproliferative effect of DSG was not affected by the continuous presence of polyamines. Therefore, the preventing effect of polyamines on 4h-treated DSG action in Fig. 4 was considered to be direct interaction between them. It is reported that DSG inhibits the spermidine transport through cell membrane. Therefore, our results supported the idea that DSG is transported through the polyamine transporter.

To explore the further possibility that DSG acts through the spermidine moiety, we prepared a DSG derivative, in
which spermidine moiety was structurally hindered, using methyldeoxyspergualin (MeDSG) (Fig. 1). As well as DSG, MeDSG inhibited the growth of EL-4 cells at IC50 0.02 μg/ml. On the contrary, a DSG derivative, cyclopropylmethylated MeDSG (3-CP-DSG) did not affect the growth (Fig. 6). This result shows that the structure in a spermidine moiety in DSG is important for its antiproliferative effect.

These results therefore suggested that DSG binds to the cells strongly through a spermidine moiety and exerts its long-lasting antiproliferative effect. It is speculated that this unique structural characteristic of DSG would confer the potent antitumor effect in vivo. Although we are now studying the precise mechanism for DSG action, it might be one of modifications to create an antitumor compound fusing spermidine and a cytotoxic lead compound.

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