SUPPLEMENTARY INFORMATION

The SRC inhibitor Dasatinib induces stem cell-like properties in head and neck cancer cells that are effectively counteracted by the mithralog EC-8042

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SUPPLEMENTARY MATERIALS AND METHODS

Western blotting Analysis

The following primary antibodies were used in overnight incubations at 1:1,000 dilution: phospho-SRC (Y418) (Invitrogen # 44660G), total SRC (Santa Cruz Biotechnology # sc-8056), phospho-FAK (Y861) (Invitrogen # 44-626G), phospho-FAK (Y397) (Invitrogen # 700255), Anti-FAK clone 4.47 (Merck Millipore # 05-537), phospho-Akt (Ser473) (Cell Signaling # 9271), Anti-ALDH1 (BD Biosciences # 611195), Anti-SOX2 (Merck Millipore # AB5603), NANOG1 mAb (Cell Signaling # D73G4), Anti-Oct4 (Abcam # ab19857), cleaved Notch1 mAb (Cell Signaling # D3B8), phospho-p44/42 MAPK (Erk1/2)AMPKα (Thr202/Tyr204) (D13.14.4E) (Cell Signaling # 4370), Anti-SP1 antibody (Sigma # WH0006667M2) and Anti-GAPDH clone 6C5 (Merck Millipore # MAB374; at dilution 1:10,000).

Analysis of synergism in drug combinations

The existence of synergy in drug combinations was determined in *in vitro* cell viability assays where FaDu cells were treated with dasatinib and/or EC-8042 alone or combined at a fixed ratio (Dasatinib:EC-8042, 10:1) for 72 h. Cell viability data were used to calculate the combination index (CI) according to the Chou and Talalay method [38] using CompuSyn software (ComboSyn). CI *vs* the fraction affected (F_a) plots were generated for 6 combinations of Dasatinib and EC-8042. CI values indicate synergistic (<1.2), additive (0.8-1.2) and antagonistic (>0.8) drug interactions.

Calculation of tumor growth and tumor growth inhibition (%TGI)

Mice were monitored daily for signs of toxicity and tumor size was measured with a caliper 2–3 times a week and tumor volume was determined using the equation $(D \times d^2)/6 \times 3.14$, where D is the maximum diameter, and d is the minimum diameter. Tumor volumes for all mice in each xenograft-treatment group were averaged to obtain the mean tumor volume for the corresponding group. Student's *t*-test was performed to determine the statistical significance between control and treated groups. Animals

were sacrificed by CO_2 asphyxiation when the tumors of the control group reached approximately 1500 mm^3. Drug efficacy was expressed as the percentage tumor growth inhibition (%TGI), calculated using the equation 100-(T/Cx100), where T is mean relative tumor volume [RTV = tumor volume at day of measurement (V_t) - tumor volume at the beginning of the treatment (V_0)] of the treated tumor and C is the mean RTV in the control group at day of measurement.

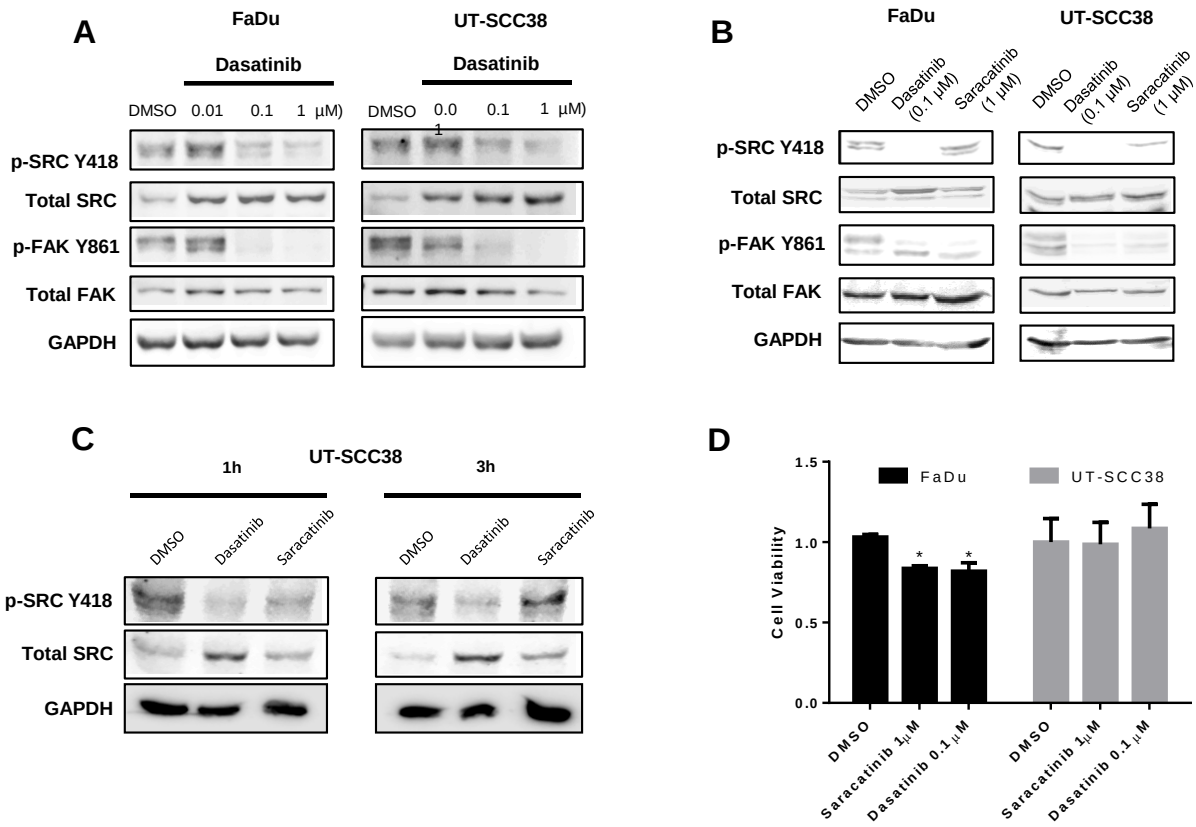
Generation of SORE6-expressing cell lines and detection of SORE6 activity by flow cytometry

Lentiviral reporter systems in which a SOX2/OCT4 response element (SORE6) coupled to a minimal cytomegalovirus (mCMV) drive the expression of GFP (SORE6-mCMVp-dsCopGFP-Puro) and the corresponding control lacking SORE6 (mCMVp-dsCopGFP-Puro) were previously generated and characterized [39] and donated by Dr. L.M. Wakefield (National Cancer Institute, Bethesda, MD). Generation of lentiviral particles expressing these constructions and transduction into HNSCC cell lines were performed as previously described [39]. Transduced cells were positively selected through a treatment with puromycin (2 µg/mL) for 7 days. The level of SORE6-driven GFP fluorescence in untreated cultures or after different drug treatments were analyzed and/or SORE6+ and SORE6- subpopulations were sorted by flow cytometry using a BD FACS Aria II Cell Sorter (BD Bioscience, Erembodegem, Belgium). Cells transduced with the minCMVp-GFP lentivirus were used as matched SORE6 negative control for gating purposes.

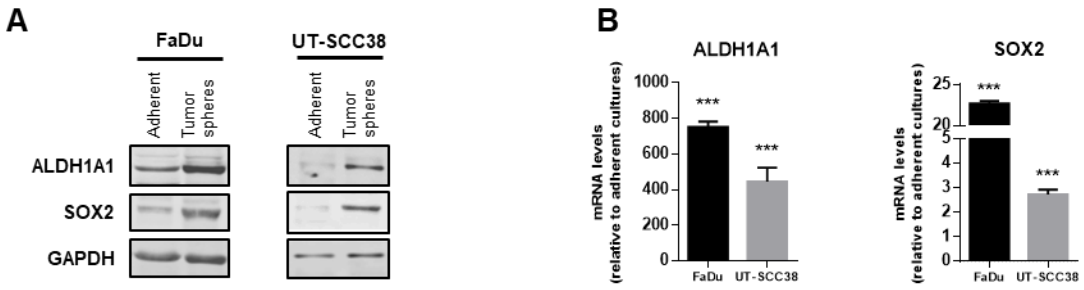
Supplementary Table S1. Primers used for real-time RT-PCR

Gene	Seq (5' → 3')
ALDH1A1-Fw	AACAGTGTGGGTGAATTGCT
ALDH1A1-Rv	GGAAACCGTACTCTCCCAGT
SOX2-Fw	TCAGGAGTTGTCAAGGCAGAGAAG
SOX2-Rv	CTCAGTCCTAGTCTTAAAGAGGCAGC
OCT4-Fw	GGCAACCTGGAGAATTTGTT
OCT4-Rv	ACTCGGACCACATCCTTCTC
NANOG1-Fw	TGCTTATTCAGGACAGCCCT
NANOG1-Rv	TCTGGTCTTCTGTTTCTTGACT
c-MYC-Fw	TGCTCCATGAGGAGACACC
c-MYC-Rv	CTTTTCCACAGAAACAACATCG
Notch1-Fw	CAAGTTCTTGGTCCCTCCAG
Notch1-Rv	TGTGTTGCTGGAGCATCTTC
E-Cadherin-Fw	TGGAGGAATTCTTGCTTTGC
E-Cadherin-Rv	CGTACATGTCAGCCAGCTTC
Vimentin-Fw	AGTCCACTGAGTACCGGAGAC
Vimentin-Rv	GGTTCCTTTAAGGGCATCCAC
Snail-Fw	CGAGCTGCAGGACTCTAAT
Snail-Rv	CCACTGTCCTCATCTGACA
Twist-Fw	CCGGAGACCTAGATGTCATTG
Twist-Rv	CACGCCCTGTTTCTTTGAAT
RPL19-Fw	GCGGAAGGGTACAGCCAAT
RPL19-Rv	GCAGCCGGCGCAAA

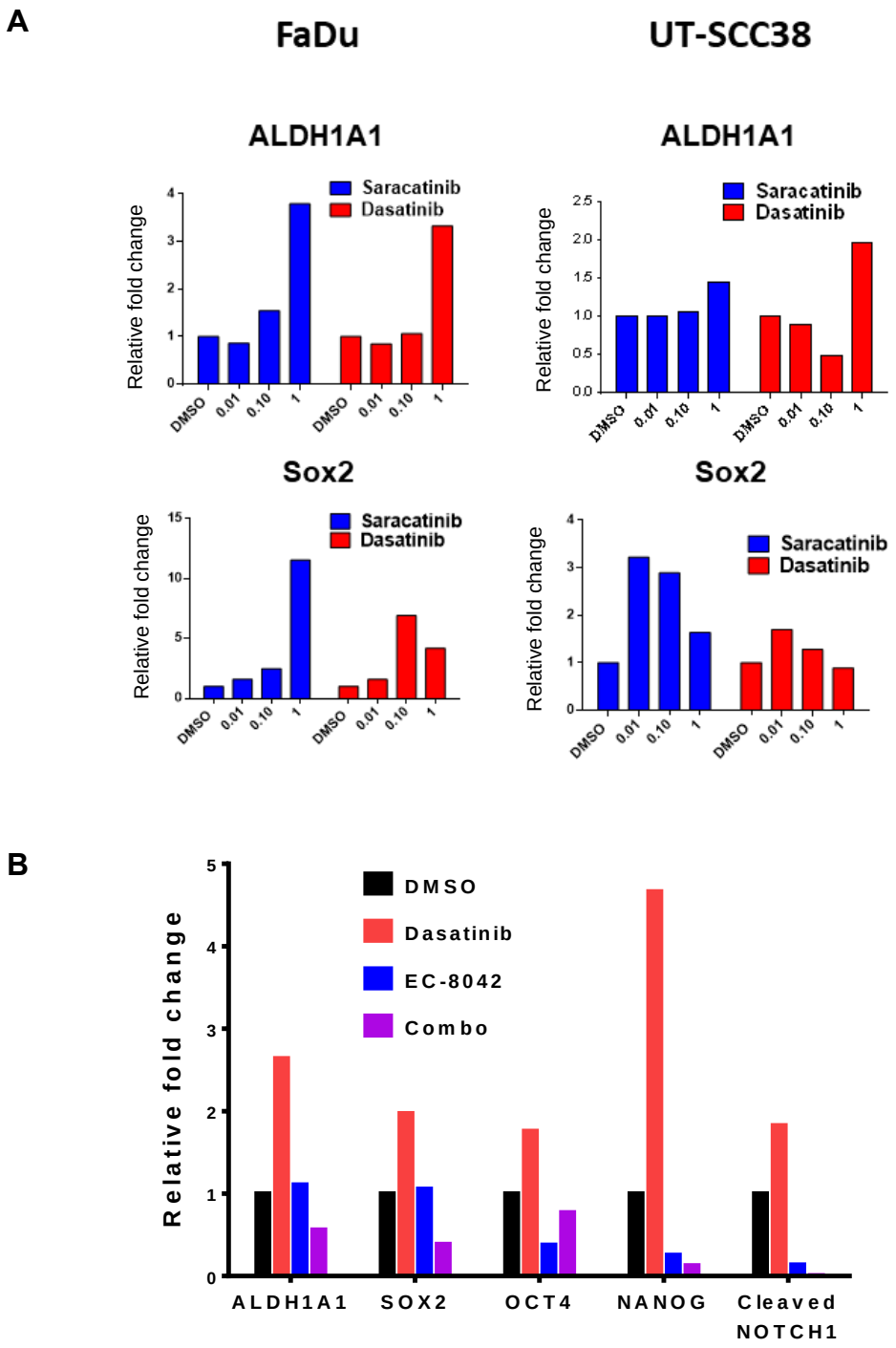
SUPPLEMENTARY FIGURES



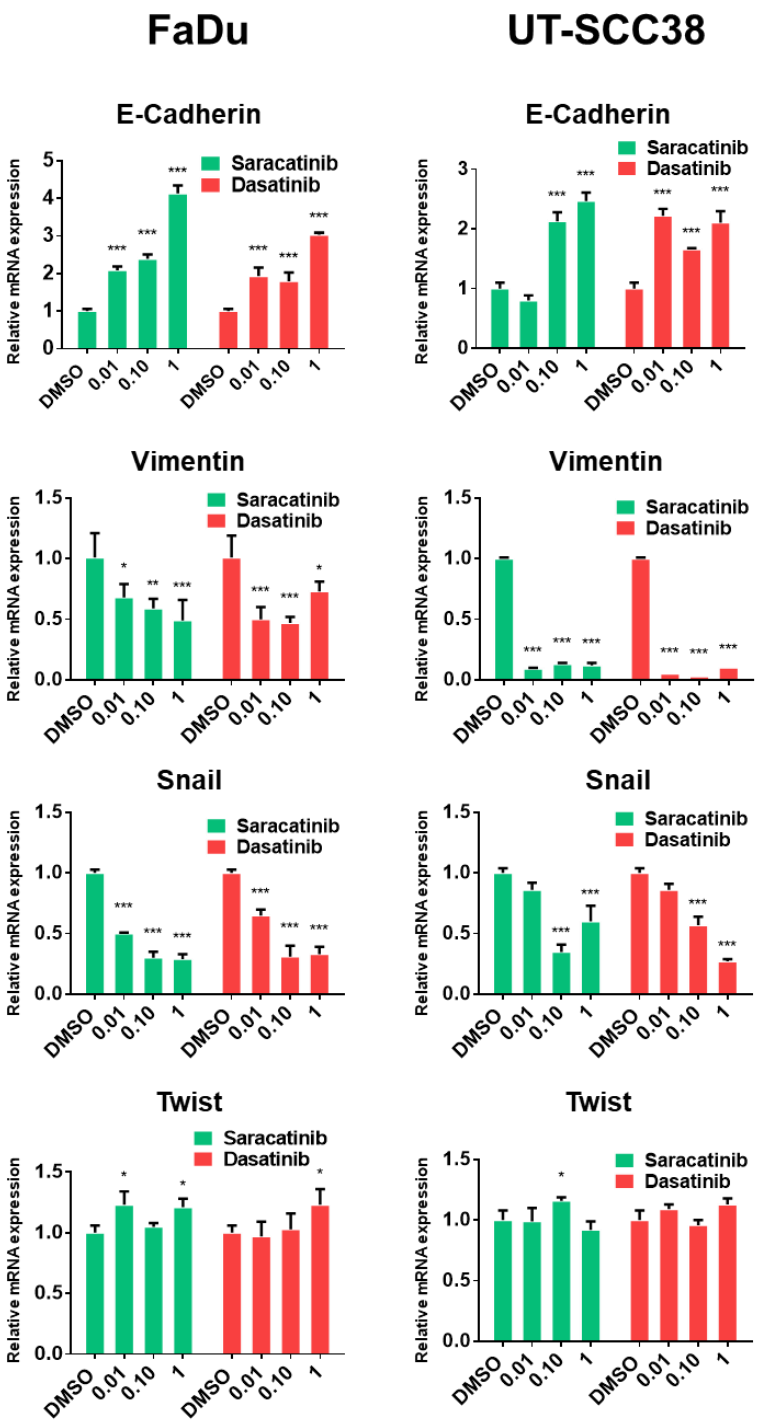
Supplementary Figure S1. (A-B) Western blot analyses of the phosphorylation/expression levels of the indicated proteins in FaDu and UT-SCC38 cells treated for 24h with increasing concentrations of dasatinib (A) or FaDu and UT-SCC38 cells treated with either DMSO (vehicle), 0.1 µM dasatinib or 1 µM saracatinib (B) or UT-SCC38 cells treated with either DMSO (vehicle), 0.1 µM dasatinib or 1 µM saracatinib for 1h and 3h (C). GAPDH levels were used as loading control. (D) Cell viability of FaDu and UT-SCC38 cells was measured by MTS assay after 24h treatment with either DMSO (vehicle), 0.1 µM dasatinib or 1 µM saracatinib. Data are expressed relative to vehicle-treated cells (mean ± SD) * $p < 0.05$ by Student's t-test.



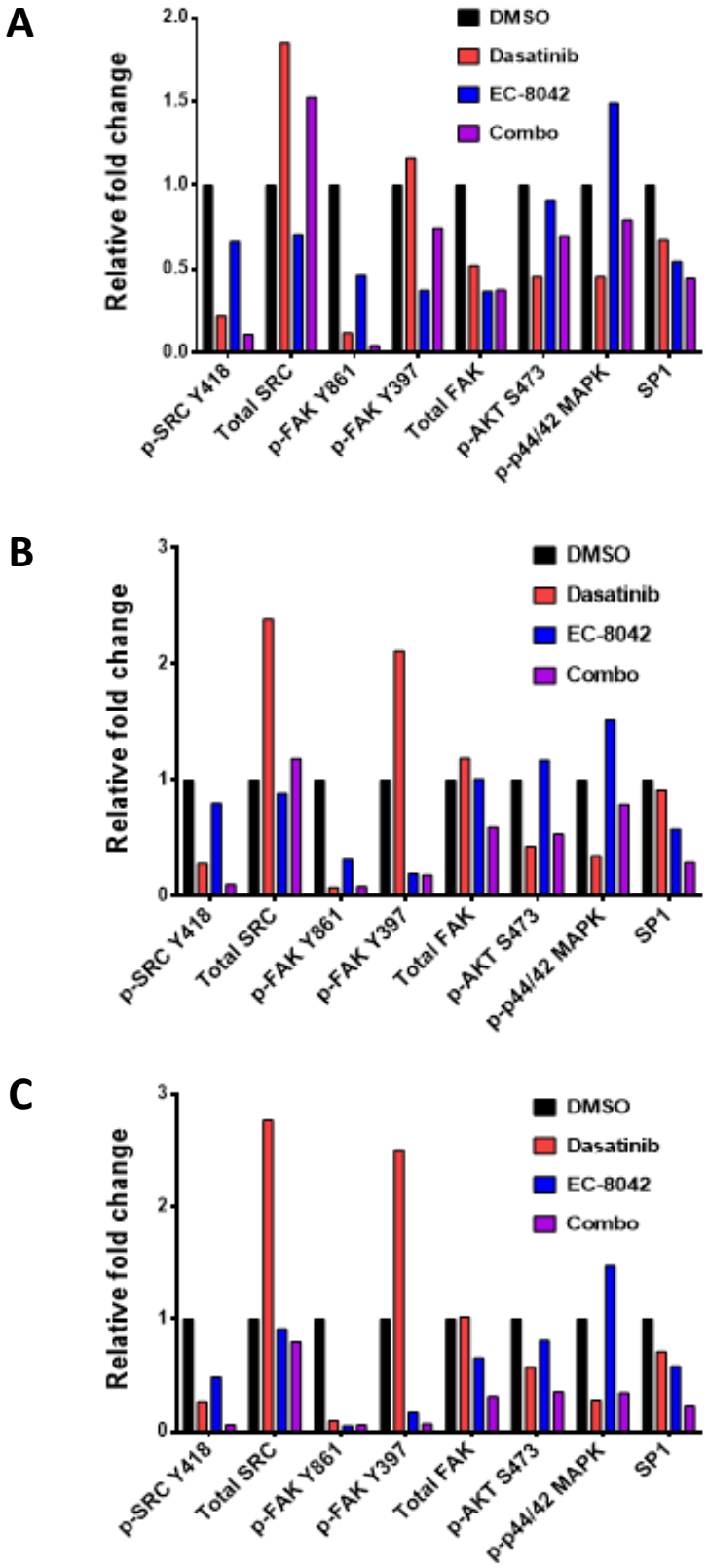
Supplementary Figure S2. Analysis of the expression of the CSC markers ALDH1A1 and SOX2 in CSC-enriched tumorspheres and adherent cultures of FaDu and UT-SCC38 cells by Western-blot (A) and RT-qPCR (B). mRNA levels were normalized and the relative fold-change (to adherent cells) ± SD plotted. *** $p < 0.001$ by Student's t-test.



Supplementary Figure S3. Quantification of the infrared fluorescent signals from the Western blot analyses shown in Figure 2C (A) and Figure 4F (B). The expression levels of the indicated proteins was measured in FaDu and UT-SCC38 cells treated for 72h with increasing concentrations of dasatinib and saracatinib (A) or in FaDu cells treated for 72h with 0.1 µM dasatinib or 0.1 µM EC-8042 alone, or in combination. Data were normalized to GAPDH levels and relative to vehicle-treated (DMSO) cells.

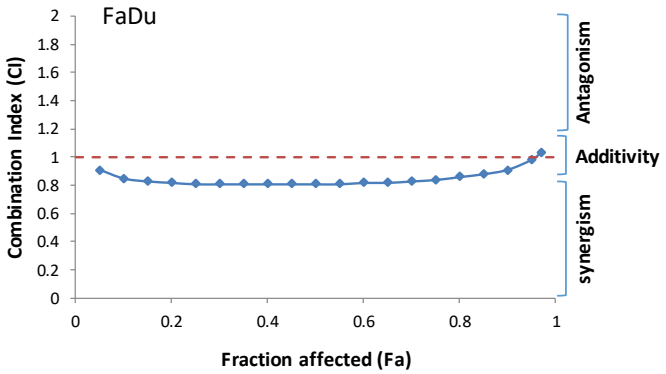


Supplementary Figure S4. Effect of dasatinib and saracatinib on the expression of EMT markers in HNSCC-derived cell lines. The expression analysis of E-Cadherin, Vimentin, Snail and Twist was performed in FaDu and UT-SCC38 cells treated for 72h with increasing concentrations of dasatinib and saracatinib by RT-qPCR. mRNA levels were normalized to RPL19 levels, and the relative fold-change to vehicle-treated (DMSO) cells ± SD plotted. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* DMSO by Student's t-test.



Supplementary Figure S5. Quantification of the infrared fluorescent signals from the Western blot analyses shown in Figure 4A. The expression/phosphorylation levels of the indicated proteins was measured in FaDu cells treated with either vehicle (DMSO), 0.1 µM dasatinib, 0.1 µM EC-8042 or combination for 24h (A), 48h (B) and 72h (C). Data were normalized to GAPDH levels and relative to vehicle-treated (DMSO) cells.

A



B

Dasatinib	EC-8042: Absent (-)	EC-8042: Present (+)
Present (+)	dasatinib	both
Absent (-)	neither	EC-8042

C

Source term	Degrees of Freedom	Sum of Squares	Mean square	F-ratio	P value	Comments
dasatinib	1	120234	120234	0.58	0.4503	**Not significant interaction between dasatinib and EC-8042**
EC-8042	1	2728527	2728527	13.22	0.0009*	
Interaction	**1**	**51850**	**51850**	**0.25**	**0.6192**	
Residual (error)	36	7429022	206362			
Total	39	10329633				

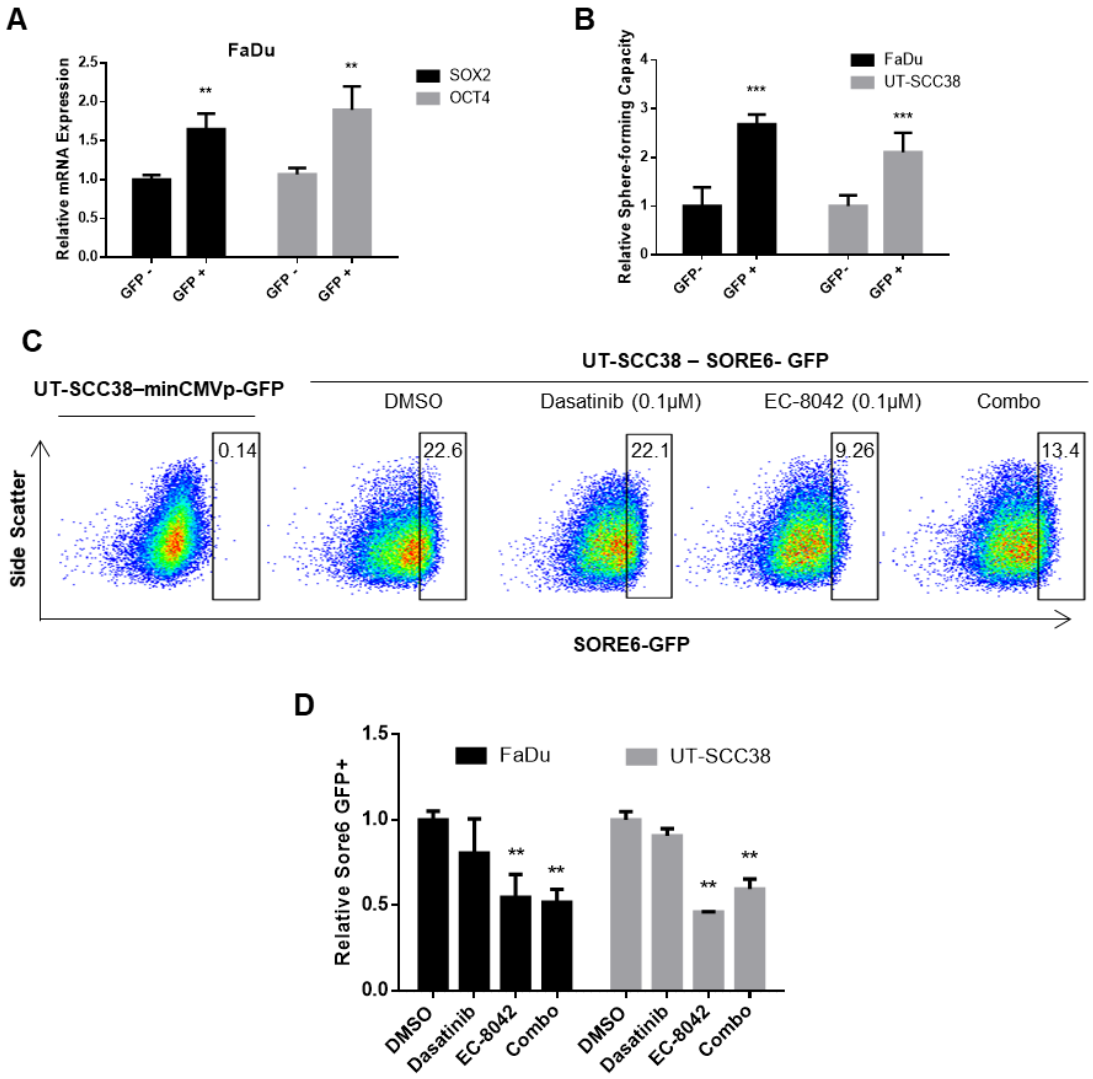
* Term significant at α=0.05

D

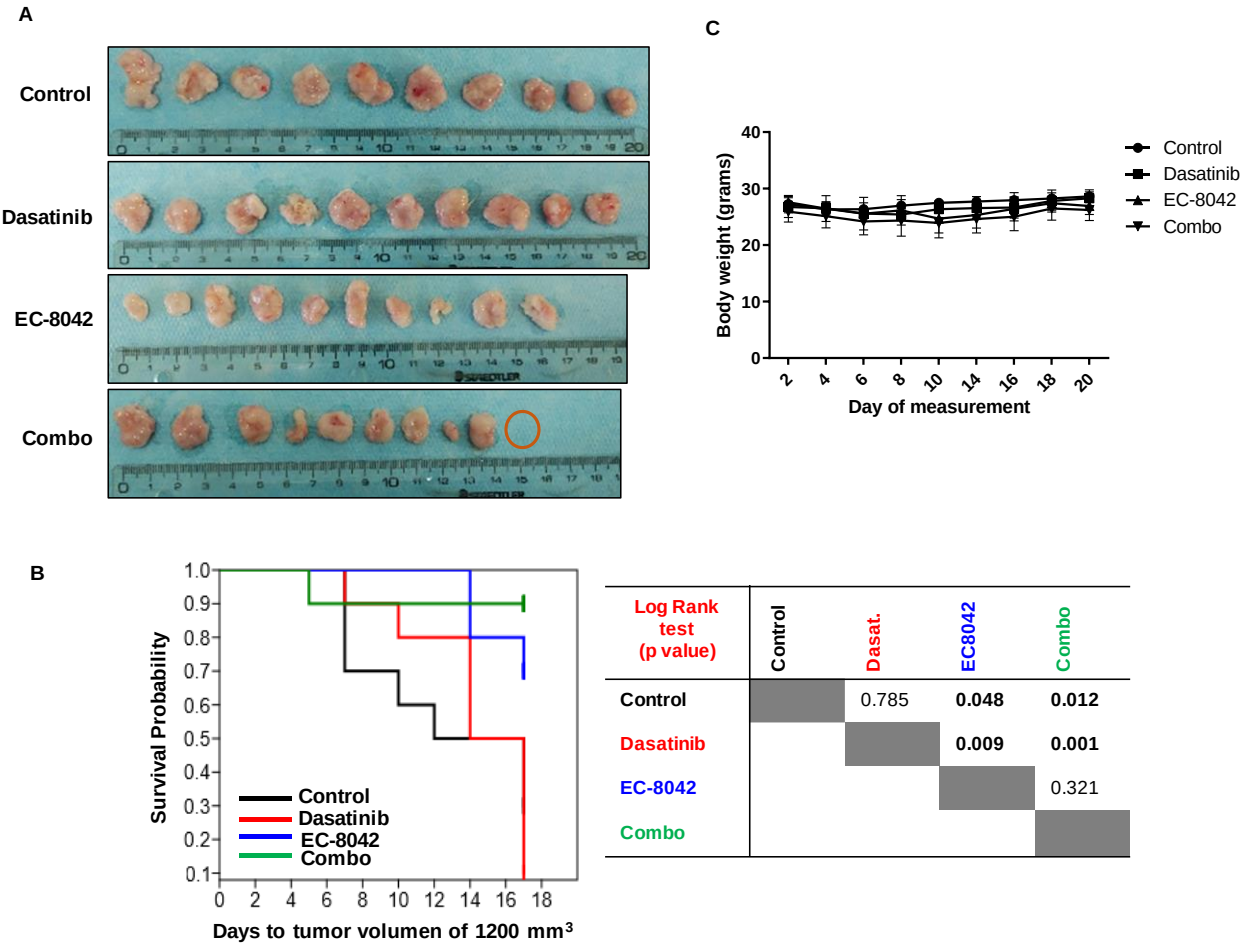
Source term	Degrees of Freedom	Sum of Squares	Mean square	F-ratio	P value	Comments
dasatinib	1	9610	9610	0.19	0.6674	**Not significant interaction between dasatinib and EC-8042**
EC-8042	1	1398760	1398760	27.32	< 0.0001*	
Interaction	**1**	**14440**	**14440**	**0.28**	**0.5986**	
Residual (error)	36	1842900	51192			
Total	39	3265710				

* Term significant at α=0.05

Supplementary Figure S6. Analysis of synergism between Dasatinib and EC-8042. (A) Analysis of synergism *in vitro*. FaDu cells were treated with dasatinib and/or EC-8042 alone or combined at a fixed ratio (Dasatinib:EC-8042, 10:1) for 72 h and the assayed for cell viability. Using these data, a combination index vs Fraction affected plot was generated for 6 constant ratio combinations of Dasatinib and EC-8042 (10:1) using the CompuSyn software according to the Chou-Talalay method [38]. Most of the combinations show CI values between 0.8 and 1.2, thus indicating an additive cytotoxic effect when both drugs were combined. (B-D) 2-Way ANOVA analysis of the interaction between dasatinib and EC-8042 *in vivo*. (B) Schematic diagram of the 2x2 factorial design employed to investigate synergism according to B.K. Slinker [40]. (C-D) Analysis of variance tables resulting from 2-way ANOVA for relative tumor volumes at the experimental end point (C) and for tumor weights (D). No significant interaction between dasatinib and EC-8042 was detected, and therefore no synergistic effect was expected**.**



Supplementary Figure S7. Effect of dasatinib, EC-8042 and combination treatment on the targeting of CSC subpopulations in HNSCC cell lines monitored by flow cytometry using the SORE6 reporter system. (A) RT-qPCR analysis of Sox2 and Oct4 levels in SORE6-GFP+ and SORE6-GFP- subpopulations sorted from FaDu-SORE6-GFP cells (mean ± SD, Student's t-test). (B) Quantification of the tumorsphere-forming capacity of SORE6-GFP+ and SORE6-GFP- subpopulations sorted from UT-SCC38-SORE6-GFP and FaDu-SORE6-GFP cell lines by MTS assay (mean ± SD, Student's t-test). (C-D) Flow cytometry analysis of the SORE6-GFP+ subpopulation in UT-SCC38-SORE6-GFP and FaDu-SORE6-GFP cells upon treatment for 72h with either DMSO, 0.1 µM dasatinib, 0.1 µM EC-8042 or combination. Data are expressed relative to DMSO-treated cells (mean ± SD, one-way ANOVA, Turkey's test, * $p < 0.05$, ** $p < 0.01$). Representative dot plots for UT-SCC38-SORE6-GFP cells and the corresponding UT-SCC38-minCMV-GFP gating control (C) and summary quantification (D) are presented.



Supplementary Figure S8. (A) Pictures of tumors extracted from FaDu xenografts after the treatments indicated in Figure 5. The circle indicates the lack of tumor growth in one of the Combo-treated mice. (B) Kaplan-Meier curves and Log Rank test *p* values generated using the reaching of a tumor volume of 1200 mm^3 as end-point event. (C) Mean body weight of mice during the *in vivo* treatments.