Heterogeneous Sensitivity to Src Inhibitors in Oral Squamous Cell Carcinoma and Its Implications for Combination Therapy with Cisplatin

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Running title; Suppression of oral cancers by Src inhibitors

Competing interests

The authors declare no competing financial interests.

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Abstract

Purpose:

Treatment options of advanced oral squamous cell carcinomas (OSCC) are limited, and cisplatin toxicity and drug resistance are major clinical issues. Src is a central kinase that integrates multiple oncogenic pathways and a promising therapeutic target. However, Src inhibitors have shown suboptimal efficacy as monotherapies and their sensitivity in OSCC remains elusive.

Experimental Design:

We examined the activation of major oncogenic signaling pathways and the antitumor effects of six Src inhibitors (dasatinib, ponatinib, vandetanib, saracatinib, PP2, bosutinib) in seven human OSCC cell lines (HSC-2, HSC-3, HSC-4, SAS, HO-1-u-1, CAL27, SCC-25). BALB/cAJcl nu/nu mice bearing CAL27 xenografts received dasatinib (30 mg/kg, intraperitoneally, daily), bosutinib (50 mg/kg, intraperitoneally, daily), cisplatin (2 mg/kg or 4 mg/kg, intraperitoneally, weekly), or combinations. Tumor volume, bioluminescence imaging, and body weight were monitored for 17 or 21 days, followed by histopathological assessment.

Results:

The activation of the key pathways, including Src and MAPK, considerably differed among the cell lines and was linked to heterogeneous sensitivity to Src inhibitors. Effective growth suppression required Src dephosphorylation and downstream MAPK pathway inhibition, which vary depending on the cell line. Additionally, combination treatment with a Src inhibitor and cisplatin showed additive antitumor effects, allowing the reduction of cisplatin doses by half without efficacy loss. Notably, dasatinib alone and in combination with cisplatin decreased tumor burden with characteristic internal tumor death *in vivo*.

Conclusions:

These findings elucidate Src signaling dependency on OSCC and the potential of Src inhibition to decrease cisplatin toxicity, paving way for Src targeted therapeutic strategies.

Introduction

Oral squamous cell carcinoma (OSCC) is a malignant disease on the mucosal surfaces of the tongue, gingiva, and lips, and is the most common subtype of head and neck malignancy(1). Approximately 390,000 new cases of OSCC were reported worldwide in 2022, and the rate of increase is predicted to reach nearly 60% by 2050, according to the Global Cancer Observatory (2).

Almost 60% of patients with oral cancer are diagnosed at advanced stages (stage III–IV), with a 5-year survival rate of less 60%(3–6). Managing patients with advanced cancer requires multimodal treatment including surgery, radiotherapy, and chemotherapy(6). Although multidisciplinary therapy is effective for managing advanced cancer, it is quite invasive, resulting in treatment-related cosmetic and functional impairments such as dysphagia and speech difficulties, which considerably compromise the quality of life(QOL) patients (7). Moreover, 10–50% of patients experience local recurrence or distant metastasis following initial therapy, and only a minority are eligible for salvage surgery or radiotherapy(8–10). Palliative or supportive care is the last option for most patients with recurrent or metastatic cancer, with a median survival of 4–9 months(3,11). Thus, improving the prognosis and QOL of patients with OSCC remains an urgent clinical challenge.

Systemic chemotherapy plays a key role, especially in patients with recurrent or metastatic cancer. Cisplatin is the standard systemic therapy in definitive chemoradiotherapy and recurrent/metastatic settings. However, owing to severe toxicities, few patients with adequate performance status (PS) can be prescribed cisplatin(12). No alternative systemic therapy with efficacy comparable to that of cisplatin has been established for patients ineligible due to age, comorbidities, or poor PS(3). In addition, cisplatin resistance occurs in approximately 30–40% of patients with OSCC, substantially limiting its therapeutic effect(13). Therefore, drugs that decrease cytotoxicity and overcome cisplatin resistance are necessary.

Several studies have explored the use of cisplatin substitutes. While carboplatin shows lower toxicity than cisplatin, its antitumor activity is inferior to cisplatin at equivalent doses(14). Cetuximab is the only molecular-targeted drug approved for OSCC. However, it has not shown clear superiority over cisplatin yet (15). Even though immune checkpoint inhibitors such as pembrolizumab and nivolumab have recently been approved for treating recurrent/metastatic OSCC, their response rates are limited to approximately 10–20%(16,17). The identification of new effective therapeutic agents for OSCC is a crucial unmet clinical need.

92 A central non-receptor tyrosine kinase within the Src family of kinases (SFKs), c-Src
93 (hereafter, Src), regulates multiple cellular processes involved in malignant progression,
94 including proliferation, survival, migration, invasion and angiogenesis(18). Src
95 hyperactivation has been noted in solid tumors, including breast, colorectal, pancreatic,
96 ovarian, gastric, esophageal, and lung cancers and is strongly liked to poor prognosis(19).
97 This indicates that Src inhibitors are potential candidates for cancer chemotherapy. Src
98 activation is correlated with poor clinical outcomes and invasive and metastatic potential
99 in head and neck squamous cell carcinoma (HNSCC)(20). Particularly, in OSCC, high
100 Src expression is markedly linked to tumor size, lymph node metastasis, advanced stage
101 and recurrence, and has been identified as an independent prognostic factor. This suggests
102 that Src plays a crucial role in OSCC progression(21).
103 Preclinical studies have revealed that FDA-approved Src inhibitors, including dasatinib
104 and saracatinib, suppress proliferation, migration and invasion, and induce apoptosis in
105 HNSCC/OSCC models(19,22,23). Despite these promising results, Src inhibitors have
106 demonstrated suboptimal efficacy as monotherapies in clinical trials(24,25). Possible
107 mechanisms behind this discrepancy include the non-lethal nature of Src inhibition in
108 HNSCC/OSCC cells, compensatory activation of alternative pathways, such as
109 PI3K/AKT and STAT3(22,26), and variations in EMT status or cell adhesion molecules

110 that affect drug sensitivity(27,28). Substantial efforts have been directed toward
111 elucidating the mechanisms of action of Src inhibitors and identifying biomarkers that
112 predict the therapeutic response in lung, colorectal, and breast cancers, where oncogenic
113 driver mutations are well characterized. However, the molecular determinants of Src
114 inhibitor sensitivity in OSCC remain elusive.

115 HNSCC/OSCC is characterized by profound molecular heterogeneity, with distinct
116 dependencies on kinases, such as EGFR, EPHA2, AURKA, WEE1, and JAK1 across
117 various cell lines(29). This heterogeneity suggests that sensitivity to Src inhibition may
118 markedly differ among the OSCCs. However, there is no report systematically
119 investigating Src inhibitor sensitivity in OSCC, especially using animal models.
120 Moreover, recent the combined effects of Src inhibitors and cisplatin may provide a
121 strategy to overcome cisplatin resistance and decrease toxicity in gastric cancer and
122 tubular epithelial cells(30,31).

123 Based on this evidence, we sought to reassess the therapeutic potential of Src inhibition
124 in OSCC and generate foundational insights that may guide the optimization of future
125 treatment strategies. We analyzed Src inhibitor sensitivity across multiple OSCC cell lines
126 and a mouse model and assessed the therapeutic effect of combining Src inhibition with
127 cisplatin.

128 **Materials and Methods**

129 **Cell culture**

130 Seven human OSCC cell lines were utilized. RIKEN BRC (Tsukuba, Japan) provided
131 HSC-2 (RCB1945), HSC-3 (RCB1975), HSC-4 (RCB1902), SAS (RCB1974), and HO-
132 1-u-1 (RCB2102). CAL27 (CRL-2095) and SCC-25 (CRL-1628) cells were purchased
133 from the American Type Culture Collection (ATCC, VA). HSC-2 and HSC-3 cells were
134 maintained in E-MEM (051-07615, Wako, Osaka, Japan). HSC-4, SAS, and HO-1-u-1
135 cells were maintained in RPMI-1640 (189-02023, Wako). Furthermore, CAL27 cells
136 were maintained in high-glucose DMEM supplemented with L-glutamine, phenol red,
137 and sodium pyruvate (041-30081, Wako). SCC-25 cells were maintained in a 1:1
138 mixture of DMEM and Ham's F-12 medium containing L-glutamine, phenol red,
139 HEPES, and sodium pyruvate (042-30555, Wako) supplemented with 400 ng/mL
140 hydrocortisone. All mediums cultivating cells mentioned above were further
141 supplemented with 10% fetal bovine serum (Gibco, CA) and 1% penicillin-
142 streptomycin (100×, Wako), and cells were maintained at 37°C in a humidified
143 atmosphere containing 5% CO_2 before use. All cell lines used were cultured within
144 three months of the second passage.

145

146 **Reagents**

147 Src inhibitors used in this study are as follows. Dasatinib (SML2589, TCI TC-I 201,
148 and D5949) was obtained from Sigma-Aldrich. Ponatinib (AMBH303C5EAB),
149 vandetanib (SML2983), and saracatinib (SML3195) were purchased from Sigma-
150 Aldrich. Additionally, PP2 (529573) was purchased from Calbiochem (MA). Bosutinib
151 (T0152) was obtained from TargetMol, MA. The Src inhibitors were dissolved in
152 dimethyl sulfoxide (DMSO). Cisplatin (033-20091) was obtained from Wako and was
153 dissolved in 0.9% NaCl.

154

155 **Antibodies**

156 The following primary antibodies were utilized: p53 (7F5) Rabbit mAb (#2527),
157 Phospho-p53 (Ser15) Antibody (#9284), Src (32G6) Rabbit mAb (#2123), Phospho-Src
158 Family (Tyr416) (D49G4) Rabbit mAb (#6943), Ras (D2C1) Rabbit mAb (#8955), p38
159 MAPK (D13E1) XP Rabbit mAb (#8690), Phospho-p38 MAPK (Thr180/Tyr182)
160 (D3F9) XP Rabbit mAb (#4511), p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb (#4695),
161 Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb
162 (#4370), SAPK/JNK Antibody (#9252), Phospho-SAPK/JNK (Thr183/Tyr185) (81E11)
163 Rabbit mAb (#4668), and β-Actin Antibody (#4967) (all from Cell Signaling

164 Technology, MA). The K-Ras Monoclonal Antibody (9.13) was purchased from
165 Invitrogen (Carlsbad, CA). For detection, Anti-rabbit IgG, HRP-linked Antibody (Cell
166 Signaling Technology) was used for all primary antibodies except K-Ras and β-actin,
167 for which HRP-conjugated anti-mouse IgG secondary antibodies (Cell Signaling
168 Technology) were used.
169
170 **Western blotting**
171 HSC-2, HSC-3, HSC-4, SAS, HO-1-u-1, CAL27, and SCC-25 cells were lysed using
172 M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, NY)
173 supplemented with 10× Complete protease inhibitor and 10× PhosSTOP phosphatase
174 inhibitor (Roche, Basel, Switzerland), mixed with an equal volume of 2× sodium
175 dodecyl sulfate (SDS) buffer, and boiled at 90 °C for 10 min. Proteins were separated
176 using SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto
177 polyvinylidene fluoride membranes. Membranes were blocked with Every Blot
178 Blocking Buffer (Bio-Rad Laboratories, Hercules, CA) and incubated with primary
179 antibodies for 1 h at 25 ℃. After washing with Tris-buffered saline containing 0.1%
180 Tween-20 (TBS-T), the membranes were incubated with secondary antibodies for 1 h at
181 25 ℃. Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad

182 Laboratories) and imaged using a ChemiDoc Toch MP Imaging System (Bio-Rad
183 Laboratories).
184 For phosphorylation analysis following Src inhibitor treatment, HSC-2, HSC-3, HSC-4,
185 SAS, HO-1-u-1, CAL27, and SCC-25 cells were treated with dasatinib, ponatinib,
186 vandetanib, or bosutinib for 18 h. The cells were seeded in 12 well plates at densities
187 that yielded approximately 80% confluence at the treatment time. Supplementary Table
188 S1 provides the concentrations utilized for each cell line. Cells were harvested using the
189 same lysis procedure described above, and protein samples were subjected to SDS-
190 PAGE and western blotting, as previously described. The following primary antibodies
191 were used: Phospho-Src Family (Tyr416) (D49G4) Rabbit mAb (#6943), Phospho-
192 p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb (#4370),
193 Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP Rabbit mAb (#4511), and Phospho-
194 SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb (#4668) (all from Cell Signaling
195 Technology). Detection and imaging were conducted as previously described.
196
197 **Cell viability assays**
198 Cell viability was evaluated using the CellTiter-Glo® 2.0 Reagent (Promega, WI). Cells
199 were seeded into Costar 96-well flat white plates (3917, Costar, NY) at the following

200 densities: HSC-2 (0.8×10^4), HSC-3 (1.0×10^4), HSC-4 (0.8×10^4), SAS (2.0×10^4),

201 HO-1-u-1 (1.5×10^4), CAL27 (1.5×10^4), and SCC-25 (0.7×10^4) cells per well, and

202 allowed to adhere overnight. HSC-2, HSC-3, HSC-4, SAS, HO-1-u-1, CAL27, and

203 SCC-25 cells were treated with the Src inhibitors dasatinib (100 nM), ponatinib (2 μM),

204 vandetanib (10 μM), saracatinib (5 μM), PP2 (50 μM), bosutinib (5 μM), or cisplatin

205 (10 μM) for 72 h. All compounds were diluted in a culture medium at the final

206 concentration of DMSO with 1%, and cell viability was calculated relative to the 1%

207 DMSO control. Following drug treatment, CellTiter-Glo® 2.0 reagent was added at a

208 1:1 ratio to the culture medium, mixed for 2 min on an orbital shaker and incubated for

209 10 min at 25 ℃ to stabilize the luminescent signal. The luminescence was measured

210 using a Tecan Spark microplate reader (TECAN, Männedorf, Switzerland). All

211 experiments were conducted in triplicates (n = 3).

212 For IC_{50} determination, cells were seeded into Costar 96-well flat white plates (3917,

213 Costar) at the same densities described above for each cell line and allowed to adhere

214 overnight. Cells were subsequently treated with serial dilutions of dasatinib, ponatinib,

215 saracatinib, or bosutinib for 36 h. Cell viability was measured using the CellTiter-Glo®

216 2.0 Reagent (Promega) as described above. IC_{50} values were calculated from dose-

217 response curves using nonlinear regression in R (version 4.5.2). All experiments were
218 conducted in triplicates (n = 3).
219 For combination treatment assays, cell viability was evaluated using the CellTiter-Glo®
220 2.0 Reagent (Promega). Cells were seeded in Costar 96-well flat white plates (3917,
221 Costar) at the cell line-specific densities described above and allowed to adhere
222 overnight. Cells were treated with cisplatin (10 μM or 5 μM), each Src inhibitor alone,
223 or their combinations for 36 h. Supplementary Table S2 provides the Src inhibitor
224 concentrations used for each cell line. All compounds were diluted in the culture
225 medium to a final concentration of DMSO (1%), and cell viability was calculated
226 relative to the 1% DMSO control. Luminescence was measured using a Tecan Spark
227 microplate reader (TECAN). All experiments were performed in triplicates (n = 3).
228
229 **Lentiviral production and generation of Renilla luciferase (RLuc)-expressing**
230 **CAL27 cells**
231 RLuc-expressing CAL27 (CAL27-Rluc) cells were generated by infecting parental
232 CAL27 cells with lentiviral particles produced by HEK293T cells. Lentiviruses were
233 produced by co transfecting HEK293T cells with the packaging plasmids pCMV-VSVG
234 -RSV-Rev (1 μg/μL) and pCAG-HIVgp-RRE (1 μg/μL), together with the transfer

235 vector CSII-EGFP-P2A-RLuc-IRES-Bsd at a ratio of 1:1:2. Viral infection of CAL27
236 cells was conducted in the presence of polybrene at a 10 μg/mL final concentration.
237 Seven independent CAL27-Rluc clones were established following infection and
238 blasticidin selection. RLuc activity in each clone was confirmed using EnduRen live
239 cell Renilla luciferase substrate (Promega) according to the manufacturer's instructions.
240 The drug responsiveness of the clones was assessed 36 h following treatment with Src
241 inhibitors and cisplatin under the same conditions used for the Cell-Titer Glo® assay.
242 Supplementary Fig. 1 shows the detailed screening results for the seven clones.
243
244 **Animal experiments**
245 CAL27-Rluc cells (clone 7; 2×10^7 cells/mouse) were subcutaneously implanted into
246 the right flank of 5-week-old male BALB/cAJcl nu/nu mice (CLEA Japan, Tokyo,
247 Japan). The mice were kept in a 12-hour light-dark cycle and were given free access to
248 food and water. Three days following implantation, the mice were randomly assigned to
249 six treatment groups based on their body weight (n = 5-6 in each group): vehicle control
250 (DMSO), dasatinib alone (30 mg/kg), cisplatin alone (4 mg/kg), cisplatin alone (2
251 mg/kg), dasatinib (30 mg/kg) + cisplatin (4 mg/kg), and dasatinib (30 mg/kg) + cisplatin
252 (2 mg/kg). Dasatinib and DMSO were dissolved in 10 mM Tris-HCl (pH 7.4), and

253 cisplatin was dissolved in 0.9% NaCl. All drugs were intraperitoneally administered at a
254 volume of 100 μL per mouse. Dasatinib and DMSO were administered once daily,
255 whereas cisplatin was administered once weekly for two consecutive weeks. This
256 dosing schedule was designed to approximate the clinical dosing frequencies in humans.
257 In a separate experiment conducted under identical implantation and monitoring
258 conditions, the mice were assigned to three groups: vehicle control (DMSO), dasatinib
259 alone (30 mg/kg), and bosutinib alone (50 mg/kg). Bosutinib was dissolved in 10 mM
260 Tris HCl (pH 7.4) and intraperitoneally administered once daily at a volume of 100 μL
261 per mouse, following the same dosing schedule as in the dasatinib experiment.
262 To detect Renilla luciferase activity, ViviRen™ In Vivo Renilla Luciferase Substrate
263 (Promega) was dissolved in dimethyl sulfoxide at 10 mg/mL to prepare a stock solution,
264 which was subsequently diluted 1,000-fold in PBS containing 0.1% bovine serum
265 albumin. A total of 100 μL (1 mg) of the diluted substrate was intravenously
266 administered. Mice were anesthetized with isoflurane, and bioluminescence imaging
267 was conducted using an IVIS system. Bioluminescence signals were quantified 10 min
268 following ViviRen injection according to the manufacturer's instructions(32). IVIS
269 image analysis was performed on days 5, 7, 10, and 13 after subcutaneous tumor
270 transplantation and on days 7, 13, and 20, respectively.

271 Tumor volumes were measured using calipers and calculated as $L \times W^2 \times 0.52$. Body
272 weight was monitored every 2-3 d throughout the treatment period. At the end of the
273 treatment, the mice were perfusion-fixed, and the tumor, kidney, and liver were
274 harvested (N＝5-6). All animal experiments were conducted in accordance with the
275 Animal Experimentation Regulations of the Jichi Medical University. The Institutional
276 Animal Care and Use Committee of Jichi Medical University reviewed and approved
277 the study protocol. The study subsequently received final authorization from the
278 University President.
279
280 **Hematoxylin and Eosin (H&E) staining**
281 Excised tumor, kidney, and liver tissues were fixed in 1% paraformaldehyde at 4 °C
282 overnight. The tissues were subsequently cryoprotected by sequential incubation in 10%
283 sucrose/PBS and 20% sucrose/PBS at 4 °C, each for 24 h. After cryoprotection, the
284 tissues were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and snap-
285 frozen in liquid nitrogen. Frozen blocks were sectioned at 8 μm thickness, and the
286 sections were stained with H&E using standard procedures. The sections were post-
287 fixed in 2% paraformaldehyde, stained with hematoxylin, rinsed under running tap
288 water, counterstained with eosin, dehydrated using graded ethanol, cleared in xylene,

289 and mounted with coverslips. Histological images were obtained using a light

290 microscope.

291

292 **Statistical analysis**

293 No statistical methods were used to predetermine sample size. Mouse studies were

294 randomized; however, the investigators were not blinded to group allocation during the

295 experiments or outcome assessment. Western blot analysis was independently

296 performed three times. IC_{50} values for the Src inhibitors were calculated from three

297 independent CellTiter-Glo assays, and mean ± standard deviation (SD) was obtained

298 using Microsoft Excel. For *in vitro* combination assays of the Src inhibitors and

299 cisplatin, data are presented as mean ± SD from n = 3, and P values were calculated

300 using paired t-tests in Microsoft Excel. Statistical significance was defined as follows: p

301 < 0.01 **, $p < 0.05$ *, and $p \geqq 0.05$ ns.

302 For *in vivo* experiments, tumor volume and body weight were analyzed using data from

303 surviving mice at each time point (n = 5–6), and mean ± SD was calculated using R

304 (version 4.5.2).

305

306 **Results**

307 **OSCC cell lines show substantial heterogeneity in the activation of major oncogenic**

308 **signaling pathways**

309 Cancer develops through the accumulation of genetic and epigenetic changes in

310 oncogenic signaling pathways, eventually resulting in the acquisition of malignant

311 phenotypes(33). In OSCC, p53 mutations frequently occur(34), and multiple oncogenic

312 pathways including EGFR, PI3K/AKT/mTOR, JAK/STAT, MET, Wnt/β-catenin and

313 RAS/RAF/MAPK are aberrantly activated and contribute to tumor progression(35).

314 Besides, the activation of MAPK signaling (ERK1/2, JNK, p38, and ERK5) is involved

315 in HNSCC oncogenicity(35). These observations suggest that the activity of signaling

316 networks in the OSCC cell lines may markedly vary.

317 To test this hypothesis, we investigated the phosphorylation status of key signaling

318 molecules by western blotting (Fig. 1A). HSC-4 and CAL27 revealed increased protein

319 levels of p53 and phosphor-p53 (p-p53) (Ser15). Total Src expression was consistent in

320 all cell lines; however, its active form, phospho-Src (p-Src) (Tyr416), was detected in all

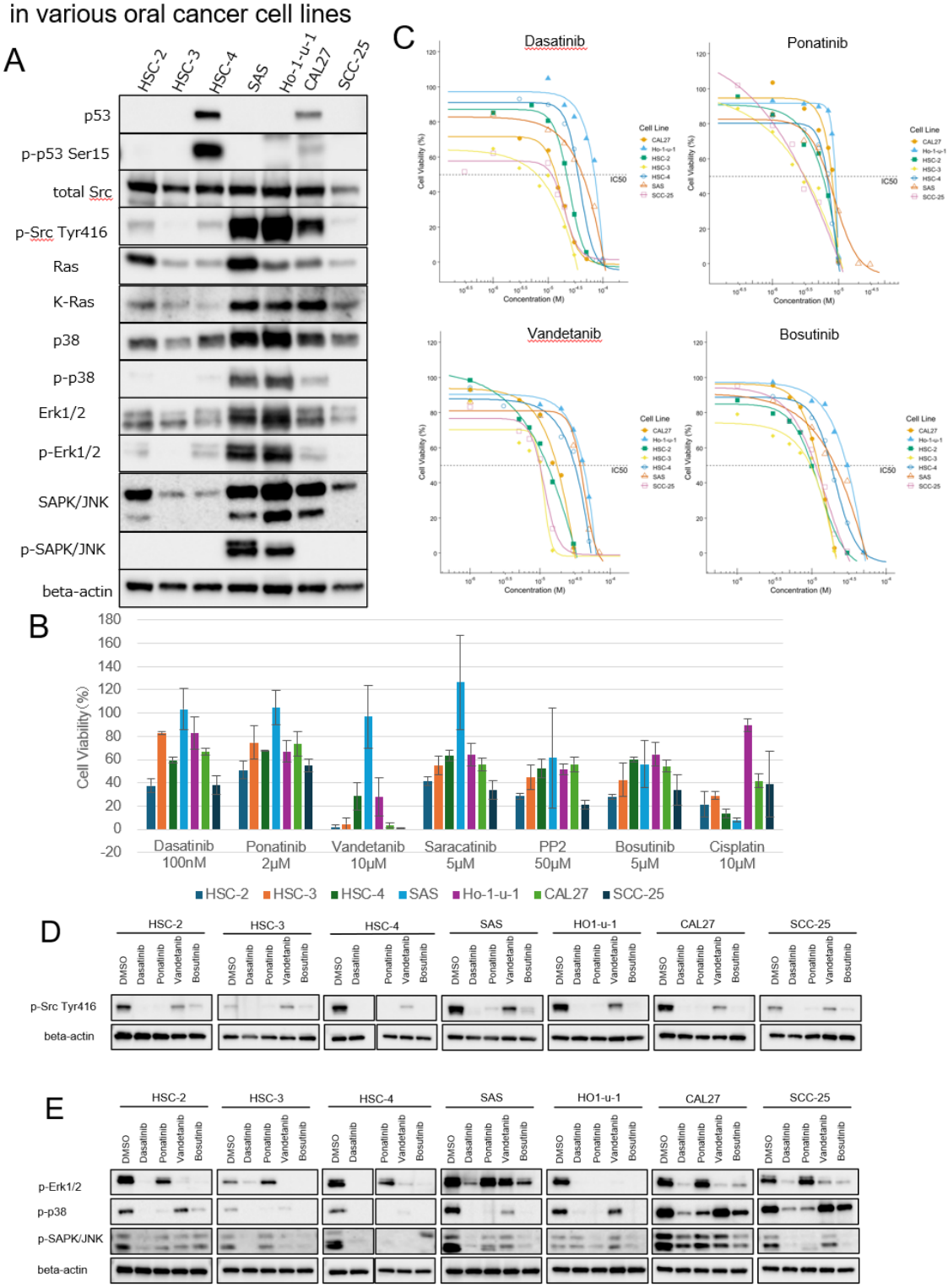
321 cell lines except HSC-3 and SCC-25. K-Ras expression was augmented in SAS, HO-1-

322 u-1, and CAL27, and the discrepancy between Ras expression and activation in HSC-2

323 and SAS suggested N-Ras or H-Ras activation. Within the MAPK pathway, all seven

324 cell lines exhibited strong p38 and ERK1/2 activation, as shown by elevated
325 phosphorylation levels (p-p38 and p-ERK1/2), which closely paralleled the activation
326 pattern of p-Src (Tyr416). This concordant activation pattern suggested that Src activity
327 was closely associated with downstream MAPK signaling in the OSCC cells.
328 Contrastingly, SAPK/JNK expression was relatively high in HSC-2, SAS, HO-1-u-1,
329 and CAL27, whereas enhanced SAPK/JNK phosphorylation was only noted in SAS and
330 HO-1-u-1, indicating a more selective activation profile. Thus, broadly activated Src,
331 together with the downstream signaling activation patterns noted in the OSCC cell lines,
332 could be a promising target for chemotherapy in OSCCs.

Figure 1 Src activity and its inhibitory effect of tumor growth by specific inhibitors in various oral cancer cell lines



333

334 **Src activity and its inhibitory effect of tumor growth by specific inhibitors in**

335 **different oral cancer cell lines**

336 Some Src inhibitors have already been approved for clinical cancer therapies(19,22,23),

337 suggesting that these Src inhibitors can be easily utilized for oral cancers if their

338 anticancer effect is prospective. Next, we evaluated the sensitivity of the OSCC cell lines

339 to FDA-approved Src inhibitors. Despite PP2 is not FDA-approved, it was included for

340 comparison because it is frequently used as a research-grade Src inhibitor. Treatment of

341 seven OSCC cell lines with each Src inhibitor at concentrations close to their mean IC_{50}

342 showed variation in cell line viability (Fig. 1B). HSC-2 and SCC-25 were broadly

343 sensitive to all the inhibitors tested, whereas SAS showed lower sensitivity (Fig. 1B).

344 Comparison of the IC_{50} values obtained following 36 h of treatment indicated differences

345 between the inhibitors (Table 1, Fig. 1C). Dasatinib, vandetanib, saracatinib, and PP2

346 revealed pronounced variability in their IC_{50} values across cell lines. In contrast, ponatinib

347 and bosutinib showed broad antitumor effects with less variability in cell lines (Table 1,

348 Fig. 1C). Notably, HO-1-u-1 showed resistance to cisplatin. Thus, OSCC cell lines show

349 distinct pharmacological vulnerabilities resulting from the differences in the activating

350 pathways for Src signaling.

351 We examined p-Src (Tyr416) levels 18 h following inhibitor treatment to determine

352 whether the differences in antitumor activity were attributable to the degree of Src

353 inhibition (Fig. 1D). All inhibitors suppressed Src activation, although vandetanib

354 exhibited a relatively weaker inhibition. This effect was especially modest in SAS and

355 HO-1-u-1, which is consistent with their reduced sensitivity (Figs. 1B and 1C).

Table 1 The half maximal inhibitory concentration (IC50) of Src inhibitors in various oral cancer cell lines after 36 h of treatment

	Dasatinib	Ponatinib	Vandetanib	Saracatinib	PP2	Bosutinib	Cisplatin
HSC-2	0.1 μM	2 μM	5 μM	5 μM	50 μM	5 μM	10 μM
HSC-3	10 μM	2 μM	8 μM	10 μM	100~250 μM	5 μM	1~5 μM
HSC-4	25 μM	5~10 μM	25 μM	100 μM	600 μM	15 μM	10 μM
SAS	10 μM	5~10 μM	50 μM	100 μM	＞600 μM	25 μM	10 μM
Ho-1-u-1	50 μM	5~10 μM	25 μM	50~100 μM	＞600 μM	25 μM	＞50 μM
CAL27	10~25 μM	5~10 μM	10~50 μM	25 μM	＞600 μM	15 μM	10 μM
SCC-25	10 μM	2~5 μM	8~10 μM	50 μM	200 μM	10 μM	10 μM

356 We further assessed whether Src inhibition suppressed the phosphorylation of MAPK

357 subpathways (ERK1/2, p38, and JNK; Fig. 1E). Dasatinib completely suppressed all

358 MAPK components in all the cell lines. Ponatinib completely inhibited p38 and largely

359 inhibited JNK phosphorylation, whereas the suppression was partial in CAL27.

360 Vandetanib inhibited distinct MAPK components, depending on the cell line used.

361 Bosutinib typically inhibited the phosphorylation of all MAPK components except ERK

362 in SAS and p38 in SCC-25. Thus, while the pathway associated with the upregulation of

363 Src varies in cells, different inhibitors targeting Src activity are effective in suppressing
364 the growth of OSCC cell lines.
365 Taken together, these results suggest that the activation status of major signaling pathways
366 involving Src significantly differs across the OSCC cell lines and that such molecular
367 differences may underlie the observed variation in sensitivity to Src inhibitors.
368

369 **Tumor inhibitory effect by combination therapy using cisplatin and Src inhibitors**

370 OSCC cell lines showed considerable heterogeneity such as variable susceptibilities to
371 Src inhibitors and cisplatin (Fig. 1). Next, we assessed the combinatorial effects of Src
372 inhibitors with cisplatin at 10 μM and 5 μM (Fig. 2).
373 Compared with cisplatin at 10 μM, Src inhibitors alone revealed equal or lower antitumor
374 activity in most OSCC cell lines, except for HO-1-u-1 and SCC-25, indicating that Src
375 inhibition was effective in the OSCC cell lines. The combination treatment showed
376 additive effects in HSC-3, HSC-4, CAL27, and SCC-25, especially with dasatinib and
377 bosutinib, and these effects increased in a cisplatin dose-dependent manner. Although
378 cisplatin at 5 μM alone showed limited activity, its combination with Src inhibitors
379 produced antitumor effects comparable to or more for 10 μM cisplatin alone in HSC-4,
380 CAL27, and SCC-25.

381 On the other hand, cisplatin-resistant HO-1-u-1 responded better to Src inhibitors alone
382 than to cisplatin, and no enhancement was noted with the combination treatment. This
383 indicates that the efficacy of Src-cisplatin combination therapy may be determined by the
384 intrinsic cisplatin sensitivity of each OSCC cell line and its degree of dependence on Src
385 signaling.
386 These results suggest that combining Src inhibitors with cisplatin may reduce the cisplatin
387 dose to half of the standard *in vitro* therapeutic concentration while maintaining its
388 efficacy in cisplatin-sensitive OSCC.
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390
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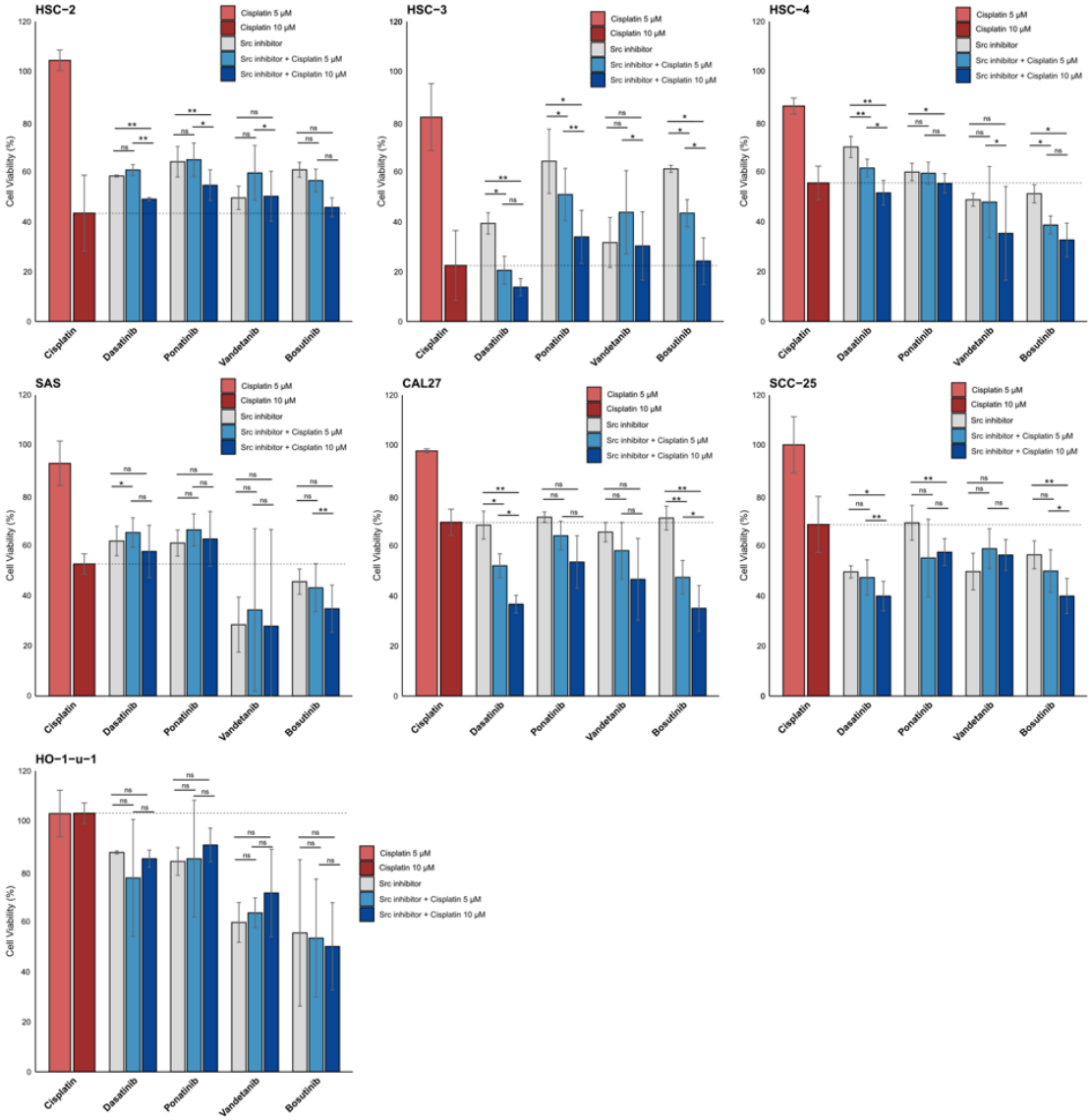
392
393

Figure 2 The tumor inhibitory effect by combination therapy using Cisplatin and Src inhibitor



394

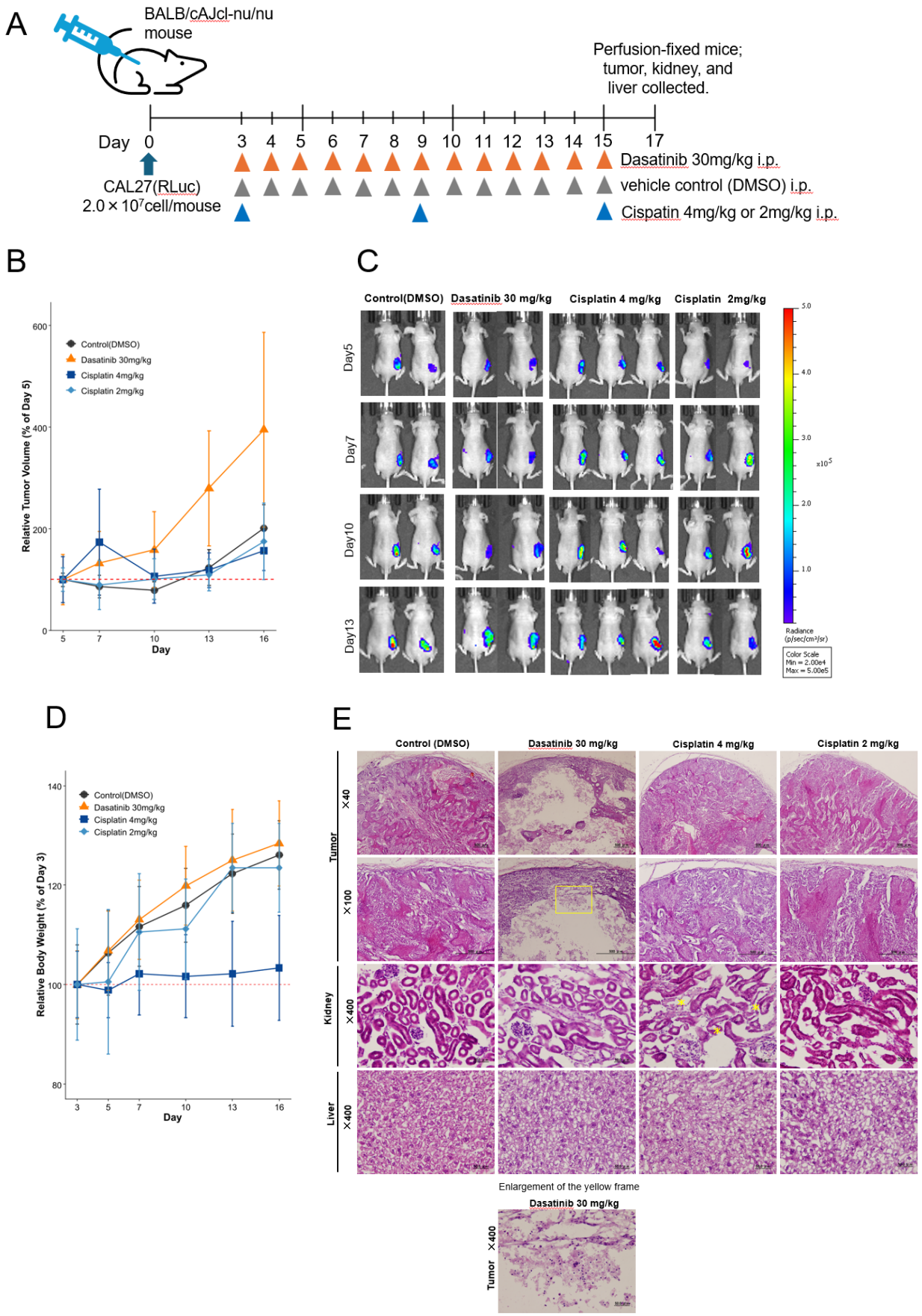
395 **Tumor growth inhibition by single treatment of dasatinib and bosutinib *in vivo***

396 Next, we examined whether dasatinib and bosutinib, which showed additive effects *in*
397 *vitro*, could suppress tumor growth as single agents *in vivo* (Fig. 3, Fig. S2, Fig. S3).
398 Since dasatinib and bosutinib demonstrated additive effects with cisplatin in more cell
399 lines than the other Src inhibitors (Fig. 2) and both are relatively inexpensive and thus
400 more suitable for clinical applications; these two inhibitors were selected for further *in*
401 *vivo* analysis. CAL27 cells were selected as the subcutaneous xenograft model because
402 their cells showed reliable tumor formation in preliminary experiments and demonstrated
403 high sensitivity to Src inhibitors with additive effects with cisplatin *in vitro*. To monitor
404 tumor size using *in vivo* imaging system, we established stable Rluc-expressing CAL27
405 (CAL27-Rluc) cells and checked their susceptibility to Src inhibitors (Fig. S1). Src
406 inhibitors suppressed the growth of CAL27-Rluc clones in a similar manner as the CAL27
407 Wt cells. Clone7, which exhibited a median susceptibility to Src inhibitors, was selected
408 for *in vivo* analysis. CAL27-Rluc cells-inoculated BALB/cAJcl nu/nu mice were treated
409 with inhibitors by indicated schedule which were designed to approximate clinically
410 relevant regimens (Fig. 3A). Furthermore, dasatinib (30 mg/kg) and bosutinib (50 mg/kg)
411 were compared in a separate experiment (Fig. S3A). Cisplatin 4 mg/kg was selected as a

412 positive control based on previous reports, and this dose is within the range of the
413 maximum repeated tolerated dose in the mouse model(36).
414 The physical tumor volumes in the cisplatin group at 4 and 2 mg/kg were decreased
415 compared to those in the control group, whereas their size in the dasatinib group continued
416 to increase (Fig 3B). In contrast, IVIS bioluminescence live imaging showed a substantial
417 reduction in the tumor signal in the dasatinib group relative to the control group. In some
418 cases, it exceeded the effect of cisplatin (Fig. 3C, Fig. S2). Body weight gain was
419 impaired in the cisplatin 4 mg/kg group, whereas mice treated with dasatinib or cisplatin
420 (2 mg/kg) maintained stable weight without apparent toxicity (Fig. 3D).
421 Bosutinib treatment led to antitumor activity; one of the three mice died, and the
422 remaining animals showed weight loss, indicating considerable toxicity (Fig. S3B–D).
423 Histological analyses on day 17 showed that tumors from the control and cisplatin groups
424 were solid, well-differentiated squamous cell carcinomas with keratin pearls (Fig. 3E).
425 Contrastingly, tumors from the dasatinib group showed cyst-like structures with hollow
426 interiors and serous fluids. Under magnification, the cells remaining in the lumen had
427 blurred outlines, lost nuclei, and died. In addition, the cancer pearls in the epithelial
428 regions disappeared. In the kidneys, normal kidney structure was maintained in the
429 control group (DMSO) and the dasatinib group. On the other hand, degeneration and

430 atrophy of the renal tubular epithelium and localized rupture were observed in the
431 cisplatin 4 mg/kg group, while only mild renal tubular epithelial atrophy was observed in
432 the cisplatin 2 mg/kg group. These results suggest that renal damage occurred in a dose-
433 dependent manner only in mice treated with cisplatin. The liver tissues retained normal
434 architecture in all groups, corresponding to no apparent tissue damage.
435 Together, these results show that dasatinib exerts antitumor activity *in vivo* by inducing
436 extensive intratumoral cell death, even in cases in which the tumor size appears to
437 increase. Especially, dasatinib achieved tumor suppression that exceeded that by cisplatin
438 under certain conditions, strongly supporting its therapeutic potential. Contrastingly,
439 bosutinib revealed antitumor efficacy comparable to that of cisplatin, but was linked to
440 toxicity, which may limit its clinical utility.

Figure 3 The inhibition of tumor growth by single treatment of Dasatinib in vivo



442 **Tumor growth inhibition by the combination treatment of cisplatin and dasatinib *in***

443 ***vivo***

444 We finally evaluated the antitumor efficacy of combination treatment with dasatinib and

445 cisplatin to determine whether the additive effects of Src inhibitors and cisplatin could be

446 reproduced *in vivo*. The dosing schedule was identical to that used for the single-agent

447 treatment (Fig. 3), and mice bearing subcutaneous tumors were assigned to three groups:

448 control, dasatinib 30 mg/kg + cisplatin 4 mg/kg, and dasatinib 30 mg/kg + cisplatin

449 2 mg/kg; Fig. 4A).

450 Tumor volumes in the dasatinib 30 mg/kg + cisplatin 4 mg/kg group showed a slight

451 decrease compared with those in the control group on day 16, whereas tumor size in the

452 dasatinib 30 mg/kg + cisplatin 2 mg/kg group increased relative to the control group and

453 single-agent cisplatin (Fig. 3B, Fig. 4B). Similar to single-agent treatment, tumor volume

454 alone might not completely capture the therapeutic effects of the combination treatment.

455 Thus, we evaluated the tumor burden using IVIS bioluminescence live imaging, which

456 showed substantially decreased tumor signals in both combination groups compared with

457 those in the control group (Fig. 4C, Fig. S4). This finding was consistent with the pattern

458 noted in the dasatinib monotherapy group. Thus, IVIS bioluminescence live imaging on

459 day 13 showed that the antitumor effect of the combination was comparable to the

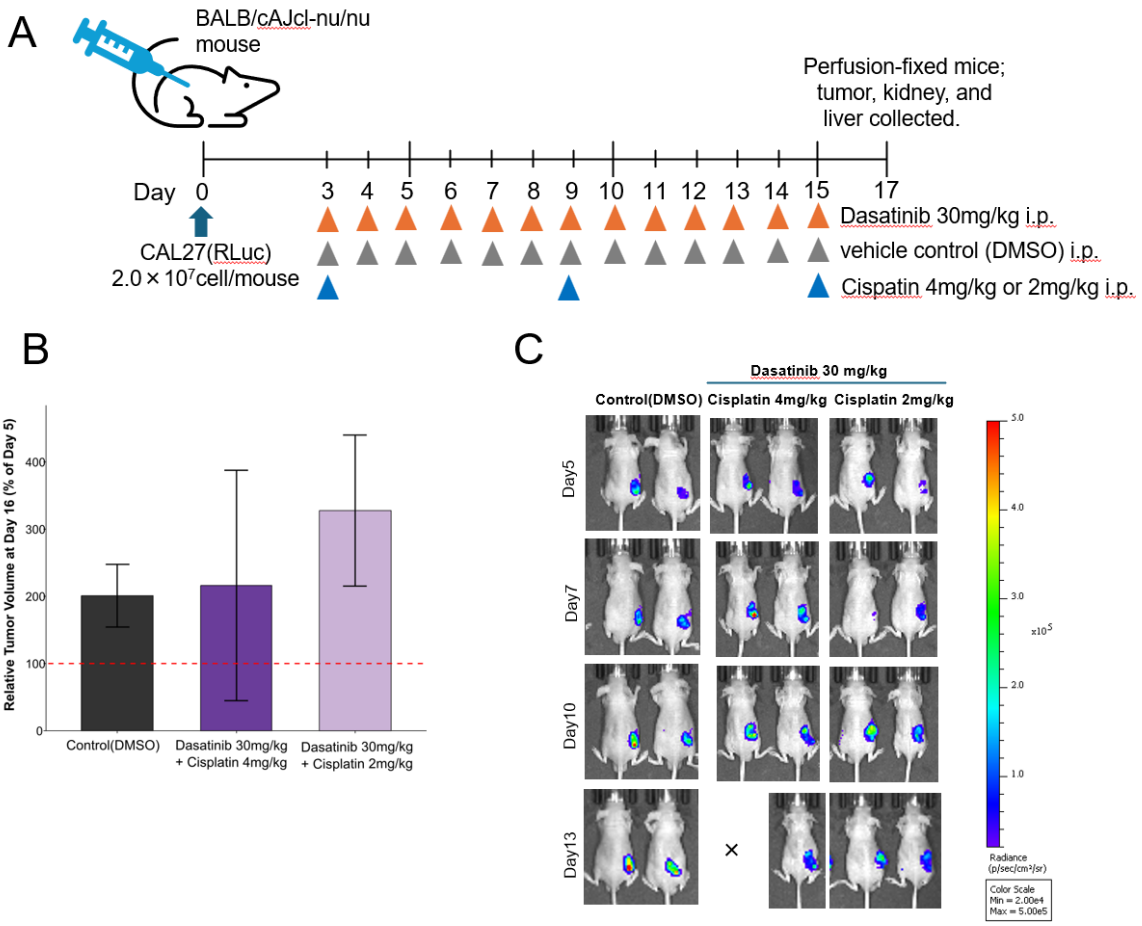
460 expected additive effect derived from each monotherapy. Body weight in the dasatinib
461 30 mg/kg + cisplatin 4 mg/kg group gradually decreased compared with that in the control
462 group, whereas that in the dasatinib 30 mg/kg + cisplatin 2 mg/kg group slightly increased,
463 especially on days 13 and 16 (Fig. 4D). This suggested that reducing the cisplatin dose
464 may mitigate its toxicity.
465 Histological analysis of the excised tumors showed extensive intratumoral cell death in
466 both combination groups, similar to that noted with dasatinib monotherapy, supporting an
467 antitumor effect that was not reflected by tumor size alone (Fig. 4E). In the kidneys, the
468 dasatinib 30 mg/kg + cisplatin 4 mg/kg group showed tubular epithelial degeneration and
469 atrophy, as well as localized rupture, similar to the cisplatin 4 mg/kg group alone. In the
470 dasatinib 30 mg/kg + cisplatin 2 mg/kg group, only mild tubular epithelial atrophy was
471 observed, like the cisplatin 2 mg/kg group. These results suggest that renal damage occurs
472 in a dose-dependent manner in cisplatin-treated mice, and that concomitant use of
473 dasatinib did not enhance toxicity. Additionally, liver sections revealed no apparent organ
474 toxicity, which is consistent with the results from the single-agent treatment.
475 Collectively, these findings indicate that combination treatment with dasatinib and
476 cisplatin additively improves the antitumor activity *in vivo*. Notably, reducing the

477 cisplatin dose in combination with dasatinib treatment preserved the antitumor efficacy

478 and may help attenuate treatment-related toxicity.

479

Figure 4 The inhibition of tumor growth by combination treatment of Cisplatin and Dasatinib in vivo



482 **Discussion**

483 Herein, we examined the signaling pathway associated with tumorigenicity in OSCC
484 (Fig. 1A), involvement of Src activity in broad oral cancer cell lines (Fig. 1A and D),
485 sensitivity of OSCC cells to Src inhibitors (Fig. 1B and C, Table-1) and, molecular basis
486 underlying this variability, and the therapeutic effect of combining Src inhibition with
487 cisplatin in *in vitro* and *in vivo* models (Fig. 2 and 4). Particularly, dasatinib showed a
488 distinctive antitumor effect, characterized by extensive intratumoral cell death, leading
489 to a substantial decrease in the tumor burden *in vivo* (Fig. 3E). Additionally, the
490 dasatinib and cisplatin combination markedly decreased tumor volume, even under
491 cisplatin-dose-reduced conditions (Fig. 4), suggesting its potential to maintain antitumor
492 efficacy while mitigating toxicity.

493 OSCC is a molecularly heterogeneous malignancy characterized by aberrant activation
494 of multiple oncogenic signaling pathways, including EGFR, PI3K/AKT/mTOR,
495 JAK/STAT, MET, Wnt/β catenin, and RAS/RAF/MAPK(35). These pathways are
496 closely linked to Src, which serves as a central signaling node that regulates diverse
497 malignant phenotypes. Screening using multiple Src inhibitors showed considerable
498 variability in Src inhibitor sensitivity among the OSCC cell lines (Fig. 1B). This
499 heterogeneity likely reflects the underlying molecular diversity of OSCC and may

500 explain why therapies targeting a single pathway, including the Src pathway, often fail
501 to achieve sufficient efficacy.
502 Previous studies on HNSCC have attributed Src inhibitors' limited efficacy to numerous
503 factors *in vivo*, including the non-lethal nature of Src inhibition in some tumor cells,
504 compensatory activation of alternative pathways such as PI3K/AKT and STAT3(22,26),
505 and differences in EMT status or cell adhesion molecules(27,28). Consistent with these
506 observations, our findings support the notion that the therapeutic response to Src
507 inhibition in OSCC is strongly influenced by molecular diversity. Our data did not
508 support a high phosphorylated-Src/total-Src ratio, although this has been proposed as a
509 predictor of dasatinib sensitivity(30). Although Src activation has been reported in
510 93.75% of OSCC tissues(37), our results suggest that assessing the Src activation status
511 and the degree of downstream pathway suppression are necessary for predicting Src
512 inhibitor sensitivity.
513 An especially striking finding in this study was that dasatinib induced extensive
514 intratumoral cell death *in vivo*, resulting in a considerable decrease in viable tumor
515 mass, even when the overall tumor size remained unchanged or increased (Fig. 3). IVIS
516 data from *in vivo* imaging and histological analyses confirmed a clear dissociation
517 between tumor size and tumor burden, showing an antitumor effect that could not be

518 captured by size-based evaluation alone in some cases. From this perspective, dasatinib
519 showed tumor-suppressive activity comparable to or exceeding that of cisplatin,
520 indicating that the effect underscores its therapeutic potential.
521 Although dasatinib-induced intratumoral cavitation and cell death have not been
522 previously reported, studies on bladder cancer cells have revealed that dasatinib
523 suppresses the phosphorylation of the PI3K/Akt/mTOR pathway and activates
524 apoptosis- and autophagy related proteins(38). These mechanisms may contribute to cell
525 death noted in OSCC. Furthermore, inhibition of angiogenesis may also play a role in
526 necrotic formation because Src activation is necessary for VEGF expression(39).
527 Whether these effects are specific to CAL27 cells or represent a broader OSCC
528 phenotype requires further *in vivo* investigations. Especially, this necrosis-inducing
529 effect was not noted with bosutinib (Fig. S3E). Although this suggests a dasatinib-
530 specific response, the current data are insufficient to determine its specificity, indicating
531 that comparisons with additional Src inhibitors are required.
532 The dasatinib and cisplatin combination markedly decreased the tumor burden, even
533 under cisplatin dose-reduced conditions, indicating its potential to maintain antitumor
534 efficacy while decreasing toxicity (Fig. 4). This combination produced additive rather
535 than synergistic effects *in vitro*, likely reflecting the independent actions of the

536 inhibition of Src downstream signaling by dasatinib and the impairment of DNA repair
537 mechanisms by cisplatin. Despite previous studies have reported that dasatinib
538 suppresses cisplatin-induced Src activation(40) and reverses Src-dependent cisplatin
539 resistance(41), such effects were not noted in the cisplatin-resistant OSCC cell line HO-
540 1-u-1 (Fig. 2E). This may be owing to the Src-independent nature of cisplatin resistance
541 in HO-1-u-1 and its low sensitivity to Src inhibitors. Nevertheless, our results suggest
542 that combining Src inhibitors with cisplatin may enable dose reduction and mitigate
543 toxicity (Fig 2 and 4), offering a clinically meaningful strategy for overcoming
544 resistance and enhancing tolerability.
545 Although bosutinib also showed antitumor activity *in vivo*, its pronounced toxicity,
546 including loss of body weight, may limit its clinical applicability (Fig. S4). The
547 considerable differences in toxicity profiles among the Src inhibitors underscore the
548 criticality of careful drug selection when considering Src-targeted therapies for OSCC.
549 Dasatinib showed a favorable balance between tumor reduction and tolerability,
550 suggesting that it may be a more suitable Src inhibitor for clinical use.
551 This study has some limitations. First, Src inhibitors were ineffective in all the OSCC
552 cell lines, and based on the seven cell lines examined, the molecular determinants of
553 sensitivity could not be conclusively defined. Future studies incorporating a larger and

554 more diverse panel, including patient-derived cell lines, are necessary for identifying the
555 molecular features that predict the responsiveness of the Src inhibitor. Second, complete
556 tumor disappearance could not be confirmed in any of the groups because the endpoint
557 of this study was defined as visible tumor enlargement in mice. Importantly, dasatinib-
558 treated tumors frequently showed extensive central cell death, raising the possibility that
559 they may eventually collapse and regress, even though the time course of this process
560 remains elusive. Further examination is necessary to optimize the combination
561 strategies and treatment schedules. Nonetheless, FDA-approved Src inhibitors are orally
562 administered and are generally less toxic than cytotoxic chemotherapies, leaving
563 considerable room for their clinical application in settings such as neoadjuvant therapy
564 or palliative treatment.
565 In summary, this study reassessed the therapeutic potential of Src inhibition in OSCC
566 and showed that Src inhibitors, especially dasatinib, are promising candidates, both as
567 monotherapy and in combination with cisplatin. Dasatinib's ability to decrease tumor
568 burden and the potential for toxicity reduction through a reduction in cisplatin dose
569 provide a foundation for developing new therapeutic strategies aimed at expanding
570 treatment options and enhancing outcomes in patients with OSCC. Thus, our results

571 provide new insights that may allow the establishment of future therapeutic strategies

572 for oral cancer.

573

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Author Contributions

K.O. conceived and designed the study. Y.O. performed all the experiments and analyzed the data. Y.O. and K.O. interpreted experimental results. Y.O. and K.O. wrote the manuscript. Y.O., T.N., H.M., and K.O. edited the manuscript. All the authors have reviewed and approved the final manuscript.

Competing Interests

The authors declare no competing interests.

Data Availability Statement

The authors declare that the data supporting the findings of this study are available in the article and its Supplementary Information files. All relevant data are available from the authors upon reasonable request.

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724

725 **Figure Legends**

726 **Figure 1 Src activity and its inhibitory effect of tumor growth by specific inhibitors**

727 **in different oral cancer cell lines**

728 **A** Western blot analysis of the oral squamous carcinoma cell lines to monitor numerous

729 signaling pathways. The phosphorylated and total protein levels were determined as

730 indicated. **B** Cell viability (%) of the oral squamous carcinoma cell lines treated with

731 each Src inhibitor for 72 h. Viability was measured using CellTiter-Glo, and values are

732 indicated relative to the 1% dimethyl sulfoxide (DMSO) control. Data represent

733 mean ± standard deviation from three independent experiments (n = 3). **C** IC_{50} curves of

734 the oral squamous carcinoma cell lines treated with each Src inhibitor for 36 h. Viability

735 was measured using CellTiter-Glo and normalized to that of the 1% DMSO control.

736 Values represent the mean of n = 3. **D, E** Western blot analysis of the oral squamous

737 carcinoma cell lines to investigate Src (D) and MAPK (E) activity induced by different

738 Src inhibitors. Oral squamous carcinoma cell lines were treated with an Src inhibitor for

739 18 h, followed by western blot analysis. D indicates the Src phosphorylation (Tyr416),

740 and E reveals MAPK pathway-related proteins. Supplementary Table S1 lists the

741 concentrations of the Src inhibitors used.

742

743 **Figure 2 The tumor inhibitory effect by combination therapy using cisplatin and**

744 **Src inhibitor.**

745 Cells were treated with cisplatin (10 μM or 5 μM), each Src inhibitor (dasatinib, ponatinib,

746 vandetanib, or bosutinib) alone, or their combinations for 36 h. Cell viability is indicated

747 as the percentage relative to the 1% dimethyl sulfoxide (DMSO) control. Data represent

748 mean ± standard deviation from three independent experiments. A–G show the data for

749 the indicated cells. P-values were calculated using paired t-tests in Microsoft Excel.

750 Statistical significance was indicated as $p < 0.01$ **, $p < 0.05$ *, and $p \geqq 0.05$ ns.

751

752 **Figure 3 Tumor growth inhibition by single treatment of dasatinib *in vivo*.**

753 **A** Experimental schedule for a single treatment with dasatinib *in vivo*. RLuc-expressing

754 CAL27 (CAL27-Rluc) cells (clone 7) (2×10^7 cells/mouse) were subcutaneously

755 implanted into the right flank of 5-week-old male BALB/cAJcl nu/nu mice. Three days

756 following implantation, the mice were randomly assigned to four treatment groups:

757 vehicle control (dimethyl sulfoxide [DMSO]), dasatinib alone (30 mg/kg), cisplatin alone

758 (4 mg/kg), and cisplatin alone (2 mg/kg).(n=5–6). **B** Tumor volumes in each time point

759 (mean ± s.d., n = 5–6). Tumor volumes were measured using calipers and calculated as L

760 × W^2 × 0.52. Measurements were conducted every 2 to 3 days. The tumor volumes were
761 normalized to the volume on day 5 for each mouse. **C** IVIS bioluminescence live imaging
762 of the tumor burden (additional mice are indicated in Fig. S2). Bioluminescence signals
763 were quantified 10 min following ViviRen injection according to the manufacturer's
764 instructions. D Body weights of nude mice from various treatment regimens implanted
765 with CAL27-Rluc xenografts. Dasatinib was intraperitoneally administered at 30 mg/kg
766 once daily and cisplatin was intraperitoneally administered at 4 or 2 mg/kg once weekly
767 for two consecutive weeks. Body weights are presented as mean ± standard deviation (n
768 = 5–6) and were normalized to the weight on Day 3 for each mouse. **E** Hematoxylin and
769 eosin staining of the tumor, kidney, and liver sections. Mice were perfusion-fixed on
770 day 17 and the harvested tissues were processed to generate frozen sections for
771 histological analysis. Representative images of each organ are presented. Tumor
772 histology: Control (DMSO) and cisplatin groups showed solid squamous cell carcinoma.
773 Dasatinib group exhibited a hollow, cyst-like structure with evidence of cell death within
774 the lumen. Kidney histology: Control (DMSO) and dasatinib groups displayed normal
775 renal architecture. Cisplatin 4 mg/kg group caused tubular epithelial degeneration and
776 atrophy with focal rupture (*), while cisplatin 2 mg/kg groups induced mild tubular
777 epithelial atrophy. Liver histology: All groups showed normal hepatic morphology

778 without detectable abnormalities. Scale bars: 500 μm (×40, ×100); 50 μm (×400).

779

780 **Figure 4 Tumor growth inhibition by combination treatment of cisplatin and**

781 **dasatinib *in vivo***

782 **A** Experimental schedule for the combination treatment with dasatinib and cisplatin *in*

783 *vivo*. CAL27-Rluc cells (clone 7) (2×10^7 cells per mouse) were subcutaneously

784 implanted into the right flank of 5-week-old male BALB/cAJcl nu/nu mice. Three days

785 following implantation, the mice were randomly assigned to three treatment groups:

786 vehicle control (dimethyl sulfoxide [DMSO]), dasatinib (30 mg/kg) plus cisplatin

787 (4 mg/kg), and dasatinib (30 mg/kg) plus cisplatin (2 mg/kg). (n=5–6). **B** Tumor

788 volumes normalized to Day 3 at the final Day 16 time point (mean ±standard deviation

789 (s.d.), n = 5–6). Tumor volumes were measured using calipers and calculated as $L \times W^2$

790 × 0.52. Measurements were conducted every 2 to 3 days. **C** IVIS bioluminescence live

791 imaging of the tumor burden (additional mice are illustrated in Fig. S2).

792 Bioluminescence signals were quantified 10 min following ViviRen injection according

793 to the manufacturer's instructions. **D** Body weight of nude mice normalized to Day 3 at

794 the final Day 16 time point (mean ± s.d., n = 5–6). **E** Hematoxylin and eosin staining of

795 the tumor, kidney, and liver sections. Mice were perfusion-fixed on day 17 and

796 harvested tissues were processed to generate frozen sections for histological analysis.
797 Representative images of each organ are indicated. Tumor histology: Control group
798 showed solid squamous cell carcinoma. Dasatinib + cisplatin 4 mg/kg and dasatinib +
799 cisplatin 2 mg/kg group exhibited a hollow, cyst-like structure with evidence of cell
800 death within the lumen. Kidney histology: Control (DMSO) group displayed normal
801 renal architecture. dasatinib + cisplatin 4 mg/kg group caused tubular epithelial
802 degeneration and atrophy with focal rupture (*), while dasatinib + cisplatin 2 mg/kg
803 groups induced mild tubular epithelial atrophy. Liver histology: All groups showed
804 normal hepatic morphology without detectable abnormalities. Scale bars: 500 μm (×40,
805 ×100); 50 μm (×400).
806

807 **Supplementary Figures and tables**

808

809 **Supplementary Table 1.**

810 **Concentrations of Src inhibitors used for the Western blot analyses.**

	Dasatinib	Ponatinib	Vandetanib	Bosutinib
HSC-2	**20 μM**	**5 μM**	**10 μM**	**9 μM**
HSC-3	**10 μM**	**2 μM**	**10 μM**	**10 μM**
HSC-4	**40 μM**	**7 μM**	**30 μM**	**15 μM**
SAS	**40 μM**	**10 μM**	**40 μM**	**15 μM**
Ho-1-u-1	**70 μM**	**9 μM**	**30 μM**	**30 μM**
CAL27	**15 μM**	**7 μM**	**15 μM**	**10 μM**
SCC-25	**10 μM**	**3 μM**	**10 μM**	**10 μM**

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812

813 **Supplementary Table 2.**

814 **Concentrations of Src inhibitors used for the cell viability and combination**

815 **treatment assays.**

	Dasatinib	Ponatinib	Vandetanib	Bosutinib
HSC-2	**0.1 μM**	**2 μM**	**5 μM**	**5 μM**
HSC-3	**10 μM**	**2 μM**	**8 μM**	**5 μM**
HSC-4	**10 μM**	**5 μM**	**25 μM**	**15 μM**
SAS	**10 μM**	**5 μM**	**50 μM**	**25 μM**
Ho-1-u-1	**10 μM**	**5 μM**	**25 μM**	**25 μM**
CAL27	**10 μM**	**5 μM**	**10 μM**	**10 μM**
SCC-25	**10 μM**	**2 μM**	**8 μM**	**10 μM**

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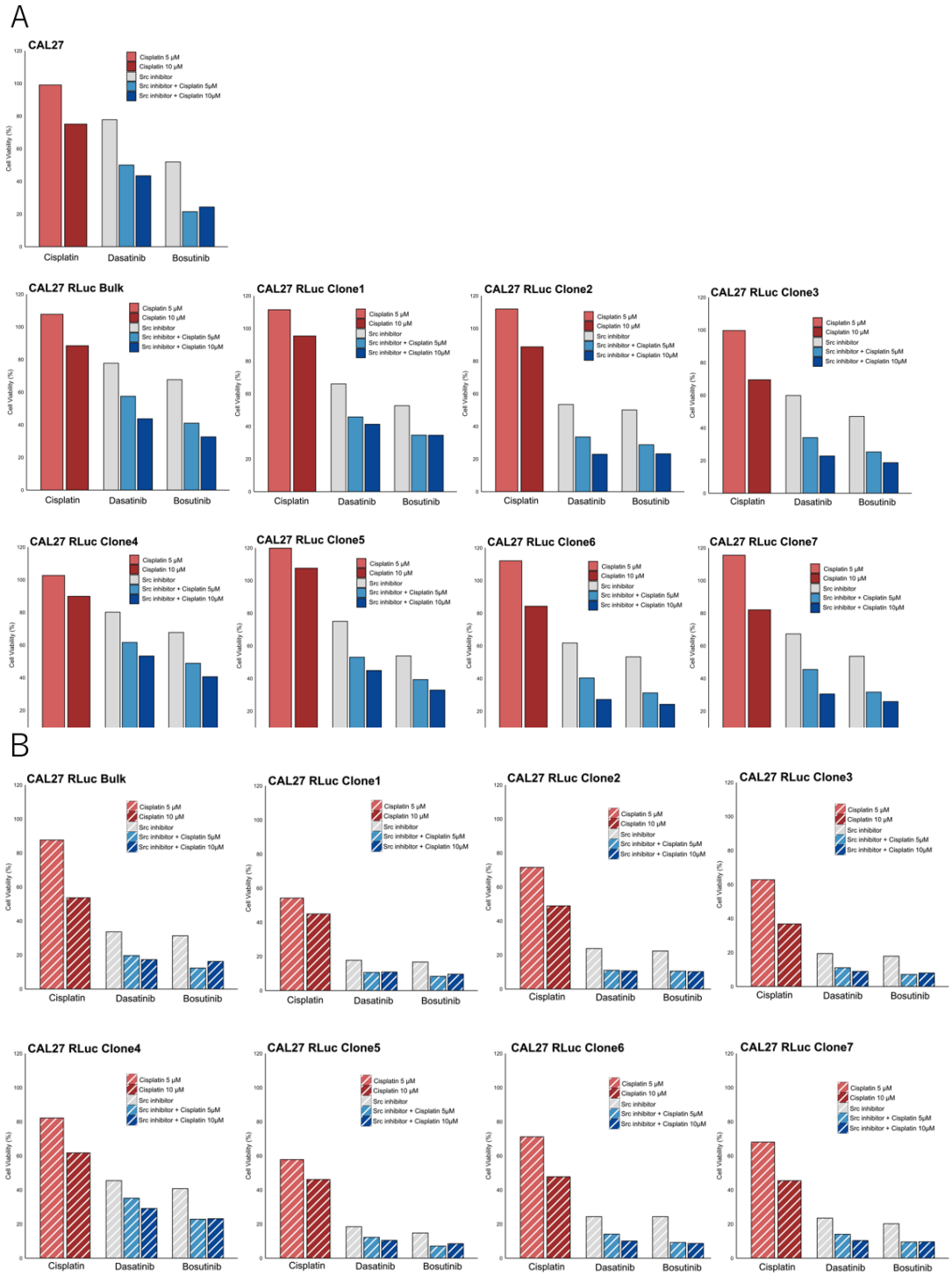
Supplementary Figure 1 Comparison of drug sensitivity in RLuc-labeled CAL27 bulk cells and clones 1–7 using CellTiter-Glo and EnduRen assays.



821 Drug sensitivity of stable Renilla luciferase (Rluc)-expressing CAL27 (CAL27-Rluc)

822 bulk cells and clones 1–7 was evaluated 36 h following treatment using CellTiter-Glo

823 (A) and EnduRen (B) assays.

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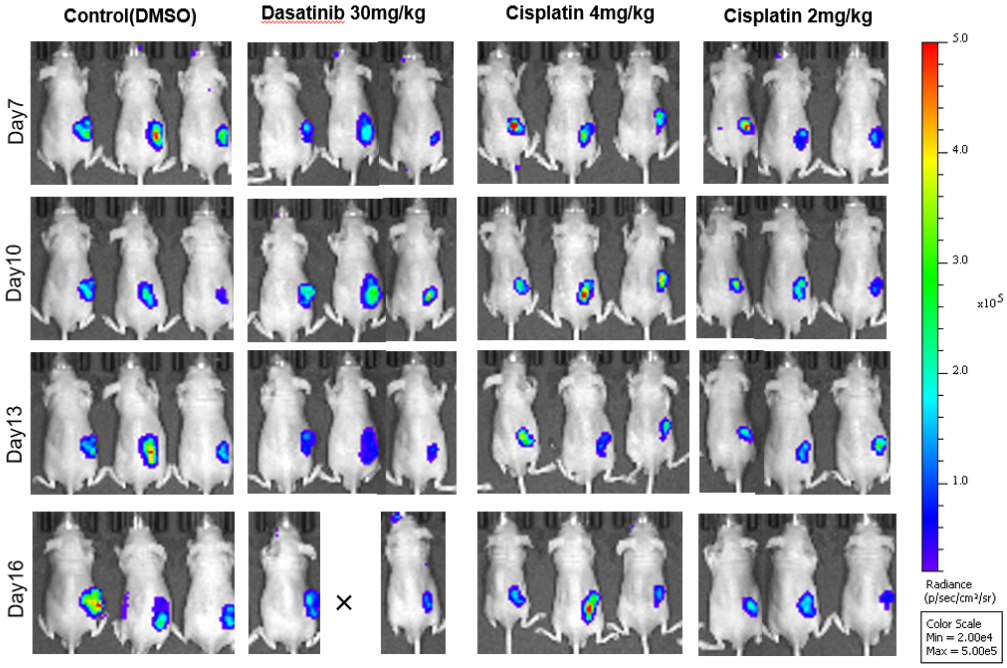
825

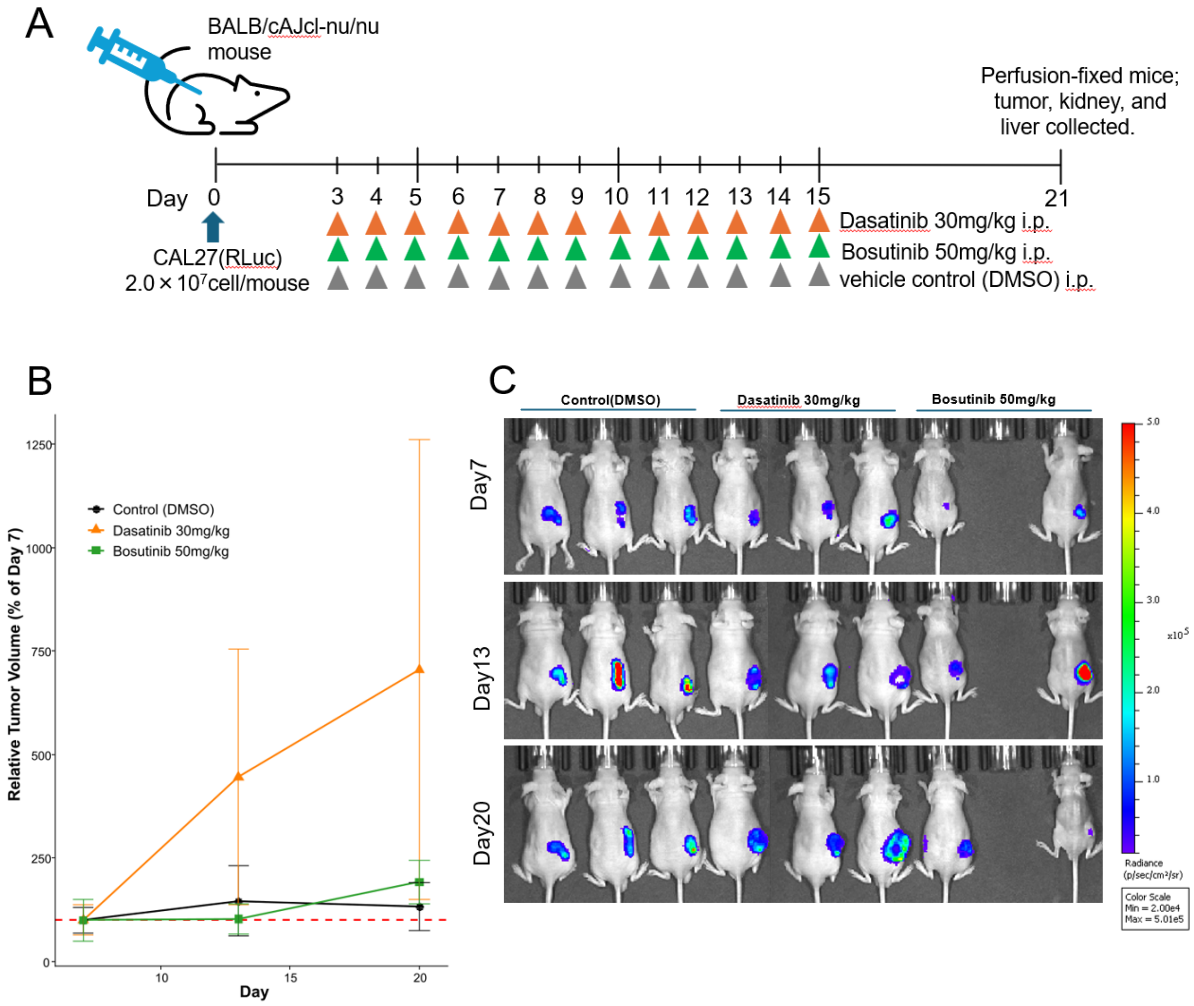
Supplementary Figure 2 Supplementary IVIS bioluminescence images corresponding to Fig. 3



Additional IVIS bioluminescence live images corresponding to Fig. 3. These images include mice scanned at various time points, which are not indicated in the main figure but contribute to the total sample size (n = 5–6). One of three mice in the dasatinib group died on day 16.

833 **Supplementary Figure 3 Tumor growth inhibition by single treatment of dasatinib**

834 **and bosutinib *in vivo***



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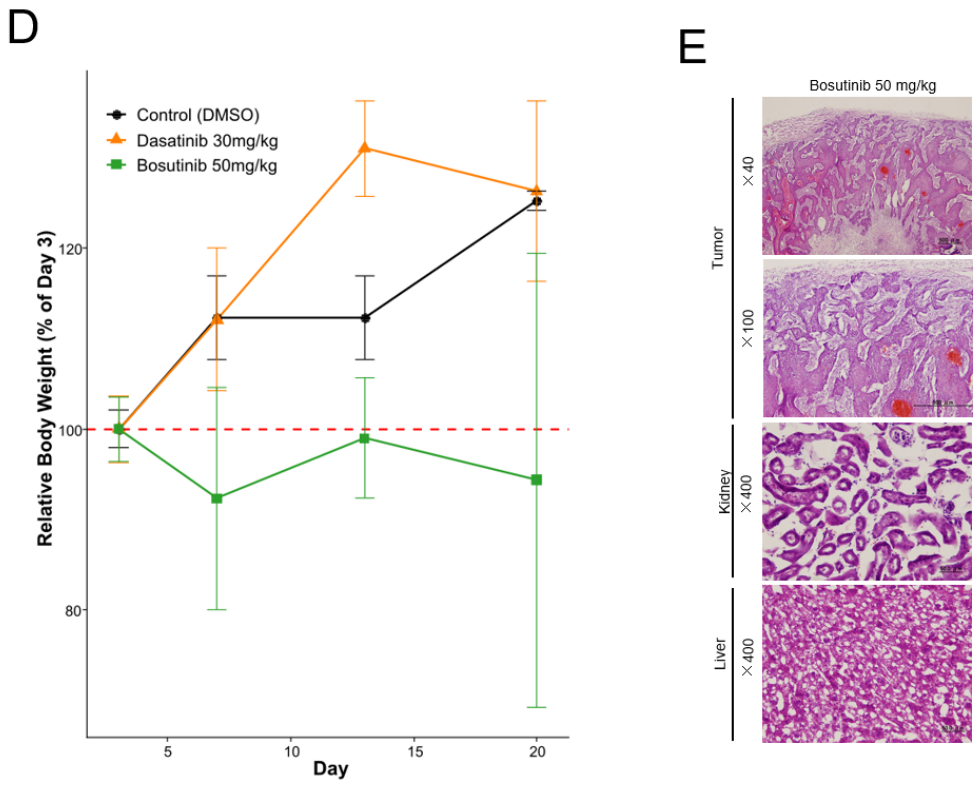


A Experimental schedule for single treatment with dasatinib and bosutinib *in vivo*. CAL27-Rluc cells (clone 7) (2×10^7 cells/mouse) were subcutaneously implanted into the right flank of 5-week-old male BALB/cAJcl nu/nu mice. Three days following implantation, the mice were randomly assigned to four treatment groups: vehicle control (dimethyl sulfoxide [DMSO]), dasatinib alone (30 mg/kg), and bosutinib alone (50 mg/kg). Dasatinib, bosutinib, and DMSO were dissolved in 10 mM Tris-HCl (pH 7.4). All drugs were intraperitoneally administered at a volume of 100 µL per mouse. Dasatinib, bosutinib, and DMSO were administered once daily (n=3). **B** Tumor volumes normalized to Day 7 at the final Day 20 time point (mean ± standard deviation [s.d.], n = 2–3). Tumor volumes were measured using calipers and calculated as $L \times W^2$

848 × 0.52. Measurements were conducted every 2–3 days. **C** IVIS bioluminescence live

849 imaging of tumor burden. One of three mice in the bosutinib group died on day 6. **D**

850 Body weights of nude mice from various treatment regimens implanted with CAL27-

851 Rluc xenografts. Body weights are presented as mean ± standard deviation [s.d.] (n = 5–

852 6) and were normalized to the wight on Day 3 for each mouse.(mean ± s.d., n = 2–3). **E**

853 Hematoxylin and eosin staining of the tumor, kidney, and liver sections. Mice were

854 perfusion-fixed on day 21 and the harvested tissues were processed to generate frozen

855 sections for histological analysis. Representative images of each organ are presented.

856 Tumor histology: Bosutinib group showed solid squamous cell carcinoma. Kidney

857 histology: Bosutinib group displayed normal renal architecture. Liver histology:

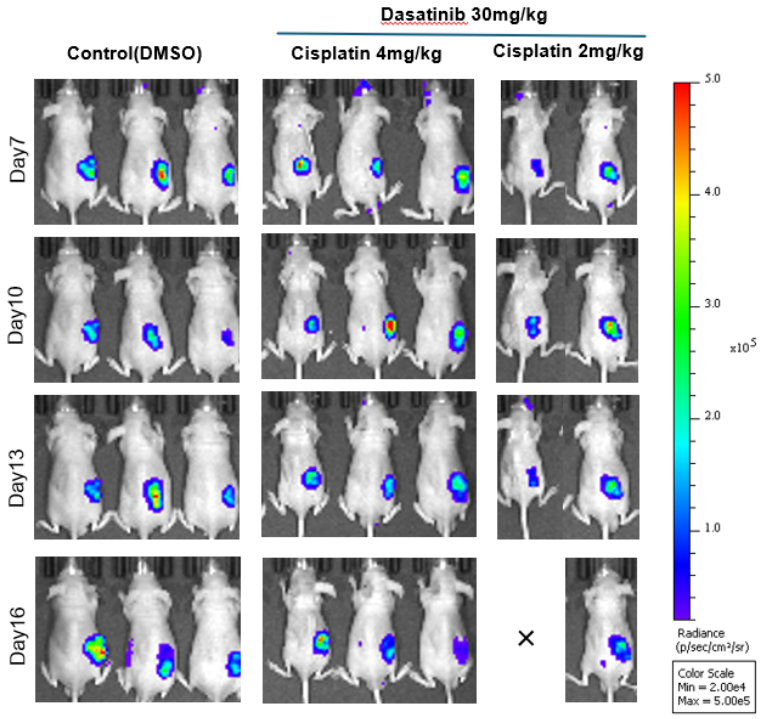
858 Bosutinib group showed normal hepatic morphology without detectable abnormalities.

859 Scale bars: 500 μm (×40, ×100); 50 μm (×400).

860

861 **Supplementary Figure 4 Supplementary IVIS bioluminescence images**

862 **corresponding to Fig. 4**



863

864 Additional IVIS bioluminescence live images corresponding to Fig. 4. These images

865 included mice scanned at various time points, which were not included in the main figure

866 but contributed to the total sample size (n = 5–6). One of three mice in the dasatinib

867 30 mg/kg + cisplatin 2 mg/kg group died on day 14.

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