Inhibition of cell growth and up-regulation of MAD2 in human oesophageal squamous cell carcinoma after treatment with the Src/Abl inhibitor dasatinib

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Abstract
Aberrant expression and/or activity of the non-receptor protein tyrosine kinase SFK (Src family kinase) members are commonly observed in progressive stages of human tumours. The aim of the present study was to investigate whether Src is a potential drug target for treating oesophageal squamous cell carcinoma. Compared with the human immortalized oesophageal epithelial cell line SHEE, oesophageal squamous cell carcinoma cells have increased tyrosine phosphorylation activities. We have explored the therapeutic potential of dasatinib, a small-molecule inhibitor that targets multiple cytosolic and membrane-bound tyrosine kinases, for the treatment of oesophageal squamous cell carcinoma. We examined that the effects of dasatinib on proliferation, invasion, apoptosis, spindle checkpoint, cell-cycle arrest and kinase activity in vitro using three human oesophageal carcinoma cell lines KYSE30, KYSE180 and EC109. In nude mouse models, dasatinib treatment effectively inhibited the expression of activated Src, resulting in the inhibition of tumour growth. Multiple drug effect isobologram analysis was used to study interactions with the chemotherapeutic drug docetaxel. As expected, the three oesophageal carcinoma cell lines were highly sensitive to dasatinib, but SHEE cells were not sensitive to this drug. Concentration-dependent anti-proliferative effects of dasatinib were observed in the three oesophageal carcinoma cell lines. Dasatinib significantly inhibited oesophageal carcinoma cell invasion and up-regulation of MAD2 (mitotic arrest-deficient 2), as well as inducing cell apoptosis and cell-cycle arrest. Additive and synergistic interactions were observed for the combination of dasatinib and docetaxel. Therefore it was concluded that dasatinib blocks the G1/S transition and inhibits cell growth. These results provided a clear biological rationale to test dasatinib as a single agent or in combination with chemotherapy in oesophageal squamous cell carcinoma. Moreover, we have shown in vitro and in vivo that dasatinib might have therapeutic benefit for patients with oesophageal squamous cell carcinoma who are not eligible for surgery.

Key words: Bcr/Abl, dasatinib, docetaxel, mitotic arrest-deficient 2 (MAD2), oesophageal squamous cell carcinoma, Src.
Abbreviations: CML, chronic myeloid leukaemia; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; MAD2, mitotic arrest-deficient 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PI, propidium iodide; SFK, Src family kinase.
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INTRODUCTION

Oesophageal carcinoma occurs worldwide with a variable geographic distribution. It ranks eighth in order of occurrence and is the sixth leading cause of cancer mortality. Approximately 462,000 new cases were diagnosed in the world in 2003 [1]. The majority of cases of oesophageal squamous cell carcinoma are infiltrating, recurrences and metastases. Biological agents, chemotherapy and/or radiotherapy or combinations of these treatments have little impact on the survival of patients with metastatic oesophageal squamous cell carcinoma. Thus novel systemic treatment approaches are urgently needed. Research on the genetic changes associated with the development of cancer has resulted in promising new agents that inhibit specific proteins up-regulated in cell signalling pathways or inhibit anti-apoptotic proteins. Src is the prototypic member of a family of non-receptor tyrosine kinases (SFKs; Src family kinases), which include Src, Lyn, Fyn, Lck, Hck, Fgr, Blk, Yrk and Yes) that has a key role in many cellular signalling pathways [2]. SFKs regulate four main cellular functions that ultimately control the behaviour of transformed cells: cell proliferation, adhesion, invasion and motility [3,4]. Src offers a particularly promising molecular target for anticancer therapy, as Src inhibition leads to inhibition of multiple signalling pathways. Src is overexpressed in oesophageal carcinoma relative to control tissues, and increased Src expression correlates with disease progression [5]. A novel drug named dasatinib (BMS-354825; Sprycel™, Bristol-Myers Squibb) is a highly potent ATP-competitive orally active dual Src/Abl kinase inhibitor with anti-proliferative activity against solid tumours and CML (chronic myeloid leukaemia) cell lines. It is thought to bind to the active and inactive conformations of the Abl kinase domains.

Dasatinib is currently entering clinical trials for various epithelial malignancies (http://ClinicalTrials.gov). In preclinical studies, it has also been shown to be active against prostate cancer cells [6], basal-like breast cancer cells [7], melanoma cells [8], colon cancer cells [9], head and neck squamous cancer cells and non-small-cell lung cancer cells [10,11]. At low nanomolar concentrations, dasatinib inhibits the progression of CML, pancreatic adenocarcinoma, colon cancer, prostate cancer, non-small-cell lung cancer and breast malignancies [6–11,27]. In the present study, we aimed to use dasatinib in oesophageal squamous cell carcinoma to target Src and provide a rationale for combination therapy with dasatinib and docetaxel.

The expression of the Bcr/Abl kinase causes CML, and it has been shown that 30% of centrosomes in the chronic phase of CML are aberrant [12]. Centrosome hypertrophy is an early event in the transformation process and precedes karyotype instability. Wolanin et al. [13] have shown that Bcr/Abl expression limits the activation of the mitotic checkpoint and compromises the post-mitotic checkpoint, which normally prevents polyploidization. Furthermore, the occurrence of chromosomal aberrations in Bcr/Abl-positive cells from patients being treated with dasatinib raises the question of whether centrosome-associated mechanisms may be responsible. MAD2 (mitotic arrest-deficient 2) is an important component of the spindle assembly checkpoint in centrosome-associated mechanisms, and it can protect cells from aberrant chromosome segregation. Thus we were interested in whether dasatinib affects the level of MAD2 in oesophageal squamous cell carcinoma cells.

To more fully understand dasatinib activity in human oesophageal squamous cell carcinoma, we studied dasatinib activity with an emphasis on invasion, proliferation, cell-cycle arrest, apoptosis and kinase activity using the three established human oesophageal squamous cell carcinoma cell lines, KYSE30, KYSE180 and EC109, and an immortalized oesophageal epithelial cell line SHEE. In the present study, we also sought to evaluate the potential of dasatinib to influence the levels of MAD2 and cyclin D1. We observed that dasatinib significantly inhibited proliferation, induced cell apoptosis and caused cell-cycle arrest in KYSE30, KYSE180 and EC109 cells via blockage of Src. Dasatinib also inhibited oesophageal squamous cell carcinoma invasion in a Matrigel invasion assay by preventing Src auto-phosphorylation and focal adhesion formation. Dasatinib induced the G1-phase arrest in the three cell lines, and this was associated with a decline in cyclin D1 levels. We have also shown that dasatinib combined with docetaxel synergistically inhibited cell proliferation. In vitro, an increase in MAD2 was also observed. It just remains to determine whether dasatinib could inhibit the development of metastases in human oesophageal squamous cell carcinoma.

MATERIALS AND METHODS

Cell lines and culture conditions

To assess the effect of dasatinib in human oesophageal squamous cell carcinoma, we used the KYSE30, KYSE180 and EC109 cell lines, and another cell line, SHEE, as a control. All cell lines were cultivated in DMEM (Dulbecco’s modified Eagle’s medium; Gibco) supplemented with 10% heat-inactivated FBS (fetal bovine serum) and 1.0% penicillin/streptomycin. Cells were incubated in a 37° C humidified incubator with a mixture of 90% air and 10% CO2 (Forma Scientific).

Reagents

Dasatinib (Mr, 488.0) was obtained from Bristol-Myers Squibb and was prepared as a 10 mmol/l stock solution in DMSO (Sigma). The stock was stored at −20°C and diluted further as needed to an appropriate final
concentration in serum-containing DMEM/F-12 (Life Technologies).

**Cellular proliferation and apoptosis studies in vitro**

Cell growth was evaluated by seeding cells in triplicate in complete medium (medium supplemented with 10 % FBS). After incubation for 18 h, fresh medium or medium containing dasatinib was added. Cell viability was determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay after 72 h [20].

Apoptosis was quantified using the Annexin V-FLUOS Apoptosis Kit (Roche) according to the manufacturer’s instructions. Treated KYSE30, KYSE180 and EC109 cells were incubated under standard culture conditions with or without drug treatment for 48 h before staining and subsequent flow cytometric analysis.

**Western blot analysis**

Cells were grown for 24 h with dasatinib (5 × 10⁵ cells in 10-cm-diameter tissue culture dishes). After incubation, cells were washed twice in ice-cold PBS (pH 7.4) and lysed for 10 min in lysis buffer [50 mmol/l Tris/HCl (pH 7.6) (Sigma), 150 mmol/l NaCl (Merck), 1 mmol/l EDTA (Sigma), 1 mmol/l EGTA (Sigma), 1 % Nonidet P40 (USB), 10 % glycerol (Sigma), 50 mmol/l NaF (Sigma) and 1 mmol/l dithiothreitol (Invitrogen)]. Cells were scraped from the plates and the cell lysates were clarified by centrifugation for 20 min at 19,000 g at 4 °C. The supernatant was collected and the total protein concentration was determined using the Bio-Rad Laboratories Protein Assay. Total protein (20–50 mg) was separated by SDS/PAGE (4–20 % gels) and transferred to PVDF membranes (Bio-Rad Laboratories). Membranes were blocked for 2 h with 5 % BSA or with 5 % non-fat milk, immunoblotted with primary antibody [rabbit polyclonal antibody to phospho-SFK (Tyr416) (Cell Signaling Technology), β-actin (Calbiochem), pro-caspase 3, caspase 3, MAD2, cyclin D1 (each from Santa Cruz Biotechnology), cytochrome c, Bcl-2 or Bax (each from Abcam Biotechnology)] and detected with a secondary antibody [horseradish-peroxidase-conjugated goat anti-(mouse IgG) antibody or goat anti-(rabbit IgG) (Santa Cruz Biotechnology)]. Electrochemiluminescence (Genscript) was used for detection of immunoreactivity.

**Flow cytometry analysis of cell cycle and apoptosis**

Cell-cycle status and apoptosis analysis was performed using flow cytometry. Apoptosis of cells was evaluated by measuring the exposure of phosphatidylserine on the cell membranes using an Annexin V Detection Kit (BD Pharmingen). Briefly, cells were plated at a density of 5 × 10⁵ cells/well in 10-cm-diameter plates and maintained in medium containing 10 % FBS overnight. The following day, cells were treated with various concentrations of dasatinib in medium containing 10 % FBS for the indicated length of time. Cells were harvested from the culture plates and washed with PBS containing 2 % FBS. For DNA analysis, cells were then fixed with ice-cold 70 % ethanol, washed and resuspended in a solution containing 10 µg/ml PI (propidium iodide) and 10 µg/ml bovine RNase (Boehringer Mannheim). After 30 min, DNA content was analysed using flow cytometry. For apoptosis analysis, the cells were resuspended in a staining solution containing 50 µg/ml PI and 25 µg/ml Annexin V–FITC for 15 min at room temperature (20 °C) in the dark. The cells were assessed by FACs equipped with Cell Quest software (BD Pharmingen).

**Matrigel invasion assay**

Invasion of oesophageal carcinoma cells in vitro was measured based on the invasion of cells through Matrigel-coated 24-well Transwell inserts. Cells (1 × 10⁶) were treated with various concentrations of dasatinib for 24 h in 10-cm-diameter plates and washed with serum-free medium. Cells (2.5 × 10⁵) were then transferred to each Transwell in duplicate and were allowed to invade for 24 h at 37 °C. Invading cells were stained with 0.1 % Crystal Violet in methanol and quantified by dissolving the Transwell inserts in a 2.0 % sodium deoxycholate solution, which was subsequently analysed spectrophotometrically at 595 nm. Experiments were carried out in triplicate.

**Tumour growth in nude mice**

The exponentially growing cells were trypsiniied and resuspended in D-Hanks solution, and 5 × 10⁵ KYSE180 cells (0.2 ml) were injected subcutaneously into the left flank of 4-week-old female BALB/c nude mice (at least six mice per group). In vivo studies were approved by the State Scientific and Technological Commission of China, and all animal care was conducted in accordance with regulations of the administration of affairs concerning experimental animals.

**Drug formulation and administration**

At 3 days after the xenograft injection, the mice were randomized to receive either the drug or the control vehicle (four mice per group). For oral administration, dasatinib was dissolved in an 80 mmol/l citrate buffer (pH 3.1), according to the manufacturer’s instructions. The animals were treated daily with 30 mg·kg⁻¹ of body weight·day⁻¹, using a 20 gauge gavage needle. The control group was given an equal volume of diluent buffer by the same gavage technique. The mice were treated for 28 days.
**Necropsy and tissue preparation**
The tumour volume was calculated as \((\text{length} \times \text{width}^2) \times 0.5\). At the end of the 4 week treatment period, the mice were killed by an overdose of sodium pentobarbital (1 mg/g of body weight) 4 h after the last drug dose or control diluent was given. The tumours were surgically excised and weighed, followed by fixation in phosphate-buffered 10% formaldehyde.

**Statistical analysis**
The means ± S.D. for the biological effect of dasatinib in the different assays were calculated. Statistical significance between groups was compared using a Student’s *t* test. One-sided tests were considered significant at a level of 0.05. For the studies examining the anti-proliferative effects of the kinase inhibitor on the cell line, the cell density values were calculated from five replicate wells per condition, and the results shown are representative of three independent experiments. The IC\(_{50}\) values were determined from a sigmoidal dose–response curve fit to the data (percentage inhibition compared with the log of the concentration). The \(P\) values were calculated using a two-way ANOVA model.

**RESULTS**

**Cell proliferation was potently inhibited by dasatinib in human oesophageal squamous cell carcinoma cell lines, but not in SHEE cells**

Previous studies have shown that dasatinib inhibits proliferation of lymphoma and prostate and breast cancer cells *in vitro* [14,15]. It also has been reported that Src has a prominent role in controlling the proliferation of prostate cancer cells *in vitro* [16]. The effects of dasatinib on an oesophageal carcinoma cell line have not yet been evaluated; therefore we first examined the effects of dasatinib on KYSE30, KYSE180, EC109 and SHEE cells *in vitro*. For these experiments, cells were plated in 96-well plates (6000 cells/well), followed by dasatinib treatment after 24 h, as described in the Materials and methods section. Viable cells were counted after 24, 48 and 72 h. Similar to other cancer cell lines, dasatinib significantly decreased proliferation of KYSE30, KYSE180 and EC109 cells in a dose-dependent manner, but was abolished in SHEE cells (\(P < 0.01\)) (Figures 1A–1D) and IC\(_{50}\) values were in the submicromolar concentration range in the three cell lines (KYSE30, 29.13 nmol/l; KYSE180, 33.15 nmol/l; and EC109, 40.30 nmol/l). Western blot analysis of phospho-Src, total Src and \(\beta\)-actin showed that phospho-Src (Tyr\(_{416}\)) levels were significantly higher in KYSE30, KYSE180 and EC109 cells, but not in SHEE cells. It was also shown that the extent of growth inhibition in the cell lines was not significantly associated with total Src expression nor its phosphorylation level (Figure 1E).

**Dasatinib treatment of the human oesophageal squamous cell carcinoma cell lines decreased Src phosphorylation**

To investigate the effect of dasatinib on Src signalling, the cells were treated with increasing doses of dasatinib for 48 h. We also used the anti-phospho-SFK (Tyr\(_{416}\)) antibody, as there are no specific antibodies for phospho-Src available. SFKs are recruited to the cytoplasmic membrane by receptor tyrosine kinases, where they undergo activation through a series of phosphorylation and dephosphorylation events [17,18]. Tyr\(_{416}\) is an autophosphorylation site that is highly conserved among SFKs [19,20]. Phosphorylation at this site has been reported to initiate a conformational re-organization of the kinase activation loop, leading to relief of the steric barrier for substrate binding and to activation of Src tyrosine kinase [21]. However, the Western blot analysis did not allow for the determination of the specific phosphorylation status of individual members of Src. Although the levels of total Src did not decrease upon dasatinib treatment, inhibition of phospho-Src (Tyr\(_{416}\)) was found after treatment with dasatinib in the KYSE30, KYSE180 and EC109 cells (Figure 2). A decrease in its phosphorylation level of >80% was observed in KYSE30 and EC109 cell lines exposed to 60 nmol/l dasatinib for 48 h.

**Dasatinib induced apoptosis in the human oesophageal squamous cell carcinoma cell lines**

The pro-apoptotic action of dasatinib has been described previously [10]. Staining for pro-caspase 3 (37 kDa) gradually increased in KYSE30, KYSE180 and EC109 cells upon treatment with various concentrations of dasatinib (20, 40 and 60 nmol/l). This suggests pro-caspase 3 cleavage and active caspase 3-mediated apoptosis [22]. Treatment with dasatinib resulted in the cleavage of caspase 3 in the three oesophageal squamous cell carcinoma cell lines (Figure 2).

We next examined evidence of apoptosis in the KYSE30, KYSE180 and EC109 cell lines after treatment with various concentrations of dasatinib. As shown in Figure 3, the three cell lines with constitutive phosphorylation of Src also underwent apoptosis after treatment with dasatinib. These results indicate that the Src/Abl inhibitor dasatinib might induce apoptosis of the human oesophageal squamous cell carcinoma cells (Figure 3, and also see Figure 6).
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Dasatinib treatment coincided with MAD2 induction

Having observed the G1/S-phase arrest induced by dasatinib, we assessed the spindle status of the KYSE30, KYSE180 and EC109 cell lines exposed to dasatinib. After dasatinib treatment, the level of MAD2 was increased. Even at low dasatinib concentrations, however, a conspicuous up-regulation in the level of MAD2 was observed (Figure 2).

Dasatinib blocked the G1/S-phase transition in the human oesophageal squamous cell carcinoma cell lines

Inhibition of cell proliferation, induction of centrosome and chromosome aberrations, and an increase in MAD2 protein in the cells treated with dasatinib raised the question of whether these phenomena were related to changes in the cell cycle. We therefore measured the
Although the levels of total Src did not decrease upon dasatinib treatment in the cells, a decrease in phospho-Src (Tyr 416) (Src Y416) was observed upon dasatinib treatment. At a 60 nmol/l dose of dasatinib, the upper band of the phospho-Src staining disappeared. Additionally, levels of Bcl-2 and cyclin D1 appeared to gradually decrease, but the level of MAD2, Bax, cytochrome c, pro-caspase3 and caspase 3 increased in the cell lines upon dasatinib treatment. Western blots were repeated three times with similar results.

cell cycle in the dasatinib-treated cells using FACS analyses. Having established that cell proliferation is the primary cellular event inhibited by dasatinib in the KYSE30, KYSE180 and EC109 cell lines, we then determined the cell-cycle stage at which dasatinib exerts its effect (Figure 4). The distribution of cells among the different cell-cycle stages was analysed following dasatinib treatment. Compared with untreated cells, a marked reduction in S-phase was observed in the three cell lines following treatment (Figure 4). This reduction was accompanied by a corresponding increase in the G0/G1-phase. A decrease in S-phase with a concomitant increase in the G2/G1-phase was also found in cells treated with dasatinib. Moreover, the results indicated that dasatinib might induce G1 arrest in human oesophageal squamous cell carcinoma cells (Figure 4).

**Figure 2** Effect of dasatinib on Src, and apoptosis, cell-cycle progression and spindle checkpoint proteins

Although the levels of total Src did not decrease upon dasatinib treatment in the cells, a decrease in phospho-Src (Tyr 416) (Src Y416) was observed upon dasatinib treatment. At a 60 nmol/l dose of dasatinib, the upper band of the phospho-Src staining disappeared. Additionally, levels of Bcl-2 and cyclin D1 appeared to gradually decrease, but the level of MAD2, Bax, cytochrome c, pro-caspase3 and caspase 3 increased in the cell lines upon dasatinib treatment. Western blots were repeated three times with similar results.

**Dasatinib enhanced docetaxel-induced cytotoxicity and apoptosis in the human oesophageal squamous cell carcinoma cell lines**

The effect of the concomitant administration of dasatinib and docetaxel in the cell lines was evaluated after 24 h of treatment by means of cell viability and apoptosis assays. The results of the MTT assays showed that the cells had an enhanced sensitivity to docetaxel in the presence of dasatinib (Figure 5A).

Furthermore, a higher rate of apoptosis in cells was found with exposure to the dasatinib/docetaxel combination than with exposure to docetaxel alone (Figure 5B). The status of Src phosphorylation was evaluated under these experimental conditions. Figures 5(C)–5(E) show that an increase in Src phosphorylation levels after docetaxel exposure was observed in the three cell lines. This increase was quantified with integrated densitometry [as the ratio of phospho-Src (Tyr 416)/pan-Src ratio, see the lower panels of Figures 5C–5E] and was found to be 18, 34 and 10% above that of the untreated cells in the KYSE30, KYSE180 and EC109 cell lines respectively. Src phosphorylation, both endogenous and induced by docetaxel, was found to be impaired by dasatinib treatment (Figures 5C–5E). Thus docetaxel and dasatinib in combination are effective in inhibiting tumour cell growth in vitro.

**Dasatinib reduced in vitro invasion, viability and proliferation in the human oesophageal squamous cell carcinoma cell lines**

The ability of dasatinib to inhibit the in vitro invasion of oesophageal carcinoma cell lines was tested using Matrigel-coated invasion chambers. The KYSE30, KYSE180 and EC109 cells were seeded in the upper chamber in medium supplemented with 10% FBS. In the lower chamber, medium supplemented with 20% FBS was used as a chemoattractant. The experiment was terminated after 24 h and the cells that had migrated to the lower chamber were stained and counted. Dasatinib used at increasing concentrations (10, 20, 40 and 60 nmol/l) inhibited invasion by the three cell lines tested.

We then tested the effect of dasatinib on the viability and proliferation of the cell lines. The cells were treated with increasing drug concentrations (10, 20, 40 and 60 nmol/l) and MTT assays were carried out after 48 h. Dasatinib reduced the viability and proliferation of the three oesophageal carcinoma cell lines tested, although to
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Figure 3  Induction of apoptosis in cells after dasatinib treatment

Apoptosis of cells was evaluated by measuring the exposure of phosphatidylserine on the cell membranes using an Apoptosis Detection Kit (BD Pharmingen). The apoptosis rates of the cell lines treated with 10, 20 or 40 nmol/l dasatinib were increased remarkably.

Figure 7 (B) shows that dasatinib effectively reduced the median tumour weight (2.07 g for control group compared with 0.53 g for dasatinib-treated group, \( P = 0.018 \)). Thus we conclude that dasatinib significantly decreased oesophageal carcinoma growth in this model.

**DISCUSSION**

Oesophageal squamous cell carcinoma is the most common cause of cancer-related deaths. Although most clinical trials are performed in patients with advanced refractory disease and the conventional paradigm for efficacy depends on demonstrating a reduction in tumour dimensions using anatomical imaging, the patient population is not likely to be appropriate for agents that inhibit cancer cell invasion, migration and the development of metastases [23]. This limitation is particularly relevant for patients with oesophageal squamous cell carcinoma, in whom cytotoxic chemotherapy and radiotherapy have limited efficacy for advanced inoperable disease. We therefore explored the existence of molecular targets for the systemic treatment of oesophageal squamous cell carcinoma. The non-receptor tyrosine kinase Src is known to promote cell proliferation, survival and
Figure 4  Effect of dasatinib on the cell-cycle progression in human oesophageal carcinoma cells

Cells were blocked at the G1/S-phase transition by dasatinib. The cells were incubated for 24 and 48 h with dasatinib and, subsequently, the cell-cycle profile was analysed by flow cytometry. The percentages of cells in the G0/G1-phase and in the S-, G2- and M-phases are shown. Dasatinib induced a G1 cell-cycle arrest in all of the cells tested.

decreased adhesion, as well as increased motility, invasiveness and angiogenesis, all of which promote the neoplastic phenotype [4,24,25]. Kinases are also excellent targets for anticancer therapy, as they work as a molecular switch, their regulation is reversible and rapid, and does not require new protein synthesis.

Mutations in Src are involved in the malignant progression of cancer [26]. Fyn and Lck, together with Yes, Fgr, Hck, Blk, Lyn and Frk, are SFK members. The Src pathway can be targeted by dasatinib. Dasatinib is well known for its efficacy in the treatment of CML and Philadelphia-chromosome-positive ALL (acute lymphoblastic leukaemia) [27], in which dasatinib inhibits the Abl kinases [28]. Dasatinib has also been shown to be effective in the treatment of cells derived from solid tumours, such as head and neck squamous cell carcinoma [9].

In the present study, we first examined oesophageal carcinoma cell cultures; by averaging their profiles, we identified the activity of the Src pathway in three oesophageal carcinoma cell lines. We defined sensitivity as IC_{50} values <100 nmol/l. Using this criterion, KYSE30, KYSE180 and EC109 cells were considered sensitive. Accordingly, the decrease in cell viability in vitro and in vivo after treatment with the Src inhibitor dasatinib confirmed these findings.

The single-agent IC_{50} value for dasatinib with standard chemotherapy agents is a potential treatment option for oesophageal squamous cell carcinoma. We found that dasatinib synergized with docetaxel, a drug often used in oesophageal squamous cell carcinoma treatment, suggesting potential therapeutic areas for further exploration. In the present study, docetaxel when used alone slowed tumour growth, but these results did not reach statistical significance (less than 20%). When docetaxel was used in combination with dasatinib, however, significant decreases in proliferation rates beyond those from the administration of docetaxel alone were observed. Additionally, Src inhibition has been reported to increase the sensitivity of other advanced cancers to several commonly used chemotherapy agents,
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**Figure 5** Effect of combined dasatinib and docetaxel treatment in the KYSE30, KYSE180 and EC109 cell lines

(A) Evaluation of cell viability, as measured using an MTT assay, after combined drug treatment. Increasing doses of docetaxel alone (continuous line) or in combination with 10 nmol/l dasatinib (broken line) were administered to the cell lines for 48 h. The bars represent the S.D. of at least eight replicates of the MTT measurements.

(B) Evaluation of the apoptosis rate, as measured by Annexin V/PI staining. The oesophageal carcinoma cell lines were treated for 48 h with docetaxel or dasatinib (DAS) alone, or a combination of docetaxel/dasatinib. The docetaxel concentrations used with the cells were 5, 10, and 20 μmol/l. *Significantly higher apoptotic rates in the dasatinib/docetaxel-treated cells compared with those treated with docetaxel alone. The cells were treated for 48 h at the indicated concentration. (C–E) Whole-cell lysates of KYSE30, KYSE180 and EC109 cells were treated for 24 h with docetaxel or dasatinib alone, or a combination of docetaxel/dasatinib at the indicated doses and immunoblotted with antibodies to phospho-Src (Tyr416), Src and β-actin (the loading control). The histograms show the integrated densitometry representing the phosphorylation levels [the phospho-Src (Tyr416)/pan-Src ratio].

including docetaxel [29,30]. This phenomenon may have important implications for patient care, as the efficacy of docetaxel therapy may be enhanced with the addition of dasatinib. Nonetheless, final conclusions regarding the effects of dasatinib in this regard will require additional clinical investigations. Whether this effect was due to the effective inhibition of Src activity or to the increased cytotoxicity of the combined chemotherapy agents remains unclear, and changes should be observed when different schedules of drug combinations (subsequent or simultaneous) are tested.

Additional experiments are needed to explore further the mechanisms underlying growth inhibition and whether the effects of dasatinib on oesophageal squamous cell carcinoma growth can be increased by combination with another cytostatic drugs and reach higher growth-inhibition rates [31].

Moreover, we found decreased cell viability and proliferation in the oesophageal squamous cell carcinoma cell lines after dasatinib treatment. Our results suggest that dasatinib induces caspase-3-mediated apoptosis in the three cell lines. This finding suggests that dasatinib inhibits oesophageal squamous cell carcinoma cell growth through certain mechanisms (by inducing G1 arrest, for example). Other studies have shown that in vitro dasatinib treatment of head and neck squamous cell
carcinoma cell lines causes a dose-dependent inhibition of cell growth [9]. In these studies, the cell lines had decreased percentages of cells in the G2/M- and S-phases and an increased number of cells in the sub-G2 population, which is consistent with cell-cycle blockade in the G1-to-S-phase transition and induction of apoptosis [9]. We have found that dasatinib affects the regulatory molecule cyclin D1, which is involved in cell-cycle progression and which can be regulated by Src [4,10]. Src inhibition by dasatinib could therefore lead to a decrease in cyclin D1 levels, resulting in G1 cell-cycle arrest and a decreased rate of spindle formation. It also indicates that dasatinib triggered some molecules in the mitochondrion pathway, such as caspase 3, cytochrome

Figure 6  Effect of dasatinib on KYSE30, KYSE180 and EC109 cell lines viability and migration in vitro

(A) KYSE30, (B) KYSE180 and (C) EC109 cells were plated on 96-well plates and treated with either DMSO (0) or dasatinib at the concentrations specified (10, 20, 40 or 60 nmol/l). The cell viability was assayed with Cell-Titer Glo. The proliferation rates were assessed by BrdU (bromodeoxyuridine) incorporation at 24 h. The apoptosis levels were analysed with a caspase 3/7 activity assay at 48 h. The cell migration through Matrigel-coated 24-well was measured at 24 h post-treatment. Triplicate samples were assayed for each condition. Results are means ± S.D.

Figure 7  Effect of oral administration of dasatinib on in vivo orthotopic tumour weight

KYSE180 cells were orthotopically injected into nude mice (see the Materials and methods section). (A) At 40 days after the injection, animals were killed, and the mean tumour volume was calculated in the untreated and dasatinib-treated groups. The P value was determined using a Student’s t test. (B) Dasatinib treatment was given at a dose of 30 mg·kg⁻¹·day⁻¹ of body weight·day⁻¹. Body weight was periodically measured until the last day of treatment (day 40). In all the groups, the body weight differences between the control and dasatinib-treated animals throughout the experiment were significant (P = 0.018). (C) The size of the transplanted tumours in untreated and dasatinib-treated groups. The volume of tumours in the dasatinib-treated group were smaller than the untreated group at day 40.
c and Bax. However, immunoblotting for phospho-Src, caspase 3, cytochrome c and Bax was performed on three responsive cell lines only, and extrapolating these results to unresponsive oesophageal squamous cell carcinoma cell lines should be attempted with caution.

The molecular mechanisms for the observed effects of dasatinib on the spindle checkpoint may relate to one or more of its known targets. MAD2 is one of the essential components of the cell-cycle checkpoint in the final phase of the mitotic spindle checkpoint [32] and it plays an important role in drug sensitivity [33]. It controls the ability of cancer cells to enter the next cell cycle of the final clearance. For the role of dasatinib in the oesophageal squamous cell carcinoma cell lines, the spindle checkpoint was the 'state' that determined whether their ultimate fate was death or survival.

In the evaluation of the efficacy of dasatinib in vivo, drug treatment in the orthotopic model utilizing KYSE180 cells produced a significant reduction in tumour burden. Nevertheless, it should be stressed that dasatinib activity in vivo appears weaker than its activity in vitro. The tumours induced by KYSE180 cells were reduced in size, but complete tumour eradication was not achieved. This partial efficacy may be explained by a suboptimal drug concentration at the tumour site. A possible reason for the partial efficacy of dasatinib in vivo may be due to differing supplies of growth factors and nutrients. Taken together, these results emphasize the importance of assessing the efficacy of dasatinib by demonstrating in vivo antitumour activity in a setting that mimics the individual microenvironment of spontaneous human cancer as closely as possible. Despite the partial efficacy, interestingly, the protein kinases upstream of ERK1/2 (extracellular-signal-regulated kinase 1/2) in the MAPK (mitogen-activated protein kinase) pathway are among those shown to interact physically with dasatinib [34]. In this regard, it has been demonstrated that dasatinib may both have limited activity when used alone and act synergistically with some cytotoxic drugs already used in therapy [35].

Briefly, our results indicate that dasatinib inhibits the growth of oesophageal carcinoma cells and that these inhibitory effects are increased in combination with docetaxel. These results support further the trial of the integration of chemotherapy agents with inhibitors of specific molecular pathways in the attempt to ameliorate the outcomes of oesophageal squamous cell carcinoma patients.

Additionally, the results of our present study demonstrate a decrease in tumour formation in dasatinib-treated animals, suggesting that dasatinib used alone or in combination for metastatic oesophageal squamous cell carcinoma should be investigated further. Furthermore, the heterogeneous behaviour of dasatinib-sensitive and dasatinib-insensitive tumour cells should be studied and the inhibitory effect of dasatinib on ERK1/2, c-Kit and Akt activation in tumours should be determined. Moreover, we have shown in vitro that dasatinib, as an inhibitor of the Src pathway, may provide a potential therapeutic benefit for oesophageal squamous cell carcinoma patients who are not eligible for surgery.

Conclusions

Our results demonstrate that dasatinib is active against oesophageal squamous cell carcinoma cells both in vitro and in vivo. On the basis of the findings of the present study, further investigations should be performed to investigate dasatinib in combination with other chemotherapy agents and the possibility of additional effects through the inhibition of angiogenesis. Such research may lead to the development of more effective and less toxic therapies for the treatment of oesophageal squamous cell carcinoma.

AUTHOR CONTRIBUTION

Li Wang cultured the cell lines, performed the flow cytometry analysis, undertook the in vivo experiments and wrote the paper. Bin Guo performed the statistical analysis and proofread the paper prior to submission. Ruwen Wang, Shukui Qin and Yaoguang Jiang provided laboratory space and directed the experiments. Shuhui Liang and Yunping Zhao performed the cellular proliferation experiments. Wei Guo performed the cellular apoptosis studies. Kun Li performed the Matrigel invasion assay. Xiaoqing Fan performed the Western blot analysis.

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