Cell Membrane Signaling as Target in Cancer Therapy II: Inhibitory Effect of N,N,N-Trimethylsphingosine on Metastatic Potential of Murine B16 Melanoma Cell Line through Blocking of Tumor Cell-dependent Platelet Aggregation

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ABSTRACT

Two phenotypic parameters, aberrant expression of protein kinase C and tumor cell-induced platelet aggregation (PA), have been correlated with abnormal growth behavior and metastatic potential of tumor cells. We recently observed that N,N,N-trimethylsphingosine (TMS) and N,N-dimethylsphingosine (DMS), but not sphingosine (SPN), had an inhibitory effect (via blocking of transmembrane signaling) on the growth of various human tumor cell lines in vitro as well as in vivo in nu/nu mice (K. Endo et al., Cancer Res., 51: 1613-1618, 1991). We therefore investigated the effects of TMS, DMS, and SPN on (a) PA induced by ADP and thrombin; (b) PA induced by melanoma cell line B16/BL6; and (c) experimental lung colonization as well as spontaneous lung metastasis of BL6 cells in syngeneic C57BL/6 mice. In experiments on agonist-induced PA, TMS inhibited PA and ATP secretion 5-fold more strongly than DMS or SPN. This effect may be based on the inhibition of M, 47,000 platelet protein phosphorylation and/or inhibition of phosphatidylinositol turnover as a transmembrane signaling pathway in platelets. Tumor cell (BL6 melanoma)-induced PA and ATP secretion were also strongly inhibited by TMS, but not by DMS or SPN. Unlike ADP- or thrombin-induced PA, BL6 cell-induced PA was not inhibited by Calphostin-C (a potent protein kinase C inhibitor) or cilostazol (a potent inhibitor of PA based on inhibition of cyclic AMP phosphodiesterase). Since many previous studies suggested that the ability of tumor cells to induce PA is related to the degree of malignancy (e.g., metastatic potential) of tumor cells, we studied the effect of TMS on lung metastatic potential. Three independent sets of experiments, as described below, all showed clear inhibition of lung metastasis by administration of TMS: (a) i.v. coinjection of BL6 melanoma cells and TMS; (b) i.v. injection of BL6 cells; (c) spontaneous metastasis to lung from s.c. BL6 tumor (TMS administered after establishment of tumor, followed by resection of tumor). In comparison to tumor growth inhibition produced by TMS or DMS, inhibition of melanoma metastasis by TMS is obvious at lower doses.

INTRODUCTION

While a number of biochemical parameters have been claimed to be associated with malignancy and metastatic potential of tumor cells (1-9), two parameters have received much attention in recent studies: (a) aberrant expression of PKC and ras protein, which play important roles in tumor cell growth, and (b) aberrant induction of PA. That is to say, metastatic potential has been correlated with the overexpression of protein kinases (10, 11), and a mutant of PKC- has been shown to induce highly malignant tumor cells with increased metastatic potential (12). Since the metastatic potential of tumor cells has also been correlated with the ability of the cells to induce PA, resulting in platelet-derived growth stimulation and endothelial adhesion (13-18), we systematically investigated the effects of TMS and SPN on agonist-dependent and tumor cell-dependent PA in relation to the inhibition of metastatic potential.

SPN functions as an inhibitor of PKC (19) as well as of PA (20), and DMS and TMS showed stronger stereospecific inhibition of PKC activity than SPN (21-23). We have also observed that DMS and TMS inhibit growth of a large variety of tumor cells in vitro and in vivo, although TMS showed much stronger inhibition than DMS on PKC activity in vitro (23). Since the metastatic potential of tumor cells can be correlated with PKC activity as well as the ability to induce PA, we were prompted to study the effect of TMS and DMS, as compared to SPN, on thrombin-, ADP-, and tumor cell-induced PA, as well as on in vitro growth and in vivo metastatic potential of B16 melanoma variants.

MATERIALS AND METHODS

Cell Lines and SPN Derivatives. B16/BL6 melanoma cells were obtained from Dr. Isiah J. Fidler (M. D. Anderson Cancer Center, University of Texas, Houston, TX) and cultured in Eagle's medium supplemented with 5% fetal calf serum, 1% nonessential amino acids, 2 mM glutamine, 1.5% vitamin solution, and 1% sodium pyruvate. Maintenance, freezing, and thawing of cells were performed strictly according to the instructions of Dr. Fidler (24). C57BL/6 mice (7-10 weeks old) were used for inoculation of melanoma cells. Lung colony metastasis was determined after tail vein injection or s.c. tumor formation followed by resection (Figs. 6 and 7). TMS, DMS, and TMS were prepared as previously described (22, 23). These preparations were free of endotoxin as determined by the Limulus amebocyte lysate assay (sensitivity limit, 0.125 ng/ml). The assay system was purchased from Associates of Cape Cod (Woods Hole, MA). Calphostin-C, a potent PKC inhibitor, was donated by Otsuka Pharmaceutical Co. (Tokyo, Japan). Cilostazol (OPC-13013), a potent inhibitor of PA through inhibition of cAMP phosphodiesterase, was donated by Otsuka Pharmaceutical Co. (Osaka, Japan).

Agonist-induced PA. Effects of various compounds were tested for three types of PA: those induced by thrombin; by ADP; and by B16 melanoma cell variant BL6. Human platelets were purchased from the American Red Cross Blood Service (Portland, OR) and diluted in Tyrode's buffer to a concentration of 3.5 x 10^9 (this was termed platelet-rich plasma, or PRP). Platelet DMS, and ADP-induced PA. Alternatively, for the study of thrombin-induced PA, washed platelets were prepared according to the method of Siess et al. (25) and suspended in Tyrode's buffer to the same concentration as for PRP. Aliquots of PRP (0.45 ml) or washed platelet suspension (3.5 x 10^9 platelets/ml) were mixed with defined concentrations of SPN, DMS, TMS, cilostazol, or Calphostin-C and incubated for 2 min. PA reaction was initiated by γ-thrombin (final concentration, 10 nM) or...
ADP (final concentration, 10 μM). PA and ATP secretions in the suspension were measured using an aggregometer (Chrono-log Corp., Havertown, PA) equipped with a computer analyzer. For the study of possible cytotoxic effects of SPN derivatives, PRP or washed platelet suspension was preincubated with TMS or DMS and supplemented with 1.5 mg/ml of ristocetin, an alkaloid which induces PA.

BL6 Melanoma Cell-induced PA. BL6 cells were detached from culture by application of 0.02% EDTA (Irvine Scientific, Santa Ana, CA), washed, and resuspended in Eagle’s medium with 5% fetal calf serum to make a suspension containing 3 × 10⁶ cells/ml. The tumor cell suspension was added to washed platelet suspension (final concentration of tumor cells, 3 × 10⁷/ml) for observation of PA. Alternatively, the tumor cell membrane fraction (100 μl) was added to the platelet suspension to induce PA. For preparation of membrane fractions, BL6 cells homogenized in 40 mM Tris-HCl (pH 7.4), 0.2 mM EDTA, and 0.25 M sucrose were centrifuged at 800 × g for 10 min. The lower phase was removed, dried, and the mixture was sonicated for 10 min for extraction. Chloroform/methanol (2:1). An aliquot of the lower phase was removed, dried, and the mixture was sonicated for 10 min for extraction. Chloroform/methanol (2:1). The chloroform/methanol extract was evaporated to dryness and then redissolved in chloroform/methanol (2:1) again for chromatography.

RESULTS

Effects of SPN, TMS, Calphostin-C, and Cilostazol on ADP- or Thrombin-induced PA. ADP- and thrombin-induced PA was completely suppressed by application of 20 and 10 μM TMS, respectively. In contrast, these processes were not inhibited by 40–50 μM DMS or 60 μM SPN (Fig. 1, A and B). ADP- and thrombin-induced ATP secretion, which is closely associated with PA, is also completely inhibited by 10–20 μM TMS (Fig. 1, D and E). Using the same system, ADP- and thrombin-induced PA was completely suppressed by the application of 5 and 2 μM cilostazol, respectively; this PA inhibitor acts through the inhibition of cAMP phosphodiesterase. However, ADP- or thrombin-induced PA was not inhibited by Calphostin-C, the potent PKC inhibitor (dose producing 50% inhibition = 50 nM) (28), possibly because of the inability of Calphostin-C to penetrate platelet membranes. TMS had a much stronger dose-dependent inhibitory effect than DMS or SPN on ADP- and thrombin-induced PA and ATP secretion (Fig. 1). A strong inhibitory effect of TMS for platelet function is probably due to its quaternary ammonium ion structure, which may enhance the basic effect of SPN structure (see “Discussion”).

Inhibitory Effect of TMS on BL6 Melanoma Cell-induced PA and ATP Secretion. Under the conditions described in “Materials and Methods,” BL6 cells induced PA and ATP secretion in about 5 min. This process was completely inhibited by 20 μM TMS but not by 60 μM DMS or SPN (Fig. 3, A and B).

Effect of TMS on Experimental and Spontaneous Lung Metastasis of BL6 Melanoma Cells. Various doses of TMS (0.1 and 0.25 mg) and SPN (0.25 mg) were injected by tail vein in C57BL/6 mice, followed after 1 h by i.v. injection of 4 × 10⁶ BL6 cells. Fourteen days after tumor cell inoculation, the mice were sacrificed, and lung colony number was determined with a dissecting microscope (experimental metastasis). Alternatively, mice were injected s.c. in the right hind foot pad with 5 × 10⁶ BL6 cells, SPN or TMS was injected i.v. on days 10, 15, and 19, and the primary tumor was resected on day 21. The mice were sacrificed 14 days after resection, and the lung colony number was counted (spontaneous metastasis).
The membrane fraction of BL6 cells also induced PA. When tumor cells were preincubated with TMS, inhibition of PA or ATP secretion was not seen (data not shown), indicating that TMS affects mainly platelets rather than tumor cells. When the membrane fraction of BL6 cells was added to platelet suspension, PA was clearly observed, although the incubation time required was about 15 min. This membrane-induced PA was completely suppressed by the application of 20 \( \mu \text{M} \) TMS (Fig. 3C).

Cilostazol, in contrast to its strong inhibitory effect on ADP- or thrombin-induced PA, produced only a weak inhibitory effect on BL6-induced PA even at high concentrations (20–40 \( \mu \text{M} \)) (Fig. 4C). Calphostin-C, which produced a strong inhibition of PKC, did not inhibit BL6-induced PA even at 5 \( \mu \text{M} \) (Fig. 4B). Thus, TMS was a much more potent inhibitor than cilostazol or Calphostin-C of tumor cell-induced PA.

Effect of SPN, DMS, and TMS on \( M_{47,000} \) Platelet Protein Phosphorylation. \( \gamma \)-Thrombin-induced \( M_{47,000} \) protein phosphorylation is shown in Fig. 5. Activation of platelets is initiated by or closely associated with phosphorylation of \( M_{47,000} \) protein, as typically shown in \( \gamma \)-thrombin-induced PA. A great enhancement of phosphorylation at the \( M_{47,000} \) band was observed (Fig. 5, Lanes 1 and 2). \( ^{32} \text{P} \) phosphorylation was inhibited in a dose-dependent manner by the addition of increasing concentrations of TMS (Lanes 3 and 4) but not by the addition of DMS (Lane 5) or SPN (Lane 6). On the other hand, Calphostin-C (Lane 7), known as a strong PKC inhibitor in in vitro enzymatic experiments (28), did not inhibit thrombin-induced \( M_{47,000} \) protein phosphorylation.

Effect of TMS on PI Turnover in Human Platelets. Sensitivity of PI turnover to thrombin-induced PA has been reported and reviewed (29). To assess whether TMS affects PI metabolism as an initial step in transmembrane signaling change, we studied the effects of 5 min of preincubation with TMS and 3 min of stimulation with thrombin on PI metabolic turnover (see "Materials and Methods"). As shown in Table 1, thrombin stimulation doubled PI turnover during the 3-min incubation, while TMS pretreatment (5 min) prior to thrombin addition reduced it back to below control level. TMS pretreatment alone had no effect on PI turnover.

Effect of SPN, DMS, and TMS on Experimental and Spontaneous Metastasis of BL6 Cells. Following i.v. coinjection of BL6 cells with TMS or SPN, lung colony number was greatly reduced (Fig. 6A). Lung metastasis was almost completely abolished in groups treated with 0.2 mg (or more) TMS. When TMS or SPN was injected i.v., followed after 1 h by i.v. injection of BL6 cells, lung colony number was still significantly reduced (Fig. 6B). Lung colony number was not significantly reduced following injection of BL6 cells that had been preincubated with 4 \( \mu \text{M} \) TMS for 24 h and washed (data not shown).

In another experiment, BL6 cells were inoculated s.c., TMS or SPN was injected i.v. three times at defined points during tumor development, the primary tumor was resected, and lung colonies were counted 5 weeks after tumor inoculation (2 weeks after primary tumor resection). Colony number, reflecting spontaneous metastasis from primary tumor, showed much greater reduction in the group treated with TMS as compared to SPN.

\[ \text{Fig. 2. Comparative dose-dependent effect of SPN, DMS, and TMS on PA and ATP secretions. PA and ATP secretions were measured as described in Fig. 1. A and C. ADP-induced PA and ATP secretion, respectively; B and D, \( \gamma \)-thrombin-induced PA and ATP secretion, respectively. O, SPN; D, DMS; \( \Delta \), TMS. Values are expressed as percentage responses relative to control experiments (no addition of SPN derivatives).} \]

\[ \text{Fig. 3. Inhibitory effect of TMS on BL6-induced PA and ATP secretion. A. PA induced by addition of 3 \times 10^7/\text{ml} BL6 cells and inhibitory effect of 20 \mu \text{M} TMS, 60 \mu \text{M} DMS, and 60 \mu \text{M} SPN. B, ATP secretion induced by addition of 3 \times 10^7/\text{ml} BL6 cells and inhibitory effect of 20 \mu \text{M} TMS, 60 \mu \text{M} DMS, and 60 \mu \text{M} SPN; C, PA induced by BL6 cell membrane (50 \mu \text{g protein/100 \mu l}) (b), as compared to that induced by BL6 cells (a), and inhibitory effect by 20 \mu \text{M} TMS of membrane-induced PA (c).} \]
effect of 0.5 and 5 mM Calphostin-C (compared to 10 mM TMS) on BL6-induced PA. Note that PA was completely inhibited by 5 mM cilostazol and 20 mM TMS on thrombin-induced PA; C, effect of 0.5 and 5 mM Calphostin-C (compared to 10 mM TMS) on BL6-induced PA. Note that Calphostin-C, which strongly inhibits PKC in the 1-10 nM range, does not inhibit BL6-induced PA even at a concentration of 5 μM.

Table 1  Effect of TMS on thrombin-induced PI turnover in human platelets

<table>
<thead>
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<th>Treatment</th>
<th>PI</th>
<th>PIP1</th>
<th>PIP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6,160 (100)</td>
<td>12,560 (204)</td>
<td>3,840 (62)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>12,560 (176)</td>
<td>18,160 (109)</td>
<td>11,360 (106)</td>
</tr>
<tr>
<td>TMS (10 μM)</td>
<td>6,400 (104)</td>
<td>11,280 (109)</td>
<td>10,320 (96)</td>
</tr>
<tr>
<td>Thrombin + TMS (10 μM)</td>
<td>3,840 (62)</td>
<td>11,920 (60)</td>
<td>5,920 (55)</td>
</tr>
</tbody>
</table>

* Values are in cpm and represent the means of two experiments. Numbers in parentheses, percentage of control.

Fig. 4. Comparison of inhibitory effects of TMS, cilostazol, and Calphostin-C on PA. A, effect of 5 μM cilostazol and 20 μM TMS on ADP-induced PA; B, effect of 2 μM cilostazol and 10 μM TMS on thrombin-induced PA; C, effect of cilostazol and TMS on BL6-induced PA. Note that PA was completely inhibited by 10 μM TMS but not by cilostazol at concentrations as high as 20-40 μM. D, effect of 0.5 and 5 μM Calphostin-C (compared to 10 μM TMS) on BL6-induced PA. Note that Calphostin-C, which strongly inhibits PKC in the 1-10 nM range, does not inhibit BL6-induced PA even at a concentration of 5 μM.

Fig. 5. Effect of TMS, DMS, and SPN on phosphorylation of M, 47,000 platelet protein. Washed platelets were labeled with [32P]phosphoric acid, preincubated with various concentrations of TMS, DMS, and SPN for 5 min, and stimulated with 10 nM γ-thrombin. The reaction was stopped by addition of Laemmli’s sample buffer, and aliquots were taken and subjected to electrophoresis. The amount of sampling is normalized according to [32P]cpm. [32P]-labeled proteins were visualized by autoradiography. Lane 1, no addition; Lane 2, 10 nM thrombin; Lane 3, thrombin + 20 μM TMS; Lane 4, thrombin + 10 μM TMS; Lane 5, thrombin + 20 μM DMS; Lane 6, thrombin + 20 μM SPN; Lane 7, thrombin + 5 μM Calphostin-C. Right ordinate, molecular weights determined using high-molecular-weight standard mixture (Sigma) for markers. A large quantity of [32P] activity associated with a low-molecular-weight component (which could be phospholipids) was run off from gels and is not shown in this figure.

In groups treated with 0.1-0.25 mg TMS, there was a significant reduction in the size of the primary tumor, but it was not as striking as the reduction in lung colony numbers (Fig. 7A).

DISCUSSION

This study is essentially an extension of our previous observation that both DMS and TMS inhibit, to roughly similar degrees, in vitro as well as in vivo tumor cell growth (23), despite the fact that TMS showed a markedly stronger inhibitory effect on in vitro PKC activity. The design of the present study was based on the hypothesis that the metastatic potential of tumor cells is closely related to their enhanced ability to induce PA (16, 30, 31). Gasic et al. (30) originally suggested that the degree of tumor cell/platelet interaction is correlated with metastatic potential. Many subsequent studies (13-17, 31, 32) have confirmed that tumor cell-induced PA is indeed an important factor in metastasis, although the underlying reason for this remains unknown.

Platelet activation is closely associated with PA and ATP secretion. The present study was therefore focused on the effects of SPN derivatives on PA and ATP secretion induced by such agonists as ADP, thrombin, and ristocetin. This study is also focused on the effect of SPN derivatives on PA induced by tumor cells and by membrane preparation therefrom. While DMS has been identified as a physiological modulator of transmembrane signal transducers (21, 22), TMS has not been identified as a physiological component. Nevertheless, TMS, and to a much lesser extent DMS and SPN, showed strong inhibition of both agonist- and tumor cell-induced PA and ATP secretion. This is probably because TMS has a quaternary ammonium ion structure which may inhibit PKC and signal transduction mechanisms with stronger pharmacological action. An important distinction was that cilostazol, a potent inhibitor of agonist-induced PA, had no effect on tumor cell-induced PA. Similarly, Calphostin-C, a potent PKC inhibitor in in vitro experiments, did not inhibit tumor cell-induced PA. In striking contrast, TMS strongly inhibited both agonist- and tumor cell-induced PA. It is also noteworthy that BL6 mem-
brane preparation also induced PA and that again this process was inhibited by TMS.

These findings suggest (a) the interesting possibility that presentation of living tumor cells to platelets is not a prerequisite for PA, since the degree of platelet aggregation induced by an equivalent membrane preparation displayed a similar response, and (b) that both tumor cell-induced and membrane-induced PA was initiated by the contact of the platelet surface with the tumor cell membrane and may require transmembrane signaling of the platelet; they also rule out the possibility (c) that ADP secretion by tumor cells is the cause of PA. However, sialic acid may not be involved in tumor cell- or membrane-induced PA, since desialylation of tumor cell or membrane did not alter the effect on PA (data not shown). Extensive further studies on the mechanism of tumor cell-induced PA are obviously needed.

The inability of Calphostin-C, a potent PKC inhibitor, to inhibit tumor cell-induced PA suggests that this reagent, in contrast to TMS, is unable to penetrate into platelets. Indeed, incubation of intact platelets with Calphostin-C for 5–20 min did not reduce 32P phosphorylation of the M₄7,000 platelet protein.

The inhibitory effect of TMS on platelet activation by agonist operates through blocking of transmembrane signaling, since both M₄7,000 platelet protein phosphorylation and PI metabolism were clearly inhibited by preincubation of platelets with TMS.

Based on this background information, we studied the effect of TMS on (a) lung colonization of BL6 cells after i.v. injection of tumor cells and (b) spontaneous lung metastasis after s.c. inoculation of tumor, with later resection of primary tumor. In both experimental systems, lung colony numbers in groups treated with TMS in the 0.1–0.5-mg range were greatly reduced relative to control or SPN-treated groups. It should be noted that the inhibitory effect of TMS on primary tumor size, although clearly observed, was not as striking as the effect on lung colony metastasis.

Expression of GPIIb/IIIa integrin and GMP-140 selectin on platelet activation through tumor cells may provide a favorable condition for adhesion of tumor cells with basement membrane or capillary endothelial cells mediated by platelets (32). F10 or BL6 cells coincubated with RGDS or YIGSR peptide sequence were previously reported to inhibit lung colony formation (13). Recent studies involving administration of peptides with repeating RGDS sequence produced much more striking effects, including the significant reduction of spontaneous metastasis (33). Obviously, the approach with peptide sequences is based on the blocking interaction of tumor cells with basement membrane components through integrin receptor. In contrast, the present approach is based on blocking tumor cell-induced PA, and possibly tumor growth per se, through the inhibition of M₄7,000 platelet protein phosphorylation and tumor cell PKC activity. These new approaches are both fundamentally distinct from established chemotherapeutic techniques which are directed against DNA and protein synthesis. The reagents used, whether specific peptide sequences or TMS, are closely related to naturally occurring cell components and are expected to have little or no cytotoxicity, in contrast to chemotherapeutic drugs. Further studies along these lines are therefore expected to provide useful tools for the prevention of cancer progression.

ACKNOWLEDGMENTS

We thank Dr. Stephen Anderson for scientific editing and preparation of the manuscript, Dr. Tatsuro Irimura for helpful advice on melanoma cell culture, and Dr. Isaiah J. Fidler (M. D. Anderson Cancer Center, University of Texas) for donating original BL6, F10, and F1 melanoma cell lines.

Note Added in Proof

The SPN used for studies of inhibition of ADP- or thrombin-induced platelet aggregation (PA) (as shown in Figs. 1–3) was in the form of SPN sulfate, while TMS used was in the form of TMS chloride. Inhibitory effect of SPN sulfate was very weak as shown in the figures; however, when free SPN was used, ADP- or thrombin-induced PA was significantly inhibited, i.e., 10 μM and 20 μM concentrations of free SPN inhibited ADP-induced PA by 25 and 50%, respectively. This effect is still much weaker than that of TMS, i.e., 5 μM and 20 μM concentrations of TMS inhibited ADP-induced PA by 55 and 80%, respectively. The inhibitory effect of TMS was consistently observed regardless of salt form, as shown in Figs. 1–3. Neither SPN sulfate nor free SPN inhibited tumor cell-induced PA, whereas TMS produced strong inhibition of this type of aggregation (Fig. 4). On the other hand, both SPN sulfate and free SPN, but not TMS, inhibited in vivo tumor cell metastasis.

REFERENCES

CELL MEMBRANE SIGNALING AS TARGET IN CANCER THERAPY


Errata

There are two errors in the article by Chang et al., entitled “A Single 3.7-Kilobase Messenger RNA Hybridizes to Immediate-Early Promoter-Enhancer of Human Cytomegalovirus in HL-60 and Acute Myeloid Leukemia Cells,” which appeared in the September 1, 1991 issue of Cancer Research (pp. 4724-4728). In the “Materials and Methods” section, the last sentence under “Cells and Cultures” reads: “The leukemic blast cells were prepared from the peripheral blood of AML patients by centrifugation on a Ficoll-Paque gradient (density, 1.097).” This sentence should have read: “The leukemic blast cells were prepared from the bone marrow of AML patients by centrifugation on a Ficoll-Paque gradient (density, 1.077).”

There is also an error in the article by Okoshi et al., entitled “Cell Membrane Signaling as Target in Cancer Therapy II: Inhibitory Effect of Melanoma Cell Line through Blocking of Tumor Cell-dependent Platelet Aggregation,” in the November 15, 1991 issue of Cancer Research (pp. 6019-6024). The last sentence of the “Note Added in Proof” reads: “On the other hand, both SPN sulfate and free SPN, but not TMS, inhibited in vivo tumor cell metastasis.” It should have read: “On the other hand, TMS, but not SPN sulfate or free SPN, inhibited in vivo tumor cell metastasis.”
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