Resistance to dasatinib is associated with the activation of Akt in oral squamous cell carcinoma

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Abstract

Objectives: Overexpression and aberrant activation of Src promote the development of oral squamous cell carcinoma (OSCC), thus therapies targeting Src-related kinases may afford an improvement in patient survival. However, limited clinical activity of the Src-targeted drug, dasatinib, in cancer patients warrants further investigation to better understand the underlying basis of resistance to dasatinib in OSCC.

Methods: Response to dasatinib was evaluated in a panel of oral cancer cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, DNA synthesis, cell cycle and apoptosis analysis. The underlying mechanism of drug response was investigated using immunoblotting. Xenograft models were used to test efficacy.

Results: All cell lines were sensitive to dasatinib (IC₅₀ < 250 nM), but this was not associated with CDKN2a/p14ARF mutations. Dasatinib-induced cell cycle arrest and apoptosis, while inhibiting Src, Akt and FAK activity in all lines tested. However, dasatinib failed to inhibit tumour growth in xenograft models and treated tissues showed Akt activity despite the loss of Src activity.

Conclusions: Our data revealed that reactivation of Akt (Ser473) could be a compensatory mechanism that bypasses Src inhibition by dasatinib, providing important clues that could improve treatment strategies to overcome dasatinib resistance.

Keywords
Oral squamous cell carcinoma, targeted therapy, cell signalling, Src kinase, xenografts

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Introduction

Oral squamous cell carcinoma (OSCC) is amongst the most prevalent cancers worldwide accounting for an estimated 300,400 new cases and 145,400 deaths annually.¹ Furthermore, recurrent disease remains a major challenge largely due to limited therapeutic options.² While chemotherapy remains the most feasible standard-of-care for advanced OSCC, partial or lack of response with these cytotoxic therapies remains a concern and compounding this, adverse side effects resulting from treatment severely impact the quality of life and patient outcome. To date, cetuximab (EGFR inhibitor), pembrolizumab and nivolumab (PD-1 inhibitor) are the only targeted drugs that
are approved by the Food and Drug Administration (FDA) to treat refractory head and neck cancer patients. However, these therapies currently benefit only a subset of these patients. To this end, recent genomic analysis of OSCC has shown that approximately 80% of samples harboured genetic alterations that are likely targetable, and in the process, afford opportunities to identify appropriate inhibitors that could be developed as anticancer therapies. Among these potential genomic targets, Src was found to be a frequently altered gene, which was also reflected in elevated (29%) protein expression.6

Src, a non-receptor tyrosine kinase, is emerging as a potential therapeutic target for several solid tumours. Elevated levels of Src protein and activation are frequently found in OSCC, prostate, breast and colorectal cancers.7–10 Aberrant Src activity is now known to impact key aspects of cell behaviour including increased proliferation, migration, invasion, angiogenesis and survival.11 In the context of OSCC, Src activity has been shown to induce the activation of STAT3, which in turn increased cell survival via abrogation of apoptosis in animal models.12 Several studies have shown that these responses may be mediated by Akt, Erk and FAK activity.13,14 Pertinently, several preclinical studies now suggest that several FDA-approved Src inhibitors (dasatinib, saracatinib, bosutinib, KX01) may be effective in treating several solid cancers.15–17 Dasatinib, initially developed as a second-generation drug treating resistant chronic myeloid leukaemia by targeting the abnormal activity of bcr-abl,18 has shown encouraging clinical benefits in metastatic prostate and recurrent ovarian cancer patients.19,20 Furthermore, dasatinib and erlotinib given in combination have also been shown to be effective in overcoming resistance to EGFR inhibitors in head and neck cancer, that is primarily conferred by activated Src kinase.21

Despite promising preclinical observations,22,23 recent data from clinical evaluation have demonstrated that dasatinib did not improve overall survival of OSCC patients, suggesting that a better understanding of the signalling pathways of the drug targets as well as the underlying mechanism of resistance is needed.24 Notwithstanding, recent findings by Garnett et al.25 showed that inactivating mutations of CDKN2a/p14ARF may be positively correlated with dasatinib sensitivity and thus providing a predictive biomarker of response to the drug.25 To this end, mutations and altered expression of a number of molecules targeted by dasatinib have been reported for OSCC,6 providing important clues for discovering novel biomarkers for dasatinib treatment. Based on these reports, we sought to improve on our understanding of dasatinib response and the underlying mechanism of action in OSCC.

Materials and methods

Cell culture

A panel of 16 ORL oral squamous cell carcinoma lines cell lines established and maintained as previously reported were used in this study.26,27 Authentication to original tumour tissues and/or blood samples from the corresponding patients was done as described previously.27 Two cell lines derived from adenocarcinoma of the lung (HCC827 and A549) were purchased from ATCC (VA, USA) and included as controls. These lines have been previously demonstrated to be sensitive (HCC827) and insensitive (A549) to dasatinib.28,29 ORL cell lines and A549 were maintained in Dulbecco’s Modified Eagle’s Medium/Nutrient mixture F-12 HAM’s medium (HyClone, Logan, UT, USA) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS; Gibco, Auckland, New Zealand), 100 IU Penicillin/Streptomycin (Gibco), 0.5 µg mL−1 hydrocortisone (Sigma-Aldrich, St Louis, MO, USA). HCC827 was maintained in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated FCS, 100 IU Penicillin/Streptomycin and 0.5 µg mL−1 hydrocortisone. All lines were maintained in a humidified atmosphere of 5% CO2 at 37°C.

Materials

Dasatinib (Selleckchem, Houston, TX, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a stock concentration of 200 µM and further diluted in culture medium to the required concentrations (1–1000 nM). Primary antibodies used in these studies included Src (#2123), pTyr416Src (#6943), AKT (#2983), pSer473AKT (#4060), pThr108AKT (#9275), Erk (#9102), pErk (#9101), FAK (#3285), pTyr567/577FAK (#3281), pTyr925FAK (#3284), p21 (#2947) (Cell Signaling Technology, Danvers, MA, USA), p53 (SC-126; Santa Cruz, Dallas, Texas, USA), p16 (550834; BD Pharmingen, San Diego, CA, USA) and pan-actin (MAB1501; Millipore Bioscience Research Reagents, Billerica, MA, USA). The secondary antibodies goat anti-rabbit IgG-HRP (SB4010-05) and goat anti-mouse IgG-HRP (SB1010-05) were purchased from Southern Biotech (Birmingham, AL, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were plated in duplicate wells in 96-well plates and treated with dasatinib at the indicated concentrations (1–1000 nM). After 72 h of incubation, cell viability was measured using 5 mg mL−1 MTT solution (Sigma-Aldrich) colorimetric method. The purple formazan was dissolved in 100 µL of 100% DMSO and the absorbance at 595 nM was measured. IC50 of dasatinib in cell lines was presented as the mean ± SEM of three independent experiments. DMSO (0.5%) was used as the control.

Cell proliferation

Cell proliferation was determined by the Click-iT assay (Invitrogen, Carlsbad, CA, USA) and according to the manufacturer’s instructions. Briefly, target cells grown on glass coverslips
were treated with DMSO (0.05%) and dasatinib with the indicated concentrations (1–100 nM) for 24 h. Then, cells were processed to allow incorporation of 5-ethyl-2'-deoxyuridine (EdU) for 2 h. Then, cells were fixed with 3.7% formaldehyde (in PBS) for 15 min. After washing with 3% bovine serum albumin (BSA), cells were incubated with 0.5% Triton-X solution (Sigma-Aldrich) for 20 min at room temperature. Next, cells were stained with the Click-iT reaction cocktail (1X Click-iT reaction buffer, CuSO₄, Alexa Fluor® containing azide, reaction buffer additive) for 30 min in the dark. After washing off the Click-iT reaction cocktail, cells were incubated with 1X Hoechst 33342 solution (5 μg mL⁻¹) in the dark for 30 min. After washing with PBS, coverslips were mounted with Vectashield mounting medium (Vector Laboratories Inc, Burlingame, CA, USA) and kept protected from light. For analysis, photos of EdU-positive and DAPI-stained cells were captured with a fluorescent microscope (Olympus, Japan) and quantified using ImageJ software (http://rsbweb.nih.gov/ij/). Number of EdU-positive cells and DAPI-stained cells were counted and the percentage of EdU-positive cells was calculated using the following formula: number of EdU-positive cells/number of DAPI stained cells x 100. EdU-positive cells broadly represent cells that are undergoing DNA synthesis, whereas DAPI-stained cells represent all viable cells. This assay was repeated at least twice for each cell line.

**Apoptosis**

The apoptotic effect of dasatinib on target cells was examined after treatment with 100 nM of drug for 24–72 h. Floating and adhered cells were first harvested and after pooling together, all cells were collected by centrifugation at 1200 rpm for 5 min. Next, the supernatant was discarded and the pellet was washed once with 1X PBS and after collection, the subsequent pellet was resuspended in 1X binding buffer (BD Bioscience, San Jose, CA, USA). Cells were then double stained with Annexin V-FITC (BD Bioscience) and propidium iodide (BD Bioscience) for 15 min in the dark according to the manufacturer’s instructions. Cells were analysed with FACSCalibur™ flow cytometer (BD Bioscience), and the apoptotic index was calculated as the number of apoptotic cells over the total number of cells. The data were compared with the control (0.05% DMSO) group.

**Cell cycle analysis**

Cells treated with dasatinib were harvested at the indicated time points and after centrifugation, pellets were resuspended and fixed in 70% ethanol for 15 min on ice before storage at −80°C. Prior to analysis, fixed cells were pelleted, washed in 1X PBS and stained with 10 μg mL⁻¹ propidium iodide and 20 μg mL⁻¹ RNase for 15 min in the dark on ice. DNA content of the cells was analysed with FACSCalibur™ flow cytometer (BD Biosciences) using ModFit software (Verity Software House, Australia). To determine if the cell cycle arrest was reversible, dasatinib was removed from the cells after 12-h incubation and replaced with fresh media. Cells were collected at 24 and 48 h post-treatment for cell cycle analysis by flow cytometer. Cell cycle progression was compared with cells which were continuously treated for the same time period.

**Effect of dasatinib in vivo**

All animal studies were carried out in accordance with the guidelines laid down by the National Institutes of Health (NIH) in the United States regarding the care and use of animals for experimental procedures. The protocols were approved by the Animal Ethics Committees of National University of Malaysia (UKM) (Ethical approval code: CARIF/2013/CHEONG/25-SEPT/546-NOV.-2013-NOV.-2016). Female NOD/SCID mice (BioLASCO, Taiwan) at 4–6 weeks of age were used in this study. Mice were housed in appropriate sterile filter-capped cages and fed and watered ad libitum. The subcutaneous model of OSCC was established by injecting 2 × 10⁶ either of the target cells (ORL-48 and ORL-115) to both flanks of the mice. The mice were evaluated every other day for general behavioural abnormalities, signs of illness or discomfort. Body weight and tumour volume were recorded by the same operator using digital calipers throughout the study. Tumour volume was determined using the formula: \( L \times W / 2 \), where \( L \) and \( W \) represent length and the width of the tumour, respectively. When tumour volume reached approximately 150–200 mm³, animals were randomized into treatment group \((n = 5)\) and control group \((n = 5)\). For treatment, dasatinib was dissolved with DMSO to 100 mg mL⁻¹ and then further diluted to 2 mg mL⁻¹ in diluent containing 5.1% of polyethylene glycol (PEG; Sigma-Aldrich) and 5.1% of Tween80 (Sigma-Aldrich) in PBS prior to treatment. Dasatinib was administered at 20 mg/kg/day² by intraperitoneal injection, whereas the control group was treated with equivalent concentration of DMSO (2%) dissolved in the same diluent mentioned above. All animals were treated once daily for 21 days and tumours were measured two times weekly. At the end of the study, all animals were killed and all tumours were harvested. Non-necrotic tumour tissues were snap-frozen and stored in liquid nitrogen, and in addition, a portion of the tumour tissue was fixed in formalin and processed for paraffin embedding for haematoxylin and eosin (H&E) histopathological evaluation using the Anneroth multifactorial grading system³⁰ by a board certified pathologist (KS Mun). According to this system, six parameters, including keratinization, nuclear pleomorphism, mitoses, pattern and stage of invasion, lymphoplasmacytic infiltration, are graded with each receiving a score from 1 to 4. The sum of the scores are grouped as follows: 6–12 grade I, 13–18 grade II, 19–24 grade III that represent the level of tumour malignancy and a higher score broadly reflects increased aggressiveness of the tumour.³¹

**Western blotting**

After treatment with dasatinib and control (0.05% DMSO), total cell lysates (TCL) were extracted with M-PER
mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) supplemented with halt protease and phosphatase inhibitor cocktail (Pierce Biotechnology) on ice. TCL were collected by centrifugation and protein content determined using the Bradford protein assay (Thermo Scientific, Waltham, MA, USA). For Western blot analysis, 20 μg of the subsequent TCL was used to resolve total proteins by SDS-PAGE. Next, after transferring at 400 mA for 1 h on ice, PVDF membranes (Millipore) were blocked with 5% milk in TBST (0.1% Tween 20) for 1 h. After brief washing in PBS supplemented with 0.1% Tween 20 (Sigma-Aldrich; PBST), the membranes were probed with the indicated primary antibodies (1:1000 dilution in 1% BSA) overnight at 4°C. Then, membranes were washed in PBST thrice, each for 10 min. The membranes were next incubated with the corresponding secondary antibody (1:10,000 dilution in 5% milk) for 1 h at room temperature. This was followed by washing in PBST x3, each for 10 min before detection by WesternBright Quantum HRP substrate (Advansta Inc, Menlo Park, CA, USA) and visualisation using the FluorChem™ HD2 imaging systems (Alpha Innotech, USA). To normalise for loading, the blots were reprobed with anti-pan-actin monoclonal antibody (1:1000 dilution in 1% BSA) and processed as described above. To analyse signalling pathway molecules in frozen mouse xenograft tissues, cryosections were made on clean glass slides from control and drug-treated tumours. Next, RIPA buffer (50 mM Tris pH8, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) containing protease and phosphatase inhibitor cocktail was used to lyse the tissue sections off the slides. Cell lysates then underwent sonication on ice (3 times at 10 s each) and soluble proteins were collected after centrifugation. After estimation of protein concentration, total soluble protein concentration was determined using the Bradford protein assay (Thermo Scientific, Rockford, IL, USA) supplemented with halot protease and phosphatase inhibitor cocktail. 

Statistical analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc, San Diego, CA, USA) or SPSS software (SPSS for Windows, version 16.0 (Chicago, IL, USA)). Independent t-test was used to determine the significant difference of IC50 between ORL lines with the resistant line A549, the association of CDKN2a/p14ARF mutation with dasatinib sensitivity, differences between the control and treatment cells in the apoptosis, cell cycle and proliferation assays. Significant p values were indicated as * p < 0.05, **p < 0.01 or ***p < 0.001.

Results

OSCC ORL cell lines are exquisitely sensitive to dasatinib in vitro

The MTT viability test demonstrated all of the lines are responsive to dasatinib when compared to the sensitive (HCC827, IC50 = 26 nM) and the resistant (A549, IC50 = 1.87 μM) control lines, with IC50 values ranging from approximately 50 nM (ORL-207, ORL-196, ORL-48, ORL-156) to approximately 150 nM (ORL-214) with the highest IC50 at 250 nM (ORL-204; Figure 1). Overall, our data suggest that these ORL lines are relatively sensitive to dasatinib (mean IC50 = 81.8 nM) when compared to other reported OSCC lines (mean IC50 = 763 nM; Table 1).13,14

CDKN2a/p14ARF mutations do not correlate with dasatinib sensitivity in ORL lines

As our previous data suggested that ORL lines were conferring a level of drug sensitivity that may be of clinical value, we sought to investigate the molecular basis to this observation. Based on a recent study demonstrating that mutations in CDKN2a/p14ARF positively correlated with dasatinib sensitivity in a panel of cancer cell lines that included a subset of OSCCs, we assessed if this gene status in our ORL lines contributed to their drug response.25 To this end, we have previously performed RNA-seq analysis on our panel of ORL lines and with this information we were able to determine that 10/16 (62.5%) lines harboured a CDKN2a/p14ARF missense mutation, while the others were wild-type for CDKN2a/p14ARF.28 Next, we aligned the IC50 of the cell lines to their CDKN2a/p14ARF mutation status, and in contrast to the reported study, we did not observe any association between drug sensitivity and the mutational status. The response was broadly observed to be similar between the wild-type (ORL-48, -136, -195, -207, -214 and -215) and mutant (ORL-115, -150, -153, -156, -166,
-174, -188, -196, -204 and -247) CDKN2a/p14ARF cell lines ($p = 0.65$; Figure 2). We further looked into the link regarding dasatinib sensitivity with the mutation status and expression level of p16, which is another isoform protein of CDKN2a gene. Of the lines that either harboured CDKN2a/p16 ink4a mutations (ORL-115, -150, -153, -156, -174, -188, -195, -196, -204 and -247) or wild type, no association was noted between the mutation status and drug sensitivity (data not shown). Furthermore, almost all lines failed to demonstrate p16 expression and being assessed as HPV negative, with the exception of ORL-166 and ORL-115 which expressed p16 and detected to have the presence of HPV-16 and HPV-31, respectively. Similarly, there was also no obvious association with these observations to sensitivity to dasatinib (data not shown).

Dasatinib inhibits cell proliferation and induces reversible G1 cell cycle arrest in OSCC cells

To determine the molecular basis of response of the ORL cell lines to dasatinib, we chose to use two of the lines (ORL-48 and ORL-115), which demonstrated very similar IC50 (44.5 nM and 57.1 nM, respectively; Figure 3) and previously shown to induce xenograft tumours, for further investigation. We first investigated if dasatinib would impact cell proliferation by measuring active DNA synthesis in drug-treated (1–100 nM) and control (0.05% DMSO) cells for 2 h, using the Click-iT assay. As shown in Figure 4(a) and (b), dasatinib at approximately 100 nM was observed to inhibit cell proliferation in both cell lines. The data are presented as a percentage of EdU-positive cells for each treatment or DMSO control, which broadly represents the fraction of actively proliferating cells compared to the total number of cells. From this analysis, 100 nM of dasatinib over 24 h was able to reduce proliferating cells by approximately 15% compared to DMSO control cells ($p < 0.05$). Minimal response was observed with lower levels of the drug in both lines tested (Figure 4(a) and (b)). As dasatinib was seen to inhibit cell proliferation, we next investigated if this effect was due to a cell cycle block. Using propidium iodide–stained dasatinib-treated (100 nM) cells, flow cytometry analysis demonstrated that the drug induced a G1 cell cycle arrest as early as 12 h, with a corresponding reduction in cell population in G2-M phases in both cell lines tested (Figure 5(a) and (b)). From the observation of cell morphology, we observed notable cell shrinkage of the treated cells compared to control cells, likely representing signs of cell apoptosis. Furthermore, we did not observe

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**Table 1.** Comparison of the IC50 values of dasatinib between the ORL lines and previously published data of drug sensitivity in other OSCC cell lines.

| Tumour site | Cell lines | IC50 (nM) |
|-------------|------------|-----------|
| Tongue      | Tu159 \(^a\) | 90        |
|             | Tu686 \(^a\) | 540       |
|             | SCC-25 \(^b\) | 620       |
|             | HSC3 \(^b\) | 780       |
|             | SAS \(^b\)  | >10,000   |
|             | ORL-207    | 40        |
|             | ORL-156    | 47        |
|             | ORL-150    | 48        |
|             | ORL-174    | 59        |
|             | ORL-188    | 61        |
|             | ORL-166    | 66        |
|             | ORL-136    | 98        |
|             | ORL-247    | 110       |
|             | ORL-215    | 122       |
| Gingiva     | Ca9-22 \(^a\) | 450       |
|             | ORL-48     | 45        |
|             | ORL-115    | 57        |
|             | ORL-153    | 67        |
|             | 358B \(^a\) | 2100      |
| Buccal      | ORL-196    | 43        |
|             | ORL-195    | 91        |
|             | ORL-214    | 137       |
|             | ORL-204    | 217       |

OSCC: oral squamous cell carcinoma.

\(^a\)Johnson et al. \(^b\)Lin et al.

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**Figure 2.** Independent t-test was used to determine the association of mutational status of CDKN2a/p14 with dasatinib sensitivity. Cell lines with p14 mutations are not significantly more sensitive to dasatinib compared to cell lines with wild-type p14.

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**Figure 3.** Five-parameter curve fitting shows the viability of ORL-48 and ORL-115 upon dasatinib treatment for 72 h. Both cell lines show similar killing effect by dasatinib. These two lines are selected for further in vitro and in vivo studies.
senescence-like morphology (enlarged and flattened cells) and hence we eliminated the possibility of dasatinib-induced cell senescence (Figure 6). To further assess if the G1 arrest caused by dasatinib is reversible, we removed the drug after 12 h of treatment. From our analysis, we noted a significant reduction of cells in the G1 cell population as well as cells entering S and G2-M phases between 12 and 36 h post-removal of dasatinib when compared to the cells treated continuously for 24 and 48 h (Figure 7(a) and (b)). Moreover, following drug treatment also resulted in an increase in the sub-G1 fraction, suggesting the presence of apoptotic cells (Figure 5(a) and (b)). To test if these sub-G1 fractions were indeed apoptotic cells, we next subjected similarly treated cells (24–72 h) to the Annexin V-FITC and propidium iodide assay. From the data, we observed that treatment with 100 nM of drug for 72 h readily induced apoptosis in ORL-115 and ORL-48 cells when compared to their respective controls (Figure 8(a) and (b)). Since both ORL-48 and ORL-115 cells are p16 deficient and express a mutant form of p53,27 we determined the mechanism by which G1 arrest was induced. With the treatment of dasatinib (100nM) for 12 and 24 h, expression of p21 increased significantly in a p53 independent manner, and irrespective of treatment the absence of p16 was also reconfirmed (Figure 5(c)).

**Biochemical correlates the sensitivity of OSCC cells to dasatinib**

Dasatinib is a known inhibitor of Src kinase activity. To assess if biochemical markers associated with the Src signalling pathway play a role in conferring drug sensitivity, we broadly
Figure 5. Dasatinib induces cell cycle arrest in ORL cells. Cells were treated with 100 nM dasatinib for 0, 6, 12, 24, 48 and 72 h, stained with propidium iodide and analysed with FACSCalibur™ flow cytometer. Significant difference between the control and dasatinib-treated cells is represented by * (<0.05), ** (<0.01) and *** (<0.001) (a) and (b). Protein lysate harvested from cells treated with dasatinib (0, 12 and 24 h) were analysed by Western blot. Levels of p21 are observed to increase in dasatinib-treated cells and minimal changes noted for p16 and p53 in both ORL lines (c). HeLa protein lysates were used as a positive control for p16.
analysed the key target proteins in ORL-48 and ORL-115 cells exposed to dasatinib (100 nM) for the indicated times by Western blot. As shown in Figure 9, dasatinib inhibited Src activity (Tyr416) within 2 h of drug treatment in both cell lines when compared with total Src levels where activated Src was reduced by approximately fourfold. Subsequently, we determined which of the downstream signalling targets affected by dasatinib could impact sensitivity. Using phospho-specific antibodies, Akt activity was monitored by the status of S473 and T308 phosphorylation between the two cell lines in response to dasatinib. When compared to total Akt, S473 activity in ORL-48 cells showed a reduction (approximately 1.8-fold) with prolonged treatment (24 h), while minimal differences were seen in ORL-115 cells (approximately 1.3-fold). On the other hand, T308 activity in ORL-48 cells was observed to be lower within 2 h of treatment, which became more prominent with longer exposure (approximately 1.2-fold). A similar trend was also noted in ORL-115 cells. We also assessed for Erk activity and found that this was broadly reduced approximately 1.25-fold with 2 h of treatment, but this effect was observed to revert between 6 and 24 h in ORL-115 cells. Although ORL-48 cells demonstrated a similar trend in Erk activity, with prolonged treatment a reduction was noted. Total Erk levels were similar for both lines. Activity of FAK was assessed by monitoring the phosphorylation of Tyr576/577 and Tyr925 residues in response to drug treatment in both cell lines. Phosphorylation events at these sites are broadly induced by Src activity to promote downstream signalling.34 As noted for both cell lines, drug treatment resulted in a marked and sustained reduction in FAK activity within 2 h (Tyr576/577 = approximately 1.25 – 1.5 fold; Tyr925 = approximately two- to threefold), while total levels remained similar. Actin levels were used to control for loading variations.

Dasatinib does not confer benefit in OSCC xenograft mouse models

As our in vitro data were suggesting promising anticancer properties, we next investigated if these observations could be translated into a clinical benefit in our xenograft mouse models of OSCC. Several notable studies have now reported that dasatinib when given daily in the range of 20–50 mg kg\(^{-1}\) over a 21-day period resulted in marked tumour regression in xenograft mouse models, particularly 20 mg kg\(^{-1}\) day\(^{-1}\) of dasatinib gave a response of approximately 37% in mice bearing head and neck cancer xenografts.22,23,35 Furthermore, as the ORL series cell lines demonstrated an approximately fourfold reduction of in vitro mean IC\(_{50}\) (81.8 nM) compared to 312 nM for OSC-19 cells which were used to induce xenografts, we decided to use a comparable dose of 20 mg day\(^{-1}\) kg\(^{-1}\) for our in vivo efficacy studies.22 However, following this reported treatment schedule, our in vivo efficacy study did not give the desired response in either of our xenograft model when compared with their respective control groups (Figure 10(a) and (b)). Of note, tumour growth in the ORL-115 xenograft increased in the dasatinib-treated group at late treatment time-points (\(p = 0.025; p = 0.039\)). Tumour tissues harvested from both treatment and control groups were processed for H&E and histopathological evaluated by a board certified pathologist (KS Mun). The analysis showed that 80% of the ORL-48-derived tumours in both treatment and control groups were broadly defined as grade II, based on tumour cell features and tumour–host relationship measured in the multifactorial grading system.36 ORL-115 in comparison, all the tumour tissues from the control group and approximately 67% from the treatment counterpart were scored as grade I (Table 2). We found no significant difference in the scores given for parameters in the grading system between the treatment and control groups, which are consistent with the result of efficacy study mentioned above.
The differential expression of signalling molecules in xenograft tissues

In order to determine the effect of dasatinib on Src signalling and related molecules in vivo, proteins extracted from tumours of both treatment and control groups were subjected to Western blotting. From our data, a reduction in Src, Erk and FAK activity (approximately 1.5-fold) was observed in both of the treated tumour models when compared with the control counterpart, as seen in our in vitro analysis. By contrast, no notable decrease in S473 Akt activation was seen but of note, an increase (1.5-fold) in activity of this molecule was seen in the dasatinib-treated ORL-115 xenograft model (Figure 11). Akt T308- and FAK Tyr576/577-activated protein could not be detected in these samples (data not shown).

Discussion

Aberrant Src activity is known to be pathogenic in several human cancers including OSCC, and the use of small molecule inhibitors to targeting Src affords a viable opportunity as treatment options for these cancers.37 Based on prior knowledge that dasatinib targets several other molecules that are frequently over-expressed and mutated in OSCC,6 we questioned if this drug was able to offer benefit in controlling OSCC
growth and concurrently to assess if Src kinase played a role in this process. Here, we demonstrated that OSCC cell lines were exquisitely sensitive to dasatinib in vitro, and this was achieved through the inactivation of Src and its downstream signalling pathways. However, this was not recapitulated in the in vivo studies, hence a better understanding of the molecular
signalling associated with dasatinib treatment could afford an opportunity to identify molecular events responsible for this observation.

We first demonstrated in vitro that all our oral cancer cell lines (ORL) were broadly sensitive to dasatinib with an average 50% inhibitory concentration (IC50) of 81.8 nM. It is worth mentioning that when comparing this sensitivity with similar studies, our ORL cell lines were approximately ninefold more sensitive, suggesting dasatinib could be a viable candidate for OSCC treatment.13 These similar studies revealed inhibition of cell growth and migration through Src-dependent pathways13,14 and we questioned if this effect was likely to be more robust in our model system. Of note, inactivating mutations in \textit{CDKN2a/p14ARF} have been proposed to be a predictive marker for dasatinib sensitivity in several cancer cell lines of different types including oral cavity lesions.25 However, in our ORL cell system, we noted from our RNAseq data that no correlation between dasatinib sensitivity and \textit{CDKN2a/p14ARF} mutation was found. This difference may be due to the small sample size of oral cancer lines analysed by Garnett et al.25 compared to the cohort size of the current study. Also possible is that \textit{CDKN2a/p14ARF} alterations found in our ORL cells were different from the mutations previously described by Garnett et al.25 Hence, the usefulness of \textit{CDKN2a/p14ARF} inactivating mutations as a marker for dasatinib sensitivity in OSCC remains unknown.

In pursuit of which molecules might be conferring this favourable drug sensitivity, we looked at some of the known targets of dasatinib as well as those involved in the EGFR-Akt-mTOR signalling pathway. The ORL cells treated with dasatinib showed marked inhibition of Src and its downstream pathways which are consistent with the G1 arrest and cell death observed at 24–72 h post-treatment. In the two ORL cell lines tested, Src (Tyr416) and FAK (Tyr576/577, Tyr925) activity were the most compellingly impacted by dasatinib (100 nM). Perhaps relevant, these observations were similar to those described by Johnson et al.13 using HNSCC (Tu167, Tu686) and non-small lung cancer cells (H226) treated with similar amounts of dasatinib and time duration. Of interest, we also noted from our study that prolonged treatment with dasatinib was likely inducing Erk reactivation after an initial reduction at 2 h post-treatment. This observation was also found in similar studies13,14 and likely suggests other unknown compensatory signals may be involved in regulating Erk activity after sustained inhibition of Src. Also, we noted a modest effect on Akt activity with prolonged drug treatment likely suggesting that inhibition of Src activity by dasatinib has a modest effect on downstream targets, which was also noted in previous studies.13,14 Dasatinib treatment (100 nM) in the ORL cells resulted in an increase in p21 expression and subsequent G1 cell cycle block leading to apoptosis as seen by increased sub-G1 population and Annexin V-FITC-positive cells. Other
studies have reported similar observations in other solid tumours, for example, pancreatic cancer (BxPC3) and melanoma (Lox-IMVI)\textsuperscript{38,39} and consistent with those reported for HNSCC (Tu167, JMAR).\textsuperscript{13,40} Nevertheless, the anti-proliferative properties of dasatinib were demonstrated to be reversible in the ORL cells, which was not reported in the aforementioned studies.

As our in vitro data were suggesting that dasatinib could be efficacious in vivo, we tested if this was the case in the same ORL cell lines (ORL-48 and ORL-115) which are known to induce xenograft tumours.\textsuperscript{27} Following our established treatment schedule and a dose of 20 mg kg\textsuperscript{-1}, unfortunately dasatinib demonstrated no benefit in the two models tested and this is despite observing favourable sensitivity and reduction in Src (Tyr416) and FAK (Tyr925) activity in the tumour tissues after completion of treatment. Notably, dasatinib-treated ORL-115 tumours demonstrated an increase in tumour volume compared to control-treated tissues, which may be due to the appearance of Akt activity observed in the tumour tissues compared to control tumours. Comparing observations in vitro (Figure 9) to those in vivo (Figure 11), while Akt phosphorylation was inhibited in ORL-48 in vitro, Akt phosphorylation was not affected in vivo suggesting that there is likely a feedback compensatory mechanism that could lead to the reactivation of Akt that subsequently impacted tumour growth. A distinct difference between ORL-48 and ORL-115 is that an activating mutation in PIK3CA is present in ORL-115\textsuperscript{27}; however, the role of this mutation in modulating response to dasatinib remains to be studied. Similar observations have been seen in another HNSCC tumour xenograft model using FaDu cells where dasatinib enhanced the tumour growth via activation of Akt despite Src inhibition.\textsuperscript{41} Additionally, feedback activation of Akt pathway has been suggested to be responsible for the imatinib (Src kinase inhibitor) resistance in tumour samples.\textsuperscript{42} Broadly, our results were similar to Sen et al.,\textsuperscript{43} who reported that no regression of tumour was achieved by dasatinib alone and this was irrespective of demonstrating FAK inactivation.\textsuperscript{43} However, other studies including those on head and neck cancer\textsuperscript{22,23,38,44} demonstrated approximately 37\% response with regard to tumour regression by which Src and FAK in these tumours were inhibited after the completion of treatment.\textsuperscript{22,23} This suggests that some head and neck tumours are able to activate compensatory pathway that results in resistance to dasatinib as observed in our study. Indeed, Jak/Stat pathway has been suggested to play a role in the process\textsuperscript{22,40}, however, in our study, we did not observe this from the biochemical analysis. Our findings further support those of Baro et al.\textsuperscript{41} on dasatinib-induced resistance demonstrating that Akt
reactivation could be causal. When activated, Akt goes on to phosphorylate other downstream effector proteins that promotes cell proliferation and cell survival through pro-survival and anti-apoptotic proteins.\textsuperscript{45,46} This study as well as others can likely go towards explaining in part, the limited clinical activity in patients from a recent phase II clinical trial reported by Brooks et al.\textsuperscript{24}

Notwithstanding, we acknowledge several limitations in our current study and notably among these includes the pharmacokinetics status of dasatinib. Previous preclinical studies have demonstrated that dasatinib has intermediate clearance and giving approximately 14\% of oral bioavailability in the models used, suggestive of poor absorption and high first pass metabolism, broadly defined as drug metabolism whereby the

| Group         | Mice Description | Size (cm) | LVP<sup>a</sup> | Keratin | Nucleus | Mitosis<sup>b</sup> | Pattern<sup>c</sup> | Stage<sup>d</sup> | L/P<sup>e</sup> | Total points | Grade |
|---------------|------------------|-----------|-----------------|---------|---------|---------------------|--------------------|-----------------|--------------|--------------|-------|
| ORL 48/control A | 2 nodules         | 0.6 × 0.3 | No              | 1       | 2       | 2                   | 2                  | 2               | 2            | 11           | I     |
|               |                  | 0.5 × 0.4 | No              | 1       | 2       | 2                   | 1                  | 3               | 10           | I             |       |
| B             | 2 nodules         | 1.8 × 1.1 | Yes, neural     | 1       | 2       | 4                   | 2                  | 2               | 3            | 14           | II    |
|               |                  | 1.9 × 1.1 | Yes             | 1       | 2       | 3                   | 2                  | 2               | 3            | 13           | II    |
| C             | 2 nodules         | 1.7 × 1   | No              | 1       | 2       | 4                   | 2                  | 3               | 3            | 15           | II    |
|               |                  | 1.7 × 1.2 | Susp<sup>f</sup> | 1       | 2       | 4                   | 3                  | 3               | 3            | 16           | II    |
| D             | 2 nodules         | 2.2 × 1.2 | No              | 1       | 2       | 3                   | 2                  | 2               | 3            | 13           | II    |
| E             | 2 nodules         | 2.1 × 0.8 | No              | 1       | 2       | 3                   | 2                  | 2               | 3            | 13           | II    |
|               |                  | 2 × 1.2   | No              | 1       | 2       | 3                   | 2                  | 3               | 3            | 13           | II    |
| ORL 48/dasatinib A | 2 nodules        | 2 × 1.1   | No              | 1       | 2       | 4                   | 3                  | 3               | 3            | 16           | II    |
|               |                  | 2.1 × 1   | No              | 1       | 2       | 4                   | 3                  | 3               | 3            | 16           | II    |
| B             | 2 nodules         | 1.7 × 1.1 | No              | 1       | 2       | 3                   | 2                  | 2               | 2            | 12           | I     |
|               |                  | 1.8 × 0.8 | Yes             | 1       | 2       | 3                   | 2                  | 2               | 2            | 12           | I     |
| C             | 2 nodules         | 2.8 × 1.1 | No              | 1       | 2       | 3                   | 3                  | 3               | 3            | 15           | II    |
|               |                  | 1.8 × 1   | No              | 1       | 2       | 3                   | 3                  | 3               | 3            | 15           | II    |
| D             | 2 nodules         | 1.8 × 1.1 | Susp<sup>f</sup> | 1       | 2       | 4                   | 3                  | 3               | 3            | 17           | II    |
|               |                  | 2.3 × 0.6 | Susp            | 1       | 2       | 3                   | 3                  | 3               | 3            | 16           | II    |
| E             | 2 nodules         | 2 × 0.9   | No              | 1       | 2       | 3                   | 3                  | 3               | 3            | 13           | II    |
|               |                  | 1.8 × 1   | No              | 1       | 2       | 3                   | 3                  | 3               | 3            | 14           | II    |
| ORL 115/control A | 2 nodules        | 0.6 × 0.5 | No              | 1       | 2       | 2                   | 2                  | 2               | 9            | 1             | I     |
|               |                  | 1.1 × 0.5<sup>e</sup> | No | 1       | 2       | 2                   | 2                  | 2               | 9            | 1             | I     |
| B             | 2 nodules         | 0.9 × 0.6 | No              | 1       | 2       | 1                   | 3                  | 3               | 3            | 8             | I     |
|               |                  | 1 × 0.8   | No              | 1       | 2       | 1                   | 3                  | 3               | 3            | 8             | I     |
| C             | 2 nodules         | 0.6 × 0.5<sup>h</sup> | No | 1       | 2       | 1                   | 3                  | 3               | 3            | 8             | I     |
|               |                  | 1.8 × 0.2<sup>e</sup> | No | 1       | 2       | 2                   | 2                  | 2               | 3            | 12            | I     |
| D             | 3 nodules         | 0.6 × 0.6<sup>h</sup> | No | 1       | 2       | 1                   | 3                  | 3               | 3            | 8             | I     |
|               |                  | 0.5 × 0.4<sup>h</sup> | No | 1       | 2       | 1                   | 3                  | 3               | 3            | 8             | I     |
|               |                  | 0.3       | No              | 1       | 2       | 2                   | 2                  | 2               | 3            | 11            | I     |
| ORL 115/dasatinib A | 2 nodules      | 1.2 × 0.6 | No              | 1       | 2       | 2                   | 2                  | 2               | 3            | 11           | I     |
|               |                  | 1.2 × 0.7 | No              | 1       | 2       | 2                   | 2                  | 2               | 3            | 11           | I     |
| B             | 2 nodules         | 1 × 0.6   | No              | 1       | 2       | 2                   | 3                  | 3               | 3            | 14           | II    |
|               |                  | 1 × 0.8<sup>e</sup> | No | 1       | 2       | 3                   | 3                  | 3               | 3            | 14           | II    |
| C             | 3 nodules         | 0.6 × 0.4<sup>h</sup> | No | 1       | 2       | 2                   | 2                  | 3               | 3            | 10            | I     |
|               |                  | 0.8 × 0.6<sup>h</sup> | No | 1       | 2       | 3                   | 3                  | 2               | 2            | 12           | I     |
|               |                  | 1 × 0.5<sup>e</sup> | No | 1       | 2       | 3                   | 3                  | 2               | 2            | 12           | I     |
| D             | 4 nodules         | 0.3<sup>h</sup> | No | 1       | 2       | 2                   | 3                  | 2               | 3            | 13           | II    |
|               |                  | 0.4<sup>h</sup> | No | 1       | 2       | 2                   | 3                  | 2               | 3            | 13           | II    |
|               |                  | 1 × 0.4<sup>h</sup> | No | 1       | 2       | 2                   | 3                  | 2               | 3            | 13           | II    |
|               |                  | 1.2 × 1.1  | No              | 1       | 2       | 2                   | 2                  | 3               | 2            | 12           | I     |
| E             | 1 nodule          | 0.5       | No              | 1       | 2       | 2                   | 2                  | 1               | 2            | 9             | I     |

<sup>a</sup>Lymphovascular permeation.
<sup>b</sup>Per HPF (×40 objective, in the worst area).
<sup>c</sup>Pattern of invasion.
<sup>d</sup>Stage of invasion.
<sup>e</sup>Lymphoplasmacytic cell infiltrates.
<sup>f</sup>Suspicious of lymphovascular permeation.
<sup>g</sup>Still fragmented despite several deeper levels.
<sup>h</sup>Separate yet adherent nodules.
concentration of drug is greatly reduced before it reaches the systemic circulation. In human phase 1 clinical trials, it was shown that CYP3A4 could be metabolizing dasatinib and consequently, co-administration of enzyme inhibitor, improved the plasma concentration of dasatinib. Finally, compensatory signalling pathways that get activated upon drug treatment are now known to confer resistance and with dasatinib, likely through bypassing the blockade of Src. In the case of Akt reactivation as seen in this current study, several reports have now shown that by adding Akt pathway inhibitors, for example, rapamycin, erlotinib and lapatinib, have essentially resulted in a synergistic effect and conferred benefit. Further evaluation to fully understand the molecular targets and drug metabolism is needed to improve the clinical usefulness of this drug in OSCC treatment to improve patient outcome.

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Declaration of Conflicting Interests

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**Translational value**

This study provides clues to the possible mechanism of resistance to the Src inhibitor dasatinib which could be useful in designing future treatment strategies for better clinical outcome. Akt reactivation could be a biomarker of resistance, and testing dasatinib in future clinical trials may require a combination of drugs that could inhibit Akt reactivation to overcome drug resistance to dasatinib.