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“ Src inhibitors act through different mechanisms in Non-Small Cell Lung Cancer Models depending on EGFR and RAS mutational status”

Roberta Marciano

University of Naples Federico II
Dipartimento di Medicina Molecolare e Biotecnologie Mediche

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. Rosa R, Marciano R, Malapelle U, Formisano L, Nappi L, D'Amato C, D'Amato V, Damiano V, Marfe G, Del Vecchio S, Zannetti A, Greco A, De Stefano A, Carlomagno C, Veneziani BM, Troncone G, De Placido S, Bianco R. Sphingosine Kinase 1 (SphK1) overexpression contributes to cetuximab resistance in human colorectal cancer models. Clin Cancer Res. Clin Cancer Res. 2013 Jan 1;19(1):138-47

ABSTRACT

Lung cancer is the leading cause of cancer death worldwide.EGFR tyrosine kinase inhibitors (TKIs)gefitinib, erlotinib and afatinib, are approved in first line for metastatic Non-Small Cell Lung Cancer (NSCLC) with sensitizing epidermal growth factor receptor (EGFR) mutations. Intrinsic or acquired resistance to EGFR often related to RAS or secondary EGFR mutations, is a relevant clinical issue in NSCLC management. Although Src TK has been involved in such resistance in preclinical models, clinical development of Src inhibitors has been so far limited and unsuccessful in NSCLC therapy.

To better define the molecular targets of the Src TKIs saracatinib, dasatinib and bosutinib, we used a variety of techniques including kinase assays, molecular modelling analysis, and *in vitro*/*in vivo* studies on NSCLC cell lines with different EGFR/RAS mutational profile and EGFR sensitivity to EGFR TKIs.

Kinase inhibition assays supported by docking analysis demonstrated that all the compounds are able to directly inhibit not only Src, but also EGFR TK variants. However, in cell lysates only saracatinib efficiently reduced EGFR activation, while dasatinib was the most effective agent in inhibiting Src TK. Consistently, in EGFR-activating mutant, erlotinib sensitive cells, saracatinib showed anti-proliferative effects due to possible EGFR inhibition.

In EGFR wt/RAS mutant cells, poorly dependent on EGFR activation and thus erlotinib resistant, Src inhibition by dasatinib interfered with cell proliferation and signal transduction. Based on these assumptions, we tested the following combinations: in EGFR-addicted cells, saracatinib with anti-EGFR drugs. (erlotinib or cetuximab), and in RAS mutant, erlotinib resistant models, dasatanib with the MEK inhibitor selumetinib. These combinations were effective both *in vitro* and in nude mice, inhibiting tumor growth, prolonging mice survival and interfering with signal transduction. Importantly, the combination of saracatanib and cetuximab was effective also in condition of T790M dependent EGFR resistance.

In conclusion, Src inhibitors may act with different mechanisms, in NSCLC cell lines, depending on EGFR/RAS mutational profile. Integration of anti-Src agents with EGFR or MEK inhibitors could represent an effective therapeutic option for different cohorts of NSCLC patients.

BACKGROUND

Lung cancer (NSCLC) is the leading cause of cancer-related death worldwide (Siegel RL Cancer J Clin 2015). approximately 85% of lung cancer are histologically defined as non-small cancer (NSCLC) and the majority of patients present an advanced disease for which no curative treatments are available.

The epidermal growth factor receptor (EGFR) is a well characterized mutated oncogene in NSCLC. Activation of the EGFR pathway may promote

tumor growth and progression, stimulating cancer cell proliferation, invasion and metastasis and inhibiting apoptosis. The most common activating mutations are in-frame-deletion in exon 19 or in exon 18 and the L858R point-mutation in exon 21. Both types of mutationincrease the sensitivity of the tumor to anilinoquinazoline inhibitors of EGFR, mostlikely byrepositioning critical residues surrounding the ATP-binding cleft of the tyrosine kinase domain of the receptor, thereby stabilizing their interactions with both ATPand its competitive inhibitor(Lynch NEJM 2004; Paez Science 2004; Pao PNAS 2004).Indeed, mutant EGFR kinase binds the TKIs more tightly than the wild-type. These constitutive kinase activation are predominantly with adenocarcinoma histology and the EGFR tyrosine kinase inhibitors (TKIs) gefitinib, erlotinib and afatinib represent a relevant therapeutic option for NSCLC patients with EGFR activating mutations (Rosell Lancet Oncol 2011; Mok NEJM 2009; Sequist JCO 2013). Unfortunately,*de novo* resistance to TKIs is often observed and virtually all patients who initially respond ultimately develop acquired resistance. Mechanisms of *de novo* resistance include K-RAS (15-25%) or B-RAF (2-3%) mutations, alterations in the exon 20 of the EGFR (~50%), such as the T790M substitution, activation of phosphoinositide-3-Kinase (PI3K)/Akt or insulin-like growth factor 1 receptor (IGF-1R) signaling. Acquired resistance may depend on second-site EGFR mutations (50%; i.e. T790M), MET or HER2 amplification, PI3K mutations, activation of AXL, PI3K or IGF-1R pathways, small cell transformation.In about half of the cases, tumors biopsied after disease progressioncontain a

second-site mutation in the EGFR kinase domain. The most common (>90%) alteration involves a C→Tchange at nucleotide 2369 in exon 20, which results in substitution of methionine for threonine at position 790 (T790M). Thissubstitution is analogous to the BCR-ABL T315I change foundin patients with chronic myelogenousleukemias, who have developedacquired resistance to imatinib(GorreScience 2001). Based uponcrystal structure analyses, the EGFRT790M substitution may impairbinding of either gefitinib or erlotinib to the EGFR ATP-bindingpocket.The change could also alter the relative affinity of ATP versus drug.Like other drug-sensitizing *EGFR* mutations, the T790M changeby itself has been shown to increase kinase activity and oncogenicpotential when compared with wild-type protein(Yun PNAS 2008; PaoPLoS Med 2005; Zhang Nat Gen 2012; Gainor JCO 2013; Yu CCR 2013; Balak, Clin. Cancer Res. 2006; Kobayashi. N. Engl. J. Med. 2005; Godin-HeymannCancer Res. 2007). Alternative strategies for the treatment of patients after failure of EGFR TKIs are considered high-priority areas of research (GridelliCLin Lung Cancer 2013).

Although EGFR is generally activated through ligand binding and autophosphorylation of its cytoplasmic tail, it is well established that Src non-receptor TK, one of the EGFR downstream transducers, is able to transactivate EGFR by phosphorylating tyrosine 845 (Y845); this event may contribute to full receptor activation (Tice PNAS 1999). Src is a member of SFKs that are non receptor tyrosine kinases (nRTKs) including Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk and Yrk. Src can physically associate with activated EGFR, thus resulting

in conformational changes and Srcautophophorylation at tyrosine 416 (Y416) and transient activation. In turn, this activation leads to phophorylation of several targets including EGFR on tyrosine 845 (Y845), that results in the receptor ability to enhance EGFR-mediated signaling. Elevated SFK activity is found in human tumors, including lung cancer. Although its frequency of detection varied among the different subgroups, SFK phosphorylation was detected in all three histological subtypes examined (adenocarcinoma, squamous cell, and bronchioloalveolar cell), in both genders, and in all smoking categories.

Based on this evidence, TKIs acting on Src family kinases (SFKs) such as saracatinib and dasatinib have been proposed as therapeutic agents for NSCLC; these drugs are small molecules ATP competitors that can target kinases beyond the SFKs, and these off-target effects could have biological relevance. Particularly, some studies have described the effect of saracatinib(Green MolOncol 2009; Mc Dermott PNAS 2007) or dasatinib (Li Nat ChemBiol 2010;Yoshida Clin Cancer Res 2014) on cancer cell lines carrying EGFR TK variants, either wild-type or mutants. SFK inhibition usually affects invasion of NSCLC cells but has variable effects on apoptosis. Inhibiting SFKs in NSCLC cell lines that have activating EGFR mutations leads to significant apoptosis. However, apoptosis is rarely observed in NSCLC cell lines that express wild-type EGFR. However, disappointing results from phase I/II clinical trials have so far delayed the clinical development of these drugs. Although all studies, conducted in prospectively unselected patients with

advanced NSCLC, showed poor activity of Src inhibitors either in first and subsequent lines of therapy, some isolated clinical response have been reported (Johnson JCO 2010; Haura JCO 2010; Johnson J Thor Oncol 2011; Laurie Clin Lung Cancer 2013).

AIMS OF THE STUDY

In the present study, we attempted to clarify the possible role of Src inhibitors in the context of NSCLC therapy. To this purpose, we used a variety of *in vitro*/*in vivo* assays aimed at better defining the molecular targets of saracatinib, dasatinib and bosutinib, the three most clinically investigated Src TKIs. Moreover, we tried to suggest the optimal combination regimens and the clinical settings where the different anti-Src agents may better exert their antitumor activity.

MATERIALD AND METHODS

Compounds. Cetuximab was kindly provided by ImClone Systems. Erlotinib, saracatinib, dasatinib, bosutinib and selumetinib were purchased from Selleck Chemicals, Germany.

***In vitro* EGFR kinase inhibition assay.** The EGFR kinase inhibition assay on saracatinib, dasatinib and bosutinib was performed with an EGFR kinase mutant profile screening service by ProQinase (ProQinase GmbH, Freiburg, Germany). Briefly, compounds were tested at 10 different concentrations (standard range: $3x10^{-10}$M–$1x10^{-5}$M; semilog dilutions) against

wild type EGFR and then EGFR mutants and IC_{50} values were calculated. IC_{50}values of EGFR reference inhibitor (gefitinib or erlotinib) were determined side-by-side. All assays were performed at the corresponding app. ATP Km of each protein kinase using the radiometric ^{33}PanQinase Assay™.

Docking of the Src inhibitors saracatinib, dasatinib and bosutinib in the EGFR tyrosine kinase domain. The three-dimensional (3D) structures of saracatinib, dasatinib, and bosutinib were drawn using the Builder tool and generated with Ligprep module within the Schrodinger package (Schrödinger, LLC: New York, NY, 2009). The Glide program, of the same package, was used to dock these compounds into the X-ray crystal structure of the EGFR tyrosine kinase domain (PDB 2ITT) (Yun Cancer Cell 2007). The receptor grid generation was performed for the box with a center in the putative binding site. The size of the box was determined automatically. The extra precision mode (XP) of Glide was used for the docking. The ligand scaling factor was set to 1.0. The geometry of the ligand binding site of the complex between the selected ligands and the enzyme was then optimized. The binding site was defined as the ligand and all amino acid residues located within 8 Å from the ligand. All the protein residues located within 2 Å from the binding site were used as a shell. The following parameters of energy minimization were used: OPLS2005 force field; water was used as an implicit solvent; a maximum of 5000 iterations of the Polak–Ribier conjugate gradient minimization method was used with a convergence threshold of 0.01 kJ 3 mol^{-1} $Å^{-1}$. Molecular graphics were performed with the UCSF Chimera package. Chimera is

developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311). (PettersenJ Comput Chem. 2004).

Cell cultures. Human HCC827, Calu3, H460, H1299, A549 and NSCLC cell lines were obtained from the American Type Culture Collection (ATCC). PC-9 and GLC82 cell lines were kindly provided by Dr F. Morgillo.Calu3-ER (Erlotinib Resistant) cells were established as previously described (Morgillo, Lung Can 2011). All cell lines were authenticated using DNA fingerprinting and maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, pH 7.4, penicillin (100 IU/ml), streptomycin (100 µg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell survival assay (MTT). Cells (10^4 cells/well) were grown in 24-well plates and exposed for 72 hours to increasing doses of erlotinib, cetuximab, saracatinib, dasatinib or bosutinib, alone or in combination. The percentage of cell survival was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The drug concentration producing 50% survival inhibition was used as a marker of drug effect.

Combination effect. The combination effect of two drugs was evaluated based on the combination index (CI), calculated using Calcusyn software (Biosoft, Cambridge, UK) and defined as follows: $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2 + (D)_1(D)_2/(Dx)_1(Dx)_2$, where: $(Dx)_1$ is the dose of Drug 1 alone required to produce an X% effect; $(D)_1$ is the dose of Drug 1 required to

produce the same X% effect in combination with Drug 2; $(Dx)_2$ is the dose of Drug 2 alone required to produce an X% effect; and $(D)_2$ is the dose of Drug 2 required to produce the same X% effect in combination with Drug 1. The combination effect was defined as follows: CI < 1, synergistic effect; CI < 0.5, highly synergistic effect; CI = 1, additive effect; and CI > 1, antagonistic effect.

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 EGFR, monoclonal pMAPK, MAPK (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal pEGFR Y1173, pAkt, Akt, pSrc and Src (Cell Signaling Technologies, Beverly, MA), monoclonal actin (Sigma-Aldrich, Milan). Immunoreactive proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA). Densitometric analyses were performed with Image J software (NIH).

Analysis of EGFR phosphorylation on Tyr845. Levels on pEGFR Y845 in cell protein extracts was analyzed by using PathScan® Phospho-EGF Receptor (Tyr845) Sandwich ELISA Kit (Cell Signaling Technologies, Beverly, MA), according to manufacturer's instructions.

RNA interference. Small interfering RNA (siRNA) against Src was obtained from Ambion Life Technology (Grand Island, NY, USA). A nonsense sequence was used as negative control. For siRNA validation, cells were transfected with Src siRNAs (5 and 50 nmol/L) using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen); 24 or 48 hours after transfection,

Western blot analysis for Src protein expression was done. For the assessment of siRNA effects on cell survival and signaling, cells were transfected with Src siRNA for 24 hours, then treated with saracatinib, dasatinib or bosutinib for 3 additional hours.

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 HCC827 or H1975 cells (10^7 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 35 mg/kg intraperitoneally (i.p.) three times a week for 3 weeks, erlotinib 20 mg/kg i.p. three times a week for 3 weeks, saracatinib 50 mg/kg via oral gavage five times a week for 3 weeks,dasatinib 20 mg/kg i.p. three times a week for 3 weeks. Animals treated with DMSO vehicle served as controls. Tumor volume (cm^3) was measured using the formula $\pi/6$ x larger diameter x (smaller diameter)^2 as previously reported (Rosa Clin Can Res 2013).

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 significancewas determined by one-way ANOVA and Dunnett's multiple comparison post-test regarding tumor growth, by log-rank test concerning mice survival (Rosa Clin Cancer Res 2013). Linear regression analysis for the

correlation between drug concentration producing 50% survival inhibition and Src activation was performed by using Sigma Plot ver. 11.0, as reported (Chang BMC Cancer 2013). 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).

RESULTS

Src inhibitors saracatinib, dasatinib and bosutinib are able to inhibit EGFR tyrosine kinase activation.

Based on conflicting reports concerning the capability of Src inhibitors to directly affect EGFR tyrosine kinase activity (Green MolOncol 2009; Li Nat ChemBiol 2010; Mc Dermott PNAS 2007; Zhang Am J Pathol 2007; BoschelliEur J cancer 2010), we attempted to elucidate this point through an *in vitro* kinase assay comparing the effect of the Src inhibitors saracatinib, dasatinib and bosutinib with that of erlotinib on different EGFR tyrosine kinase variants, both wild-type and mutant. As indicated in **Table 1**, all the compounds were able to inhibit EGFR variants with IC_{50} values similar to erlotinib.Dasatinib seemed to be slightly less efficient than the other agents.

Table 1. Effect of Src inhibitors on EGFR TK catalytic activity

	EGFR d746-750	EGFR d747-749 A750P	EGFR d747-752 P753S	EGFR d752-759	EGFR G719C	EGFR G719S	EGFR L858R	EGFR L861Q	EGFR T790M	EGFR T790M/L858R	EGFR wt
Compound	**IC_{50} (nM)**	**IC_{50} (nM)**	**IC_{50} (nM)**	**IC_{50} (nM)**	**IC_{50} (nM)**	**IC_{50} (nM)**	**IC_{50} (nM)**	**IC_{50} (nM)**	**IC_{50} (nM)**	**IC_{50} (nM)**	**IC_{50} (nM)**
Saracatinib	13.1	13.3	10.0	36.2	9.77	13.5	9.40	12.3	769	802	11.0
Dasatinib	54.7	64.0	61.2	138	24.8	21.7	28.1	25.5	3100	2790	25.4
Bosutinib	2.53	5.80	3.47	23.7	2.59	2.25	2.29	3.95	145	214	3.17
Erlotinib	1.56	2.40	1.57	2.33	2.30	2.22	1.62	1.24	200	999	1.01

Compound concentrations in the assay from 0.3 nM to 10μM, semi-log steps; singlicate measurement
Ranking of IC_{50} values:

IC_{50} (nM) between 10000 and 1000
IC_{50} (nM) between 1000 and 100
IC_{50} (nM) below 100

To better clarify how Src inhibitors could exert a direct effect on EGFR kinase, we evaluated whether these compounds could adapt in the EGFR kinase domain binding site. As shown in **Figure 1**, docking results demonstrated that all the three compounds are able to settle in the enzyme ATP active site, establishing several interactions with the protein. Interestingly, a well-oriented hydrogen-bond with the M793 residue of the hinge region is formed, further substantiating their ability to act as typical EGFR TKIs.

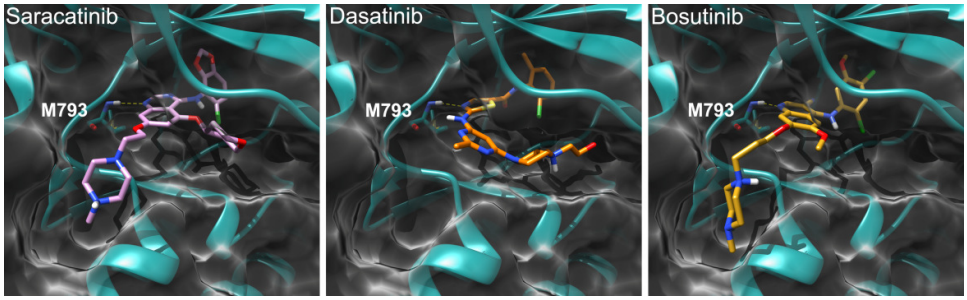


Figure 1.*Binding mode of saracatinib, dasatinib, and bosutinib in the EGFR kinase domain (PDB 2ITT) as predicted by docking calculations. The protein is depicted as cyan and white ribbons and surface, respectively. Key hinge region residue M793 is depicted as cyan sticks. Saracatinib, dasatinib, and bosutinib are represented as pink, orange, and yellow sticks, respectively. H-bond interactions are represented as dashed yellow lines.*

Src inhibitors exert different effects on human Non-small Cell Lung Cancer (NSCLC) cell lines.

In order to confirm the *in vitro* data, we used a panel of NSCLC cell lines with different levels of EGFR-dependent signaling activation and different degree of sensitivity against EGFR inhibitors. As shown in **Table 2**, this panel includes two cell lines with EGFR sensitizing mutations (A746_A750del), PC-9 and HCC827 (Terai H, Mol Cancer Res 2013); cell lines with wild-type EGFR, some of which harboring Ras or PI3K mutations and thus resistant to anti-EGFR drugs (Calu3, H1299, H460, A549, GLC-82); and H1975 cells, showing a double EGFR mutation (L858/T790M) conferring resistance to EGFR inhibitors (Regales JCI 2009). We also included Calu3-ER, a cell line with acquired resistance to erlotinib that we obtained from Calu3 cells through a validated protocol of *in vivo*/*in vitro* selection (Morgillo F, Lung Can 2011); Calu3-ER cells display a significant increase in the expression of activated, phosphorylated MAPK compared to Calu-3 (Morgillo F, Br J Cancer 2011).

The above described NSCLC cells showed different levels of activation of EGFR-dependent signaling, as proven by the variable expression of EGFR and activation of Src, Akt and MAPK (**Figure 2**). The observed levels are consistent with the mutational status of the cell lines and with previous data (Guo A, PNAS 2008; Morgillo F, Br Cancer 2011).

Table 2. Mutational status and sensitivity to EGFR inhibitors of NSCLC cell lines

Cell line	EGFR	K-Ras	N-Ras	PI3K	Erlotinib (concentration producing 50% survival inhibition, μM)	Cetuximab (concentration producing 50% survival inhibition, μM)
PC9	A746_A750del *				0.01 - 0.1	1.4 – 3.5
HCC827	A746_A750del *				0.1	0,07
Calu3					2,5 - 5	3.5
Calu3-ER					> 5	> 3.5
H1299			Q61K		5	1.4
H460		Q61H		E545K	> 5	> 3,5
A549		G12S		Q546K	> 5	> 3.5
GLC-82				H1047R	> 5	3.5
H1975	L858R/T790M				> 5	3.5

* Exon 19 deletion

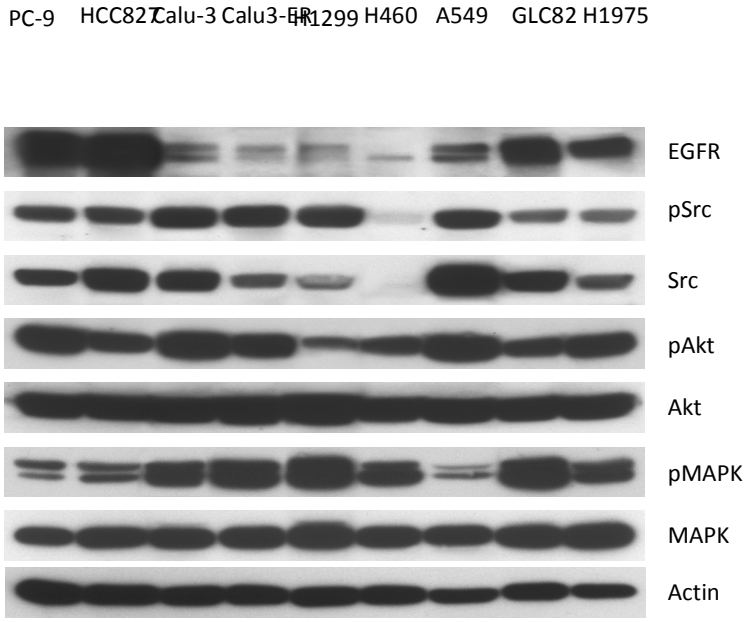
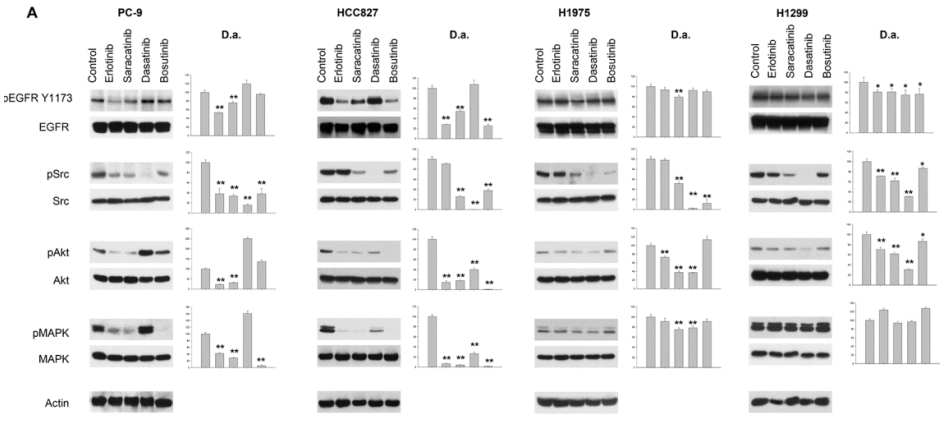


Figure 2. EGFR-dependent pathway activation in a panel of human NSCLC cell lines. *Western blot analysis of protein expression in a panel of human NSCLC cell lines.*

When we tested Src inhibitors on these NSCLC models in comparison with erlotinib, we found that the three compounds have different effects on EGFR-

dependent signal transduction. As shown in **figure 3A**, saracatinib was the most efficient, among the tested compounds, in reducing EGFR phosphorylation on Y1173, a well-known autophosphorylation site; this effect was more evident in EGFR mutant (PC-9 and HCC827) than in EGFR wild-type (H1299) cells, similarly to that observed with erlotinib. Surprisingly, the effect was detected also in the EGFR double mutant H1975 cells, although at a very slight degree. Similar results were obtained also in the other NSCLC cells tested (**Fig. 3B**); H460 cells were poorly sensitive to all the tested drugs because of low EGFR and Src activation (data not shown). In all cell lines, dasatinib was the most effective agent in inhibiting Src TK activity (**Fig. 3A and Fig. 3B**). In EGFR wild-type cells, Src inhibition by dasatinib seemed to interfere with Akt and MAPK activation, while in the EGFR mutant cells a more relevant inhibition of these transducers was obtained with saracatinib and/or bosutinib (**Fig. 3A and Fig. 3B**).



B

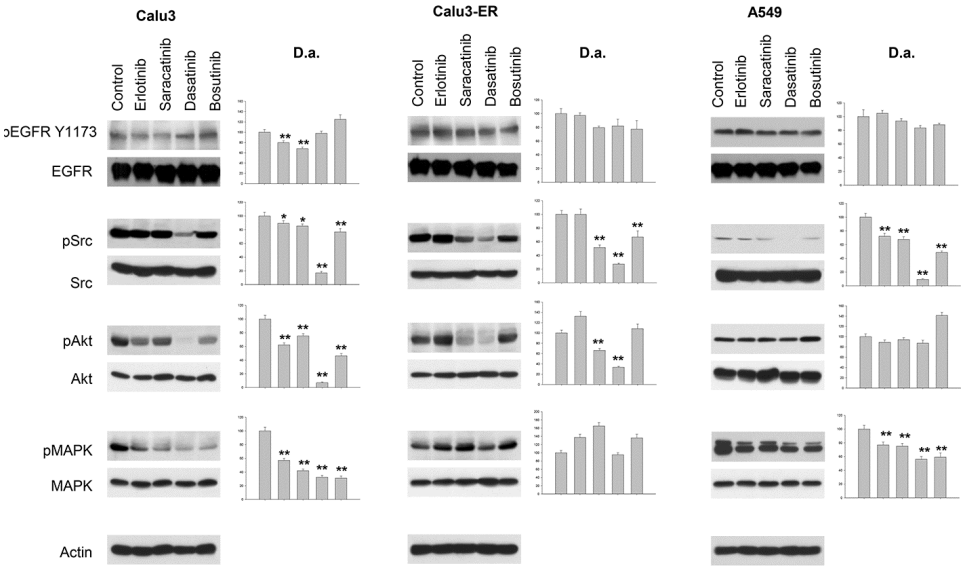


Figure 3. Effects of Src inhibitors on signal transduction and survival of human NSCLC cell lines sensitive or resistant to erlotinib. *(**A**) Western blot analysis of protein expression in PC-9, HCC827, H1975 and H1299 cells treated for 3 hours with erlotinib, saracatinib, dasatinib or bosutinib (1 μM). Densitometric analysis (D.a.): The relative optical density of phospho-protein levels normalized to actin level is shown as histograms. *, 2-sided P< 0.05 versus control; **, 2-sided P< 0.01 versus control.* ***(B)*** *Western blot analysis of protein expression in Calu3, Calu3-ER and A549 cells treated for 3 hours with erlotinib, saracatinib, dasatinib or bosutinib (1 μM). Densitometric analysis (D.a.): The relative optical density of phospho-protein levels normalized to actin level is shown as histograms.*, 2-sided P< 0.05 versus control;**, 2-sided P< 0.01 versus control.*

Consistently with data from Western blot analysis, in EGFR-activating mutant, erlotinib sensitive PC-9 and HCC827 cells, saracatinib showed anti-proliferative effects probably related to simultaneous EGFR/Src inhibition. Also in the EGFR double mutant model, saracatinib was slightly more effective than the other two compounds. Conversely, in EGFR wt/Ras mutant cells, poorly dependent on EGFR activation and thus erlotinib resistant, efficient Src inhibition by dasatinib interfered with cell proliferation (**Fig. 3C, Fig. 3D and**

Table 3). As a further confirmation, Src activation in NSCLC cells correlateswith sensitivity to dasatinib more than to saracatinib and bosutinib. Linear regression analysis (Chang and Wang BMC Cancer 2013) shown in **figure 3E** demonstrated that high levels of Src activation in NSCLC cells significantlycorrelate with high sensitivity to dasatinib (P = 0.0382). This correlation was not found with sensitivity to saracatinib and bosutinib (P> 0.05).

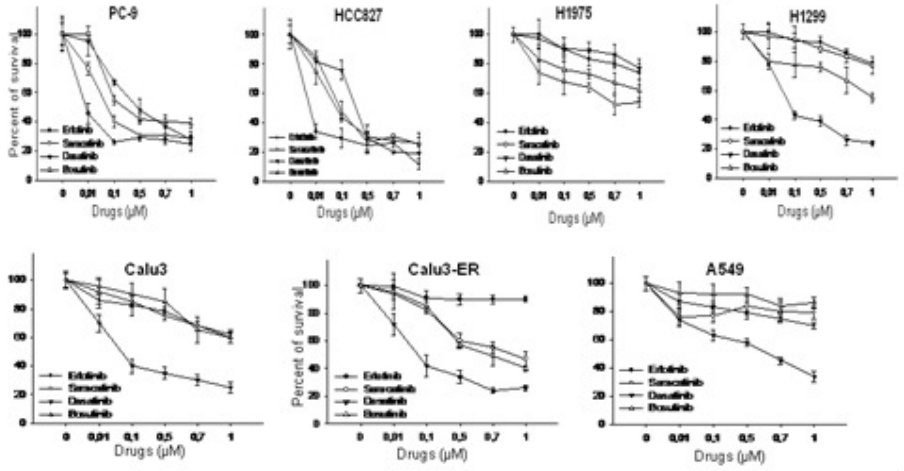


Figure 3D*. *Effects of Src inhibitors on signal transduction and survival of human NSCLC cell lines sensitive or resistant to erlotinib. (C) *Percent of survival of PC-9, HCC827, H1975 and H1299 cells treated for 72 hours with erlotinib, saracatinib, dasatinib or bosutinib (1 μM), as measured by MTT assay. Data represent the mean (±SD) of three independent experiments, each performed in triplicate. Bars, SDs.* ***(D)****Percent of survival of Calu3, Calu3-ER and A549 cells treated for 72 hours with erlotinib, saracatinib, dasatinib or bosutinib (1 μM), as measured by MTT assay. Data represent the mean (±SD) of three independent experiments, each performed in triplicate. Bars, SDs.*

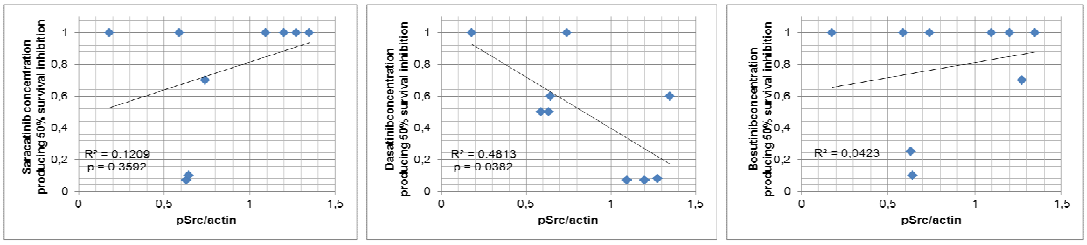


***Figure 3E*.Correlation between growth inhibition by saracatinib, dasatinib or bosutinib and Src baseline activation.** *The correlation between the concentration of saracatinib, dasatinib or bosutinib producing 50% growth inhibition and the ratio of p-Src/actin in 9 human NSCLC cell lines was shown. Protein expressions were measured by western blot (see Figure 2) and analysed by ImageJ software.Linear regression analysis was performed by using Sigma Plot ver. 11.0*

Table 3. P values for survival inhibition by saracatinib, dasatinib and bosutinib (treatment vs control) in human NSCLC cell lines sensitive or resistant to erlotinib, as measured by MTT assay.

PC-9

Drug	0.01 µM	0.1 µM	0.5 µM	0.7 µM	1 µM
Erlotinib	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Saracatinib	0.032	0.001	< 0.001	< 0.001	< 0.001
Dasatinib	0.536	0.002	0.001	< 0.001	< 0.001
Bosutinib	1.000	< 0.001	< 0.001	< 0.001	< 0.001

HCC827

Drug	0.01 µM	0.1 µM	0.5 µM	0.7 µM	1 µM
Erlotinib	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Saracatinib	0.023	< 0.001	< 0.001	< 0.001	< 0.001
Dasatinib	0.009	0.003	< 0.001	< 0.001	< 0.001
Bosutinib	0.032	< 0.001	< 0.001	< 0.001	< 0.001

H1975

Drug	0.01 µM	0.1 µM	0.5 µM	0.7 µM	1 µM
Erlotinib	0.463	0.140	0.012	0.004	0.012
Saracatinib	0.009	0.006	< 0.001	< 0.001	< 0.001
Dasatinib	1.000	0.041	0.068	0.051	0.002
Bosutinib	0.033	0.016	0.001	0.003	0.002

H1299

Drug	0.01 µM	0.1 µM	0.5 µM	0.7 µM	1 µM
Erlotinib	1.000	0.216	0.131	0.011	0.002
Saracatinib	0.640	0.354	0.018	0.009	0.007
Dasatinib	0.002	< 0.001	< 0.001	< 0.001	< 0.001
Bosutinib	0.009	0.016	0.002	0.005	< 0.001

Calu3

Drug	0.01 µM	0.1 µM	0.5 µM	0.7 µM	1 µM
Erlotinib	0.038	0.041	0.005	0.001	< 0.001
Saracatinib	0.233	0.015	0.002	< 0.001	< 0.001
Dasatinib	0.003	< 0.001	< 0.001	< 0.001	< 0.001
Bosutinib	0.375	0.14	0.065	0.004	< 0.001

Calu3-ER

Drug	0.01 µM	0.1 µM	0.5 µM	0.7 µM	1 µM
Erlotinib	0.819	0.092	0.054	0.041	0.032
Saracatinib	0.145	0.005	< 0.001	< 0.001	< 0.001
Dasatinib	0.007	< 0.001	< 0.001	< 0.001	< 0.001
Bosutinib	0.606	0.014	< 0.001	< 0.001	< 0.001

A549

Drug	0.01 µM	0.1 µM	0.5 µM	0.7 µM	1 µM
Erlotinib	0.033	0.009	0.006	0.002	< 0.001
Saracatinib	0.005	0.005	0.024	0.016	0.005
Dasatinib	0.003	< 0.001	< 0.001	< 0.001	< 0.001
Bosutinib	0.268	0.183	0.122	0.017	0.019

We attempted to confirm these data in HCC827 tumor xenografts. On day 63 (9 weeks after tumor cells injection) all mice in the control group reached the maximum allowed tumor size of about 2 cm^3. At this time point, dasatinib produced a tumor growth inhibition of about 27%, while erlotinib- and

saracatinib-treated mice appeared to be tumor free. These latter agents maintained a potent antitumor activity until the end of the experiment, with about 99% and 83% of growth inhibition for erlotinib and saracatinib, respectively. Comparison of tumor sizes among different treatment groups was statistically significant **(Fig. 4A).** Consistently, as shown in **figure 4B**, mice treated with erlotinib and saracatinib did not reach a median survival, since 70% and 50% of the mice were still alive at the end of the experiment, respectively. Treatments were well tolerated; no weight loss or other signs of acute or delayed toxicity were observed. Western blot analysis on tumor samples from mice sacrificed on day 14, after 1 week of treatment, demonstrated that saracatinib is able to reduce EGFR phosphorylation similarly to that observed with erlotinib. Dasatinib was unable to do that **(Fig. 4C).**

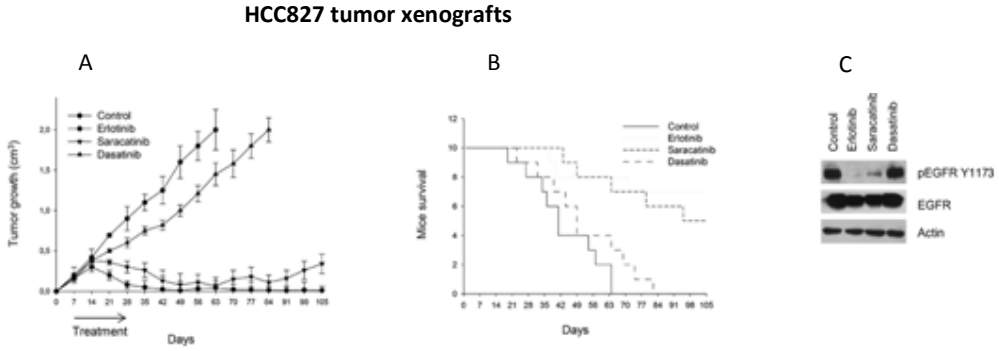


Figure 4. Effects of Src inhibitors on signal transduction and survival of human NSCLC cell lines sensitive or resistant to erlotinib. ***(A)*** *After 7 days following subcutaneous injection of HCC827 cells, mice were randomized (10/group) to receive erlotinib, saracatinib or dasatinib, as described in the Methods section. The one-way ANOVA testwas used to compare tumor sizes among treatment groups at the median survival time of the control group (42 days). They resulted statistically significant for all the drugs vs control (P< 0.0001).* ***(B)*** *Median survival did not result statistically significant for dasatinib versus control (log-rank test); mice groups treated with erlotinib or saracatinib did not reach a median survival, since 70% and 50% of animals were still alive at the end of the experiment, respectively. (**C**) Western blot analysis was performed on total lysates from tumor specimens of two mice sacrificed on day 14. Data*

represent the mean (±SD) of three independent experiments, each performed in triplicate, and are presented relative to control. Bars, SDs.

Saracatinib and bosutinib exert a direct, not Src-mediated effect on EGFR.

Based on the evidence that saracatinib and bosutinib are able to interfere with EGFR phosphorylation on Y1173 in NSCLC cell lines, we attempted to exclude that this effect could be mediated by Src inhibition.

Since it is well known that Src can phosphorylate EGFR on Y845, we first verified the capability of Src inhibitors to interfere with this event through an ELISA assay measuring pEGFR Y845 levels. Saracatinib and dasatinib moderately inhibited EGFR phosphorylation on Y845 in PC-9, HCC827 and H1299 cells, while no effect was detected in H1975 cells **(Fig.5A)**. However, it has been reported that Src phosphorylation on Y845 does not affect EGFR kinase activity (Tice PNAS 1999): so EGFR Y1173 phosphorylation should not be affected by Src inhibition, unless the inhibitors have cross-reactivity with the EGFR. Consistently, when we silenced Src expression in HCC827 cells by using a specific siRNA, we found that phosphorylation of EGFR on Y1173 was not affected **(Fig.5B).** Treatment with saracatinib in presence of Src siRNA maintained its capability to reduce EGFR Y1173 phosphorylation, thus confirming that the effect on EGFR was direct and not Src-mediated; conversely, dasatinib had no effect on EGFR phosphorylation/activation also in presence of Src silencing (**Fig.5B).**

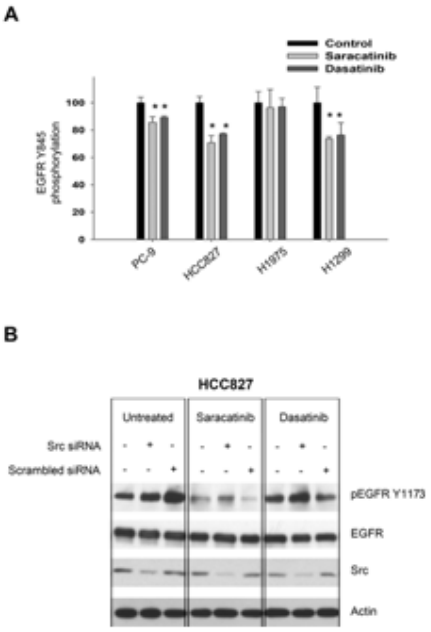


Figure 5. Correlation between drugs effect on human NSCLC cell lines and Src inhibition. *(**A**) Percent of EGFR Tyr845 phosphorylation in PC-9, HCC827, H1975 and H1299 cells treated for 3 hours with erlotinib, saracatinib or dasatinib (1 μM), as measured by using the PathScan® Phospho-EGF Receptor (Tyr845) Sandwich ELISA Kit. *, 2-sided P< 0.05 versus control. Data represent the mean (±SD) of three independent experiments, each performed in triplicate. Bars, SDs. (**B**) Western blot analysis of protein expression in HCC827 cells transfected with a Src specific siRNA or with a negative control, and then treated (24 hours after transfection) with saracatinib or dasatinib for 3 hours.*

Cetuximab plus saracatinib is an effective combination in EGFR-addicted cells.

In order to better define the role of Src and EGFR dependence, we tested Src inhibitors in combination with different classes of EGFR inhibitors. We first evaluated the combination of the most effective Src inhibitor (saracatinib for PC-9, HCC827 and H1975; dasatinib for H1299 cells) with the anti-EGFR drugs erlotinib(**Figure 6A, B and Table 4)** or cetuximab (**Figure 6C, D and Table 4)**, by MTT assays and Western blot analysis. In EGFR-addicted cells

(both erlotinib sensitive PC-9 and HCC827, and erlotinib resistant H1975 cells), the best combination was saracatinib plus cetuximab, which is able to efficiently reduce cell survival **(Figure 6C and Table 4)** and to interfere with EGFR and EGFR-dependent signal transducers activation (**Figure 6D**). The same effect was not obtained with dasatinib plus cetuximab in EGFR wild-type H1299 cells (**Figure 6C, D and Table 4**).

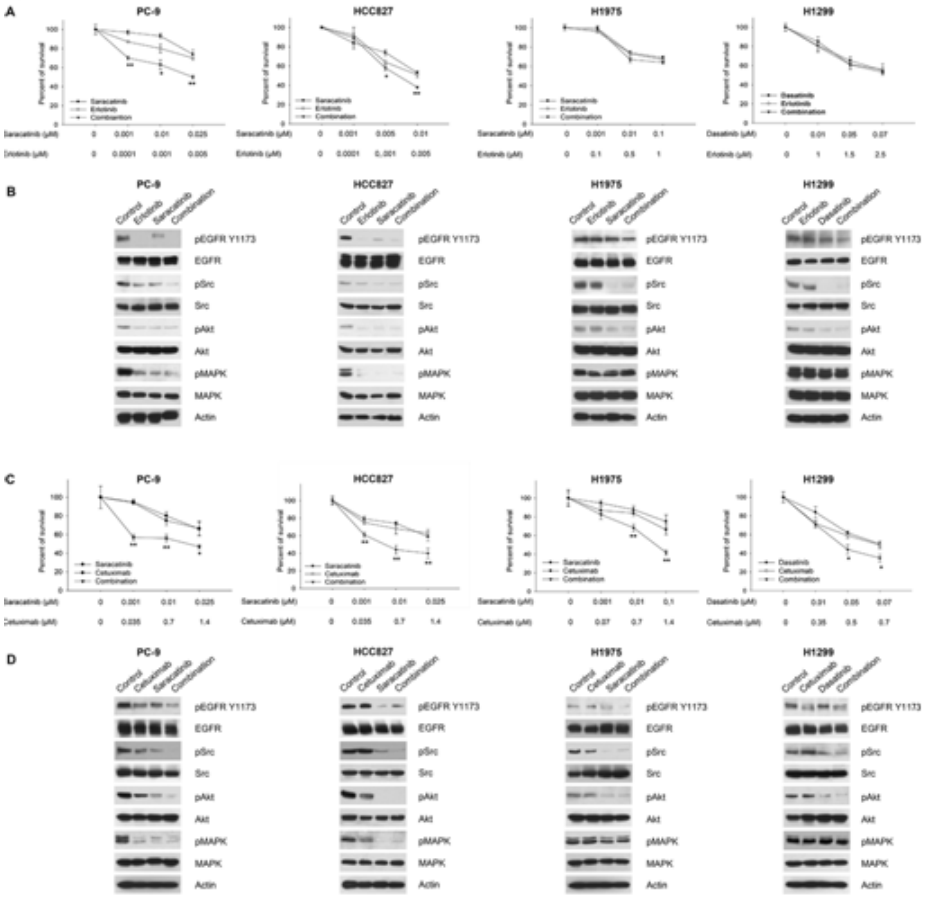


Figure 6. Effects of the combinations of EGFR and Src inhibitors on signal transduction and survival of human NSCLC cell lines.*(**A**) Percent of survival of PC-9, HCC827, H1975 and H1299 cells treated for 72 hours with different concentrations of saracatinib (for PC-9, HCC827, H1975) or dasatinib (for H1299), alone or in combination with erlotinib, as measured by MTT assay. *, 2-sided P< 0.05 versus erlotinib alone; **, 2-sided P< 0.01 versus erlotinib alone. (**B**) Western blot analysis of PC-9, HCC827, H1975 and H1299 cells treated for 3 hours with saracatinib (for PC-9, HCC827, H1975) or dasatinib (for H1299), alone or in*

*combination with erlotinib. (**C**) Percent of survival of PC-9, HCC827, H1975 and H1299 cells treated for 72 hours with different concentrations of saracatinib (for PC-9, HCC827, H1975) or dasatinib (for H1299), alone or in combination with cetuximab, as measured by MTT assay. *, 2-sided P< 0.05 versus cetuximab alone; **, 2-sided P< 0.01 versus cetuximab alone. (**D**) Western blot analysis of PC-9, HCC827, H1975 and H1299 cells treated for 3 hours with saracatinib (for PC-9, HCC827, H1975) or dasatinib (for H1299), alone or in combination with cetuximab.*
Data represent the mean (±SD) of three independent experiments, each performed in triplicate. Bars, SDs.

Src inhibitor plus erlotinib

Cell line	D1	D2	D3
PC-9	< 0.001	0.012	< 0.001
HCC827	0.666	0.045	0.002
H1975	0.176	0.070	0.140
H1299	0.330	1.000	0.448

Src inhibitor plus cetuximab

Cell line	D1	D2	D3
PC-9	< 0.001	0.008	0.015
HCC827	0.003	0.006	0.004
H1975	0.276	0.006	0.002
H1299	0.739	0.011	0.013

Table 4. P values for survival inhibition by Src inhibitor plus erlotinib (combination vs erlotinib) or cetuximab (combination vs cetuximab) in humans NSCLC cell lines, as measured by MTT assay

More interestingly, as shown in **figure 7**, the combination of cetuximab plus saracatinib resulted effective *in vivo*, in erlotinib resistant, EGFR double mutant H1975 tumor xenografts. 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. At this time point, saracatinib produced a tumor growth inhibition of about 66%, while cetuximab and the combination reduced tumor growth of about 95%. Saracatinib-treated mice reached the size of 2 cm^3 on day 119, while mice treated with cetuximab or cetuximab plus saracatinib never reached this size. The combination of cetuximab and saracatinib caused a long-lasting cooperative antitumor activity, with about 95% of growth inhibition (vs 53% of cetuximab alone) until the end of the experiment. Comparison of tumor sizes among different treatment groups was statistically significant (**Fig.7A**). Consistently, as shown in **figure 7B**, mice treated with the combination did not reach a median survival, since 80% of the mice were still alive at the end of the experiment. Treatments were well tolerated; no weight loss or other signs of acute or delayed toxicity were observed. Western blot analysis on tumor samples from mice sacrificed on day 14, after 1 week of treatment, demonstrated that cetuximab plus saracatinib efficiently interferes with EGFR-dependent signal transduction, by reducing phosphorylation/activation of EGFR, Src, Akt and MAPK. The effect on EGFR is probably potentiated by EGFR down-regulation due to cetuximab-induced internalization **(Fig. 7C),** as previously reported (Regales JCI 2009).

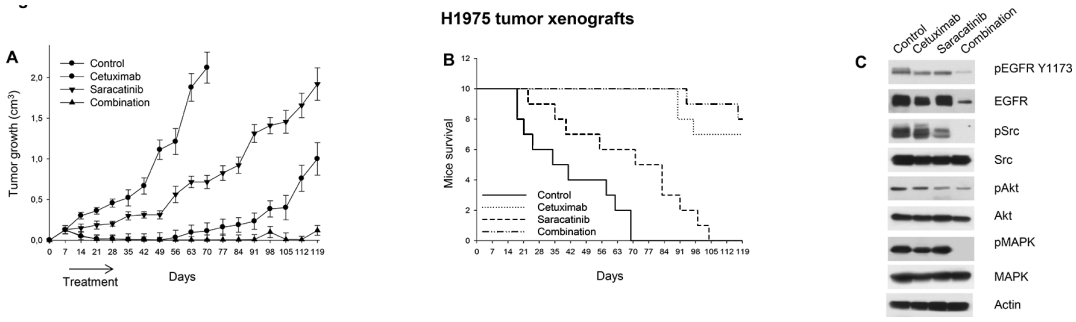


Figure 7. Effects of saracatinib plus cetuximab combination on tumor growth, survival and signal transduction of mice xenografted with H1975 erlotinib resistant tumors. *(**A**) After 7 days following subcutaneous injection of H1975 cells, mice were randomized (10/group) to receive cetuximab, saracatinib or their combination, as described in the Methods section. The one-way ANOVA testwas used to compare tumor sizes among treatment groups at the median survival time of the control group (38.5 days). They resulted statistically significant for the combination versus control or saracatinib (P< 0.0001). (**B**) Median survival resulted statistically significant for the saracatinib versus control (log-rank test); mice groups treated with cetuximab or with the combination did not reach a median survival, since 70% and 80% of animals were still alive at the end of the experiment, respectively. (**C**) Western blot analysis was performed on total lysates from tumor specimens of two mice sacrificed on day 14. Data represent the mean (±SD) of three independent experiments, each performed in triplicate, and are presented relative to control. Bars, SDs.*

Selumetinib plus dasatinib is an effective combination in Ras-dependent cells.

Since the combinations of EGFR and Src inhibitors are poorly effective in EGFR wild-type, Ras mutant models, we hypothesized that MAPK signalling blockade could potentiate Src pharmacological inhibition. In fact, we found that combination of dasatanib with the MEK inhibitor selumetinib strongly inhibits cell survival in H1299 cells. Similar effects were observed in Calu3-ER, a cell line with acquired resistance to erlotinib showing a significant increase in the levels of activated, phosphorylated MAPK (**Fig. 8A and Table 5**). The combination seemed to be highly synergistic, as demonstrated by the very low values of combination index (CI) devised by Chou and Talalay (Chou

and Talalay, Adv Enzyme 1984) using an automated calculation software (**Table 6**). Consistently, since dasatinib was able to reduce phosphorylation of Src and Akt, while selumetinib suppressed MAPK phosphorylation/activation, this effect was paired with a strong interference with signal transduction in both cell lines (**Fig. 8B**). The effectiveness of selumetinib was not observed in the Ras mutant A549 cell line (data not shown), as previously reported (Troiani Br J Cancer 2012).

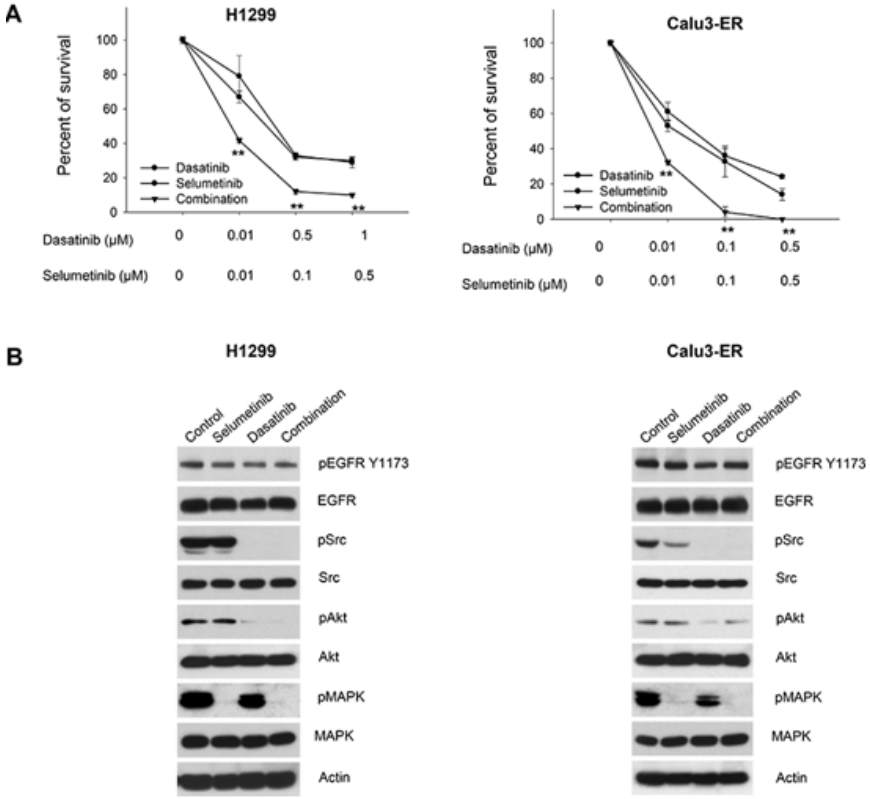


Figure 8. Effects of the combination dasatinib plus selumetinib on Ras-dependent human NSCLC models. *(**A**) Percent of survival of H1299 and Calu3-ER cells treated for 72 hours with different concentrations of dasatinib, alone or in combination with selumetinib, as*

*measured by MTT assay. **, 2-sided P< 0.01 versus selumetinib alone. (**B**) Western blot analysis of H1299 and Calu3-ER cells treated for 3 hours with dasatinib, alone or in combination with selumetinib.*

Src inhibitor plus selumetinib

Cell line	D1	D2	D3
H1299	< 0.001	< 0.001	< 0.001
Calu3-ER	< 0.001	0.006	0.002

Table 5. P values for survival inhibition by Src inhibitor plus selumetinib (combination vs selumetenib) in Ras-dependent human NSCLC cell lines, as measured by MTT assay.

Src inhibitor plus selumetinib

Cell line	D1	D2	D3
H1299	0.151	0.084	0.147
Calu3-ER	0.201	0.011	0.003

Table 6. P values for survival inhibition by Src inhibitor plus selumetinib (combination vs selumetenib) in Ras-dependent human NSCLC cell lines, as measured by Chou and Talalay method.

DISCUSSION

Src inhibitors have been suggested as promising agents for NSCLC, but disappointing results from clinical trials have so far delayed their clinical development (Johnson JCO 2010; Haura JCO 2010; Johnson J Thor Oncol 2011; Laurie Clin Lung Cancer 2013). A major limitation of these studies may have been the enrolment of molecularly unselected patients with advanced NSCLC and the lack of predictive biomarkers of response. In fact, populations of patients with different molecular features could benefit from the treatment with different Src inhibitors: despite these trials have been considered negative, some clinical responses have been reported. In the present study, we attempted to better define the mechanisms of action of the three Src TKIs (saracatinib, dasatinib and bosutinib) more extensively investigated in human cancer malignancies, in order to evaluate how these agents could be integrated in the context of NSCLC therapy.

To this purpose, we used a variety of *in vitro*/*in vivo* assays aimed to clarify the molecular targets of the above cited agents. In fact, several studies have suggested that these ATP competitors can target kinases beyond the SFKs, including EGFR TK variants, either wild-type or mutants(Green MolOncol 2009; Mc Dermott PNAS 2007; Li Nat ChemBiol 2010); however, this issue still remains controversial.By using an *in vitro* kinase assay we demonstrated that Src inhibitors are able to inhibit EGFR TK activation. Moreover, docking

studies suggested that all the three compounds are able to settle in the enzyme binding site, further confirming their ability to act as typical EGFR TKIs.

When tested on human NSCLC cell lines, Src inhibitors showed different effects. In EGFR-activating mutant, erlotinib sensitive cells, saracatinib showed anti-proliferative effects probably related to simultaneous EGFR/Src inhibition. In fact, this agent seems the most efficient in inhibiting EGFR phosphorylation on Y1173 (a well-known autophosphorylation site), especially in presence of activating mutations. This result was confirmed also *in vivo*, in HCC827 tumor xenografts, where saracatinib showed a potent and long-lasting antitumor activity. This finding is of interest: in a recently published phase II clinical trial (Laurie SA, Clin Lung Cancer 2014), two partial responses were observed out of 31 NSCLC patients treated with saracatinib monotherapy; a long lasting responder had an EGFR exon 19 deletion. Surprisingly, in contrast to erlotinib, saracatinib was proven to be also effective in the EGFR T790M mutant model. Conversely, in all cell lines, dasatinib was the most effective agent in inhibiting Src TK. In EGFR wild-type cells, Src inhibition by dasatinib seemed to interfere with Akt and MAPK activation, thus efficiently inhibiting cell proliferation.

Saracatinib has been proven able to inhibit purified EGFR variants in a kinase assay; therefore, we attempted to rule out whether its effect on EGFR Y1173 phosphorylation observed in NSCLC cell lines may be mediated by Src inhibition. It is well known that Src can phosphorylate EGFR on Y845, but it has been reported that Src phosphorylation on this residue does not affect

EGFR kinase activity (Tice PNAS 1999): so, EGFR Y1173 phosphorylation should not be affected by Src inhibition, unless the anti-Src agents have cross-reactivity with EGFR. Consistently, when we silenced Src expression in HCC827 cells, we found that phosphorylation of EGFR on Y1173 was not affected. Treatment with saracatinib in presence of Src silencing maintained its capability to reduce EGFR Y1173 phosphorylation. These data suggest that the effect observed in NSCLC cell lines after saracatinib treatment is not strictly Src-mediated.

Altogether, these data suggest that the three compounds may act with different mechanisms in NSCLC cell lines: while dasatinib, as expected, functions via Src inhibition, saracatinib showed an additional mechanism of action based on direct EGFR inhibition. Bosutinib seemed to have an intermediate behavior, with a certain degree of variability among the different cell lines. As a further confirmation, Src activation in NSCLC cells correlated with sensitivity to dasatinib more than to saracatinib and bosutinib.

In order to overcome resistance to EGFR inhibitors, we explored the optimal combination regimens and the settings where the different anti-Src agents may better exert their antitumor activity. In EGFR-addicted cells (either erlotinib sensitive or resistant), a very effective combination was saracatinib plus cetuximab. This result was confirmed also in erlotinib resistant, EGFR double mutant H1975 tumor xenografts, where this combination caused a strong cooperative antitumor activity and prolonged mice survival. Interestingly, the effect of saracatinib on EGFR was probably potentiated by

EGFR down-regulation due to cetuximab-induced internalization, as previously reported (Regales JCI 2009). Targeting EGFR by using two different approaches, namely a monoclonal antibody directed against the extracellular portion of the receptor and a TKI able to interfere with ATP binding, has been proven effective in models of NSCLC harboring the T790M resistance mutation: together, these agents efficiently deplete both phosphorylated and total EGFR (Regales JCI 2009). The robust antitumor activity of afatinib and cetuximab combination has been demonstrated in a phase Ib clinical trial in advanced EGFR mutated NSCLC cancers, which progressed after EGFR TKI therapy: among 126 patients, high objective response rate (overall 29%) was reported, and resulted comparable in T790M-positive and T790M-negative tumors (Janjigian, Cancer Discovery 2014). This study supports the hypothesis that a significant proportion of tumors with acquired resistance to anti EGFR TKI remains dependent on EGFR signaling, and that combination therapies could have a significant impact in the clinical arena. However, it should pointed out that afatinib and cetuximab possess overlapping toxicities (mainly diarrhea and skin rush) that could limit their use in patients; by contrast, saracatinib, at least based on data from completed clinical trials, seems to have a different toxicity profile (Molina, Lung Can 2014). In addition, saracatinib offers the advantage of simultaneous Src inhibition, possibly providing a further benefit in terms of tumor growth control and preventing the onset of Src-mediated resistance to EGFR inhibitors, as previously reported (Zhang Nat Med 2011; Rexer Oncogene 2011; Formisano Breast Can Res 2014).

Therefore, our study may have relevant therapeutic implications for lung cancer patients, suggesting an effective strategy to overcome EGFR drug resistance.

Conversely, in EGFR wild-type, Ras mutant models, combination of dasatanib with the MEK inhibitor selumetinib strongly inhibited cell survival and signal transduction, producing a highly synergistic effect. These data are of relevance based on the promising efficacy of selumetinib plus docetaxel combination reported in a randomized phase 2 clinical trial for K-Ras mutant advanced NSCLC (Janne, Lancet Oncology 2013). However, the higher number of adverse events observed with the combination than with docetaxel alone could suggest the search of other MEK inhibitors-based combination. In this respect, the relevance of Src in the onset of pharmacological resistance as well as the effectiveness of saracatinib in combination regimens has been recently highlighted also in ALK-positive NSCLC models (Crystal AS, Science 2014).

CONCLUSIONS

We demonstrated that different Src inhibitors act through different mechanisms in NSCLC models sensitive or resistant to erlotinib. This evidence may partially justify the failure of clinical trials with Src inhibitors in unselected NSCLC patients (Johnson JCO 2010; Haura JCO 2010; Johnson J Thor Oncol 2011; Laurie Clin Lung Cancer 2013). The off-target effect, particularly on EGFR mutant variants, could be a main strength of saracatinib in the setting of erlotinib resistant patients with EGFR mutations. Moreover, based on their differential effects, Src inhibitors may better cooperate with EGFR or MEK inhibitors in NSCLCs. In this respect, since few therapeutic options are available for EGFR wild-type, Ras mutant NSCLC, the combination of dasatinib plus selumetinib may be a novel strategy potentially valuable in the clinical setting.

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Clinical Cancer Research

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Cancer Therapy: Preclinical

Clinical Cancer Research

Sphingosine Kinase 1 Overexpression Contributes to Cetuximab Resistance in Human Colorectal Cancer Models

Roberta Rosa[1], Roberta Marciano[1], Umberto Malapelle[2], Luigi Formisano[1], Lucia Nappi[1], Claudia D'Amato[1], Valentina D'Amato[1], Vincenzo Damiano[1], Gabriella Marfè[6], Silvana Del Vecchio[3], Antonella Zannetti[3], Adelaide Greco[2,4], Alfonso De Stefano[1], Chiara Carlomagno[1], Bianca Maria Veneziani[5], Giancarlo Troncone[2], Sabino De Placido[1], and Roberto Bianco[1]

Abstract

Purpose: Although the anti–EGF receptor (EGFR) monoclonal antibody 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 signaling 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 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 resensitization 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. *Clin Cancer Res; 19(1); 138–47. ©2012 AACR.*

Introduction

In the last few years, cetuximab and panitumumab, 2 monoclonal antibodies (mAb) targeting the EGF 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 has been widely observed (2). Molecular alterations such as mutations in genes codifying for EGFR-dependent signal transducers [*K-Ras*, *B-Raf*, phosphoinositide 3-kinase (*PI3K*), and *PTEN*] have been related to primary refractoriness to cetuximab in colorectal cancer. Other mechanisms, such as alternative signaling by different tyrosine kinase receptors 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 signaling 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

Authors' Affiliations: Dipartimenti di [1]Endocrinologia ed Oncologia Molecolare e Clinica and [2]Scienze Biomorfologiche e Funzionali, Università di Napoli "Federico II"; [3]Institute of Biostructures and Bioimages, National Research Council; [4]CEINGE, Biotecnologie Avanzate; [5]Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano", Università di Napoli "Federico II", Naples; and [6]Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Università di Roma "Tor Vergata", Rome, Italy

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Roberto Bianco, Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Università di Napoli "Federico II", Via S. Pansini, 5, 80131 Napoli, Italy. Phone: 39-081-7462061; Fax: 39-081-7462061; E-mail: robianco@unina.it

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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 receptor, in cetuximab-resistant colorectal cancer models was also shown, 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.

growth factors, cytokines and mitogens, is upregulated 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*-dimethylsphingosine (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 showed that alterations of ceramide/S1P rheostat may be involved in the regulation of resistance to both chemotherapeutics and targeted agents (9–14).

On the basis of this evidence, and as several reports showed cross-talks between SphK1 and EGFR-dependent signaling 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.

Materials and Methods

Compounds

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. 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 MTT.

Apoptosis assay

Apoptosis was quantified using the Annexin V-FITC apoptosis kit (BD Biosciences).

Immunoprecipitation and Western blot analysis

Total cell lysates from cell cultures or tumor specimens were resolved by 4% to 15% SDS-PAGE and probed with anti-human, polyclonal pEGFR, EGFR and SphK1, monoclonal phospho mitogen-activated protein kinase (pMAPK), MAPK and S1PR1/EDG-1 (Santa Cruz Biotechnology), polyclonal pAkt, Akt and SphK1 phospho-Ser225 (Cell Signaling Technologies), and monoclonal actin (Sigma-Aldrich). Immunoreactive proteins were visualized by enhanced chemiluminescence (Pierce). Densitometry analysis was conducted with Image J software (NIH).

Sphingosine kinase assay

SphK1 activity was measured by using Sphingosine Kinase Activity Assay Kit (Echelon Biosciences; refs. 18, 19).

Quantification of S1P

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

Hairpin siRNA construct for Sphk1

Endogenous Sphk1 expression was downregulated 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 no. AF200328) cDNA was cloned into pCMV6-AC-GFP vector (OriGene Rockville). Transient transfections were conducted using the Lipofectamine 2000 (Invitrogen).

Nude mice cancer xenograft models

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

Morphologic and immunohistochemical analysis of mouse and human tumor samples

The morphologic 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; and 3: high level). The presence of apoptotic cells was

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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).

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 SDs for at least 3 independent experiments conducted in triplicates. The statistical significance was determined by 1-way ANOVA and Dunnett's multiple comparison posttest about tumor growth, by log-rank test concerning mice survival. All reported *P* values were 2-sided. Analyses were conducted with the BioMeDical Package (BMDP) New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software).

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 conducted to evaluate the correlation of SphK1 levels and other clinical/pathologic variables with response rate to cetuximab-based therapy (responders vs. nonresponders). Patients were classified as "responders" in case of complete or partial response, and as "nonresponders" in case of stable or progressive disease. κ^2 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. HR and OR were assessed by multivariate analysis. A *P* value of 0.05 or less was considered statistically significant. All the analyses were conducted using IBM SPSS Statistics 18 package software (SPSS Inc.).

Results

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

On the basis of 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, harboring a wild-type *K-Ras* gene; GEO cells, positive for a *K-Ras* mutation (Gly12Ala); the cetuximab-resistant derivative GEO-CR cells; SW480, LS174T, HCT116, and LoVo cells, harboring *K-Ras* mutations (Gly12Val for SW480; Gly12Asp for LS174T, and Gly13Asp for HCT116 and LoVo); and HT29 cells, positive for a *B-Raf* mutation (Val600Glu). As shown in Supplementary Fig. S1A and S1B, cetuximab inhibited survival and induced apoptosis more efficiently in SW48 and GEO than in the other cell lines. These data are consistent with the *K-Ras* or *B-Raf* status of the cell lines, except for GEO cells which are sensitive to cetuximab despite the *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), were observed to be higher in cetuximab-resistant cells compared with 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 and 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 2-fold increase in apoptosis after combined treatment compared with ceramide alone (Fig. 1F).

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

On the basis of the overexpression of SphK1 in resistant cells, we investigated the involvement of this kinase in cetuximab resistance by conducting 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, whereas 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 show 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 Fig. 2C, SphK1 silencing increased sensitivity to cetuximab-induced apoptosis in resistant cells. Conversely, SphK1 overexpression in GEO and SW48 sensitive cells

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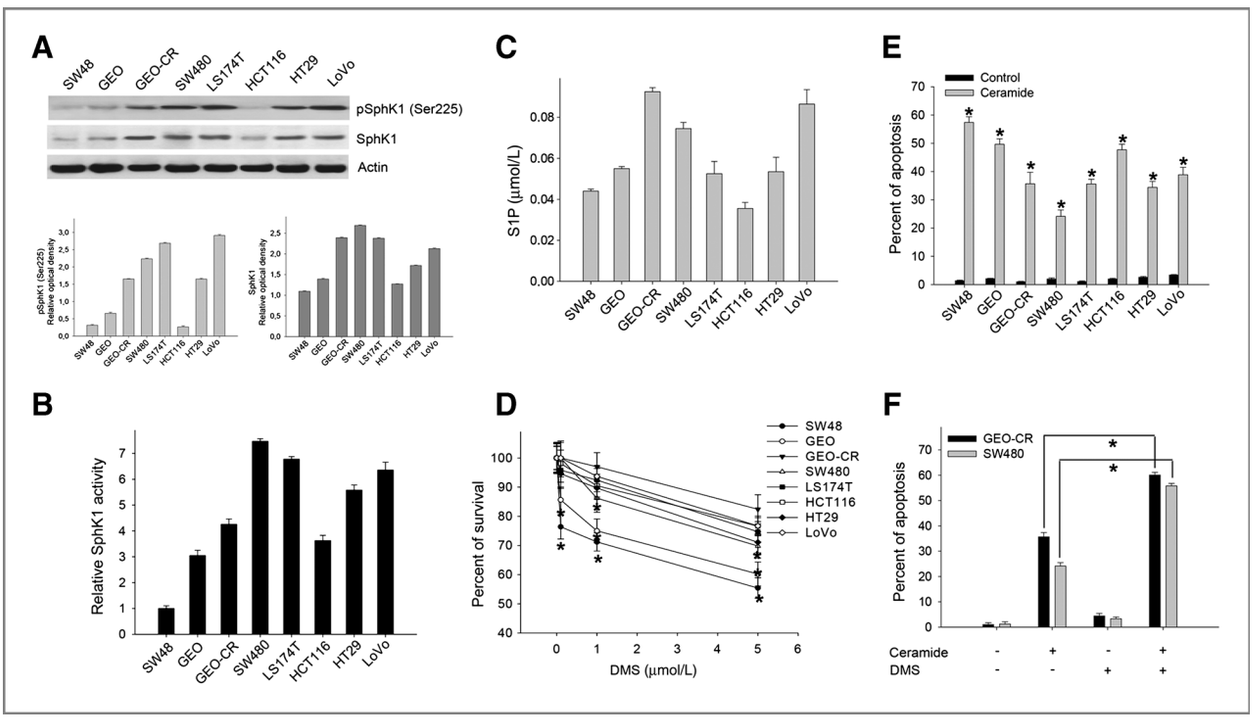

Figure 1. Overexpression and overactivation of SphK1 in human colorectal cancer cell lines resistant to cetuximab. A, Western blot analysis of SphK1 and SphK1 phospho-Ser225 expression. Bottom shows the relative optical density of pSphK1 (Ser225) and SphK1 normalized to the actin level. B, SphK1 activity was measured by using SphK1 Activity Assay Kit. C, levels of S1P production (μmol/L), as measured by ELISA assays on cell lysates. D, percentage of survival of cells treated with increasing doses of DMS (0.1–5 μmol/L), as measured by the MTT assay. *, 2-sided $P < 0.005$ versus control. Bars, SDs. E, percentage of apoptosis of cells treated with ceramide (10 μmol/L), as measured by the annexin V assay. *, 2-sided $P < 0.005$ versus control. F, percentage of apoptosis of GEO-CR and SW480 cells treated with ceramide (10 μmol/L), DMS (1 μmol/L), or the combination, as measured by the annexin V assay. *, 2-sided $P < 0.005$ versus ceramide alone. Data represent the mean (±SD) of 3 independent experiments, each conducted in triplicate. Bars, SDs.

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 with 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 $IC_{50} \geq 5$ μmol/L (data not shown). Then we evaluated fingolimod capability to restore cetuximab activity in resistant cells. As shown in Fig. 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 4 different resistant cells, cetuximab was ineffective in inhibiting Akt and/or MAPK phosphorylation. Fingolimod produced a variable effect, whereas 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

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

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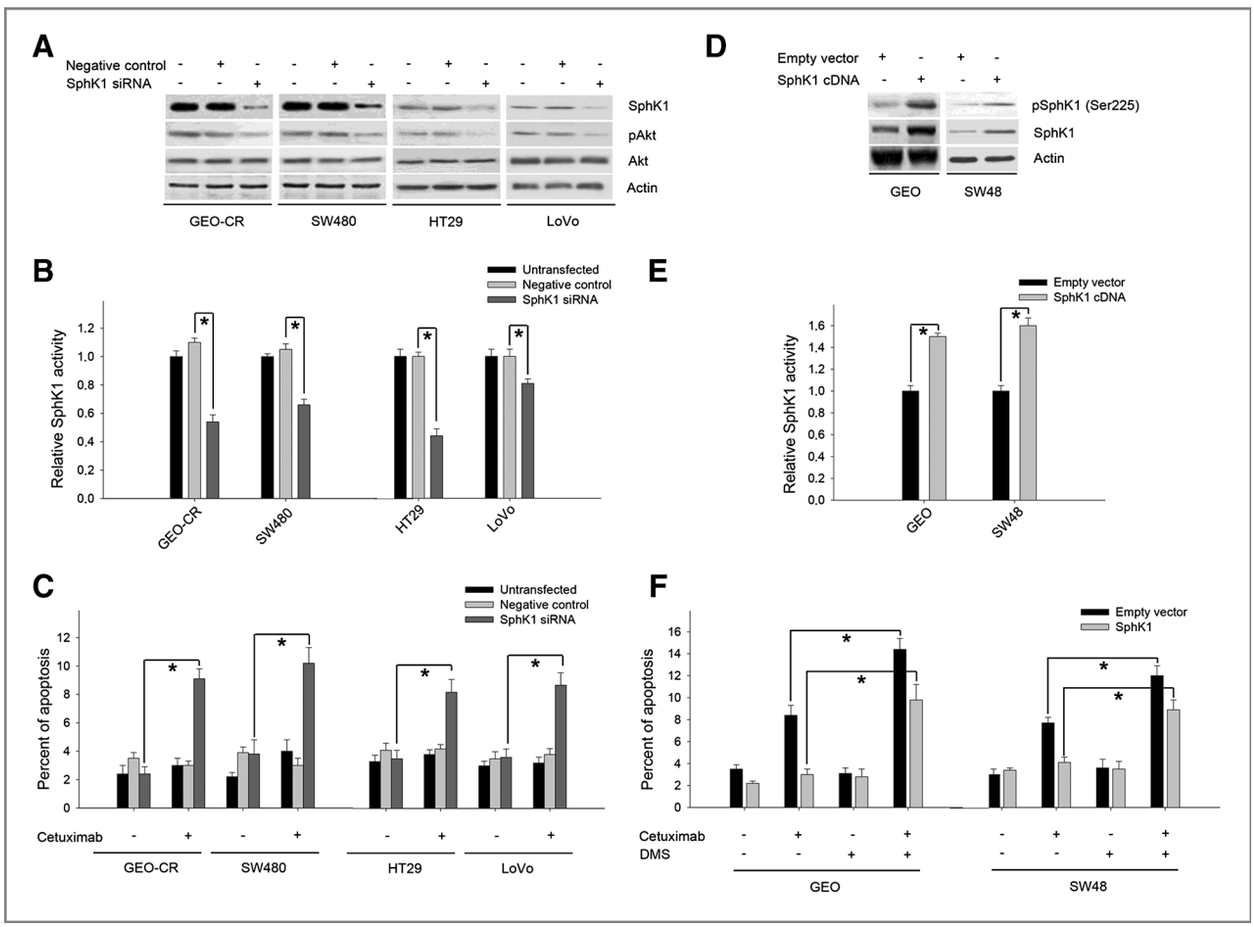

Figure 2. Effects of SphK1 modulation on cetuximab sensitivity in resistant colorectal cancer cell lines. A, Western blot analysis of protein expression in GEO-CR, SW480, HT29, and LoVo cells transfected with an SphK1-specific siRNA or with a negative control. B, SphK1 activity was measured in cells 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, percentage of apoptosis of cells transfected with a SphK1 specific siRNA or a negative control and treated with cetuximab (140 nmol/L), 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 SW48 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, percentage of apoptosis of cells transfected with human SphK1 cDNA or an empty vector and treated with cetuximab (140 nmol/L), DMS (1 μmol/L), or the combination, as measured by the annexin V assay. *, 2-sided P < 0.005 versus cetuximab alone. Data represent the mean (±SD) of 3 independent experiments, each conducted in triplicate. Bars, SDs.

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 Fig. 4B, mice treated with the combination showed a statistically significantly prolonged median survival duration compared with the controls [median survival 102.5 vs. 37.50 days; HR, 0.07552; 95% confidence interval (CI), 0.02130–0.2677; $P < 0.0001$] or to mice treated with cetuximab as single agent (median survival 102.5 vs. 57; HR, 0.1570; 95% CI, 0.04962–0.4966; $P = 0.0016$). 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 showed 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).

On the basis of the proapoptotic effect observed *in vitro* with the combination of cetuximab and fingolimod, we conducted imaging of apoptosis in GEO-CR tumor-bearing nude mice, 72 hours after the first treatment. As shown in

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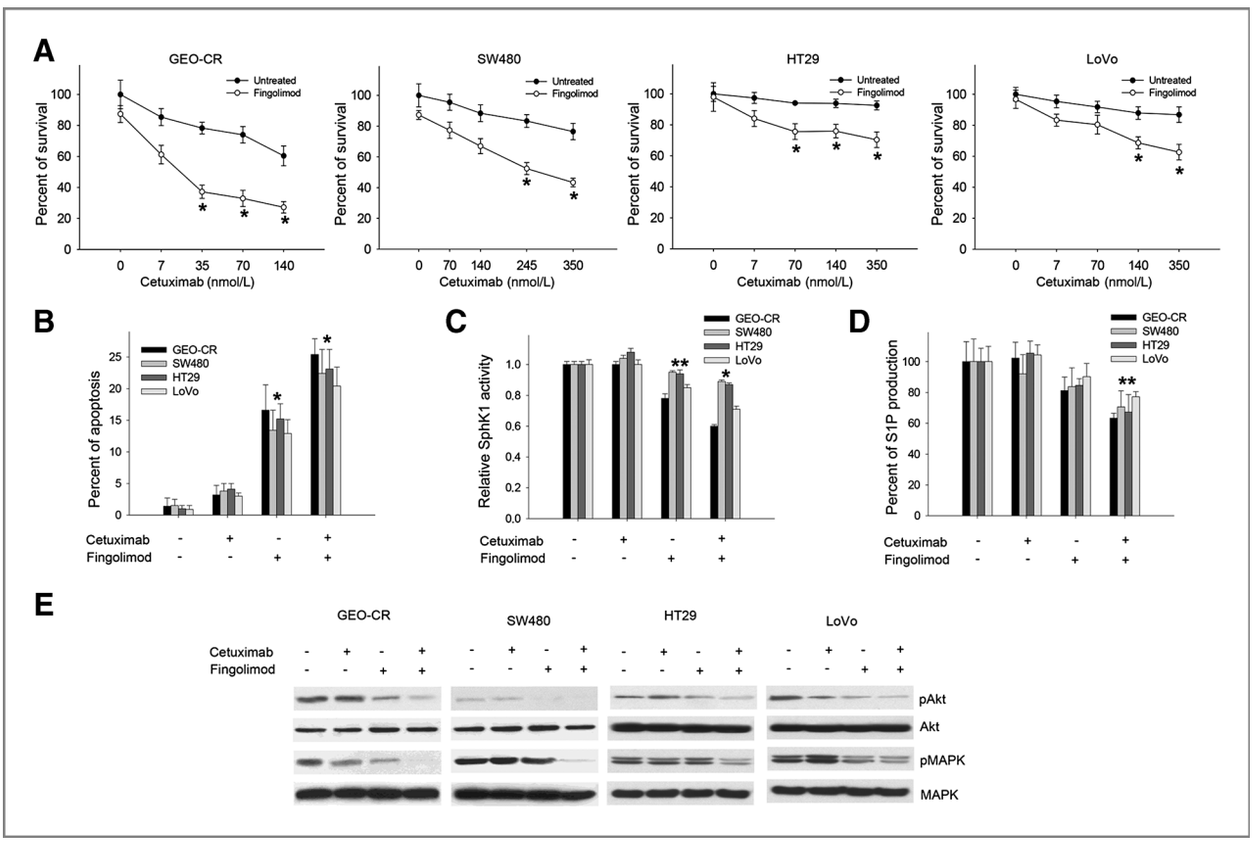

Figure 3. Effects of the S1PR antagonist fingolimod on cetuximab sensitivity in colorectal cancer cell lines. A, percentage of survival of cells treated with increasing doses of cetuximab (7–350 nmol/L), in presence or not of fingolimod (1 μmol/L), as measured by the MTT assay. *, 2-sided $P < 0.005$ versus cells treated with cetuximab alone. B, percentage of apoptosis of cells treated with cetuximab (140 nmol/L), fingolimod (5 μmol/L), 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, percentage 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 nmol/L), fingolimod (1 μmol/L), or the combination. Data represent the mean (±SD) of 3 independent experiments, each conducted in triplicate. Bars, SDs.

Supplementary Fig. S4, a relevant apoptosis induction was detected in mice treated with the combination compared with mice treated with cetuximab or fingolimod as single agents. We also investigated the long-term induction of apoptosis by conducting 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

On the basis of this body of data, and as 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, 3 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 containing control tissues, and was reproducible between different runs and lots (Supplementary Fig. S5B and S5C).

Representative pictures of samples with different SphK1 expression levels are shown in Supplementary Fig. S6A. As reported in Table 1, 22 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 showed that the correlation between high levels of SphK1 expression and poor response to cetuximab was significant ($P = 0.03$). Moreover, as shown in Supplementary Fig. S6B, progression free survival (PFS) seemed in favor of patients with low levels of SphK1 expression, with an advantage of 2.4 months

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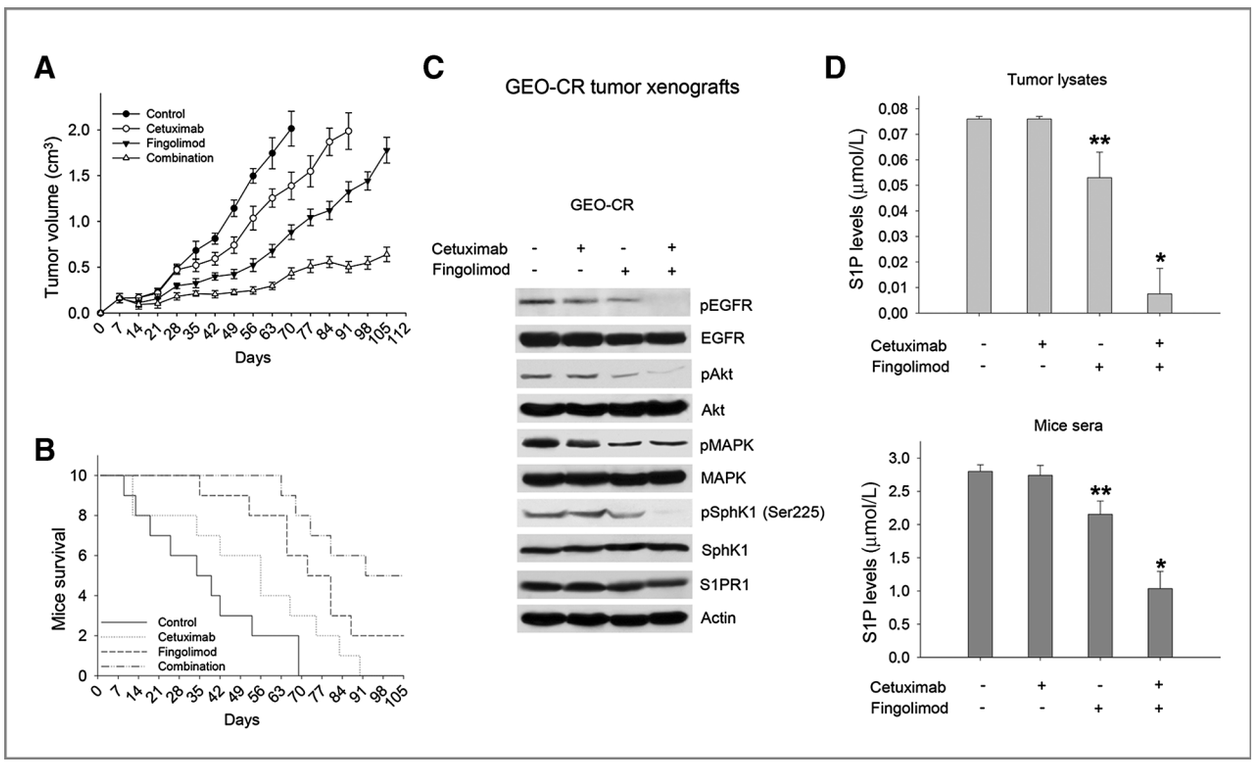

Figure 4. Effects of the combination cetuximab plus fingolimod on tumor growth, survival, and signal transduction of mice xenografted with GEO-CR resistant tumors. A, after 7 days following subcutaneous injection of GEO-CR cells, mice were randomized (10/group) to receive cetuximab, fingolimod, or their combination, as described in the Materials and Methods section. The 1-way ANOVA test was used to compare tumor sizes among treatment groups at the median survival time of the control group (35 days). The results were statistically significant for the combination versus single agents ($P < 0.0001$). B, median survival was statistically significant for the combination versus control or cetuximab (log-rank test). C, Western blot analysis was conducted on total lysates from tumor specimens of 2 mice sacrificed on day 25. D, quantification of S1P (μmol/L) 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 3 independent experiments, each conducted in triplicate, and are presented relative to control. Bars, SDs.

(median PFS 23.6 vs. 14.0 weeks, $P = 0.05$). Despite a trend in favor 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 conducted 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).

On the basis of the evidence that alterations of ceramide/S1P rheostat may be involved in resistance to biologic agents (9, 13, 14) and as 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

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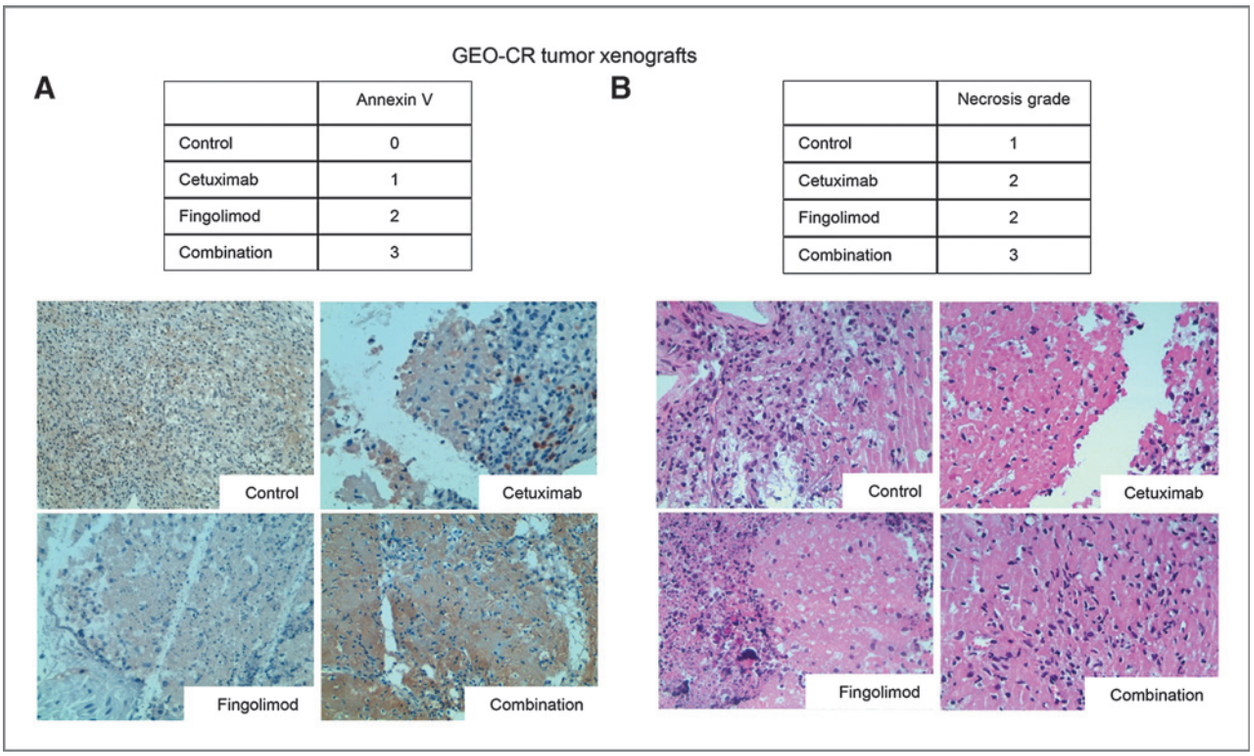

Figure 5. Effects of the combination cetuximab plus fingolimod on apoptosis and necrosis of GEO-CR tumor xenografts. A, immunohistochemical analysis of the apoptotic marker annexin V on FFPE 5-μm tissue slides. B, morphologic evaluation of necrosis-grade on hematoxilin/eosin stained 5-μm slides.

specific for SphK1. By combining each of these tools with cetuximab on resistant cells, we showed 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/mitogen-activated protein–extracellular signal-regulated kinase/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 showing the capability of S1P to induce cell proliferation and survival through activation of the Akt pathway (34–36). Most importantly, the resensitization 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).

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

Cross tabulation response versus SphK1

		SphK1		
		Low expression	**High expression**	**Total**
Response	Responders	11	3	14
	Nonresponders	17	19[a]	36
Total		28	22	50

[a]$P = 0.03$ vs. responders group.

It has been described that S1P produced by SphK1 may function as a second messenger inside the cell or may be secreted to bind to S1PRs on the cell surface. This signaling, involved in several human diseases including cancer, is defined as "inside-out" S1P signaling (37, 38). Therefore, the resensitization 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 signaling 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

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antagonistic activity on S1PRs. However, it has been described that fingolimod can act also as a SphK1 inhibitor (29). Therefore, the resensitization to cetuximab in our cell models may depend on cross-talks of EGFR pathway with intracellular S1P signaling rather than with extracellular S1P/S1PRs signaling pathways. Further investigations are needed to clarify this point.

As 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 confirmation 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 with 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 biologic marker of resistance to cetuximab.

Taken together, we show for the first time that SphK1 inhibition is effective in restoring cetuximab antitumor activity in colorectal cancers resistant to this agent. Moreover, as 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R. Rosa, B.M. Veneziani, S. De Placido, R. Bianco
Development of methodology: R. Rosa, V. D'Amato, V. D'Amato, A. Greco, S. De Placido, R. Bianco
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Rosa, R. Marciano, U. Malapelle, L. Formisano, L. Nappi, C. D'Amato, G. Marfe, S. Del Vecchio, A. Zannetti, A. De Stefano, C. Carlomagno, R. Bianco
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Rosa, R. Marciano, L. Formisano, L. Nappi, C. D'Amato, V. D'Amato, A. De Stefano, G. Troncone, S. De Placido, R. Bianco
Writing, review, and/or revision of the manuscript: R. Rosa, V. D'Amato, C. Carlomagno, B.M. Veneziani, R. Bianco
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Marciano, L. Formisano, L. Nappi, C. D'Amato, A. De Stefano, R. Bianco
Study supervision: S. De Placido, R. Bianco

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