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

Roberta Rosa1, Roberta Marciano1, Umberto Malapelle2, Luigi Formisano1, Lucia Nappi1, Claudia D’Amato1, Valentina D’Amato1, Vincenzo Damiano1, Gabriella Marfè1, Silvana Del Vecchio2, Antonella Zannetti3, Adelaide Greco2,4, Alfonso De Stefano1, Chiara Carlomagno1, Bianca Maria Veneziani5, Giancarlo Troncone6, Sabino De Placido1, and Roberto Bianco1

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.

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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...
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 signaling pathways (15, 16), we analyzed the contribution of sphingolipid rheostat alterations to cetuximab resistance (17, 18). 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.

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.

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.

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 phosho 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 (BD Biosciences). 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 (cm³) was measured using the formula π/6 × larger diameter × (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 hematoxin/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...
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 disease.

**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 reduced production (Supplementary Fig. S3B). Moreover, as shown in Fig. 2C, SphK1 silencing increased sensitivity to cetuximab 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 and resistant GEO-CR and SW480 cells, with a 2-fold increase in apoptosis after combined treatment compared with ceramide alone (Fig. 1F).

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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} \approx 5 \mu mol/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 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 cm³) 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
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.
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 cancers. 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 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 \( \text{K-Ras} \) or \( \text{B-Raf} \) 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 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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**Table 1.** Correlation of SphK1 expression with response to cetuximab-based therapy in colorectal cancer patients

<table>
<thead>
<tr>
<th>Cross tabulation response versus SphK1</th>
<th>SphK1 Low expression</th>
<th>SphK1 High expression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response Responders</td>
<td>11</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Nonresponders</td>
<td>17</td>
<td>19(^a)</td>
<td>36</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>22</td>
<td>50</td>
</tr>
</tbody>
</table>

\(^a\)P = 0.03 vs. responders group.
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
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References
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