Cell Membrane Signaling as Target in Cancer Therapy: Inhibitory Effect of N,N-Dimethyl and N,N,N-Trimethyl Sphingosine Derivatives on in Vitro and in Vivo Growth of Human Tumor Cells in Nude Mice

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ABSTRACT

Sphingosine (SPN) has been claimed to be a negative modulator of transmembrane signaling through protein kinase C (PK-C) or some other unidentified mechanism (for review see Y. A. Hannun and R. M. Bell, Science (Washington DC), 243: 500-507, 1989). N,N-Dimethylsphingosine (DMS) was recently found to be a physiological cellular component and, in comparison to SPN, to show a stronger and stereospecific inhibitory effect on PK-C activity of A431 cells (for review see Y. Igarashi, Trends Glycosci. Glycotechnol., 2: 319-332, 1990; and S. Hakomori, J. Biol. Chem., 265: 18713-18716, 1990). (4E)-N,N,N-Trimethyl-D-erythro-sphingosine (TMS) is not detectable as a normal cellular component; however, it is expected to exhibit potent activity because of its quaternary ammonium ion structure, and in fact it showed much stronger inhibitory effect than DMS or SPN on PK-C activity (which plays an important role in cell growth regulation) in vitro. In view of these findings, we investigated the effects of SPN, DMS, and TMS on in vitro growth of various human carcinoma cell lines and on in vivo tumor growth in athymic nu/nu mice. Both DMS and TMS showed similar in vitro and in vivo growth inhibitory effects on tumor cells, despite the fact that TMS showed a much stronger inhibitory effect than DMS on PK-C activity of A431 cells. In contrast, SPN showed only a weak effect on in vitro cell growth and no effect on in vivo tumor growth. Tumor growth following s.c. inoculation of mice with human gastric carcinoma cell line MKN74 was inhibited in a dose-dependent manner by DMS, and tumor size was decreased after three or four consecutive daily injections of 0.5-mg doses of DMS or TMS. Increased tumor growth occurred after administration of these compounds was stopped; however, size of tumor remained significantly smaller than in groups treated with SPN or control saline. The effect of DMS or TMS on in vitro or in vivo MKN74 cell growth was stronger than that of 8-chloro-adenosine-cyclic 3',5'-monophosphate dihydrate, the most promising agent currently being used in clinical trials for inhibition of tumor growth by induction of differentiation. These results suggest that DMS or TMS could be useful anticancer agents through modification of transmembrane signaling related to cancer cell growth.

INTRODUCTION

Promotion of carcinogenesis, and loss of growth control in tumor cells, have been correlated with dysfunction or improper coordination of various membrane-associated signaling mechanisms controlled by protein kinases, particularly receptor-associated kinase and PK-C, both of which are modulated by a variety of sphingolipids (1-3). PK-C is the primary receptor for the class of tumor-promoting agents which includes 12-O-tetradecanoylphorbol-13-acetate, and may have a key role in the process of tumor growth and development (4-6). Recent studies have shown that increased tumorigenicity is correlated with overexpression of PK-C, and PK-C, in Rat-1 and NIH 3T3 cells inoculated into nude mice (6,7), and that a mutant of PK-C, induces highly malignant tumor cells with increased metastatic potential (8), suggesting that aberrant expression or overexpression of PK-C may relate to stage of tumor progression. SPN and its derivatives MMS and DMS have recently been shown to be modulators of PK-C and EGF tyrrosine kinase activity (9-11), subsequent to the proposal by Bell, Merrill, and associates that PK-C activity is inhibited by sphingosine but promoted by diacylglycerol (1,12,13). We previously observed that DMS showed a much stronger stereospecific inhibitory effect than SPN, MMS, or other dimethyl stereoisomers on PK-C activity of human epidermoid carcinoma A431 cell line, which is characterized by high expression of EGF receptor and PK-C (10,11). SPN or DMS may regulate cell growth through modulation of growth factor receptor kinase or by some other unidentified mechanism (11,14). These findings prompted us to study the effects on human tumor growth of SPN, MMS, DMS, and TMS, as compared with other reagents and DI known to induce tumor cell differentiation and affect tumor growth. Our results show clearly that DMS and TMS show significant inhibitory effects on in vitro as well as in vivo tumor cell growth.

MATERIALS AND METHODS

Reagents, Cells, and Animals. SPN was purchased from Sigma Chemical Co. (St. Louis, MO) and shown to be usually homogeneous on high-performance thin-layer chromatography. Detection was made by using fluorescamine, ninhydrin, and primulin (15). If an extra band not exactly corresponding to D-erythro-sphingine was found, further purification on high-performance liquid chromatography was performed. The final compound was dissolved in ethanol and a 10 mM stock solution was stored at -20°C. TMS was synthesized by reductive methylation with formaldehyde and sodium borohydride as previously described (9), and purified on high-performance liquid chromatography. The final compound was dissolved in ethanol and a 10 mM stock solution was stored at -20°C. TMS was synthesized by quaternization of DMS by iodomethane under anhydrous conditions, as will be described elsewhere. B-Chloro-cAMP was purchased from ICN Biochemicals (Cleveland, OH). Dibutylryl cAMP, 5-bromo-2'-deoxyuridine and N,N'-hexamethylene bisacetamide were purchased from Sigma.

Human gastric cancer cell lines MKN45, MKN74, and Kato III were obtained from Dr. Masakazu Adachi, Japan Immunoresearch Laboratories; the MKN lines were originally established by Motoyama et al. (16). Human lung squamous cell line LUS65 was established (17) and donated by Dr. Seiizo Hirohashi, National Cancer Center Research Institute, Tokyo, Japan. Human lung small cell carcinoma line NC1-H69 and various human colon cancer cell lines (Coho205, SW48, SW403, SW1116, SW1417, HT29, LS174T, LS180) were obtained from Dr. Setsuo Hirohashi. National Cancer Center Research Institute, Tokyo, Japan.

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3 The abbreviations used are: PK-C, protein kinase C; cAMP, cyclic adenosine monophosphate; 8-chloro-cAMP, 8-chloro-adenosine-cyclic 3',5'-monophosphate dihydrate; DI, differentiation-inducer; dibutyryl cAMP, N',N'-O-dibutyryladenosine-cyclic 3',5'-monophosphate; DMS, (4E)-N,N-dimethyl-D-erythro-sphingine; EGF, epidermal growth factor; MMS, monomethyl-D-erythro-sphingine; SPN, sphingosine; TMS, (4E)-N,N,N-trimethyl-D-erythro-sphingine.

4 M. Nisar, T. Toyokuni et al., submitted for publication.
from American Type Culture Collection (ATCC), Washington, DC; their origins are described in the ATCC catalogue which is updated yearly. Human melanoma line M1733 was from Dr. Karl-Eric Hellström, then at Fred Hutchinson Cancer Research Center, Seattle, WA. Human epidermoid carcinoma line A431 was from Dr. Carol MacLeod (University of San Diego, San Diego, CA). Mouse lymphoma lines Esb and Eb83 were from Dr. Volker Schirrmacher, Institut für Immunologie und Genetik, Heidelberg, West Germany. Variants of B16 mouse melanoma (F10, F1, and BL6) were from Dr. Jean Starkey, Department of Microbiology, Montana State University, Bozeman, MT. All cells were cultured in vitro in Dulbecco’s modified Eagle’s medium supplemented with 1% fetal calf serum and 20 mM glutamine.

For all experiments, 8- to 10-week-old female nu/nu athymic mice were used for inoculation of human tumor cells and evaluation of effects of tumor growth inhibiting agents.

Cell Growth and Its Inhibition. Cells were seeded (2 x 10^4 cells/well) in flat-bottomed 96-well plates (Corning Glass Works, Corning, NY), cultured for 1–2 days, and supplemented with SPN or DMS dissolved in phosphate-buffered saline. These sphingoid bases are soluble in water as chloride at slightly acidic pH on addition of HCl, but tend to form an opaque suspension at neutral (7.0) or physiological (7.2–7.4) pH. TMS gave a clear aqueous solution at neutral and physiological pH. Suspensions or solutions were supplemented by [3H]thymidine (0.5 μCi/well) and cultured for 6 h. Cells were collected by PHD cell harvester (Cambridge Technology, Cambridge, MA), and [3H]thymidine incorporation was determined by Beckmann LS3801 scintillation counter with ScintiVerse BD solution (Fisher Scientific, Scientific, Fair Lawn, NJ).

Tumor Growth and Its Inhibition in Vivo. Tumor cells (2–4 x 10^4) were inoculated s.c. at the flank region of mice, and treatment was initiated after tumors reached an estimated volume of ~100 mm³. For SPN and DMS, appropriate concentrations (from stock ethanol solution) in 140 mM NaCl were prepared; for TMS, aqueous solution was prepared directly. Final concentration of ethanol was ~5%. Solutions (50 μl) were injected i.v. through tail vein; injections were made on a daily basis for 4 or 5 consecutive days. In the control groups, NaCl/ ethanol or NaCl solution without addition of sphingoid base was injected by the same procedure. At 3-day intervals, tumor diameter was measured and tumor volume was estimated by longest diameter x (shortest diameter)/2. Body weight of mice was measured at day 0 (when treatment was initiated), day 6, and day 12. Mice were killed on day 12, tumors were excised, and tumor weight was directly measured. Values were statistically compared by t test.

In Vitro PK-C Activity Assay. PK-C from human epidermoid carcinoma line A431 cells was prepared as previously described (9). A431 cells have only the α isoform of the enzyme (PK-Cα), which is abundant in various human cancer-derived cell lines such as MKN74, LU65, and Colo205 (18). PK-C from mouse brain, which is composed mainly of α, β, and γ forms, was prepared according to the method of Kikkawa et al. (19).

PK-C activity was determined as described previously (9). Briefly, in conical tubes (1.5-ml content; Sarstedt), phosphatidylserine (5 μg/tube), and 1.2-diolein (0.05 μg/tube), with or without appropriate quantities of sphingosines and derivatives, were added in organic solvent (ethanol or chloroform/methanol), and the mixture was evaporated under N₂ stream. The lipid mixture was sonicated in 30 μl of 20 mM Tris-HCl (pH 7.5) for 30 min. Liposomes in the tube were supplemented with the reaction mixture, consisting of 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 400 μM EDTA, 50 μM ethylenerglycol bis(β-aminoethoxyethy)-N₂,N₂,N₂,N₂-tetraacetic acid, 500 μM CaCl₂, 200 μg/ml histone III-S, and 20 μM [γ³²P]ATP (2 x 10⁶ cpm); final volume was 90 μl. The reaction was initiated by addition of 10 μl of PK-C fraction (1–2 μg protein) prepared as previously described (19), and the reaction mixture was incubated for 10 min at 30°C. The reaction was terminated by addition of 1 ml of 25% trichloroacetic acid with 200 μl of 1% bovine serum albumin in 1 mM ATP solution (pH 7.5). The precipitate was centrifuged, washed twice with 1 ml of 25% trichloroacetic acid, dissolved in 1 ml of 1 M NaOH containing 0.1% deoxycholate with slight heating (80°C for 10 min), and counted in a scintillation counter.

Fig. 1. Inhibition of in vitro [3H]thymidine uptake in MKN74 and LU65 cells by SPN and DMS. Cells were seeded (2 x 10⁴ cells/well) and cultured for 2 days. SPN or DMS was added as 100x concentrated solution in phosphate-buffered saline. [3H]Thymidine was added, cells were cultured, and radioactivity was counted as described in “Materials and Methods." Points, means of quadruplicate determinations. ○, MKN74 cells with DMS; ●, MKN74 cells with SPN; △, LU65 cells with DMS; ▲, LU65 cells with SPN.

The value without phosphatidylserine, 1.2-diolein, or Ca²⁺ was used as a reference blank.

RESULTS

Effect of SPN, DMS, and TMS on Tumor Growth and PK-C Activity in Vitro. Inhibition of [3H]thymidine uptake in MKN74 and LU65 cells is shown in Fig. 1. In comparison to SPN, DMS showed much stronger inhibitory effect on growth of both cell lines. For MKN74 and LU65, the concentrations of DMS producing 50% growth inhibition were 15 times and 7 times smaller (respectively) than those of SPN. The comparative effect of SPN, DMS, and TMS on MKN74, LU65, and Colo205 cells is shown in Fig. 2. TMS and DMS showed a similar range of inhibitory effect, which was much stronger than that of SPN. Similar trends for growth inhibition by SPN and DMS were observed for 17 other lines of human and murine tumor cells (listed under “Materials and Methods") (data not shown). However, the inhibitory effect of TMS on growth of some tumor cells (e.g., B16 mouse melanoma and T-cell leukemia cells) was 8–10 times stronger than that of DMS.

Dependence on concentration and incubation time of DMS needed to produce 50 or 95% growth inhibition of MKN74 cells, as reflected by inhibition of [3H]thymidine uptake, is illustrated in Fig. 3. As incubation time increases from 0 to 12 h, the necessary concentration of DMS for both 50 and 95% inhibition falls sharply. However, for times from 12 to 72 h, necessary concentrations were essentially constant.

Comparative effects of SPN, MMS, DMS, and TMS on PK-C activity of A431 cells (PK-Cα; commonly found in various human cell lines) were measured, and results are shown in Fig. 4. TMS showed a much stronger effect than DMS on PK-C activity, regardless of whether myelin basic protein (Fig. 4A) or histone III-S (Fig. 4B) was used as substrate. MMS and SPN showed much weaker inhibitory effects under the same conditions. Very similar results were obtained in experiments with...
PK-C from mouse brain (composed mainly of PK-Cα, PK-Cβ, and PK-Cγ) (data not shown).

Inhibition by SPN, DMS, and TMS of Human Tumor Growth in Mice. In vivo growth curves of human lung cancer LU65 tumors in nude mice following treatment with SPN and DMS are shown in Fig. 5. Growth curves of human gastric cancer MKN74 tumors are shown for treatment with SPN, DMS (Fig. 6A), and TMS (Fig. 6B). The results clearly indicate a relatively small effect by SPN, even when a dose of 0.5 mg was used. In contrast, growth of both LU65 and MKN74 tumors was clearly inhibited in the groups receiving 0.5 mg DMS. Tumor size stopped increasing, or even decreased, when these compounds were administered by 3 or 4 consecutive daily injections (Figs. 5A and 6A). After DMS treatment was stopped, tumor growth slowly returned to control levels. Comparative effect of different doses of DMS on MKN74 tumor growth is shown in Fig. 7, and tumor weights on day 12 in the DMS-treated groups are shown in Table 1. Inhibition of tumor growth was most pronounced for treatment with 0.5 mg DMS, smaller but still significant for 0.13 mg DMS, and marginal for 0.03 mg DMS or 0.5 mg SPN (Table 1; Fig. 7). In this experiment, significant reduction in tumor size was again observed following 3 consecutive injections of 0.5 mg DMS.

The effect of TMS on in vivo growth of MKN74 tumor cells in nu/nu mice was similar to that of DMS; i.e., four consecutive i.v. injections resulted in reduction of tumor size (Fig. 6B). Increased tumor size was observed after treatment was stopped, although tumor size did remain significantly below control levels. Thus, inhibitory effect of TMS on tumor growth was clearly greater than that of control saline or SPN; i.e., tumor weights at day 12 after treatment with TMS, control saline, and SPN were 229 ± 36 (SD), 325 ± 59, and 281 ± 35 mg, respectively (n = 10/group).

Side Effects of i.v. Injection. Administration of SPN, DMS, and TMS was via tail vein injection. When this was not carefully performed (i.e., when extravascular leakage occurred), edematous inflammatory reactions were observed, particularly following injection of high concentrations of the compounds (i.e., 0.5 mg/50-100 μl). Thrombosis and local necrosis were observed in cases in which a 0.5-mg/50-100 μl dose was injected and significant leakage occurred. No significant inflammatory response was observed when 0.03-mg doses were injected. In experimental groups in which special care was taken to avoid extravascular leakage, no such side effects were observed, even after injection of 0.5-mg doses.

Effects of systemic toxicity were evaluated by changes in body weight (Table 2). Mice receiving daily injections of 0.5 mg DMS or SPN showed significant reduction of weight by day 6. If treatment was stopped at day 6, weight returned to normal (i.e., day 0) level by day 12. Mice treated with 0.13- or 0.03-mg doses of DMS showed insignificant changes in weight.

DISCUSSION

In previous studies on effects of SPN and DMS on PK-C, only DMS showed a clear stereospecific inhibitory effect on in vitro activity of PK-C, the essential membrane-associated component in regulation of cell growth (4-6). Since aberrant expression of PK-C is closely associated with tumor progression and metastatic potential (6,8), the observed effect of DMS on in vitro tumor growth is of great interest. Our results indicate that...
DMS had a pronounced inhibitory effect on growth of human tumor cell lines MKN74 and LU65 in nu/nu mice. However, even at high doses, effect of SPN was similar to that of control solution (140 mM NaCl containing 5% ethanol, the solvent used for SPN and DMS). TMS was synthesized chemically and its effects on tumor growth were tested in vitro and in vivo. It should be emphasized that TMS is not chemically detectable in cells and tissues, whereas DMS has been detected by metabolic labeling (10,11) and chemical analysis. Inhibitory effect of TMS on PK-C activity in vitro was much stronger than that of DMS or SPN; however, its inhibitory effect on growth of MKN74, LU65, and Colo205 was about the same as that of DMS in vitro. The effect of TMS on MKN74 tumor growth was also very similar to the in vivo effect of DMS. TMS is readily soluble in water and therefore gave a better preparation for administration.

Although both DMS and TMS showed a clear inhibitory effect on in vitro and in vivo tumor growth, the effective doses were considerably higher than that for existing anticancer chemotherapeutic drugs, many of which are in the nM range. The use of DMS or TMS as possible reagents for modification of tumor growth through the transmembrane signaling mechanism of tumor cells is an approach similar to that with cAMP derivatives (20–22). The compound 8-chloro-cAMP was found to be the most potent activator of cAMP-dependent protein kinases, and inhibited tumor growth and induced differentiation at doses similar to or greater than those used for DMS. This compound has been found to induce megalakaryocytic differentiation (21), and growth of human lung carcinoma in nu/nu mice was inhibited by differentiation induction (22). Various compounds classified as DIs (8-chloro-cAMP, dibutyrylf AAMP, hexamethylene bisacetamide, and sodium butyrate) were compared in terms of their effect on MKN74 tumor growth. Each of these compounds required ≈16−64 μM concentration to produce a significant inhibitory effect (Fig. 8). n-Butyrate, a well-known DI, requires much higher concentration (1–5 mM) (data not shown), unless a specific targeting method with monoclonal antibody covalently linked to liposomes encapsulating sodium butyrate is used (23,24). In contrast, DMS required only ≈8 μM concentration to produce 50% inhibition of MKN74 growth. Thus, the effect of DMS in inhibition of tumor growth and possibly in modification of tumor cell physiology is equally or more potent than that of 8-chloro-cAMP. In fact, as calculated from our experiments, the effective dose of DMS or TMS for in vivo growth inhibition was ≈20 mg/kg/day or

\[ \text{Estimated Tumor Volume (mm}^3\text{)} \]

\[ \text{Days After Treatment} \]

**Fig. 5.** Effect of DMS and SPN on growth curves of in vivo LU65 tumors in mice. Arrows at the top indicate times of injection. A, •, saline (control); O, 0.5 mg DMS; 4 mice/group. B, •, saline (control); Δ, 0.5 mg SPN; 6 mice/group.

\[ \text{Estimated Tumor Volume (mm}^3\text{)} \]

\[ \text{Days After Treatment} \]

**Fig. 6.** Effect of SPN, DMS, and TMS on growth curves of in vivo MKN74 tumors in mice. Arrows at the top indicate times of injection. Treatment was initiated at day 0 as described in "Materials and Methods." A, •, 140 mM NaCl containing 5% ethanol (control); Δ, 0.5 mg SPN; O, 0.5 mg DMS; 10 mice/group. B, •, Δ same as in A; O, 0.5 mg TMS; 9 mice/group. Bars, SD.
Fig. 7. Effect of various doses of DMS on MKN74 tumor growth in mice. When MKN74 tumors reached a size of $\approx 100$ mm$^3$, daily injection of various doses of DMS was performed on 3 consecutive days. Data for growth curves were collected as described in "Materials and Methods." and on day 12 mice were killed, tumors excised, and tumor weight directly measured (Table 1). O, 0.5 mg DMS; A, 0.13 mg DMS; •, 0.03 mg DMS; @, 0.5 mg SPN; C, control.

less. In contrast, this effective dose of 8-chloro-cAMP for inhibition of human lung carcinoma LX-1 grown in nu/nu mice was found to be $\approx 60$ mg/kg/day (22). Some tumor cells, particularly highly invasive types, could be much more sensitive to TMS than to DMS, and further studies with TMS or its derivatives are expected to open up new trends of anticancer therapy through modification of signal transduction.

The general trend in development of anticancer drugs has been focused on killing of tumor cells, and many such drugs are blockers of transcription or translation. In contrast, the present study represents a new approach based on modification of transmembrane signal transduction via PK-C and possibly some yet unknown mechanism. A number of new approaches are under investigation which focus on known mechanisms of cell growth and differentiation control through growth factor receptors, protooncogenes, and other membrane-associated signaling mechanisms (25). One example involved blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors having a tyrosine analog (26), although the inhibitors may not be specific to tumor cell growth. Various structurally unrelated compounds which inhibit PK-C have been claimed to provide a basis for a new anticancer drug design (27), although no data for actual suppression of tumor growth in vivo were presented. An approach using 8-chloro-cAMP as described above (22) is one of the most successful examples, although the approach in the present study appears equally promising. On the other hand, results of the present study indicate only partial success; i.e., we were not able to inhibit or eliminate tumor growth by administration of DMS or TMS. However, these compounds may inhibit or alter malignant phenotype to a great extent, and tumors grown in animals treated with DMS or TMS may be qualitatively different (e.g., in metastatic potential) from those grown in untreated animals. Further studies along this line are in progress.

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Table 1 Weights of MKN74 tumors in nu/nu mice following treatment with SPN or DMS

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Tumor wt (mg)$^b$</th>
<th>Significance$^c$</th>
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<tr>
<td></td>
<td>2</td>
<td>3</td>
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<tr>
<td>1. Control</td>
<td>317 ± 78</td>
<td>NS</td>
</tr>
<tr>
<td>2. SPN (0.5 mg)</td>
<td>254 ± 70</td>
<td>NS</td>
</tr>
<tr>
<td>3. DMS (0.03 mg)</td>
<td>245 ± 50</td>
<td>NS</td>
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<tr>
<td>4. DMS (0.13 mg)</td>
<td>219 ± 34</td>
<td>+</td>
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<tr>
<td>5. DMS (0.5 mg)</td>
<td>172 ± 54</td>
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$^a$ MKN74 tumor cells were inoculated s.c. When estimated tumor volume reached 100 mm$^3$, mice received i.v. injections of saline (control), SPN, or DMS in the doses shown, 4 times on a daily basis. Mice were killed on day 12, tumors were excised, and actual tumor weight was determined. Each group consisted of 10 mice.

$^b$ Values represent mean ± SD.

$^c$ Significance level (P value from t test) from pairwise comparison of means for the various groups (2, 3, 4, and 5 correspond to the group numbers shown in the left-hand column). NS, not significant. +, 0.01 < P < 0.05; ++, 0.01 < P < 0.001; ++++, P < 0.001.


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