Sphingosine Kinase 1 (SphK1) overexpression contributes
to cetuximab resistance in human colorectal cancer models

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STATEMENT OF TRANSLATIONAL RELEVANCE

In this study we investigated the contribution of sphingosine kinase 1 (SphK1) overexpression to cetuximab resistance, both intrinsic or acquired, in colorectal cancer. In preclinical models with intrinsic or acquired resistance, SphK1 inhibition through different approaches partially restored sensitivity to cetuximab. The effect of fingolimod, a clinically available antagonist of sphingosine-1-phosphate (S1P) receptor, in cetuximab resistant colorectal cancer models was also demonstrated, both in vitro and in vivo. Moreover, we observed a correlation between SphK1 expression and cetuximab response in metastatic colorectal cancer patients. Therefore, our data support cetuximab plus fingolimod as a novel therapeutic combination to be tested in the clinical setting for cetuximab resistant colorectal cancer patients.
ABSTRACT

**Purpose.** Although the anti-Epidermal Growth Factor (EGFR) monoclonal antibody (mAb) cetuximab is an effective strategy in colorectal cancer therapy, its clinical use is limited by intrinsic or acquired resistance. Alterations in the ‘sphingolipid rheostat’ - the balance between the proapoptotic molecule ceramide and the mitogenic factor sphingosine-1-phosphate (S1P) - due to sphingosine kinase 1 (SphK1) overactivation have been involved in resistance to anticancer targeted agents. Moreover, cross-talks between SphK1 and EGFR-dependent signalling pathways have been described.

**Experimental design.** We investigated SphK1 contribution to cetuximab resistance in colorectal cancer, in preclinical in vitro/in vivo models and in tumor specimens from patients.

**Results.** SphK1 was found overexpressed and overactivated in colorectal cancer cells with intrinsic or acquired resistance to cetuximab. SphK1 contribution to resistance was supported by the demonstration that SphK1 inhibition by N,N-dimethyl-sphingosine (DMS) or silencing via siRNA in resistant cells restores sensitivity to cetuximab, whereas exogenous SphK1 overexpression in sensitive cells confers resistance to these agents. Moreover, treatment of resistant cells with fingolimod (FTY720), a S1P receptor (S1PR) antagonist, resulted in re-sensitization to cetuximab both in vitro and in vivo, with inhibition of tumor growth, interference with signal transduction, induction of cancer cells apoptosis and prolongation of mice survival. Finally, a correlation between SphK1 expression and cetuximab response was found in colorectal cancer patients.

**Conclusion.** Our data could contribute to clarify SphK1 role in cetuximab resistance and may suggest SphK1 inhibition as a part of novel targeting strategies potentially effective also in resistant colorectal cancer patients.
INTRODUCTION

In the last years, cetuximab and panitumumab, two monoclonal antibodies (mAbs) targeting the Epidermal Growth Factor Receptor (EGFR), have proven to be effective in combination with chemotherapy or as single agents for treatment of metastatic colorectal cancer (1). However, as is common in cancer therapy, intrinsic or acquired resistance to anti-EGFR drugs by different mechanisms have been widely observed (2). Molecular alterations such as mutations in genes codifying for EGFR-dependent signal transducers (K-Ras, B-Raf, PI3K, PTEN) have been related to primary refractoriness to cetuximab in colorectal cancer. Other mechanisms, such as alternative signalling by different TKRs or induction of angiogenesis by tumor-derived factors, could be also involved, particularly in the onset of resistance over prolonged treatment (3). Therefore, there is an urgent need to identify novel predictive markers of response to cetuximab as well as to develop novel therapeutic strategies for colorectal cancer patients with intrinsic or acquired resistance to this agent.

Sphingolipids are a family of molecules enriched in lipid rafts that contribute to their unique biochemical properties. Sphingolipid metabolites including ceramide, sphingosine, ceramide-1-phosphate (C1P) and sphingosine-1-phosphate (S1P) have emerged as bioactive signalling molecules, with ceramide and sphingoid bases serving as activators of cell death pathways whereas S1P and C1P primarily exert mitogenic effects (4). Altered regulation of the S1P/ceramide ratio can lead to an imbalance in the 'sphingolipid rheostat' through which these sphingolipid metabolites influence cell fate and tissue homeostasis. The balance of these molecules is critically regulated by sphingosine kinase (SphK), which converts sphingosine to S1P by phosphorylation (5). Two SphK isoforms have been cloned and characterized to date. SphK1, activated by a variety of growth factors, cytokines and mitogens, is up-regulated in many cancers, often correlating with higher clinical grade and resistance to standard therapy (6; 7). Consistently, interference with SphK1 activity by dominant-negative mutants or competitive inhibitors such as N,N-dimethyl-sphingosine
(DMS), as well as inhibition of S1P by mAbs or S1P receptors antagonists such as fingolimod (FTY720, Novartis), blocks tumorigenesis and tumor angiogenesis in cancer models (8). Moreover, recent studies demonstrated that alterations of ceramide/S1P rheostat may be involved in the regulation of resistance to both chemotherapeutics and targeted agents (9-14).

Based on this evidence, and since several reports showed cross-talks between SphK1 and EGFR-dependent signalling pathways (15; 16), we analyzed the contribution of sphingolipid rheostat alterations to cetuximab resistance in human colorectal cancer models. Moreover, we investigated the combination of cetuximab and fingolimod as a therapeutic strategy potentially effective in colorectal cancers resistant to cetuximab.
METHODS

Compounds. N,N-dimethyl-sphingosine (DMS) was purchased from Sigma. Cetuximab was kindly provided by ImClone Systems. Fingolimod was kindly provided by Novartis International AG (Basel, Switzerland).

Cell cultures. Human SW48, GEO, SW480, LS174T, HCT116, HT29 and LoVo colorectal carcinoma cell lines were obtained from the American Type Culture Collection (ATCC). GEO-CR (Cetuximab Resistant) cells were established as previously described (17).

MTT survival assay. Cells (10^4 cells/well) were grown in 24-well plates and exposed to increasing doses of cetuximab, DMS or fingolimod, alone or in combination. The percentage of cell survival was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Apoptosis assay. Apoptosis was quantified using the Annexin V-FITC apoptosis kit (BD Biosciences, Franklin Lakes, NJ, USA).

Immunoprecipitation and Western blot analysis. Total cell lysates from cell cultures or tumor specimens were resolved by 4-15% SDS-PAGE and probed with anti-human, polyclonal pEGFR, EGFR and SphK1, monoclonal pMAPK, MAPK and S1PR1/EDG-1 (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal pAkt, Akt and SphK1 phospho-Ser225 (Cell Signaling Technologies, Beverly, MA), monoclonal actin (Sigma-Aldrich, Milan). Immunoreactive proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA). Densitometry analysis were performed with Image J software (NIH).
**Sphingosine kinase assay.** SphK1 activity was measured by using Sphingosine Kinase Activity Assay Kit (Echelon Biosciences, Salt Lake City, UT, USA) (18; 19).

**Quantification of S1P.** Quantification of S1P from cell protein extracts, tumor lysates or mice sera by ELISA assay was performed using a validated S1P Assay Kit (Echelon Biosciences) (20; 21).

**Hairpin siRNA construct for Sphk1.** Endogenous Sphk1 expression was down-regulated with sequence-specific pSilencer-siSphK1 851 (Clone1) and 1118 (Clone2), as previously described (22), as well as with Silencer® Select Validated siRNA s16957 and s16959. A nonsense sequence was used as a negative control.

**Transfection of human SphK1 in human cell lines.** Human SphK1 (GenBank accession n° AF200328) cDNA was cloned into pCMV6-AC-GFP vector (OriGene Rockville, MD USA). Transient transfections were performed using the Lipofectamine 2000 (Invitrogen).

**Nude mice cancer xenograft models.** Five weeks old Balb/c athymic (nu+/nu+) mice (Charles River Laboratories, Milan, Italy) maintained in accordance with institutional guidelines of the University of Naples Animal Care Committee were injected subcutaneously (s.c.) with GEO-CR cells (10⁷ cells/mice) re-suspended in 200 µL of Matrigel (CBP, Bedford, MA). After 7 days, tumors were detected and groups of 10 mice were randomized to receive: Cetuximab 10 mg/kg intraperitoneally (i.p.) three times a week for 3 weeks, fingolimod 2.5 mg/kg i.p. three times a week for 3 weeks, or the combination. Tumor volume (cm³) was measured using the formula \( \pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2 \) as previously reported (23).

**Morphological and immunohistochemical analysis of mouse and human tumor samples.**
The morphological evaluation of necrosis-grade was done on hematoxilin/eosin stained 5-μm slides by a semiquantitative score (0: Absence; 1: Low level; 2: Intermediate level; 3: High level). The presence of apoptotic cells was determined immunohistochemically on formalin fixed paraffin embedded (FFPE) 5-μm tissue slides by analyzing the expression of annexin V by the alkaline phosphatase system (EnVision, Dako Carpenteria, Calif).

SphK1 immunohistochemistry was carried out on FFPE 5-μm tumor tissue sections. Validation of antibodies (24; 25) and analysis of SphK1 expression on FFPE colorectal cancer tissues is described in the Supplementary Methods section.

**Statistical analysis.** The results of *in vitro* experiments were analyzed by Student’s *t* test and expressed as means and standard deviations (SDs) for at least three independent experiments performed in triplicates. The statistical significance was determined by one-way ANOVA and Dunnett’s multiple comparison post-test regarding tumor growth, by log-rank test concerning mice survival. All reported *P* values were two-sided. Analyses were performed with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

The results of immunohistochemical analysis on colorectal cancer tissue specimens and the clinical parameters were evaluated for statistical significance. SphK1 expression pattern was analyzed as following: Level 3 (high expression) *versus* levels 0 (no expression), 1 (low expression) and 2 (intermediate expression). A multivariate analysis was performed to evaluate the correlation of SphK1 levels and other clinical/pathological variables with response rate to cetuximab-based therapy (responders *versus* non-responders). Patients were classified as “responders” in case of complete or partial response, and as “non-responders” in case of stable or progressive disease. Chi-square test and logistic regression were used. Estimation of likelihood events for disease progression or death was calculated according to Kaplan-Meier. Statistical differences between curves were calculated using the log-rank test. Hazard and Odds ratio were assessed by multivariate
analysis. A $P$ value $\leq 0.05$ was considered statistically significant. All the analysis were performed using IBM SPSS Statistics 18 package software (SPSS Inc. Chicago, IL).
RESULTS

SphK1 is overexpressed and overactivated in human colorectal cancer cell lines resistant to cetuximab.

Based on the suggested correlation between SphK1 and resistance to anticancer targeted agents (9-14), we analyzed SphK1 expression in a panel of human colorectal cancer cell lines, both sensitive or resistant to cetuximab. They include SW48 cells, harbouring a wild-type \textit{K-Ras} gene; GEO cells, positive for a \textit{K-Ras} mutation (Gly12Ala); the cetuximab resistant derivative GEO-CR cells; SW480, LS174T, HCT116 and LoVo cells, harbouring \textit{K-Ras} mutations (Gly12Val for SW480; Gly12Asp for LS174T, Gly13Asp for HCT116 and LoVo); HT29 cells, positive for a \textit{B-Raf} mutation (Val600Glu). As shown in supplementary figures S1A and B, cetuximab inhibited survival and induced apoptosis more efficiently in SW48 and GEO than in the other cell lines. These data are consistent with the \textit{K-Ras} or \textit{B-Raf} status of the cell lines, except for GEO cells which are sensitive to cetuximab despite the \textit{K-Ras} mutation, as reported by our and other groups (19; 26).

When analyzed by Western blot analysis, levels of SphK1 protein, as well as of its activated/phosphorylated form (SphK1 phospho-Ser225), resulted higher in resistant compared to cetuximab sensitive cells. Among the resistant cell lines, low levels of SphK1 expression/activation were detected only in HCT116 cells (Fig.1A), as previously reported (27). Consistently, SphK1 enzymatic activity (Fig.1B), as well as levels of S1P measured in cell lysates (Fig.1C), were higher in cells with intrinsic or acquired resistance to cetuximab. Lower SphK1 activity and S1P levels were detected in HCT116 cells (Fig.1B, C).

We then tested sensitivity of colorectal cancer cell lines to DMS, a potent competitive inhibitor of SphK1. Consistently with the previous finding, higher doses of DMS were needed to achieve complete enzyme saturation and survival inhibition in resistant cells (Fig.1D). Moreover, the proapoptotic molecule ceramide, a precursor of sphingosine, induced apoptosis less efficiently
in resistant than in sensitive cells, consistently with the idea that increased SphK1 levels mediate S1P synthesis by ceramide in resistant cells. Also in this case, the only exception was the HCT116 cell line (Fig.1E). As a confirm of SphK1 overactivity, SphK1 inhibition by DMS potentiated the effects of ceramide in resistant GEO-CR and SW480 cells, with a two-fold increase in apoptosis after combined treatment compared to ceramide alone (Fig.1F).

SphK1 inhibition partially restores sensitivity to cetuximab in resistant colorectal cancer cell lines.

Based on the overexpression of SphK1 in resistant cells, we investigated the involvement of this kinase in cetuximab resistance by performing combined treatment of GEO-CR, SW480, LS174T, HT29 and LoVo cells with DMS and cetuximab. DMS was able to significantly restore sensitivity to cetuximab in resistant cells (Supplementary Fig.S2A). Most interestingly, while neither DMS or cetuximab were able to produce significant apoptosis induction in resistant cells, the combination of these agents was effective; this result was comparable to that obtained with cetuximab alone in sensitive cells (Supplementary Fig.S2B). These data demonstrate that SphK1 blockade may restore cetuximab activity in resistant cancer cells.

We also studied the effects of modulating SphK1 expression in resistant cancer cell lines. SphK1 gene silencing via siRNA in GEO-CR, SW480, HT29 and LoVo SphK1-overexpressing resistant cells caused a marked decrease in SphK1 protein expression (Fig.2A and Supplementary Fig.S3A). SphK1 silencing was not achieved in LS174T cells due to low transfection efficiency (data not shown). In all the tested cells, treatment with SphK1 siRNA led to reduced Akt phosphorylation/activation (Fig.2A and Supplementary Fig.S3A), SphK1 enzyme activity (Fig.2B) and S1P production (Supplementary Fig.S3B). Moreover, as shown in figure 2C, SphK1 silencing increased sensitivity to cetuximab-induced apoptosis in resistant cells. Conversely, SphK1 overexpression in GEO and SW48 sensitive cells through a full-length expression vector increased SphK1 and SphK1 phospho-Ser225 protein levels (Fig.2D and Supplementary Fig. S3C), enzyme
activity (Fig.2E) and S1P production (Supplementary Fig.S3D). As a further confirm, SphK1 overexpression significantly prevented cell death in response to cetuximab in GEO/SphK1 and SW48/SphK1 cells compared to controls. Moreover, in these cells, SphK1 inhibition by DMS significantly restored sensitivity to cetuximab-induced apoptosis (Fig.2F).

**The S1PR antagonist fingolimod restores sensitivity to cetuximab in colorectal cancer cell lines.**

To further assess the role of SphK1 in cetuximab resistance, we used fingolimod (FTY720), a S1PR antagonist that could also act as a SphK1 inhibitor. This drug is currently available for the treatment of multiple sclerosis (28) and has showed anticancer properties in different models of human cancers (8; 29; 30). This agent moderately inhibits survival of all cell lines, with an IC$_{50}$ $\geq$ 5µM (data not shown). Then we evaluated fingolimod capability to restore cetuximab activity in resistant cells. As shown in figures 3A and B, fingolimod significantly potentiated survival inhibition and apoptosis induction by cetuximab in GEO-CR, SW480, HT29 and Lovo cells. Therefore, we analyzed the effect of the combination cetuximab plus fingolimod on SphK1 enzymatic activity and S1P production. In all cell lines, cetuximab did not affect SphK1 activity and S1P levels, while fingolimod reduced them. Statistically significant reductions of SphK1 enzymatic activity and S1P levels were detected with the combined treatment (Fig.3C, D). Finally, we investigated the activation of EGFR signal transducers mainly involved in the onset of resistance to cetuximab. In four different resistant cells, cetuximab was ineffective in inhibiting Akt and/or MAPK phosphorylation. Fingolimod produced a variable effect, while the combined treatment strongly interfered with both Akt and MAPK phosphorylation/activation in all cell lines (Fig.3E).

**Fingolimod restores sensitivity to cetuximab in colorectal cancer xenografts in nude mice.**

In order to confirm the antitumor effect of the combination fingolimod plus cetuximab also in *in vivo* models of cetuximab resistance, we xenografted GEO-CR cells in nude mice.
On day 70 (10 weeks after tumor cells injection) all the mice in the control group reached the maximum allowed tumor size of about 2 cm$^3$. GEO-CR tumors treated with cetuximab initially responded to this agent, but then resumed an exponential growth rate, reaching 2 cm$^3$ on day 91. Fingolimod inhibited growth of tumors, that did not reach the size of 2 cm$^3$ until the end of the experiment, on day 105. The combination of cetuximab and fingolimod caused a potent and long-lasting cooperative antitumor activity, with about 70% of growth inhibition (tumor size of 0.6 cm$^3$) until day 105, 11 weeks after treatment withdrawal. Comparison of tumor sizes among different treatment groups was statistically significant (Fig. 4A). Consistently, as shown in figure 4B, mice treated with the combination demonstrated a statistically significantly prolonged median survival duration compared to the controls (median survival 102.5 vs 37.50 days, hazard ratio = 0.07552, 95% CI = 0.02130 to 0.2677, $P < 0.0001$) or to mice treated with cetuximab as single agent (median survival 102.5 vs 57, hazard ratio = 0.1570, 95% CI = 0.04962 to 0.4966, $P = 0.0016$). Treatments were well tolerated; no weight loss or other signs of acute or delayed toxicity were observed (Supplementary table S1).

Western blot analysis on tumor samples from mice sacrificed on day 25 demonstrated that fingolimod not only inhibits phosphorylation of SphK1 on Ser225, but efficiently interferes also with EGFR-dependent signal transduction, by reducing EGFR, Akt and MAPK activated forms. The combination was more efficient than fingolimod alone, producing an almost total suppression of EGFR and Akt phosphorylation/activation. No alterations in expression of SphK1 and S1PR1 were detected (Fig. 4C). ELISA assays on tumor lysates and mice sera revealed that fingolimod significantly reduces S1P production by tumor cells, but the combined treatment was much more effective (Fig. 4D).

Based on the proapoptotic effect observed in vitro with the combination of cetuximab and fingolimod, we performed imaging of apoptosis in GEO-CR tumor-bearing nude mice, 72 hours after the first treatment. As shown in supplementary figure S4, a relevant apoptosis induction was detected in mice treated with the combination compared to mice treated with cetuximab or
fingolimod as single agents. We also investigated the long-term induction of apoptosis by performing immunohistochemical analysis of annexin V on tumor samples from mice sacrificed on day 25: a relevant staining was found only in tumors treated with both cetuximab and fingolimod (Fig. 5A). Moreover, the necrosis-grade was low in the control, intermediate in the cetuximab- or fingolimod-treated tumors, and high in the combination-treated tumors (Fig. 5B).

**SphK1 expression is related to cetuximab response in colorectal cancer patients.**

Based on this body of data, and since alterations of ceramide/S1P rheostat mediated by SphK1 overexpression have been involved in reduced response to therapy in human cancers (9-12; 14), we evaluated SphK1 expression in paraffin-embedded, archived clinical tumor tissue specimens obtained from 50 cases of K-Ras wild-type colorectal cancer patients enrolled in controlled clinical trials and treated with cetuximab-containing regimens. To evaluate SphK1 expression, three different Abs were tested, but only one yielded a strong signal intensity (Supplementary Fig. S5A). This polyclonal Ab was validated for specificity and reproducibility by using a previously described algorithm (25). Briefly, it showed a band of the expected molecular weight in Western blot analysis, produced a specific and localized staining when titered on tissue microarray (TMA) containing control tissues, and was reproducible between different runs and lots (Supplementary Fig. S5B, C).

Representative pictures of samples with different SphK1 expression levels are shown in Supplementary figure S6A. As reported in table 1, 22 out of the 50 tumor samples (44%) expressed high levels of SphK1. Among them, 19 (87%) derived from patients who did not respond to cetuximab-based therapy. Statistical analysis demonstrated that the correlation between high levels of SphK1 expression and poor response to cetuximab was significant \( P = 0.03 \). Moreover, as shown in Supplementary figure S6B, progression free survival (PFS) seemed in favour of patients with low levels of SphK1 expression, with an advantage of 2.4 months (median PFS 23.6 vs 14.0 weeks, \( P = 0.05 \)). Despite a trend in favour of patients with low SphK1 levels, the difference in
terms of overall survival (OS) did not reach the statistical significance (median OS 144.9 vs 82.7 weeks, \( P = 0.5 \); data not shown). To exactly delineate the role of SphK1 expression as a predictive/prognostic marker in the cohort of patients presented, a multivariate analysis was performed including other potential confounding factors, such as chemotherapy line, type of cetuximab-containing regimen, tumor stage at the time of diagnosis. As reported in supplementary table S2, SphK1 expression was the only factor showing a statistically significant correlation with response to cetuximab \( (P = 0.03) \).

Overall, these data support the hypothesis that SphK1 expression may correlate with resistance to EGFR targeted therapy in colorectal cancer patients.
DISCUSSION

Although a number of molecular alterations have been identified as responsible for resistance to EGFR inhibitors available in clinical practice, such as cetuximab for colorectal cancer, each of these mechanisms only partially justifies the lack of response in patients. Therefore, the search for further determinants of resistance may help to better select patients potentially responsive to cetuximab and to develop novel therapeutic strategies for resistant cancers (1; 2).

Based on the evidence that alterations of ceramide/S1P rheostat may be involved in resistance to biological agents (9; 13; 14) and since some reports showed cross-talks between SphK1 and EGFR-dependent pathways (15; 16; 31), we investigated the role of SphK1 in the onset of resistance to cetuximab in colorectal cancers. To this aim, we analyzed SphK1 expression and activation in preclinical models of colorectal cancer, both sensitive or with intrinsic/acquired resistance to cetuximab. We found that SphK1 is overexpressed and overactivated in colorectal cancer cell lines resistant to cetuximab. In fact, high expression of the activated form of SphK1, produced by MAPK-mediated phosphorylation on Ser225 (32), as well as high levels of enzyme activity and S1P production, were detected in resistant cells. Then, we investigated whether SphK1 inhibition could restore cetuximab sensitivity in resistant cancer cells. To this aim, we used different approaches, including DMS, a potent, even if not specific (33), competitive inhibitor of SphK1, the S1PRs antagonist fingolimod, and siRNAs specific for SphK1. By combining each of these tools with cetuximab on resistant cells, we demonstrated that SphK1 inhibition was able to partially restore cetuximab capability to affect cell survival, apoptosis and EGFR-dependent signal transduction. We particularly focused on signal transducers classically involved in the development of resistance to EGFR inhibitors, such as the PI3K/Akt/mTOR and the Ras/MEK/MAPK pathways: The combination of cetuximab with fingolimod produced a strong suppression of Akt and MAPK phosphorylation/activation in resistant cells. These results are consistent with other studies demonstrating the capability of S1P to induce cell proliferation and survival through activation of...
the Akt pathway (34-36). Most importantly, the re-sensitization to cetuximab induced by SphK1 inhibition was observed in different models of resistance: SW480, HT29 and LoVo cells, whose intrinsic resistance to cetuximab is related to *K-Ras* or *B-Raf* mutations and consequent overactivation of the Ras/MAPK pathway, and GEO-CR cells, whose acquired resistance is due to PI3K/Akt overactivation (17).

It has been described that S1P produced by SphK1 may function as a second messenger inside the cell or may be secreted in order to bind to S1PRs on the cell surface. This signalling, involved in several human diseases including cancer, is defined as “inside-out” S1P signalling (37; 38). Therefore, the re-sensitization to cetuximab that we observed in our cell models may depend on cross-talks of EGFR pathway with both intracellular S1P and/or extracellular S1P/S1PRs signalling pathways. By comparing the effects of SphK1 and S1PRs inhibitors we attempted to explain this point and discriminate between intracellular and extracellular effects of S1P. Fingolimod has been initially defined as a S1PRs antagonist: Its phosphorylated form, produced by SphK2, binds to S1PRs and elicits their polyubiquitination, endocytosis and degradation (39). In this study, we observed no alteration in S1PR1 protein expression after treatment of GEO-CR xenografts with fingolimod. This result seems to rule out that the effect of fingolimod depends on its antagonistic activity on S1PRs. However, it has been described that fingolimod can act also as a SphK1 inhibitor (29). Therefore, the re-sensitization to cetuximab in our cell models may depend on cross-talks of EGFR pathway with intracellular S1P signalling rather than with extracellular S1P/S1PRs signalling pathways. Further investigations are needed to clarify this point.

Since several preclinical studies reported the activity of fingolimod in human cancer models (8), the antitumor effect of this agent in colorectal cancers with acquired resistance to cetuximab was investigated also *in vivo*, in nude mice xenografted s.c. with GEO-CR cells. The combination of cetuximab and fingolimod caused a potent and long-lasting cooperative antitumor activity, with inhibition of tumor growth, interference with signal transduction, induction of apoptosis and prolongation of mice survival.
As a further confirm of our preclinical data, we examined SphK1 expression in tumor specimens from colorectal cancer patients. To date, SphK1 overexpression has been described in colorectal cancers compared to corresponding normal tissues (40), but we reported for the first time an interesting correlation between SphK1 expression and poor response to cetuximab therapy. This result should be interpreted with caution due to the limited number of patients included in the analysis and it could be used to design dedicated, prospective clinical trials to explore the potential role of SphK1 as a biological marker of resistance to cetuximab.

Taken together, we demonstrate for the first time that SphK1 inhibition is effective in restoring cetuximab antitumor activity in colorectal cancers resistant to this agent. Moreover, since fingolimod is a clinically available drug (28), the results of the present study suggest cetuximab plus fingolimod as a novel therapeutic strategy to be tested in the clinical setting for colorectal cancer patients with resistance to cetuximab.
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REFERENCES


TABLES

Table 1. Correlation of SphK1 expression with response to cetuximab-based therapy in colorectal cancer patients.

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<thead>
<tr>
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\(^a\)P=0.03 vs Responders group
FIGURE LEGENDS

Figure 1. Overexpression and overactivation of SphK1 in human colorectal cancer cell lines resistant to cetuximab.
(A) Western blot analysis of SphK1 and SphK1 phospho-Ser225 expression. Lower panels show the relative optical density of pSphK1 (Ser225) and SphK1 normalized to the actin level. (B) SphK1 activity was measured by using SphK1 Activity Assay Kit. (C) Levels of S1P production (µM), as measured by ELISA assays on cell lysates. (D) Percent of survival of cells treated with increasing doses of DMS (0.1 to 5 µM), as measured by the MTT assay. *, 2-sided P < 0.005 versus control. Bars, SDs. (E) Percent of apoptosis of cells treated with ceramide (10 µM), as measured by the annexin V assay. *, 2-sided P < 0.005 versus control. (F) Percent of apoptosis of GEO-CR and SW480 cells treated with ceramide (10 µM), DMS (1 µM), or the combination, as measured by the annexin V assay. *, 2-sided P < 0.005 versus ceramide alone.

Data represent the mean (±SD) of three independent experiments, each performed in triplicate. Bars, SDs.

Figure 2. Effects of SphK1 modulation on cetuximab sensitivity in resistant colorectal cancer cell lines.
(A) Western blot analysis of protein expression in GEO-CR, SW480, HT29 and LoVo cells transfected with a SphK1 specific siRNA or with a negative control. (B) SphK1 activity was measured in cells transfected with a SphK1 specific siRNA or with a negative control by using SphK1 Activity Assay Kit. *, 2-sided P < 0.005 versus cells transfected with the negative control. (C) Percent of apoptosis of cells transfected with a SphK1 specific siRNA or a negative control and treated with cetuximab (140 nM), as measured by the annexin V assay. *, 2-sided P < 0.005 versus cells transfected with the SphK1 specific siRNA, but untreated with cetuximab. (D) Western blot analysis of protein expression in GEO and SW480 cells transfected with human SphK1 cDNA or an
empty vector. (E) SphK1 activity was measured in cells transfected with human SphK1 cDNA or an empty vector by using SphK1 Activity Assay Kit. *, 2-sided $P < 0.005$ versus cells transfected with the empty vector. (F) Percent of apoptosis of cells transfected with human SphK1 cDNA or an empty vector and treated with cetuximab (140 nM), DMS (1 µM) or the combination, as measured by the annexin V assay. *, 2-sided $P < 0.005$ versus cetuximab alone.

Data represent the mean ($±$SD) of three independent experiments, each performed in triplicate. Bars, SDs.

**Figure 3. Effects of the S1PR antagonist fingolimod on cetuximab sensitivity in colorectal cancer cell lines.**

(A) Percent of survival of cells treated with increasing doses of cetuximab (7 to 350 nM), in presence or not of fingolimod (1 µM), as measured by the MTT assay. *, 2-sided $P < 0.005$ versus cells treated with cetuximab alone. (B) Percent of apoptosis of cells treated with cetuximab (140 nM), fingolimod (5 µM), or the combination, as measured by the annexin V assay. *, 2-sided $P < 0.005$ versus control. (C) SphK1 activity was measured by using SphK1 Activity Assay Kit. **, 2-sided $P < 0.05$ and *, 2-sided $P < 0.005$ versus untreated control cells. (D) Percent of S1P production, as measured by ELISA assays on cell lysates. **, 2-sided $P < 0.05$ versus control. (E) Western blot analysis of protein expression on GEO-CR, SW480, HT29 and LoVo cells treated for 24 hours with cetuximab (140 nM), fingolimod (1 µM), or the combination.

Data represent the mean ($±$SD) of three independent experiments, each performed in triplicate. Bars, SDs.

**Figure 4. Effects of the combination cetuximab plus fingolimod on tumor growth, survival and signal transduction of mice xenografted with GEO-CR resistant tumors.**

(A) After 7 days following subcutaneous injection of GEO-CR cells, mice were randomized (10/group) to receive cetuximab, fingolimod or their combination, as described in the Methods
section. The one-way ANOVA test was used to compare tumor sizes among treatment groups at the median survival time of the control group (35 days). They resulted statistically significant for the combination versus single agents ($P < 0.0001$). (B) Median survival resulted statistically significant for the combination versus control or cetuximab (log-rank test). (C) Western blot analysis was performed on total lysates from tumor specimens of two mice sacrificed on day 25. (D) Quantification of S1P (µM) on tumor lysates and mice sera by ELISA assays. **, 2-sided $P < 0.05$ and *, 2-sided $P < 0.005$ versus control. Data represent the mean (±SD) of three independent experiments, each performed in triplicate, and are presented relative to control. Bars, SDs.

**Figure 5. Effects of the combination cetuximab plus fingolimod on apoptosis and necrosis of GEO-CR tumor xenografts.**

(A) Immunohistochemical analysis of the apoptotic marker annexin V on FFPE 5-µm tissue slides. 

(B) Morphological evaluation of necrosis-grade on hematoxilin/eosin stained 5-µm slides.
GEO-CR tumor xenografts

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Sphingosine Kinase 1 (SphK1) overexpression contributes to cetuximab resistance in human colorectal cancer models

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