Debio 0617B Inhibits Growth of STAT3-Driven Solid Tumors through Combined Inhibition of JAK, SRC, and Class III/V Receptor Tyrosine Kinases

Maximilien Murone1, Anne Vaslin Chessex1, Antoine Attinger1, Raghuveer Ramachandra2, Shankar J. Shetty2, Girish Daginakatte2, Saumitra Sengupta2, Sivapriya Marappan2, Samiulla Dhodheri2, Stefania Rigotti1, Yogeshwar Bachhav1, Silvano Brienza1, Peter Traxler1, Marc Lang1, Michel Auge1,5, Vincent Zoete4, Olivier Michielin4, Courtney Nicholas5, Faye M. Johnson6, Murali Ramachandra2, and Andres McAllister1

Abstract

Tumor survival, metastases, chemo-resistance, and escape from immune responses have been associated with inappropriate activation of STAT3 and/or STAT5 in various cancers, including solid tumors. Debio 0617B has been developed as a first-in-class kinase inhibitor with a unique profile targeting phospho-STAT3 (pSTAT3) and/or pSTAT5 in tumors through combined inhibition of JAK, SRC, ABL, and class III/V receptor tyrosine kinases (RTK). Debio 0617B showed dose-dependent inhibition of pSTAT3 in STAT3-activated carcinoma cell lines; Debio 0617B also showed potent antiproliferative activity in a panel of cancer cell lines and in patient-derived tumor xenografts tested in an in vitro clonogenic assay. Debio 0617B showed in vivo efficacy by inhibiting tumor growth in several mouse xenograft models. To increase in vivo efficacy and STAT3 inhibition, Debio 0617B was tested in combination with the EGFR inhibitor erlotinib in a non–small cell lung cancer xenograft model. To evaluate the impact of in vivo STAT3 blockade on metastases, Debio 0617B was tested in an orthotopic tumor model. Measurement of primary tumor weight and metastatic counts in lung tissue demonstrated therapeutic efficacy of Debio 0617B in this model. These data show potent activity of Debio 0617B on a broad spectrum of STAT3-driven solid tumors and synergistic activity in combination with EGFR inhibition. Mol Cancer Ther; 15(10): 2334–43. ©2016 AACR.

Introduction

STAT proteins are transcription factors involved in cytokine signaling. These proteins are in a latent monomeric state in the cytoplasm of cells and become activated upon tyrosine phosphorylation, typically through cytokine receptor–associated kinases (JAK family), cytoplasmic tyrosine kinases (SRC and ABL families of kinases; refs. 1, 2), or certain membrane-bound growth factor RTKs (3, 4). Phosphorylated STAT proteins form homodimers or heterodimers, which translocate to the nucleus and modulate STAT target genes transcription (3, 5). Among the seven members of the STAT family, persistent activation of STAT3 and STAT5 has been reported in a number of malignancies (6–9).

In solid tumors, STAT3 plays a key oncogenic driver role in multiple tumorigenic processes, including tumor cell proliferation, survival, migration, angiogenesis, drug resistance, and immune evasion (5, 10). Unfortunately, challenges in developing direct STAT3 inhibitors have so far prevented the development of promising anticancer drug candidates (10, 11). For this reason, we targeted kinases directly upstream of STAT3. It has been demonstrated that cytoplasmic tyrosine kinases of the JAK–SRC families play a crucial role in phosphorylation of STAT3 (Y705) and STAT5 (Y694; refs. 12, 13) and that inhibition of SRC family kinases (SKF) leads to compensatory STAT3 activation by JAK family kinases, outlining the importance for at least a dual JAK–SRC blockade when targeting pSTAT3 in solid tumors (14–16).

In this article, we describe the design and characterization of a novel kinase inhibitor, Debio 0617B, selected to maximize the efficacy of STAT3 inhibition by targeting key kinases upstream of STAT3. Debio 0617B potently blocked STAT3 phosphorylation in solid tumor cells in vitro and in vivo. The compound was shown to suppress the growth of cells from various cancer cell lines and patient-derived tumors and to demonstrate in vivo antitumor activity in various mouse xenograft models as a single agent. Debio 0617B does not target EGFR kinase activity, which is abundantly expressed and active in most epithelial cancers, and one of the key RTKs able to directly phosphorylate STAT3 on Y705 (17, 18). Moreover, STAT3 blockade with a STAT3 decoy significantly enhanced response to EGFR inhibitors (19, 20). Therefore, our study was conducted to determine whether combined STAT3 blockade in vivo by Debio 0617B and the EGFR inhibitor erlotinib...
would result in improved antitumor activity. Finally, because STAT3 signaling not only affects tumor growth and survival but also promotes metastasis formation, we explored the ability of Debio 0617B to inhibit metastases \textit{in vivo}.

**Materials and Methods**

**In silico docking of Debio 0617B**

Docking of Debio 0617B to JAK1, JAK2, c-SRC, and ABL was performed with use of the Autodock 4.2 program (21). Default values were used for the Autodock parameters except for the sampling parameters: each docking experiment consisted of 250 genetic algorithm runs with a maximum of 25,000,000 energy evaluations. The search space was defined as a cubic box with an edge length of 25 Å, centered on the geometrical center of the ATP-binding site. The following x-ray structures were selected from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB; www.rcsb.org; ref. 22) to perform the docking: PDB ID 4E4L (23) for JAK1, 4GMY (24) for JAK2, 1Y57 (25) for c-SRC (26), and 2G2I for ABL (27).

**In vitro binding activity**

The binding activity (% remaining activity at 100 nmol/L and \( K_D \)) was evaluated with use of the Ambit KINOMEscan RTK panel. KINOMEscan is based on a competition binding assay that quantitatively measures the ability of a compound to compete with an immobilized, active-site–directed ligand. The assay is performed by combining three components: DNA-tagged kinase, immobilized ligand, and a test compound. The ability of the test compound to compete with the immobilized ligand is measured via quantitative PCR of the DNA tag.

**In vitro cell-based activity**

Inhibition of RTK activity in cells was determined by using a cell-based protein tyrosine kinase assay (Advanced Cellular Dynamics). Kinases are individually expressed in a common lymphoid cell line (BaF3), and each cell line is dependent upon activity of the recombinant kinase for survival. Inhibition of kinase activity leads to cell death, which is monitored via ATP concentration with use of CellTiter-Glo (Promega).

**Cell lines**

Cell lines were obtained from the ATCC. A431 and A2058 cells were grown in DMEM (Thermo Fisher Scientific) containing 10% FBS (HyClone). A549 were grown in Ham F-12K Medium (Thermo Fisher Scientific) containing FBS to a final concentration of 10%. 4T1 cells were grown in RPMI1640 (Thermo Fisher Scientific) containing 10% FBS (HyClone). Cell lines were authenticated using short-term repeat analysis at the ATCC. All revived cells were used within 20 passages, and cultured for less than 6 months.

**Analysis of total and phosphorylated STAT3 and SRC in Debio 0617B-treated cancer cell lines**

Immunoblotting was performed on lysates from TU167 cells treated for 2 hours with increasing concentrations of Debio 0617B, as described previously (28). Cells were harvested by trypsinization and lysed in Tritis/EDTA lysis buffer plus SDS and β-mercaptoethanol for subsequent SDS-PAGE and followed by immunoblotting on nitrocellulose. Immunoblots were blocked with 5% milk or 5% BSA in TBS/Tween (for phospho proteins) and incubated overnight with the following primary antibodies (all from Cell Signaling Technology): SRC (#2110), pSrc family Y416 (#2101), STAT3 (#9139), and pSTAT3 Y705 (#9131).

**In vitro In-Cell Western assays**

A549 cells were seeded at \( 2 \times 10^4 \) cells per well in a 96-well, black-wall clear-bottom plate. Six hours after seeding, the medium was replaced with serum-free medium. After overnight serum starvation, Debio 0617B or fedratinib (SAR302503) was added to the cells in serum-free medium. Compounds were added at 10-point half-log dilutions from 30 to 0.001 μmol/L. Three hours after incubation with the compound, cells were stimulated with 100 μL of IL6 (50 ng/mL) for 20 minutes. Cells were washed and fixed in 100% methanol. Cells were permeabilized by washing three times with PBS containing 0.1% (v/v) Triton X-100 and blocked for 2 hours in 5% BSA in PBS containing 0.1% Triton X-100. Cells were incubated overnight with phospho-STAT3 antibody (1:200) on a rocker at 4°C. Cells were washed four times and incubated with europium-labeled anti-rabbit secondary antibody (1:3,000) for 2 hours at room temperature. The wells were washed four times, and enhancement solution was added. The plate was read on the Victor instrument at the europium setting. The wells were washed and incubated with Hoechst 33258 (1:10,000 in PBS). The plate was read with use of the umbellifereone setting (exc. 355 nm/emission 460 nm, 0.1 seconds). The Hoechst readings were used to normalize for cell number. IC\textsubscript{50} values were calculated with the normalized europium values by using GraphPad Prism Software.

**In vitro cytotoxicity in cancer cell lines**

Cells from a panel of cancer cell lines were seeded at 10,000 cells per well in 96-well plates and treated in triplicate with increasing concentrations of Debio 0617B for 72 hours. The proliferation assay via colorimetric MTT (magnesium tetrazolium; ATCC) was performed according to the manufacturer's instructions, with the following specifications: at 72 hours, an MTT substrate was added to each well and incubated at 37°C for 2 hours. All media were then aspirated from wells, and the cells were lysed in 50 μL of DMSO for 10 minutes at 37°C. Absorbance was read at 570 nm. The percentage of growth rates were calculated relative to the untreated control and were graphed in comparison with the log\textsubscript{10} concentration of Debio 0617 (μmol/L). Curve fit analysis was performed by using GraphPad Prism Software to determine the IC\textsubscript{50} concentration in μmol/L for each cell line.

**Ex vivo activity on patient-derived human tumor xenografts**

Debio 0617B was characterized for its ability to inhibit anchorungependent growth and \textit{ex vivo} colony formation of tumor cells in semisolid medium by using a 3D soft-agar clonogenic assay. The compound was tested in 206 human tumor xenografts of 22 different histotypes at concentrations ranging from 0.001 to 30 μmol/L in half-log increments.

Solid human tumor xenografts growing subcutaneously in serial passages in thymus-aplastic nude mice (NMRI nu/nu strain) were removed under sterile conditions. All xenograft materials were mechanically disaggregated and subsequently incubated with an enzyme cocktail consisting of collagenase type IV (41 U/mL), DNase I (125 U/mL), hyaluronidase (100 U/mL), and dispase II (1.0 U/mL) in RPMI1640 medium at 37°C for 45 minutes. Cells were passed through sieves of 100-μm and 40-μm...
mesh size (Cell Strainer, BD Falcon) and washed twice with RPMI1640 medium. The percentage of viable cells was determined in a Neubauer-hemocytometer by using Trypan blue exclusion. Aliquots of the cells were frozen and stored in liquid nitrogen. The clonogenic assay was performed in a 96-well plate format in a two-layer soft agar assay as described previously (29).

Migration assay
MDA-MB-231-Luci-Z1 cells were harvested with Accutase (PAA) and seeded (40,000 cells in 100 μl of medium/well) on a Collagen I-precoated ORIS-96-well plate (Ambio). After 24 hours, cell seeding stoppers were removed. The medium was exchanged with 150 μl of fresh medium. After 48 hours of migration, the medium was substituted with 75 μl of DMEM without phenol red containing calcein AM for 10 minutes at 37°C. Finally, fluorescent cells in the insert-defined area were detected by Fluostar (BMG) with FITC-settings (Ex: 485 nm/Em: 520 nm). Subsequently fluorescence photos were taken at ×40 magnification. Raw fluorescence data were converted into percent migration relative to high controls (solvent 0.1% DMSO) and low controls (stopper inserts removed before the addition of calcein AM), which were set to 100% and 0%, respectively.

Pharmacokinetic and pharmacodynamic profiles
To determine the relationship between pharmacokinetic and pharmacodynamic profiles of Debio 0617B in the A549 xenograft model, 5 × 106 A549 tumor cells (ATCC CCL-185TM) in 150 μl of 1:1 Hank’s balanced salt solution and Matrigel were injected subcutaneously in the left flank of athymic nude mice. Randomization of mice was done 18 days after tumor cell injection into four groups of four mice each with a mean tumor volume of approximately 200 mm3. The animals were randomized according to tumor volume and body weight. At 1, 4, and 12 hours after oral gavage administration of the single dose of Debio 0617B (30 mg/kg) in EAD-Lip1 solution formulation, samples were collected for plasma and tumor drug concentration estimations. Tumor samples were analyzed for inhibition of pSTAT3 and pSRC by immunoblot at the same time points (below).

Quantification of pSTAT3 and pSRC in tumors
Resected tumors (∼200 mg) were collected and immediately dipped into liquid nitrogen. Tumor powder was later crushed in a liquid nitrogen-prechilled pestle and mortar. Powdered tissues were immediately stored at −80°C. CST lysis buffer (10X) from Cell Signaling Technology (product # 9803S) was diluted 2X; protease (P-8340, Sigma) and phosphatase inhibitor cocktails (product # 5725, Sigma) were added to 1X, and 300 μl of cell lysis buffer was added to tumor samples weighing 50 mg or less. Next, 600 μl of cell lysis buffer was added to 100 to 200 mg of tumor samples. The tumor powder was homogenously resuspended in cell lysis buffer and incubated on ice for 1 hour. During incubation, the tumor samples were sonicated three times for 10 s each. After incubation, the samples were centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was collected in a fresh tube. If the samples were turbid, they were centrifuged twice. Tumor lysates were subjected to protein quantitation by using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, # 23227). Protein (50–60 μg) was separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% milk and incubated overnight at 4°C with the following antibodies (all from Cell Signaling Technology): pSTAT3 Y705 (#9131), STAT3 (#9132), pSRC family Y416 (#2101), and SRC (#2110). Membranes were washed and incubated with horseradish peroxidase–conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence. The percentage of inhibition in each group compared with vehicle control was calculated after normalizing for blank values and total protein.

Determination of plasma and tumor drug concentration
Plasma samples were deproteinized with acetonitrile and centrifuged. The supernatant was evaporated to dryness and reconstituted with the mobile phase. The samples were later analyzed for drug concentration by liquid chromatography–tandem mass spectrometry (LC/MS-MS) in the multiple reaction monitoring mode. Tumor samples were homogenized and later subjected to the same procedure as plasma. A set of calibration standards and quality control samples were used for both plasma and tumor samples.

In vivo antitumor assays
Athymic mice (Harlan Laboratories), 6- to 8-weeks-old and weighing 24 to 28 g, were used for A549, A431, and A2058 models. Balb/c mice (Harlan Laboratories), 6- to 8-weeks-old and weighing 20 to 25 g, were used in the 4T1 model. A working solution of Debio 0617B was prepared in EAD-Lip1 solution formulation (Debiopharm’s EAD-Lip1: 0.2% Debio 0617B, 3% Acconon MC-8, 3% ethanol, 2% Labrasol, 6% Polysorbate-80, and 6% Solutol HS-15) for all studies, except for the A431 xenograft model, which was run with a prototype formulation containing 20% HPC, 2% ethanol in PBS. The EAD-Lip1 vehicle was stored at room temperature until used. Debio 0617B was a clear solution in the EAD-Lip1 formulation. Because Debio 0617B was found to be stable in this EAD-Lip1 formulation for 2 days at room temperature, formulations were prepared once every second day for dosing. Debio 0617B in the EAD-Lip1 was administered by oral gavage. A solution of 0.9% saline was used as the vehicle for erlotinib, and water was the vehicle for saracatinib and ruxolitinib. To determine the antitumor activity of Debio 0617B in the A431, A2058, and A549 xenograft models, 5 × 106 tumor cells in 150 μl of 1:1 Hank’s balanced salt solution and Matrigel were injected subcutaneously into the left flank of athymic nude mice. For the A431 xenograft model, mice were randomized 7 days after tumor cell injection into four groups of eight mice each with a mean tumor volume of 82 ± 1 mm3. For the A2058 xenograft model, mice were randomized 7 days after tumor cell injection into four groups of eight mice each with a mean tumor volume of 223 ± 12 mm3. For the A549 xenograft model, mice were randomized 15 days after tumor cell injection into eight groups of eight mice each with a mean tumor volume of 155 ± 16 mm3. The animals were randomized according to tumor volume and body weight. To determine the antitumor and antimetastatic activity of Debio 0617B in the 4T1 mouse mammary tumor model, a total of 100,000 4T1 cells (0.1 × 106; ATCC CRI-2539) were injected into the mammary fat pad on day 0. Dosing of Debio 0617B, ruxolitinib, or saracatinib started from day 2. Primary tumors were resected on day 14. Dosing continued until day 35, when the animals were euthanized and metastatic foci on the lungs were counted with the naked eye.
Determination of tumor volumes

Tumor sizes were measured three times weekly with use of digital calipers, and the tumor volume was calculated by using the formula \((\text{length} \times \text{width}^2 \times 0.5)\). The calculation for the percentage of tumor growth inhibition (TGI) was the mean tumor volume of the treated group at day \(D\) divided by the mean tumor volume of the control group at \(D\) times 100. The effective criteria of the percentage of TGI according to NCI standards is \(\geq 58\%\) (30).

Results

Rational design of Debio 0617B

A new series of SRC/JAK/ABL inhibitors (diamino-pyrimidines) were rationally designed and synthesized on the basis of structural elements in the ATP binding pocket of c-SRC, JAK1, JAK2, and ABL (Fig. 1A). In brief, the amino-pyrimidine group makes two canonical hydrogen bonds with the backbone of the hinge loop (Leu959 of JAK1, Leu932 of JAK2, Met341 of c-SRC or Met318 of ABL). In addition, in the cases of JAK1 and JAK2, the amino group of the left-hand side benzamide function (Fig. 1B) also makes a hydrogen bond with a backbone carbonyl in the hinge region; that is, from Ser961 for JAK1 or Tyr934 for JAK2. The nonpolar part of the pyrido-pyrimidine bicycle makes nonpolar interactions with several residues of the binding site (Leu881, Val889, Ala906, and Leu1010 of JAK1; Leu855, Val863, Ala880, and Leu983 of JAK2; Leu273, Val281, Ala293, and Leu393 of c-SRC; Leu248, Val256, Ala269, and Leu370 of ABL). The chloride atom occupies a hydrophobic pocket formed by residues Val938, Met956, Ala906, and Leu1010 in the case of JAK1; Val911, Met929, Ala880, and Leu983 for JAK2; Val323, Thr338, Ala293 and Leu393 for c-SRC; Val299, Thr315, Ala269, and Leu370 for ABL. The right-hand side pyridine nitrogen atom receives a

Debio 0617B shows combined inhibition of JAK, SRC, ABL, and class III/V RTKs, and inhibits phosphorylation of STAT3 in cancer cells. A, in silico docking of Debio 0617B in the ATP-binding pocket of c-SRC, JAK1, JAK2, and ABL. B, structure of Debio 0617B, N-(4-chloro-3-(2-((4-(methylcarbamoyl)phenyl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)yl)phenyl)-4(trifluoromethyl)picolinamide. C, dose-dependent inhibition of pSRC and pSTAT3 in cancer cells. A STAT3-activated head and neck squamous cell carcinoma cell line (TU67) was treated for 2 hours with increasing concentrations of Debio 0617B. Cell lysates were analyzed via SDS-PAGE and subsequent immunoblotting in equal microgram amounts for the indicated proteins. D, network effect is required for improved STAT3 phosphorylation inhibition in A549 cells. Inhibition of STAT3 phosphorylation was measured by In-Cell Western (ICW). Debio 0617B EC50 in ICW assay computed from three independent experiments was 175 ± 21 nmol/L.
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drugs with the use of medicinal chemistry efforts led to the discovery and selection of Debio 0617B (Fig. 1B). The calculated binding modes of Debio 0617B to c-SRC, JAK1, JAK2, and ABL suggested a type I kinase inhibition binding mode, which we validated experimentally (Supplementary Table S1).

The selectivity of Debio 0617B and related analogs with promising activity and physicochemical properties was evaluated according to an extensive orthogonal approach, combining in vitro and cellular kinome profiling (Table 1). Debio 0617B was selected for further development, in part, because in addition to inhibiting the SRC, JAK, and ABL kinase families, the compound also potently targeted class III (FLT3, c-KIT, CSF1R, PDGFRα) and class V (VEGFR1, 2, 3) RTKs, which have also been reported to contribute to STAT3/5 activation. We postulated that integration of the simultaneous inhibition of SRC, JAK, ABL, and RTK class III and class V kinase families should result in efficient inactivation of the STAT3 signaling pathway in a spectrum of STAT3-driven tumors. In this article, we have focused on characterizing the activity of Debio 0617B in solid tumors. In terms of physicochemical and pharmacokinetic properties, Debio 0617B is characterized by good metabolic stability, low kinetic and thermodynamic solubilities, moderate blood clearance, and good oral bioavailability, making it suitable for in vivo use (Supplementary Table S2).

Table 1. Tyrosine kinase selectivity profile of Debio 0617B

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Family</th>
<th>IC50 (nmol/L)</th>
<th>KIC50 (nmol/L)</th>
<th>Activity on cell lines (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-SRC</td>
<td>Src</td>
<td>0.2</td>
<td>2.9</td>
<td>100</td>
</tr>
<tr>
<td>FYN</td>
<td>Src</td>
<td>11</td>
<td>22</td>
<td>100</td>
</tr>
<tr>
<td>YES</td>
<td>Src</td>
<td>5</td>
<td>6.5</td>
<td>90</td>
</tr>
<tr>
<td>LYN</td>
<td>Src</td>
<td>4.8</td>
<td>8.5</td>
<td>70</td>
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<td>Src</td>
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<td>Src</td>
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<td>&gt;1,000</td>
<td>80</td>
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<td>RTK class III</td>
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<td>50</td>
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<td>ABL</td>
<td>Abl</td>
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NOTE: Binding activity (% remaining activity at 100 nmol/L and IC50) was measured with the use of the Ambit KINOMEscan panel, and cellular activity (IC50, nmol/L) with the use of in vitro stable BaF3 cells transfected with tyrosine kinases. Relative inhibition is described here.

Debio 0617B potently inhibits STAT3 and SRC activation in cancer cells

The effect of Debio 0617B with respect to the inhibition of SRC and STAT3 phosphorylation was investigated first in the STAT3-activated head and neck squamous cell carcinoma (HNSCC) cell line Tu167. Dose-dependent inhibition of phospho-SRC (pSRC) and pSTAT3 was observed for Debio 0617B by immunoblotting (Fig. 1C). To evaluate the impact of targeting multiple kinases involved in STAT3 phosphorylation versus selective JAK2 inhibition, we compared inhibition of STAT3 phosphorylation by In-Cell Western for Debio 0617B versus the selective JAK2 inhibitor fedratinib (SAR302503) in non–small cell lung cancer (NSCLC) A549 cells (Fig. 1D). Despite the approximately 4-fold higher potency of fedratinib in inhibiting the enzymatic activity of JAK2 in vitro (fedratinib IC50: 1.5 nmol/L; Debio 0617B IC50: 6 nmol/L), Debio 0617B demonstrated superior potency in pSTAT3 inhibition (In-Cell Western fedratinib IC50: 389.6 nmol/L; Debio 0617B IC50: 175.3 nmol/L). Consistent with the inhibition of pSTAT3 in A549, Debio 0617B was also able to downregulate a set of STAT3 target genes in these cells (data not shown) and affect cell viability and expansion (Supplementary Table S3).

Debio 0617B has antitumor activity in solid tumors from different histotypes in vitro and in vivo

The cellular potency of Debio 0617B was first determined by measuring the IC50 values in a panel of 21 cancer cell lines derived from solid tumors with various levels of activated STAT3 (6, 9), including NSCLC, HNSCC, melanoma, and pancreatic and breast cancers with use of MTT assays (Supplementary Table S3). In most cases, the IC50 values were less than 2 μmol/L (31). We extended the evaluation of the cellular activity of Debio 0617B in a panel of 206 patient-derived tumor xenografts tested in an in vitro clonogenic assay. The mean IC50 of Debio 0617B in this panel of tumors was 1.167 μmol/L (Fig. 2; ref. 29).

From the panel of 21 cancer cell lines (Supplementary Table S3), three human cancer cell lines with an activated STAT3 pathway were selected for in vivo testing of Debio 0617B in subcutaneous mouse xenograft models (Supplementary Fig. S1A–S1D). Oral administration of Debio 0617B to tumor-bearing mice resulted in TGI activity in the three in vivo tumor models driven by pSTAT3 with no significant treatment-related body weight changes (Table 2; Supplementary Fig. S2). TGI ≥ 58%...
was considered an active antitumoral response (30), and TGI > 58% was observed in all three tumor models indicating good in vivo efficacy.

Pharmacokinetic and pharmacodynamic profiles of Debio 0617B in A549 tumors

To monitor in vivo target engagement by Debio 0617B, we analyzed the relationship between pharmacokinetic and pharmacodynamic profiles of the compound in athymic mice carrying established subcutaneous A549 tumors. We monitored intratumoral pSRC and pSTAT3 inhibition as pharmacodynamic markers of Debio 0617B activity. Pharmacodynamic analysis of pSRC and pSTAT3 inhibition by Debio 0617B demonstrated pSRC (98%) and pSTAT3 (74%) inhibition (Supplementary Table S4). The total levels of STAT3 and SRC did not change in the course of treatment. The concentration of Debio 0617B in tumor xenografts peaked at 4 hours and was then reduced by about half after 12 hours. A sharp decrease in Debio 0617B plasma levels was observed at 12 hours after administration. Of interest, although robust pSRC inhibition (94%–98%) was already detectable after 1 hour of exposure, pSTAT3 inhibition reached maximal levels after only 12 hours of exposure, suggesting different kinetics between direct target (pSRC) and downstream target (pSTAT3) inhibition.

Table 2. Antitumor activity of Debio 0617B in various in vivo tumor models with activated pSTAT3

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Cell line</th>
<th>Dose (mg/kg)</th>
<th>Schedule</th>
<th>TGI</th>
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</thead>
<tbody>
<tr>
<td>Epidermoid carcinoma of the vulva</td>
<td>A431</td>
<td>15</td>
<td>Daily for 14 days</td>
<td>27%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td></td>
<td>67%</td>
</tr>
<tr>
<td>Melanoma</td>
<td>A2058</td>
<td>15</td>
<td>5 days on/2 days off; 14 days</td>
<td>49%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td></td>
<td>64%</td>
</tr>
<tr>
<td>NSCLC</td>
<td>A549</td>
<td>15</td>
<td>5 days on/2 days off; 14 days</td>
<td>71%</td>
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</tbody>
</table>

NOTE: TGI was determined in 3 subcutaneous xenograft models treated with various doses of Debio 0617B as indicated. In the A431 xenograft model (n = 8), Debio 0617B was administered daily by oral gavage at 15 and 30 mg/kg for 14 consecutive days (Q1D14). Treatment with Debio 0617B caused a statistically significant antitumor effect when administered at 15 and 30 mg/kg, with TGI of 27% and 67%, respectively. In the A2058 xenograft model (n = 9), Debio 0617B was administered 5 days on 2 days off for 2 cycles by oral gavage at 15 and 20 mg/kg in EAD-Lip1. Treatment with Debio 0617B caused statistically significant antitumor effects when administered at 15 and 20 mg/kg, with TGI of 49% and 64%, respectively. Saracatinib was used as the benchmark in this model and was administered once daily for 14 consecutive days (Q1D14) by oral gavage at 25 mg/kg. Treatment with saracatinib alone did not result in statistically significant antitumor effects with TGI of 15%, and the dose was well tolerated (Supplementary Fig. S2B). In the A549 xenograft model (n = 8), Debio 0617B was administered at 5 days on 2 days off for 2 cycles by oral gavage at 15 mg/kg in EAD-Lip1. In this model, Debio 0617B was benchmarked and combined with erlotinib (for details, see Fig. 3). Treatment with Debio 0617B caused statistically significant antitumor effects at the end of the treatment period with TGI of 71%. Treatment-related gross pathologic changes and body weight losses were not observed in Debio 0617B–treated groups (see also Supplementary Fig. S3A). The statistical significance of TGI was analyzed by comparing treatment groups with the vehicle-treated group using one-way ANOVA. NCI guidelines: TGI ≥ 58% is considered as an active antitumoral response (30).

Figure 2. Debio 0617B suppresses tumor growth of patient-derived tumors. Debio 0617B is active in patient-derived tumor xenografts in an in vitro clonogenic assay. Scatter plot presentation of relative IC50 values obtained for Debio 0617B in 206 patient-derived tumor xenografts.
In vivo efficacy in combination with erlotinib in an NSCLC xenograft model

Because EGFR signaling contributes to STAT3 activation in tumors of epithelial origin (17, 18), combination therapy of Debio 0617B and the EGFR inhibitor erlotinib was evaluated in an athymic nude mouse NSCLC (A549) xenograft model (32). Both Debio 0617B and erlotinib used as single agents over two cycles of treatment showed TGI in the range of 53% to 75%, depending on drug concentration, whereas the combination therapy resulted in 95% to 102% TGI (Fig. 3A). To further evaluate the benefit of the combined treatment, we monitored tumor regrowth after two cycles of treatment (2 weeks) over a period of 21 days (Fig. 3B). The combination of Debio 0617B with erlotinib showed statistically significant tumor regrowth inhibition (74%–75%) versus single-agent treatments (26%–62%).

Debio 0617B inhibits migration of cancer cells

STAT3 and SRC signaling have been reported to play a key role in promoting promigratory programs leading to metastasis (5, 33, 34). Therefore, we tested the activity of Debio 0617B in blocking in vitro migration and in vivo metastasis. In an in vitro assay, Debio 0617B demonstrated inhibition of migration of MDA-MB-231-Luci-Z1 breast cancer cells with an IC50 of 169 nmol/L, compared with the SFKs inhibitor bosutinib (SKI-606; ref. 35), for which the IC50 was 416 nmol/L (Fig. 4A).

Debio 0617B reduces metastases in vivo

We next evaluated the antimetastatic effect of Debio 0617B in vivo in a neoadjuvant and adjuvant setting. The compound was tested in an orthotopic 4T1 mouse mammary tumor model in BALB/c mice. In this model, Debio 0617B was compared with the JAK1/2-specific inhibitor ruxolitinib (INCBO18424; ref. 36) and with the SFK/ABL inhibitor saracatinib (AZD0530; ref. 37). Both compounds have partially overlapping profiles of kinase inhibition with Debio 0617B; therefore, they were used to evaluate the advantage of combined JAK-SRC inhibition by a single compound (Debio 0617B). Tumor cells were orthotopically implanted in the mammary fat pad of mice, and treatments started 48 hours later (Fig. 4B). At 14 days after tumor implantation, primary tumors were resected and weighed. Tumor weight was significantly reduced (by 60%–74%) with the Debio 0617B treatments, whereas ruxolitinib and saracatinib showed no effect compared with the control (Fig. 4C). Histologic analysis of primary tumor samples also demonstrated significant reduction in average mitotic figures in the groups treated with 10 or 20 mg/kg of Debio 0617B (Supplementary Fig. S3A). Metastatic counts in the lungs at the end of the experiment showed significant decrease after treatment with Debio 0617B, but not with ruxolitinib (Fig. 4D). Saracatinib, although inefficient in reducing primary tumor growth, was able to reduce mitotic figures and metastatic counts. No significant treatment-related body weight changes were observed (Supplementary Fig. S3B).

Discussion

Our drug discovery initiative has resulted in the generation of Debio 0617B, a novel kinase inhibitor with a unique inhibitory profile against key kinases from the JAK, SRC, and ABL families involved in STAT3 phosphorylation. In-depth profiling of Debio 0617B showed that the compound also demonstrated potent...
activity against a subset of RTKs, class III and V RTKs, which are also known to play a context-dependent role in STAT3 activation (6). Through its network effect on the key oncokinases involved in STAT3 activation, Debio 0617B was able to inhibit STAT3 in cell-based assays and NSCLC tumors in vivo. To better understand the contribution of the different sets of kinases on the tumor-inhibition properties of the compound, Debio 0617B potency was compared with various kinase inhibitors with overlapping specificity.

![Image of Debio 0617B and SKI-606 IC50 values]

**Figure 4.**
Effect of Debio 0617B treatment on cell migration and metastases formation. A, Debio 0617B inhibits migration of MDA-MB-231-Luci-Z1 after 48 hours. Shown are the means ± SD. The IC50 value calculation was performed by using GraphPad Prism 5 software with a variable slope sigmoidal response fitting model using 0% migration as the bottom constraint and 100% migration as the top constraint. B, in vivo efficacy in the 4T1 neoadjuvant mammary tumor model: study design. Debio 0617B was administered 5 days on, 2 days off for a total of 33 days by oral gavage at 10 and 20 mg/kg in EAD-Lip1 [5Q7Dx5; the last two days of the last cycle (2 days off) were skipped, and animals were euthanized on day 35]. Saracatinib and ruxolitinib were used as benchmarks in this model and administered once daily for 33 consecutive days (Q1D33) by oral gavage at 25 and 12.5 mg/kg. Primary tumors were resected on day 14. Lung metastases were counted on day 35.

C, primary tumor weight after 12 days of treatments. Treatment with Debio 0617B caused statistically significant antitumor effects when administered at 15 and 20 mg/kg, with TGI of 49% and 64%, respectively. Average mitotic figures in primary tumor samples were determined by histologic analysis (see also Supplementary Fig. S3A). D, metastatic foci and discrete metastatic nodules on lungs from all groups were counted and compared with vehicle control after animals were euthanized on day 35, and results were reported in the table as the percentage of reduction in lung metastases (*, P < 0.05; ***, P < 0.001).
selectivity profiles, including the JAK2-specific inhibitor fedratinib, the JAK1/2 inhibitor ruxolitinib (35), and the SRC/ABL inhibitor saracatinib (36). Debio 0617B efficiently inhibited in vitro proliferation and survival of various cancer cells, including cancer cell lines and patient-derived tumor samples, with a mean IC_{50} in the range of 1 μmol/L whereas the oral pharmacokinetic parameters in mice indicated that at C_{max} the concentration of Debio 0617B was 7-fold above this mean IC_{50} value (data not shown), suggesting adequate \textit{in vivo} exposure to the compound.

In a clonogenic assay on a panel of 206 patient-derived tumors from different origins, a number of samples from different cancer histotypes responded with IC_{50} values below the mean value. Those included HNSCC, melanoma, and cancer of the stomach, lung, kidney, pancreas, and ovary. Four cancer cell lines (A2058, melanoma; A431, epidermoid carcinoma; A549, NSCLC; 4T1, mammary tumor) responding to Debio 0617B were used to evaluate the \textit{in vitro} efficacy of the compound. Debio 0617B demonstrated antitumoral responses in mouse xenograft models bearing subcutaneous or orthotopic tumors derived from the four cell lines, with TGI values ranging from 64% to 71%. To determine whether the \textit{in vivo} antitumoral response to Debio 0617B was linked, at least in part, to STAT3 inhibition, we ran a dedicated pharmacokinetic/pharmacodynamic analysis of Debio 0617B in mice bearing A549 tumors, and demonstrated \textit{in vivo} target engagement. Concomitant treatment with Debio 0617B and the EGFR inhibitor erlotinib significantly enhanced \textit{in vivo} antitumor activity and delayed tumor regrowth after treatment cessation in the NSCLC A549 xenograft model. These data suggest that combining EGFR inhibition with Debio 0617B could significantly affect both the response to EGFR inhibitors and the duration of the response in NSCLC (18, 31).

To expand our characterization of the properties of Debio 0617B, we also tested the antitumoral potential of the compound in a syngeneic mouse mammary tumor model of spontaneous metastasis. The activity of Debio 0617B was tested in neoadjuvant and adjuvant settings, and benchmarked to saracatinib and ruxolitinib. We demonstrated that Debio 0617B was able to inhibit primary tumor growth in this model, whereas neither ruxolitinib nor saracatinib had any effect. Because STAT3 signaling in cancer cells also contributes to tumor evasion from the immune system, it is possible that the observed antitumor activity in this syngeneic model was also caused, in part, by the effect of Debio 0617B on the antitumor immune activity. We then examined metastases in the lungs 3 weeks after primary tumor removal, and Debio 0617B showed a 57% reduction in metastases.

In summary, our data indicate that through combined inhibition of key kinases involved in STAT3 activation, Debio 0617B abrogated STAT3 phosphorylation in STAT3-activated tumor cells both \textit{in vitro} and \textit{in vivo}. We have also shown that the combination of Debio 0617B with the EGFR inhibitor erlotinib greatly enhanced the \textit{in vivo} response and delayed tumor regrowth. This combination is particularly interesting because resistance to RTKs inhibitors has recently been shown to be due to STAT3 reactivation by a feedback loop, and inhibition of this STAT3 feedback loop by Debio 0617B could be exploited as a therapeutic strategy to increase the response to a broad spectrum of targeted agents (33).

Disclosure of Potential Conflicts of Interest
M. Aguet is a consultant/advisory board for Debiopharm. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Concept and design: M. Murone, A. Attinger, S.J. Shetty, G. Daginakatte, S. Sengupta, F.M. Johnson, M. Ramachandra, A. McAllister
Development of methodology: M. Murone, S. Maratpan, S. Rigotti, Y. Bachhav, S. Brienza, A. McAllister
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Murone, R. Ramachandra, S. Dhhodheri, Y. Bachhav, C. Nicholas, F.M. Johnson, A. McAllister
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Murone, A. Vaslin Chesser, A. Attinger, R. Ramachandra, G. Daginakatte, S. Dhhodheri, M. Lang, M. Aguet, V. Zoete, O. Michielin, C. Nicholas, F.M. Johnson, M. Ramachandra, A. McAllister
Writing, review, and/or revision of the manuscript: M. Murone, A. Vaslin Chesser, R. Ramachandra, S. Rigotti, P. Traxler, O. Michielin, C. Nicholas, F.M. Johnson, M. Ramachandra, A. McAllister
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Bachhav, C. Nicholas
Study supervision: M. Murone, A. Vaslin Chesser, R. Ramachandra, G. Daginakatte, S. Dhhodheri, Y. Bachhav, M. Aguet, A. McAllister
Other (conceptualization and synthesis of Debio 0617B): S. Sengupta
Other (consultant for the project): P. Traxler

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References


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Maximilien Murone, Anne Vaslin Chessex, Antoine Attinger, et al.


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