VOL. 52 NO. 9, SEPT. 1999 THE JOURNAL OF ANTIBIOTICS pp. 803 — 808

The Decrease of Cytochrome *c* Oxidase Activity by 15-Deoxyspergualin Results in Enhancement of XTT Reduction in Cultured Cells

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(Received for publication April 13, 1999)

Exposure to immunosuppressant, 15-deoxyspergualin (DSG) induced enhanced formazan producing activity from XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide, sodium salt) in cultured cells, but not from MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Formazan generation from XTT is known to be stimulated by cytochrome *c* oxidase (COX) inhibitors such as KCN and NaN_3, whereas MTT reduction is not affected by them. So, the effect of DSG on COX was examined. DSG did not directly inhibit the enzyme, but the cellular enzyme activity was decreased by exposure to DSG. It was thought that stimulation of XTT reduction by DSG resulted from the decrease of cellular cytochrome *c* oxidase activity.

DSG[1] is a more active derivative of spergualin (SG), an antitumor antibiotic produced by a strain of *Bacillus laterosporus*[2]. DSG has strong suppressive effects on both humoral and cell-mediated immune responses in animals[3~5] and has been used in renal transplantation as a novel immunosuppressant, named gusperimus. In spite of its therapeutic effectiveness, the biochemical mechanism of action of DSG has not been clearly understood. We had found in the assay of cytotoxicity of DSG and SG that they gave inconsistent results depending on the type of tetrazoliums[6] used for assay of cell growth. In this paper we describe that cellular XTT reducing activity is stimulated by DSG treatment and is caused by the decrease of COX, the terminal enzyme in the mitochondrial respiratory chain.

Materials and Methods

Materials

DSG, methyldeoxyspergualin, and N-30 were provided from Takara Shuzou Co., Ltd. and Nippon Kayaku Co., Ltd., respectively. SG and its analogs were prepared by us[2,7]. Fetal bovine and horse sera were purchased from ICN Biomedicals, Inc., USA, and Grand Island Biochemical Co., USA, respectively. MTT, XTT, phenazine methosulfate (PMS), and cytochrome *c* from horse heart were obtained from Sigma Chemical Co., USA.

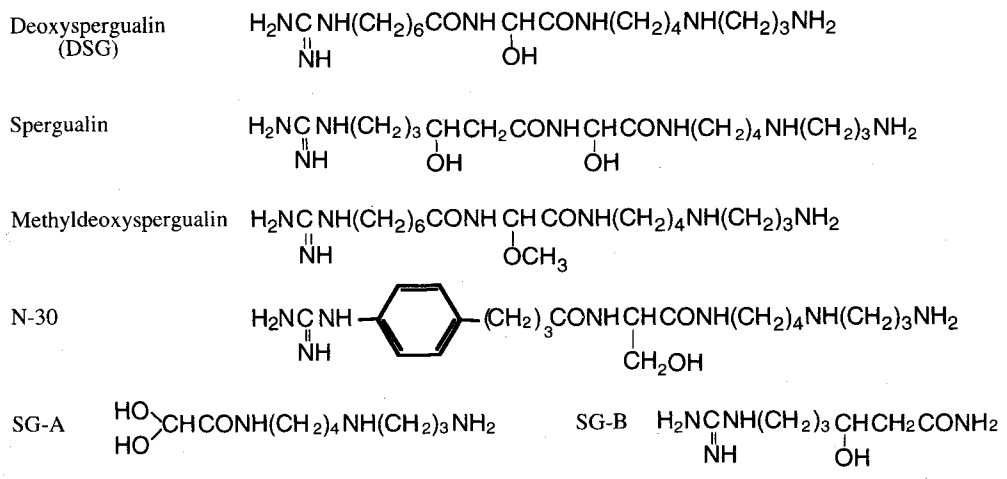
Cell Culture

Chinese hamster ovary (CHO) cells were cultured in α-MEM (Gibco Laboratories, Life Technologies, Inc., Grand Island, N.Y., USA) supplemented with 10% fetal bovine serum. L1210 and J774A.1 cells were cultured in a RPMI1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) medium supplemented with 10% fetal bovine serum. L5178Y cells were cultured in a RPMI1640 medium supplemented with 10% horse serum. Cells were inoculated in 96-well microplates at 10^4 cells/100 μl medium, adhered for 4 hours for CHO and J774A.1 cells and used for XTT or MTT assay. Cell growth was determined by counting cell number with a Coulter Counter.

XTT and MTT Assays

The XTT and MTT assays were performed using the methods described by SCUDIERO *et al.*[8] and ALLEY *et al.*[9], respectively. Briefly 25 μl of XTT-PMS solution (1 mg/ml XTT solution supplemented by 25 μM of PMS) or 10 μl of MTT solution (5 mg/ml) were added to the cells in each well on the microplates. After incubating for 3 hours at 37°C for XTT-reduction, absorbancy at 490 nm was measured by a Biorad model 3550 microplate reader. MTT-

Fig. 1. Structure of DSG and related compounds.



DSG, SG, methyldeoxyspergualin, and N-30 are active as immunosuppressant, but SG-A and SG-B are inactive decomposed fragments of SG.

formazan produced by CHO cells was solubilized by the addition of 100 μl of DMSO after removal of supernatant fluid from each well and measured at 525 nm. MTT-formazan generated by L5178Y and L1210 cells was solubilized by the addition of 50 μl of 20% SDS solution to each well and measured at 595 nm after standing overnight. The results were shown as the means obtained from duplicate microcultures.

Cytochrome *c* Oxidase Activity

COX was measured by the spectrophotometric assay[10], in which the rate of oxidation of ferrocytochrome *c* was traced by the decrease in the absorbancy of 550 nm every 15 seconds. Ferrocytochrome *c* was prepared from cytochrome *c* dissolved in 0.01 M phosphate buffer, pH 7.0 by reduction with ascorbic acid, and dialyzed for the same buffer for 12 hours before the assay. The reaction was initiated by the addition of an enzyme solution to the reaction mixture (1 ml) containing 0.07% ferrocytochrome *c* equilibrated at 37°C. Supernatant fraction at 1,000 rpm for 10 minutes from Ehrich carcinoma cell homogenate was used as the enzyme. The cells were collected from the ascites fluid of an ICR mouse inoculated intraperitoneally with the carcinoma cells, washed twice in PBS, suspended with PBS supplemented with 1 mM EGTA, and disrupted by sonication with Physcotron (Nichion Rika, Japan). For the assay of COX activity in L1210 cells after DSG treatment, the cells (10^8 cells/ml) were treated as in the case of Ehrlich carcinoma cells. Protein was measured by the Bio-Rad protein assay kit.

Results

Effect of DSG and SG on Cell Proliferation and Tetrazoliums Reducing Activities

As shown in Fig. 2, the MTT assay gave an almost equivalent result to cell counting, but XTT reduction was stimulated about 1.5 times by incubation with DSG or SG in CHO and L5178Y cells. The same phenomenon was observed in the L1210 and macrophage cell line, J774A.1 cells. XTT-formazan generation itself by cells was not affected by simultaneous addition of XTT and DSG or SG. Pretreatment of these cells for more than 24 hours was needed for the enhancement. It was thought that incubation with DSG caused the change of XTT reducing activity in the cells.

Decrease of Cellular COX Activity

COX inhibitors, NaN_3 or KCN, stimulate only XTT reduction without any effect on MTT reduction as previously reported by us[11]. Inhibitory effect on the enzyme by DSG was examined. DSG did not directly inhibit COX as shown in Table 1 as was expected from the necessity of long incubation with DSG for the enhancement, however COX activity was determined for the homogenates from the cells pretreated with DSG. As

Fig. 2. Comparison of results with XTT, MTT, or cell-counting assays in DSG- or SG-treated cells.

The growth inhibitory effect by DSG (a, c, d, and e) or SG (b) after 48 hours treatment were evaluated by XTT (○) and MTT (●) assays and cell-counting (□) using CHO (a and b), L5178Y (c), L1210 (d), and J774A.1 (e) cells.

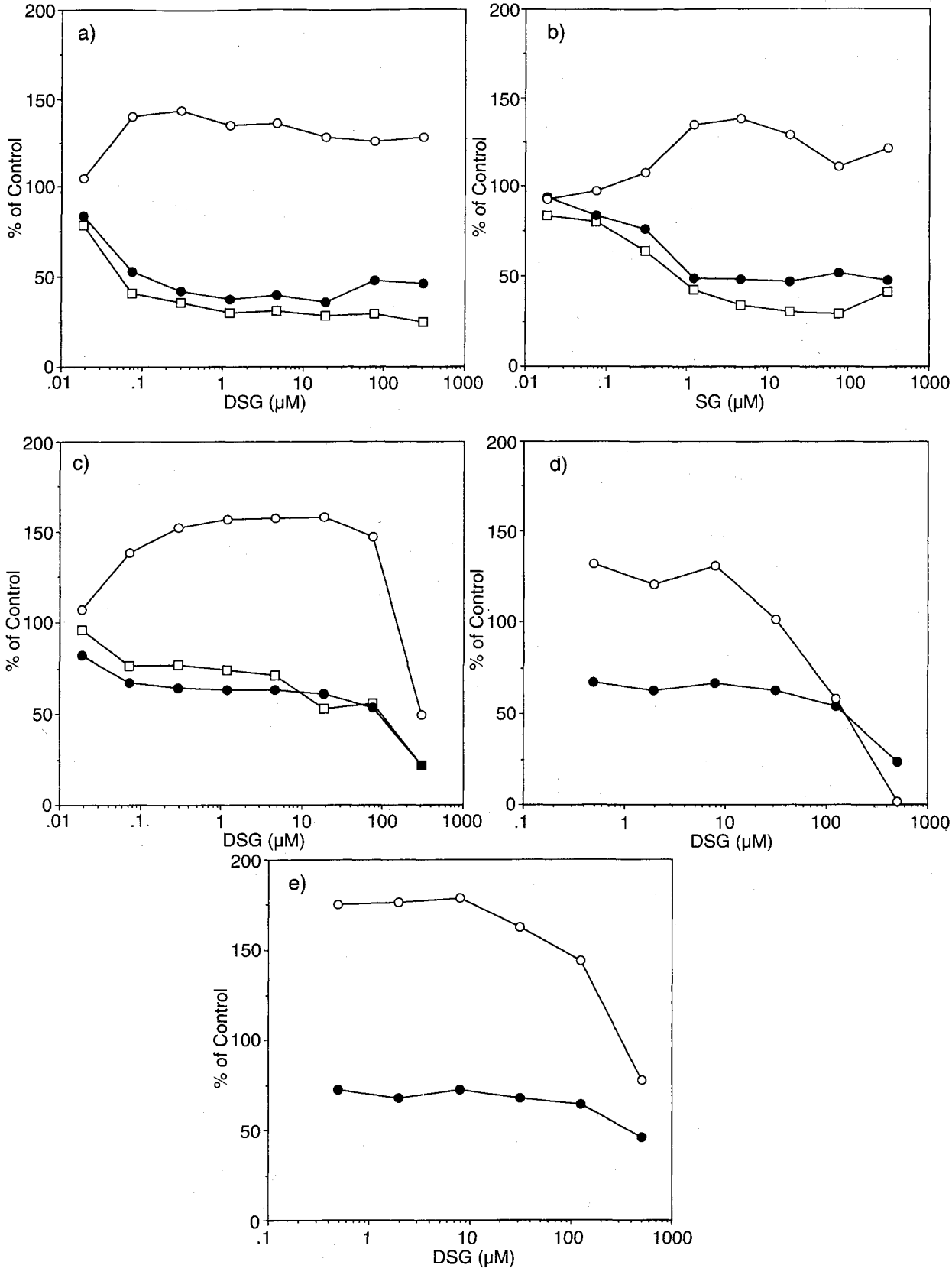


Table 1. Effect of DSG and NaN_3 on COX.

		COX activity (ΔOD_{550}/min.)	% inhibition to Control
Control		0.1669	
DSG	1 mM	0.1661	0.48
	2 mM	0.1468	12.0
NaN_3	1 mM	0.0217	87.0

COX activity in the homogenates prepared from Ehrlich carcinoma cells was determined in the absence or the presence of DSG or NaN_3.

Table 2. Decrease of COX activity after DSG treatment.

		Specific activity of COX	% inhibition to Control
Control		0.0363	
DSG	0.001 μM	0.0276	23.8
	0.01	0.0235	35.1
	1	0.0149	58.8
	100	0.0131	63.9

L1210 cells treated by DSG for 48h were determined for COX activity. The cell homogenates were prepared as described in Materials and Methods. Specific activity of COX were expressed as ΔOD_{550}/min/mg protein.

shown in Table 2, the specific activity of COX decreased to 36% of non-treated control cells by DSG treatment. Uptake of rhodamine 123 was not affected though data were not shown, indicating that the number and structural integrity of mitochondria was not affected by DSG.

Relationship between Immunosuppression and Enhancement of XTT Reduction in DSG Derivatives

To know whether the enhancement of XTT reduction is related to immunosupressive activity of DSG, active analogs, inactive degradation products of SG (SG-A and SG-B), and polyamines as to immunosuppression were tested on XTT reducing activity.

As shown in Fig. 3, methyldeoxyspergualin and N-30 having immunosuppressive activity enhanced XTT reduction. SG-A and SG-B did not show the enhancement, while their cytotoxic activity was lost. Polyamines such as spermidine, putrescine, and spermine showed almost the same inhibition in MTT and XTT assays. The enhancement of XTT reduction is brought about only by immunosuppressive substances.

Discussion

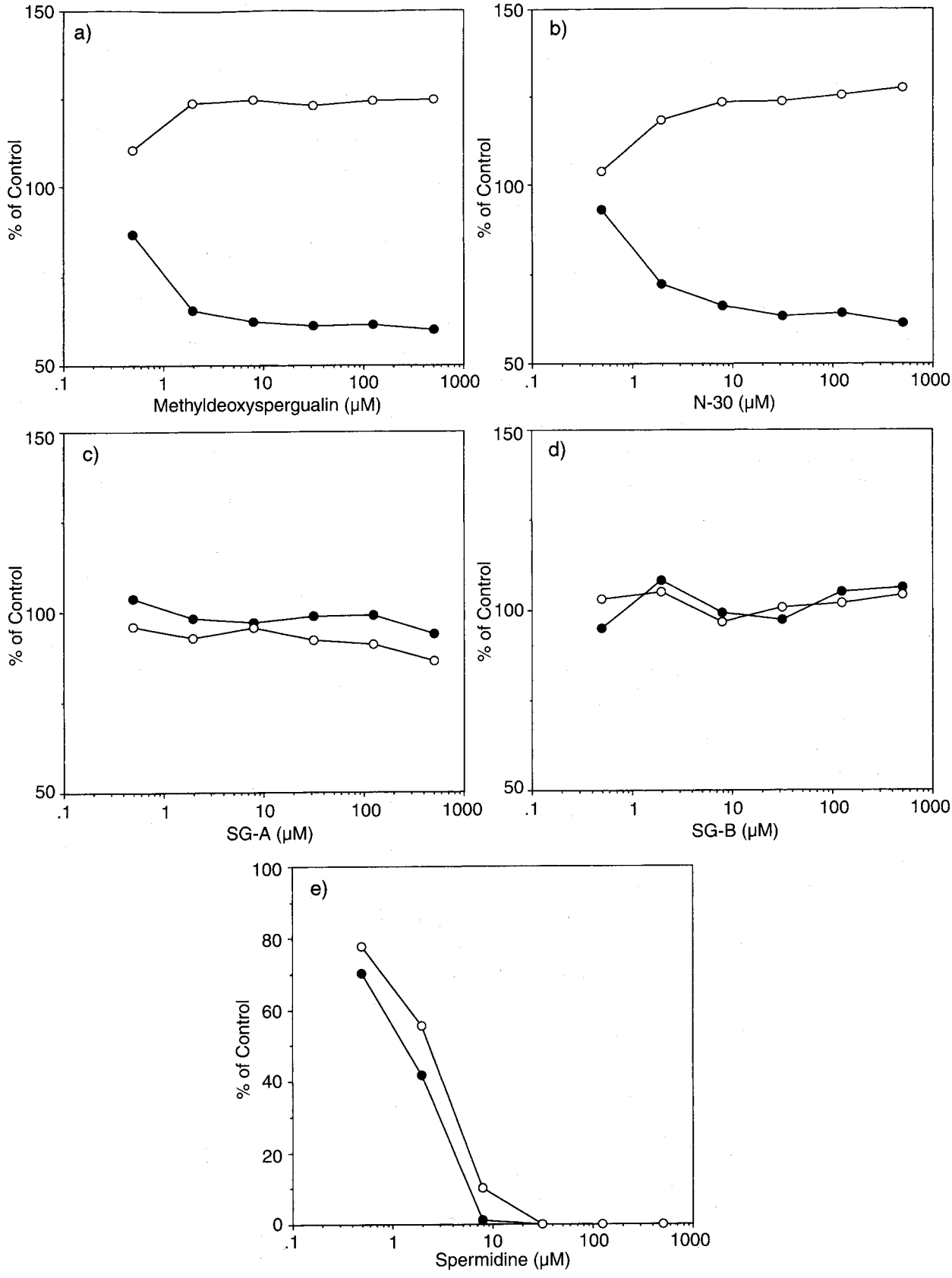
DSG or SG treatment stimulated XTT reduction without any effect on MTT reduction. This result is similar to that obtained using COX inhibitors, NaN_3 and KCN[11]. The effect on COX of DSG was examined. DSG did not directly inhibit COX, but cells preincubated by the drug showed decreased activity of COX. From structure-activity relationships, the enhancement of XTT reduction is brought about only by immunosuppressive substances. Previously we reported that DSG inhibited induction of inducible nitric oxide synthase by IFN-γ and LPS in macrophage cell line, J774A.1 cells[12]. The structure-activity relationships is similar to the case of the decrease of COX activity. The decrease of iNOS by DSG is easily understandable, because expression of iNOS is activated by NF-κB[13]. DSG binds to HSc70 and HSP90[14,15] and inhibits the translocation of NF-κB to nucleus from cytosol and antigen processing by inhibiting chaperoning activity of HSc70[16,17]. Since COX is one of hemoproteins inhibited by NO[18,19], it is difficult to explain how DSG reduces COX activity. Studies on the mechanism of action of DSG will clarify the regulation network of respiration by NO and COX.

There are several colorimetric assay methods which are based on the bioreduction of a variety of tetrazolium salts to intensely colored formazan. It is now evidenced that MTT reduction occurs extramitochondrially and involves NADH and NADH-dependent mechanisms that are insensitive to respiratory chain inhibitors[20]. Our result showed that XTT reduction involved the mitochondrial respiratory chain, unlike MTT.

During the preparation of this manuscript, it was reported by ODAKA *et al.* that DSG induced an enhanced MTT reduction in Raji cells, J774.1 cells, and NIH3T3 cells[21] using the Celltiter 96™ aqueous nonradioactive cell proliferation assay kit (Promega, Madison, WI, USA). In our experiments MTT reduction was not enhanced, but inhibited proportionally to inhibition of cell proliferation.

Fig. 3. The structure-activity relationship.

The structure-activity relationship of DSG's analogs was studied on a correlation between the activity of enhancing XTT reduction and immunosuppressive one. CHO cells were treated by methyldeoxyspergualin (a), N-30 (b), SG-A (c), SG-B (d), and spermidine (e) as described in the legend of Fig. 2. After 48 hours treatment XTT (○) and MTT (●) assays were practiced.



Only XTT reduction was enhanced by DSG treatment. This apparent discrepancy may come from the fact that they used Celltiter 96™ aqueous nonradioactive cell proliferation assay kit as the MTT assay. The manual for the kit states the method is based on the reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt) with PMS[22]. So we suppose that they observed the enhanced reduction of MTS by DSG instead of MTT. Since MTS is structurally related to XTT and also requires PMS as an electron coupler, the reduction of MTS might involve COX which is inhibited by DSG, resulting in enhanced reduction as with the case of XTT.

Acknowledgment

The authors thank Japanese Cell Bank for providing J774A.1 macrophage cell line. The authors are grateful to Takara Shuzou Co., Ltd. for providing DSG, and to Nippon Kayaku Co., Ltd. for providing methyldeoxyspergualin and N-30.

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