Sphingosine kinase 1 is a relevant molecular target in gastric cancer

Thorsten Fuereder[a], Doris Hoeflmayer[a], Agnes Jaeger-Lansky[a], Doris Rasin-Streden[a], Sabine Strommer[a], Niels Fisker[c], Bo J. Hansen[c], Richard Crevenna[b] and Volker Wacheck[a]

Sphingosine kinase 1 (Sphk1), a lipid kinase implicated in cell transformation and tumor growth, is overexpressed in gastric cancer and is linked with a poor prognosis. The biological relevance of Sphk1 expression in gastric cancer is unclear. Here, we studied the functional significance of Sphk1 as a novel molecular target for gastric cancer by using an antisense oligonucleotide approach *in vitro* and *in vivo*.

Gastric cancer cell lines (MKN28 and N87) were treated with Sphk1 with locked nucleic acid–antisense oligonucleotides (LNA–ASO). Sphk1 target regulation, cell growth, and apoptosis were assessed for single-agent Sphk1 LNA–ASO and for combinations with doxorubicin. Athymic nude mice xenografted with gastric cancer cells were treated with Sphk1 LNA and assessed for tumor growth and Sphk1 target regulation, *in vivo*.

***In vitro*, nanomolar concentrations of Sphk1 LNA–ASO induced an approximately two-fold reduction in Sphk1 mRNA in both the cell lines. This resulted in a 1.6-fold increase in apoptosis and inhibited the growth of gastric cancer cells by more than 50% ($P < 0.05$). The combination of Sphk1 LNA–ASO with doxorubicin resulted in significant chemosensitization. *In vivo*, Sphk1 LNA–ASO displayed neither mRNA target regulation in xenografts nor antitumor activity in two independent nude mouse xenograft models.**

In conclusion, the potent single-agent activity and the synergistic effect of Sphk1 LNA–ASO in combination with chemotherapy *in vitro* highlight Sphk1 as a biologically relevant molecular target for gastric cancer. Further studies are warranted to overcome the challenge of delivering Sphk1-targeting RNA-therapeutics to solid tumors *in vivo*. *Anti-Cancer Drugs* 22:245–252 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Departments of [a]Clinical Pharmacology, [b]Physical Medicine and Rehabilitation, Medical University, Vienna, Austria and [c]Santaris Pharma A/S, Horsholm, Denmark

Correspondence to Dr Volker Wacheck, Department of Clinical Pharmacology, Section of Experimental Oncology and Molecular Pharmacology, Medical University of Vienna, Waehringer Guertel 18-20, Vienna 1090, Austria
Tel: +43 1 40 400 2981; fax: +43 1 40 400 2998;
e-mail: volker.wacheck@meduniwien.ac.at

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Introduction

Sphingolipids comprise a family of membrane lipids, which have been recently identified as effector molecules for angiogenesis, motility, and multidrug resistance of cancer cells [1,2]. One of these membrane sphingolipids is ceramide, which is N-acylated sphingosine [3] that is further metabolized to sphingosine. Numerous studies support the hypothesis that ceramide is important in the apoptotic response of cancer cells to death inducers. For example, Fas ligand, hypoxia and chemotherapeutics, such as doxorubucin, irinotecan, etoposide, and gemcitabine [3] induce the accumulation of ceramide [1]. Although ceramide and sphingosine inhibit cell proliferation and promote apoptosis, sphingosine 1 phosphate (S1P) promotes cell proliferation and blocks many forms of apoptosis [1]. In essence, the fate of a cell is determined by the balance of ceramide/sphingosine and S1P [4].

The rate-limiting enzyme of S1P synthesis is sphingosine kinase (Sphk), which thereby critically regulates the ceramide/sphingosine–S1P rheostat [5]. Sphk is a highly conserved lipid kinase. To date, two mammalian Sphks, Sphk1 and Sphk2, have been identified [4]. Although the function of Sphk1 has been intensively studied, the role of Sphk2 is far less understood. Sphk1 has been implicated in cell transformation and tumor growth and can be activated by a variety of growth factors, cytokines, and mitogens [6]. As Sphk mRNA is significantly overexpressed compared with the normal tissue in several tumor entities including gastric cancer, Sphk1 seems to be a promising molecular target for novel tumor treatment strategies [7]. Recently it has been shown that Sphk1 mRNA overexpression is associated with gastric cancer progression and poor survival of gastric cancer patients [8,9]. Compounds known to inhibit Sphk1, such as *N,N*-dimethylsphingosine, *N,N,N*-trimethylsphingosine, or L-threo-dihydrosphingosine (Safingol), have shown anticancer activity *in vivo*, but they do not inhibit Sphk1 specifically [7,10,11].

An alternative approach for specifically targeting a protein of interest is represented by antisense oligonucleotides (ASO). ASO are chemically modified single-strand nucleotides capable of inhibiting protein expression by

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complexing with their complementary target mRNA, and thus preventing their translation to proteins [12,13]. Third-generation locked nucleic acid (LNA)–ASO have shown promising in-vivo activity in several animal models, as they display high target specificity and affinity for the target *in vivo*.

The aim of this study was to explore the biological relevance of Sphk1 as a molecular target for gastric cancer *in vitro* and *in vivo* by using an LNA antisense approach.

Materials and methods

Cell lines

NCI-N87 human gastric cancer cells (intestinal type) were purchased from American Type Culture Collection. MKN28 human gastric cancer cells (diffuse type) were kindly provided by Professor H. Yokozaki, First Department of Pathology, Hiroshima University School of Medicine. Both cell lines show prominent expression of the Sphk1 protein ([8], own data) and have been used for a number of in-vitro and in-vivo ASO studies [14–16]. Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and an antibiotic mixture containing penicillin, streptomycin, and amphotericin B (all Gibco, Carlsbad, California, USA; Invitrogen, Carlsbad, California, USA). All cell lines were cultured in a humidified 5% CO_2, 95% ambient air atmosphere at 37°C.

Compounds

All oligonucleotides were kindly provided by Santaris Pharma (Horsholm, Denmark). All oligonucleotides were phosporothioated LNA gapmers. The sequence of Sphk1 ASO was GGGcactggctcCTCc. Capital letters indicate LNA-modified riboses whereas small letters indicate deoxyriboses. A scramble control oligonucleotide (SCO) with no target in the human transcriptome was used as negative control. FAM-labeled random control oligonucleotides were used for uptake studies. For the in-vitro experiments, stock solutions with a final concentration of 6 μmol/l were prepared and further diluted to the desired concentration. For transfection, the cells were incubated with a mixture of lipofectamine 2000 (Invitrogen) and the respective oligonucleotides were diluted in Opti-MEM (Gibco), as described earlier [14]. For the combination experiments, doxorubicin (Ebewe, Unterach, Austria) at a concentration of 10 nmol/l was added.

Electronic cell counting

Tumor cells in exponential growth were harvested and seeded at 10 000 cells per well in 24-well plates and incubated overnight at 37°C. The cells were then transfected with or without oligonucleotides. Forty eight to 96 h after transfection, cell counts were measured using a Coulter Counter (Beckman, Brea, California, USA).

Fluorescence-activated cell sorting

For the detection of apoptosis, the cells were treated with therapeutics of interest and harvested at 24 h. The cells were harvested, washed, and stained with Annexin V (BD, Franklin Lakes, New Jersey, USA) and 7-AAD (BD) according to the manufacture's instructions and subsequently analyzed by flow cytometry.

For the uptake experiments, the cells were transfected with the labeled oligonucleotide for 4 h and analyzed using fluorescence-activated cell sorting.

Real-time PCR

Total RNA was isolated from the cells using the TRI Reagent (Sigma, St Louis, Missouri, USA). cDNA was synthesized using a first-strand cDNA synthesis kit (Fermentas, Ontario, Canada). Real-time PCR (RT-PCR) was performed using the TaqMan Universal Master Mix and an ABI Prism 7700 Sequence Detection system, according to the manufacturer's instructions (Applied Biosystems, Foster City, California, USA). Sphk1 primers and TaqMan probes, included in the Sphk1 TaqMan Gene Expression Assay, #hs00184211 m1, were purchased from Applied Biosystems. Relative gene expression was quantified using the comparative threshold cycle method and glyceraldehyde 3-phosphate dehydrogenase as an internal standard.

Western blot

Proteins from the transfected cells were extracted as described earlier [17]. A total of 10 μg protein per lane were loaded on a 10% SDS-polyacrylamide gel and electrophoretically separated followed by blotting on polyvinylidene fluoride membranes. After blocking for 1 h, the membranes were incubated with primary antibodies diluted in blocking solution at 4°C over night. Antibodies were directed against Sphk1 (dilution 1:500, Abgent, San Diego, California, USA) and actin (purified from rabbit serum, dilution 1:10 000; Sigma). Primary antibodies were detected by horseradish peroxidase-conjugated secondary antirabbit antibody (Santa Cruz, California, USA; 1:4000) and visualized by chemiluminescence using ECL (Thermo Fisher Scientific, Waltham, Massachusetts, USA) substrate.

Fluorescence and phase contrast microscopy

For phase contrast and fluorescence microscopy, the cells were seeded in two-well chamber slides and transfected with a FAM-labeled oligonucleotide, as described above. After 4 h, the cells were washed and evaluated immediately under an Axioscope (Zeiss, Vienna, Austria). Images were merged using the cell^F imaging software (Olympus Europe, Hamburg, Germany).

Tumor xenograft model

Pathogen-free, 4–6-week-old female athymic nude mice (Harlan Winkelmann, Borchen, Germany) were housed

under sterile conditions. The mice ($n = 4$/group) were inoculated with MKN28 cells bilaterally. The mice were assigned randomly to one of the following treatment groups:

(1) Sphk1 LNA–ASO 20 mg/kg solved in NaCl
(2) SCO 20 mg/kg solved in NaCl
(3) Carrier control (saline): NaCl

The mice were treated thrice weekly intraperitoneally (i.p.) with the respective treatments. Tumor volume was assessed biweekly by calliper measurements and calculated according to the approximation formula: volume (mm^3) = 4/3 π × (long diameter^2 × short diameter)/2. Treatment was initiated when tumor volumes reached approximately 150 mm^3 and continued until the tumor volume reached the abortion criteria judged by an independent veterinarian as the predefined surrogate endpoint for terminal disease. The study was approved by the local animal welfare committee and was performed in accordance with the local regulations.

Statistical analysis

The analysis started with analysis of variance, followed by posthoc testing.

Statistical data in this study represent the results of the Bonferroni posthoc test. P values less than 0.05 were considered to be of statistical significance.

Results

Downregulation of Sphk1 induces apoptosis and reduces tumor cell proliferation

Modulating the expression of a gene of interest can be achieved by different approaches. We evaluated the potency of phosphorothioate LNA gapmer oligonucleotides to specifically silence Sphk1 expression in gastric cancer cell lines. To test for transfection efficacy, uptake of FAM-labeled LNA oligonucleotides in gastric cancer cells was assessed by fluorescence microscopy and fluorescence-activated cell sorting analysis. In the presence of lipofectamine as an uptake enhancer, fluorescence microscopy showed the cellular uptake of FAM-labeled LNA oligonucleotides (Fig. 1a). Although in the absence of lipofectamine only approximately 10% of the gastric cancer cells displayed LNA cellular uptake (Fig. 1b, medium panel), LNA oligonucleotide uptake was detected in approximately 70% of the gastric cancer cells in the presence of lipofectamine (Fig. 1b, lower panel). Targeted downregulation of Sphk1 by LNA–ASO was studied in the mRNA and protein level by RT-PCR and western blotting. As shown in Fig. 1c, Sphk1 ASO treatment in the nanomolar range resulted in an approximately two-fold reduction in Sphk1 mRNA at 24 h compared with control LNA that did not reduce the Sphk1 mRNA levels in MKN28 cells ($P < 0.05$). Similar results were observed in other gastric cancer cell lines (i.e. N87). Downregulation of Sphk1 mRNA was accompanied with a reduction in the Sphk1 protein levels (Fig. 1d). Further dose escalation or prolongation of the incubation period did not result in a more pronounced target modulation in both the cell lines (data not shown).

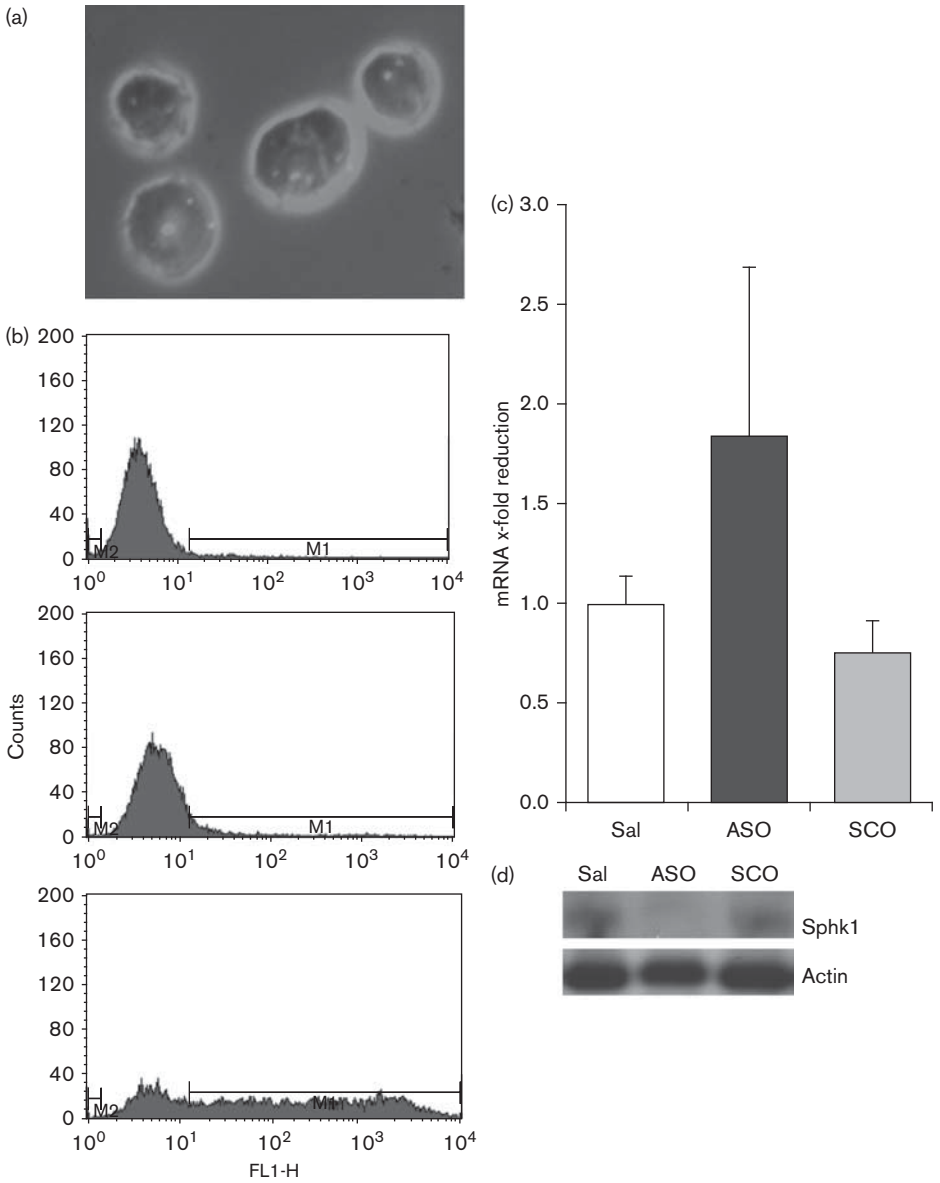
We next assessed the biological relevance of Sphk1 downregulation by LNA with respect to apoptotic cell death. Depending on the cell type, inhibition of Sphk1 has been described to influence the propensity of cells to undergo apoptotic cell death without any additional apoptotic stimulus [18]. Thus, we explored the induction of apoptotic cell death after transfection with Sphk1 ASO. Cell death analyzed by annexin V staining showed a clear increase in apoptosis (Fig. 2a). Compared with the control treatments, gastric cancer cells treated with Sphk1 LNA showed a 60% higher number of apoptotic cells. This increase in apoptotic cell death was reflected by a significant decrease in the number of gastric cancer cells. Forty-eight hours after transfection, Sphk1 LNA–ASO led to significant growth inhibition of gastric cancer cells with a more than 50% reduction in cell counts (MKN28 Fig. 2b, N87 not shown). Scramble control LNA did not result in a significant reduction in the cell number compared with saline control, at any time point.

Synergistic chemosensitization by Sphk1 LNA oligonucleotides

To assess the biological relevance of Sphk1 as a chemoresistance factor for gastric cancer cells, we pretreated gastric cancer cells with Sphk1 LNA–ASO and exposed gastric cancer cells to the ceramide-inducing chemotherapeutic doxorubicin. As shown in Fig. 3a, the combination of Sphk1 LNA and doxorubicin clearly facilitated the induction of apoptosis in gastric cancer cells. Although doxorubicin alone at a concentration of 10 nmol/l promoted apoptosis by approximately 20%, pretreatment of gastric cancer cells with Sphk1 LNA–ASO raised the fraction of apoptotic cell death by 140% as compared with the saline control. This clear increase in apoptosis differed statistically significantly from any other treatment group. Of note, SCOs did not enhance the production of apoptotic cells compared with doxorubicin monotreatment.

The lowering of the apoptotic threshold by Sphk1 LNA–ASO translated in a synergistic chemosensitization of gastric cancer cell proliferation (Fig. 3b). Although Sphk1 LNA–ASO and doxorubicin reduced cell numbers by −38 and −32%, respectively, the combination of Sphk1 LNA–ASO and doxorubicin lowered gastric cancer cell numbers by −60% compared with saline control. After the notion of specific target regulation by Sphk1 LNA–ASO, treatment with SCO plus doxorubicin did not show any statistically significant difference to the doxorubicin monotherapy. The synergistic chemosensitization by Sphk1 LNA–ASO to doxorubicin (combination index:

Fig. 1



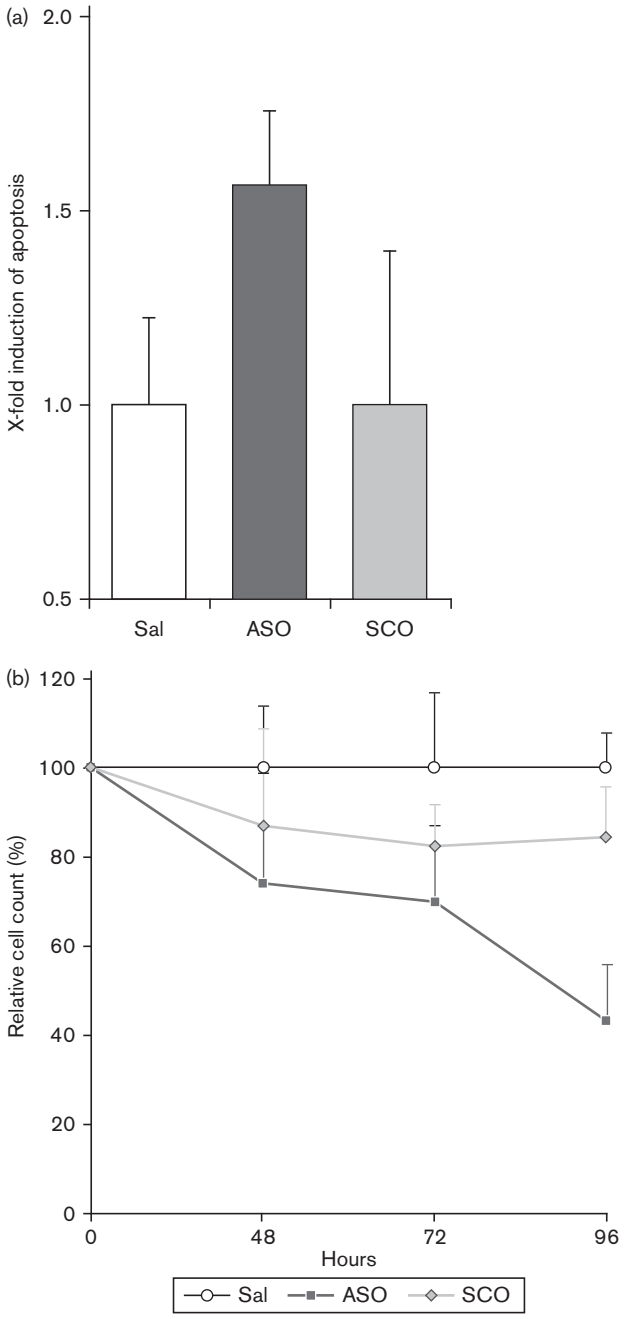
Uptake and target regulation by sphingosine kinase 1 (Sphk1) locked nucleic acid (LNA) in gastric cancer cells. (a) Uptake of FAM-labeled oligonucleotides by gastric cancer cells. MKN28 cells were incubated with 12.5 nmol/l FAM-labeled oligonucleotides in the presence of lipofectamine. Images captured by a fluorescence microscope and a phase-contrast microscope were merged. A representative example is presented. (b) Quantification of antisense oligonucleotide uptake in gastric cancer cells. MKN28 were incubated with lipofectamine only (upper panel) or with 12.5 nmol/l FAM-labeled oligonucleotides in the absence (medium panel) and in the presence of lipofectamine (lower panel) for 48 h. Cells with a fluorescence intensity above the autofluorescence of lipofectamine-only-treated cells (i.e. upper panel) were defined as positive for oligonucleotide uptake. A marker (M1) was set to quantify the number of cells above the threshold of fluorescence (FL-1) indicating FAM-labeled oligonucleotide uptake. (c) Gastric cancer cells (MKN28) were transfected with Sphk1 LNA–antisense oligonucleotides (ASO) (12.5 nmol/l), scramble control oligonucleotide (SCO) (12.5 nmol/l), and saline (Sal). Twenty-four hours after incubation, mRNA was extracted and Sphk1 mRNA was analyzed by real time-PCR. Bars represent the mean of three independent experiments, ± standard deviation. (d) Downregulation of Sphk1 protein by Sphk1 LNA–ASO. MKN28 were transfected with Sphk1 LNA–ASO (12.5 nmol/l), SCO (12.5 nmol/l), and Sal. Protein expression was assessed after 48 h by western blotting.

0.9) underlines the relevance of Sphk1 as a chemoresistance factor in gastric cancer cells. Similar results for target regulation, apoptosis induction, and chemosensitization were observed in a second gastric cancer cell line (i.e. N87, data not shown).

Sphk1 locked nucleic acid–antisense oligonucleotides *in vivo*

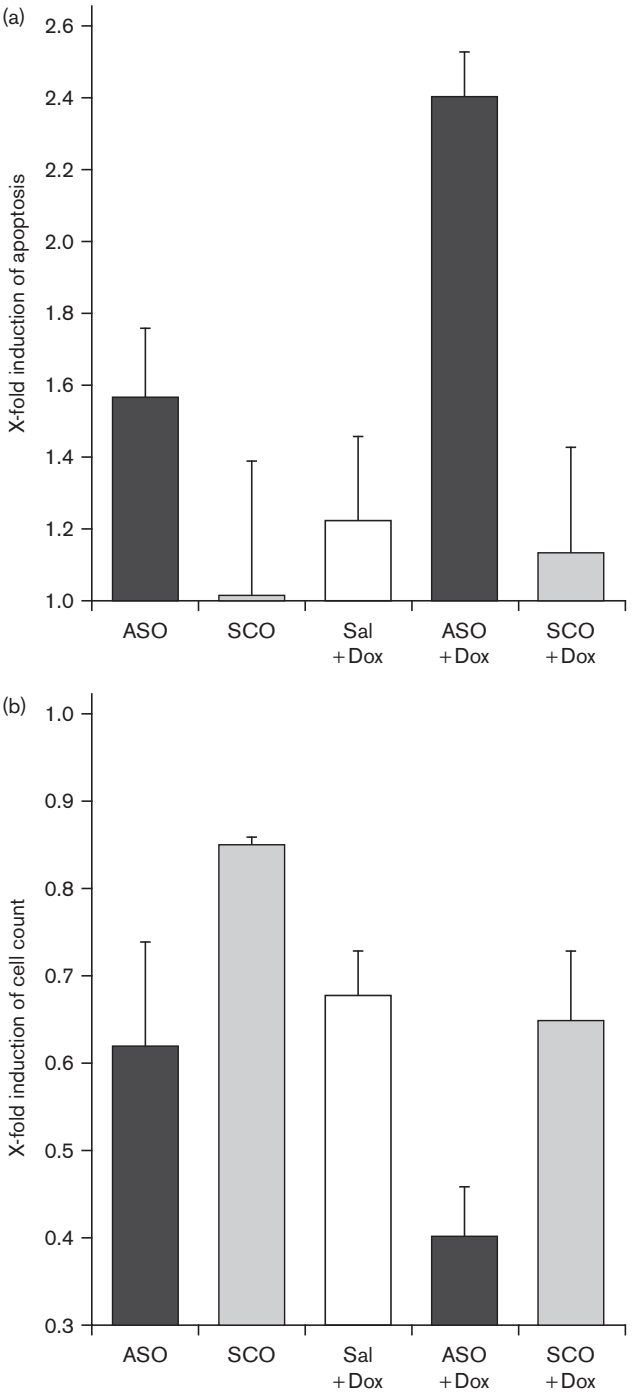
On the basis of the promising in-vitro data, we conducted in-vivo studies investigating the effect of Sphk1 LNA–ASO in two independent gastric cancer xenograft models

Fig. 2



Sphingosine kinase 1 (Sphk1) locked nucleic acid (LNA) inhibits gastric cancer cell growth and triggers apoptosis *in vitro*. (a) Annexin V staining of gastric cancer cells (MKN28) treated with Sphk1 LNA–antisense oligonucleotides (ASO) (12.5 nmol/l) or scramble control oligonucleotide (SCO) (12.5 nmol/l). Bars represent the mean of three independent experiments, ± standard deviation. (b) Gastric cancer cells (MKN-28) were transfected with Sphk1 LNA–ASO (12.5 nmol/l), SCO (12.5 nmol/l), and saline (Sal). Cell count was measured for up to 96 h by electronic cell counting. Bars represent the mean of three independent experiments, ± standard deviation.

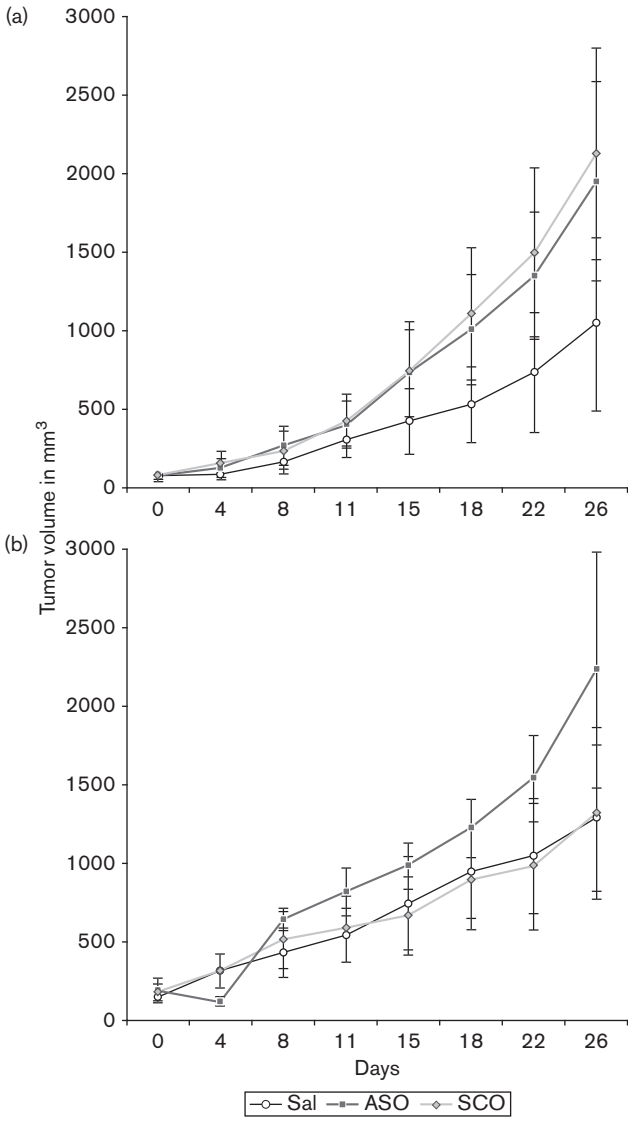
Fig. 3



Sphingosine kinase 1 locked nucleic acid chemosensitizes gastric cancer cells to (DOX). Gastric cancer cells (MKN28) were transfected with sphingosine kinase 1 locked nucleic acid–antisense oligonucleotides (ASO) (12.5 nmol/l) and/or 10 nmol/l doxorubicin and apoptosis (a) and proliferation (b) was measured at 48 h. Bars represent the mean of three independent experiments, ± standard deviation. Dox, doxorubicin; Sal, saline; SCO, scramble control oligonucleotide.

(MKN28 and N87). After the establishment of palpable tumor disease, mice were treated with Sphk1 LNA–ASO, SCO, or saline control for 3 weeks with 20 mg/kg i.p. thrice weekly. All treatments were well tolerated without any obvious signs of toxicity monitored by weekly weight measurements and clinical assessment. In contrast to the in-vitro findings, Sphk1 LNA–ASO treatment did not result in any inhibition of tumor growth, neither in the MKN28 nor in the N87 nude mouse xenograft model (Fig. 4). Analysis of human Sphk1 mRNA expression by RT-PCR in tumor xenografts excised at the end of the experiment showed heterogeneous expression of Sphk1 mRNA across all the treatment groups with no statistically significant difference among the treatment groups (Fig. 5).

Fig. 4



Sphingosine kinase 1 locked nucleic acid therapy does not inhibit gastric cancer tumor growth *in vivo*. Gastric cancer xenografts of MKN28 cells (a) and N87 cells (b) were established in athymic nude mice. Mice treated with 20 mg/kg sphingosine kinase 1 locked nucleic acid–antisense oligonucleotides (ASO) or 20 mg/kg scramble control oligonucleotide (SCO) for 4 weeks. Mice were treated thrice weekly till the tumor size reached the abortion criteria. Tumor volume was assessed twice weekly by calliper measurement. Tumor volumes of are presented as means, ± standard deviation. Sal, saline.

Discussion

Gastric cancer is one of the most common cancers worldwide [3]. Despite the fact that surgical techniques, chemotherapy, and supportive care have improved during the last decades, the overall survival time has not increased significantly [3]. The standard chemotherapy regime currently used is based on platins, anthracyclins, and pyrimidine analogs [19,20]. Very recently, Sphk, which was found to be overexpressed in gastric cancer tissue, has been shown to be involved in gastric cancer progression and is associated with poor survival [7,8]. Therefore, downregulating Sphk1 using LNA oligonucleotides in gastric cancer seems to be a valuable approach to evaluate the biological relevance of Sphk1 in gastric cancer.

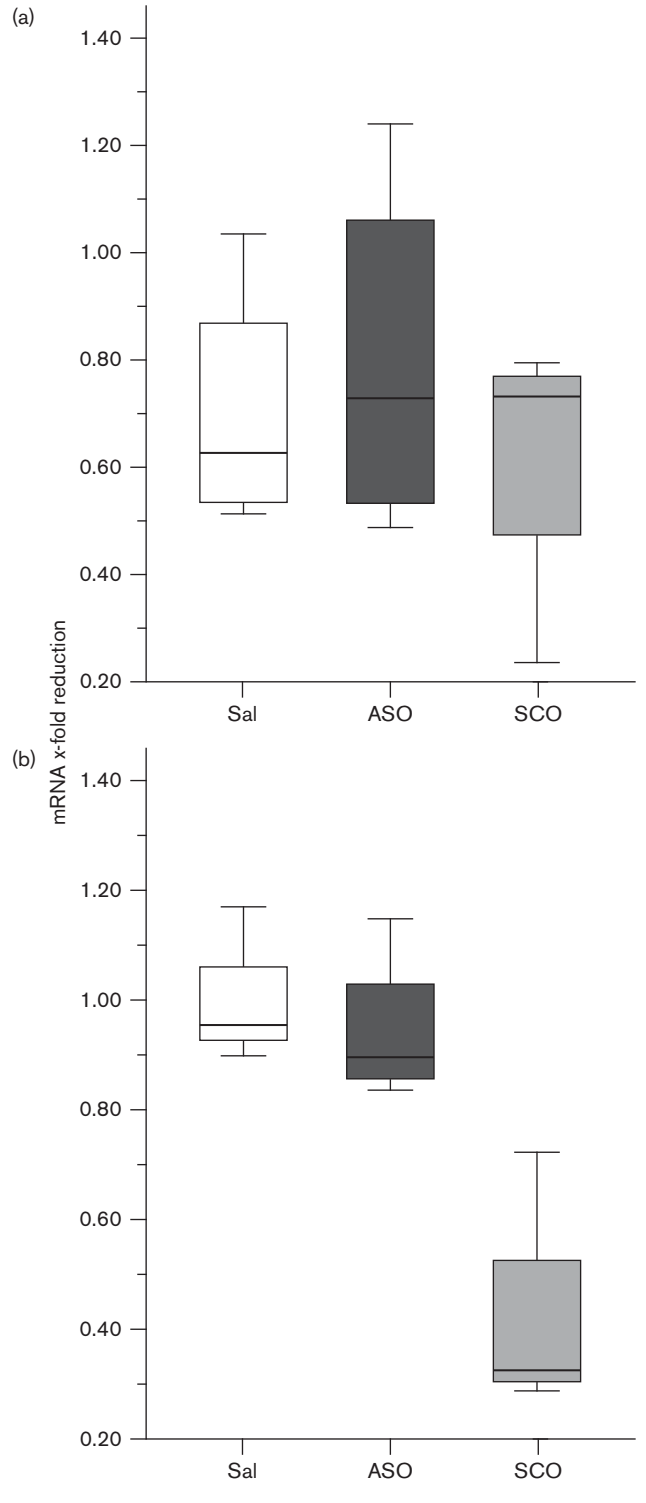
In this study, we showed that Sphk 1 is a relevant target for gastric cancer therapy *in vitro*. Sphk1 downregulation by LNA–ASOs resulted in a significant inhibition of gastric cancer cell growth, triggered apoptosis, and led to sensitization to doxorubicin. Systemic treatment with Sphk1 LNA–ASOs did not affect tumor proliferation and Sphk1 mRNA expression, *in vivo*. Thus, the promising in-vitro findings were not mirrored *in vivo*. What are the reasons for the observed discrepancy?

For the biological activity of ASOs there are two essential preconditions. First the ASO used must have sufficient target affinity and potency for downregulating their specific target mRNA. We showed profound and specific mRNA target downregulation of the Sphk1 isoenzyme by LNA–ASO in the nanomolar range *in vitro*, underlining the high affinity and potency of this class of ASO. This finding was confirmed on the protein level. Sphk1 protein was abrogated 24 h after Sphk1 mRNA reduction. This result is in line with the estimated half-life of Sphk1 mentioned in the literature, which is approximately 24 h [21].

Besides affinity, the second precondition for the successful use of ASO is the adequate delivery of these compounds. For a specific ASO effect, cellular uptake is necessary to bind to the targeted mRNA sequence [22]. We showed *in vitro* that the intracellular uptake of LNA–ASO in gastric cancer cells resulted in target downregulation and clear antiproliferative activity.

The fact that we neither observed target regulation nor antiproliferative activity in the two independent tumor models *in vivo* indicates that the lack of activity *in vivo* is likely because of insufficient intracellular Sphk1 LNA–ASO

Fig. 5



Sphingosine kinase 1 (Sphk1) locked nucleic acid therapy does not modulate Sphk1-mRNA *in vivo*. Mean x-fold reduction of human Sphk1–mRNA of gastric cancer MKN28 (a) or N87 (b) xenografts as measured by real time-PCR, ±standard deviation. ASO, antisense oligonucleotides; Sal, saline; SCO, scramble control oligonucleotide.

delivery. It is well known that delivery is essential and often limiting when performing oligonucleotide experiments *in vivo* [23]. Several biological barriers, such as slow diffusion in the extracellular matrix or insufficient endocytosis, may hinder the delivery of oligonucleotides to the target site [23]. It is unlikely that serum degradation LNA might have contributed to the observed inactivity *in vivo*, as third-generation LNA oligonucleotides have high nuclease stability resulting in superior serum stability compared with the earlier generation of oligonucleotides [13]. Even the in-vivo pharmacokinetics of this particular Sphk1 LNA–ASO are unknown; the LNA–ASO dose (20 mg/kg) and treatment regimen (thrice weekly i.p.) applied in this in-vivo study are comparable with the successful ASO studies performed earlier.

To conclude, we showed that Sphk1 is a valuable target for gastric cancer therapy. Specific downregulation of Sphk1 LNA–ASO induces apoptosis and sensitizes gastric cancer cells to the standard-of-care chemotherapeutics *in vitro*. However, until the availability of sufficient methods for the delivery of Sphk1 RNA-therapeutics, in-vivo targeting of Sphk1 in oncology remains challenging.

Acknowledgement

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