Received: 13 September 2018 | Revised: 5 December 2018 | Accepted: 24 April 2019

DOI: 10.1111/vco.12490

ORIGINAL ARTICLE

Veterinary and Comparative Oncology WILEY

Dasatinib inhibition of cSRC prevents the migration and metastasis of canine mammary cancer cells with enhanced Wnt and HER signalling

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Human epidermal growth factor 2 (HER2) overexpression leads to aggressive mammary tumour growth. Although the prognosis of HER2$^+$ tumours in humans is greatly improved using biologicals, therapy resistance, which may be caused by increased phosphatidyl-3-kinase (PI3K), rous sarcoma proto-oncogene (cSRC) or wingless-type MMTV integration site family (Wnt) activity, is a major concern. A recent analysis of 12 canine mammary cell lines showed an association between HER2/3 overexpression and phosphatase and tensin homologue (PTEN) deletion with elevated Wnt-signalling. Wnt-activity appeared to be insensitive to phosphatidyl-3-kinase (PI3K) inhibitors but sensitive to Src-I1. We hypothesized that Wnt activation, was caused by HER2/3-activated cSRC activation. The role of HER2/3 on Wnt signalling was investigated by silencing HER2/3 expression using specific small interfering RNA (siRNAs). Next, the effect of an epidermal growth factor receptor (EGFR)/HER2 tyrosine kinase inhibitor on Wnt activity and migration was investigated and compared to other tyrosine kinase inhibitors (TKIs) of related signalling pathways. Finally, two TKIs, a cSRC and a PI3K inhibitor, were investigated in a zebrafish xenograft model. Silencing of HER1-3 did not inhibit the intrinsic high Wnt activity, whereas the HER kinase inhibitor afatinib showed enhanced Wnt activity. The strongest inhibition of Wnt activity and cell viability and migration was shown by cSRC inhibitors, which also showed strong inhibition of cell viability and metastasis in a zebrafish xenograft model. HER2/3 overexpression or HER2/3-induced cSRC activation is not the cause of enhanced Wnt activity. However, inhibition of cSRC resulted in a strong inhibition of Wnt activity and cell migration and metastasis. Further studies are needed to

ABBREVIATIONS: AKT, protein kinase B; APC, adenomatous polyposis coli; Axin 2, axis inhibitor 2; Bcl-2, B-cell leukaemia 2 oncogene; CHK1/2, cell cycle checkpoint kinase 1/2; cMET, tyrosine-protein kinase Met (or HGF) proto-oncogene; CMT, canine mammary tumour cell line; cSRC, rous sarcoma proto-oncogene; Cyclin D1, cyclin D1; DCIS, ductal carcinoma in situ; dpi, days post-injection; ECM, extracellular matrix; EGFR, epidermal growth factor receptor, HER1, ERBB1; EMT, epithelial-mesenchymal transition; ER, oestrogen receptor; FAK, focal adhesion kinase; FBS, foetal bovine serum; FYN, FYN oncogene related to SRC, FGR, YES; HER1, human epidermal growth factor receptor, EGFR, ERBB1; HER2, human epidermal growth factor 2, ERBB2, Neu; HER3, human epidermal growth factor 3, ERBB3, ErbB3; HSP90, heat shock protein 90; IC50, half-maximum inhibitory concentration; ID1/2, inhibitor of DNA binding 1/2; IGF-1, Rinsulin-like growth factor 1 (receptor); JAK2, Janus kinase 2; LCK, lymphocyte specific protein-tyrosine kinase; LEF1, lymphoid enhancer-binding factor 1; LGR5, low-density-lipoprotein related protein; mTOR, mammalian target of rapamycin; MUC1, mucin 1; NRG1, neuregulin, heregulin-alpha; PR, progesterone receptor; PI3K, phosphatidyl-3-kinase; PLCB4, phospholipase C beta-4; PP2, inhibitor for cSRC-family kinases; PTEN, phosphatase and tensin homologue; RAC1, ras-like protein; siRNA, small interfering RNA; SFK, SRC family of protein kinases; SLUG, SNAIL; TCF, T-cell-specific transcription factor; TGFα, transforming growth factor alpha; TKI, tyrosine kinase inhibitor; Wnt, wingless-type MMTV integration site family.

unravel the mechanism of cSRC activation and cSRC inhibition to restore sensitivity to HER-inhibitors in HER2/3-positive breast cancer.

KEYWORDS
cSRC, dasatinib, HER2, mammary cancer, migration, Wnt

1 | INTRODUCTION

The life-time risk of mammary cancer is high not only in humans but also in dogs.[1] Most of these carcinomas in both species are oestrogen receptor (ER) and progesterone receptor (PR)-positive and respond to endocrine therapy in humans.[2-5] However, not all canine mammary tumours are well characterized, and immunohistochemical results may vary.[4,6] Approximately, 15% to 20% of human breast cancers are characterized by a combination of human epidermal growth factor 2 (HER2) overexpression and a lack of steroid hormone receptors, leading to more aggressive tumour growth and a worse prognosis.[7,8] Despite improved treatment options using therapeutic monoclonal antibodies against HER2, the acquisition of drug resistance remains a major problem and could lead to tumour recurrence.[9] For triple negative carcinomas, no targeted therapy is available.[10] HER2 overexpression in human breast cancer indicates a risk of recurrence independent of whether the axillary node is positive or negative for micrometastases.[11] Overexpression of HER2 is often observed (50%) in ductal carcinomas.[12] A clear role for HER2 overexpression in the progression from ductal carcinoma in situ (DCIS) to invasive carcinoma has not been shown,[13] although dimerization of epidermal growth factor receptor (EGFR) and HER2 induces motility.[14]

In human breast cancer, approximately, 50% of clinical samples have an elevated level of nuclear or cytoplasmic ß-catenin, implicating active wingless-type MMTV integration site family (Wnt) signalling.[15] In contrast to colon carcinomas, where mutations in adenomatous polyposis coli (APC) or ß-catenin are the cause of activated Wnt signalling, these mutations are rarely found in breast cancer.[16] Nevertheless, in human breast cancer, Wnt/β-catenin signalling has a clear contribution to tumour progression, metastasis and a poor clinical outcome in all molecular subtypes of invasive breast cancer.[17] In addition, HER2-mediated breast cancer progression requires ß-catenin signalling[18]; therefore, inhibition of Wnt signalling may be relevant in HER2-positive tumours. HER2 also acts among other pathways through the activation of rous sarcoma proto-oncogene (cSRC) kinases.[19] Because we have shown that Wnt activity is sensitive to cSrc inhibition,[20] we investigated the hypothesis that the enhanced expression of HER2/3 causes high Wnt activity through cSrc activation in some canine mammary tumour (CMT) cell lines.

Ductal carcinomas in dogs are comparable to the most common forms of human breast cancer.[21] One-third of malignant mammary carcinomas in dogs may form life-threatening distant metastases.[22] Tumour cells with cancer stem cell properties, such as a phenotypical epithelial-mesenchymal transition (EMT), can migrate and form metastases. In this regrowth and metastasis, elevated activity of the Wnt pathway plays an important role.[23]

In a panel of 12 different canine mammary cell lines, we identified three cell lines in which the basal Wnt activity was highly elevated in a ligand-independent manner, as indicated by high T-cell-specific transcription factor (TCF) reporter activity.[24] This Wnt activity was not caused by enhanced expression of Wnt ligands, whereas overexpression of lymphoid enhancer-binding factor 1 (LEF1) messenger RNA (mRNA) could only partially explain this elevated signalling.[24] A rounded cell morphology characterized the cell lines, suggesting EMT. In general, EMT, which is characterized by alterations in cell shape and loss of attachment to other cells and the extracellular matrix (ECM), is caused by loss of E-cadherin expression.[23] However, the cell lines with high Wnt activity and rounded cell morphology had relatively high E-cadherin expression,[24] excluding the likelihood that loss of E-cadherin caused high canonical Wnt activity[25] and elevated β-catenin levels.[26-28]

Further analysis by quantitative polymerase chain reaction (qPCR) of selected genes showed that the cell lines with high Wnt signalling overexpressed *HER2*,3 mRNA with *LEF1*, heat shock protein 90 (HSP90) and ras-like protein (RAC1), among others, and silenced phosphatase and tensin homologue (PTEN) mRNA expression.[20] The loss of PTEN expression in combination with an increased expression level of *HER3*, which promotes HER2-driven mammary tumour proliferation,[29] could indicate a highly activated PI3K/mammalian target of rapamycin (mTOR) pathway. However, inhibition of this pathway with the mTOR inhibitor everolimus or the dual PI3K/mTOR inhibitor BEZ325 further upregulated the already high basal activated Wnt activity.[24] Wnt activity could only be inhibited by Src-I1, a tyrosine kinase inhibitor that targets cSRC, among others.[20] Recently, SRC has been shown to promote resistance to trastuzumab in HER2-positive breast cancer. Upregulation of cSRC stabilizes HER2 and vice versa.[30] However, to the best of our knowledge, no literature is available on HER2/3 activation of Wnt signalling through cSRC activation.

HER signalling occurs predominantly by activating the PI3K/AKT/mTOR pathway,[31] but HER dimers may also transduce signals through cSRC/focal adhesion kinase (FAK) complexes.[32] These complexes influence the Wnt pathway by interacting with β-catenin. Therefore, it is likely that a connection exists between Wnt, cSRC and HER signalling. Considering that cSRC could be activated by EGFR and HER2/3, this likely makes cSRC a key player in the activation of highly activated basal Wnt cells.[33]

The central role of cSRC signalling was further investigated in vitro by cell migration and invasion assays and in an in vivo zebrafish embryo xenograft model. This model has been validated extensively, showing that short-term experiments in zebrafish embryos correlate well with long-term rodent xenograft models of breast cancer cell lines with variable grades of malignancy.[34] In general, dye-labelled cells are injected into the yolk sac, followed by invasion into the blood stream and the formation of distant metastases. However, cells may also be inserted directly into the blood stream through injection into the duct of Cuvier to investigate metastatic properties of breast cancer cell lines.[35] This model has also been validated in our laboratory for human insulinoma cell line metastasis and compared to xenografts in mice.[36] The same model was used for investigating the effects of mTOR and cSRC inhibition on the metastatic properties of cells with high Wnt signalling in the current study. For cSRC inhibition, dasatinib was used because this selective cSRC inhibitor is FDA-approved.

The aim of this study was to investigate the role of HER signalling in enhanced canonical Wnt signalling and to investigate tyrosine kinase inhibitors (TKIs) related to HER signalling as inhibitors of Wnt activity and associated cell migration and metastasis.

2 | MATERIALS AND METHODS

2.1 | Canine mammary cell lines and culture

In our studies, a panel of 12 different canine mammary cell lines were used as described previously.[37-39] For detailed studies, the well-described canine cell lines CMT-U27 (a generous gift from Prof. Dr. Hellmen, SLU, Uppsala, Sweden) and CIPm (a generous gift from Prof. Dr. Sasaki, Laboratory of Veterinary Surgery, University of Tokyo, Japan) were used,[39,40] representing a cell line with ligand-independent high Wnt signalling (CMT-U27) and absent Wnt signalling (CIPm).[20] The cells were cultured in DMEM/F12 (Invitrogen, Bleiswijk, The Netherlands) supplemented with 10% foetal bovine serum (FBS) (Invitrogen). The cells were shown to be free of mycoplasma with a Mycosensor qPCR assay according to the manufacturer's protocol (Agilent Technologies, Middelburg, The Netherlands).

2.2 | Small interfering RNA

Canine sequence-specific EGFR (GenBank: XM_533073), HER2 (GenBank: NM_001003217) and HER3 (GenBank: XM_538226) siRNA was designed on the website http://rnaidesigner.termofisher.com (Thermo Scientific, Breda, The Netherlands). Universal MOCK siRNA (ON-Target and the non-targeting pool species H (human), M (mouse) and R (rat) (Thermo Scientific) were used as negative controls for the siRNA experiments. There was no cross-silencing of non-target genes, which was verified by BLASTing the designed siRNA sequences against the canine genome database. The sequences of the siRNA duplexes were as follows: EGFR sense 3236 CCGUCUAUCACAAUCAGCCUCUAAA and antisense UUUAGAGGCUGAUUGUGAUAGACGG, HER2 2995 sense UGCACCAUUGAUGUCUACAUGAUCA and antisense UGAUCAUGUAGACAU-CAAUGGUGCA and HER3 sense 1336 CGAGCAACAUUGAUGGAUUUGUGAA and antisense UUCACAAAUCCAUCAAUGUUGCUCG. Cell transfections were first optimized with siGLO (Dharmacon, Colorado) (data not shown). A total of 125 000 CMT-U27 cells were transfected with 3-μL Lipofectamine 2000 (Invitrogen) transfection reagent, 50 nM siRNA and 0.8 μg DNA in 24-well Primaria plates (Corning, New York). After 24, 48 and 72 hours incubation in DMEM/F12 and 10% FCS, the cells were harvested for RNA isolation, protein isolation and a transcription factor (TCF)-reporter assay.

2.3 | RNA isolation, cDNA synthesis and quantitative RT-PCR

RNA was isolated at 24, 48 and 72 hours after transfection using an RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol with a DNAse (Qiagen) treatment. cDNA synthesis was performed using an iScript kit (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's protocol. Specific primer sets were used to amplify the gene products for quantitative PCR (qPCR) (Table 1). qPCR was performed using Bio-Rad CFX manager software (version 3.1) with a SYBR Green fluorophore (Bio-Rad). Relative target gene expression was normalized to a set of seven reference genes and analysed by the geNorm implementation of the SLqPCR package[41] in R using RStudio (version 3.3.1)[42] with a pairwise variation (PV) of 0.07. Relative expression was calculated by the ΔΔCt method,[43] and the relative induction of gene expression was statistically analysed using SPSS (version 24).

2.4 | Protein isolation and Western blot

CMT-U27 cells were seeded at a density of 600 000 in 6-well Primaria plates (Corning) and transiently transfected with the siRNA after 24 hours. After 24, 48 and 72 hours, the cells were washed with Hank's balanced salt solution (Invitrogen) and lysed and scraped with RIPA buffer.[24] Twenty micrograms of protein from the total cell lysate was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by Western blot. The primary antibodies used were against EGFR (Ab2430, 1:200 dilution) (Abcam, Cambridge, UK), HER2 (PA-14635, 1:500 dilution) (Thermo Scientific), HER3 (PA1-86644, 1:2500 dilution) (Thermo Scientific) and β-Actin pan Ab-5 (MS-1295-P1, 1:2000 dilution) (Thermo Scientific) as a loading control. Goat anti-rabbit Horseradish peroxidase (HRP)-conjugated (HAF008) and donkey anti-goat HRP-conjugated (HAF109) (R&D Systems, Abingdon, UK) secondary antibodies were used at a 1:20000 dilution. HRP was visualized using Advance enhanced chemiluminescence (ECL, GE Healthcare, Eindhoven, The Netherlands) and analysed using GelDoc 2000 (Bio-Rad).

TABLE 1 Primers used for quantitative polymerase chain reaction after small interfering RNA treatment

OMIM accession	Symbol	Forward primer	Reverse primer	Ta (°C)	Size (bp)	Number
Target genes						
AXIN2	Axin2	GGACAAATGCGTGGATACCT	TGCTTGGAGACAATGCTGTT	60	128	XM_54802
APC	APC	AGTCCCAAGCAACAGAAGC	GCAGTTGAACCCTGAGCA	63	138	XM_536285
CTNNB1	βCatenin	ATGGGTAGGGCAAATCAGTAAGAGG	TAAGCATCGTATCACAGCAGGTTAC	64	106	XM_005634157.1
BNIPL	Bcl2	TGGAGAGCGTCAACCGGGAGATGT	AGGTGTGCAGATGCCGGTTCAGGT	62	87	AY_509563.1
CDH1	ECAD	CAGGAAGCTCTCCACCAGAG	CTGGGAAATGTGAGCACCTC	58	105	NM_001287125.1
CCND1	CyclinD1	GCCTCGAAGATGAAGGAGAC	CAGTTTGTTCACCAGGAGCA	60	117	NM_001005757.1
ERBB1	EGFR	CTGGAGCATTCGGCA	TGGCTTTGGGAGACG	53	107	XM_533073
ERBB2	HER2	CGTGCTGGACAATGGAGACC	CCGCTGAATCAAGACCCCTC	64	51	AB008451
ERBB3	HER3	TAGTGGTGAAGGACAACGGCAG	GGTCTTGGTCAATGTCTGGCAG	70	103	XM_538226
ERBB4	HER4	CAGTTCTTGTGTGCGTGCCTG	ATGATCCTGTGCCGATGCC	64	121	XM_545629
HSP90AA1	HSP90	CTTGACCGATCCCAGTAAGC	TATTGATCAGGTCGGCCTTC	59	127	XR_134513.2
ID1	ID1	CTCAACGGCGAGATCAG	GAGCACGGGTTCTTCTC	59.5	135	XM_847117.2
ID2	ID2	GCTGAATAAATGGTGTTCGTG	GTTGTTCTCCTTGTGAAATGG	60.5	114	XR_134413.1
LEF1	TCF1α	AGACATCCTCCAGCTCCTGA	GATGGATAGGGTTGCCTGAA	60	137	XP_863334.2
LGR5	LGR5	CTCAGCGTCTTCACCTCCT	TGGGAATGTATGTCAAAGCGT	67	115	XM_846738.2
MET	cMET	TGTGCTGTGAAATCCCTGAATAGAATC	CCAAGAGTGAGAGTACGTTTGGATGAC	56	159	NM_001002963.1
MUC1	MUC1	CTATGAGGAGGTTTCTGCAG	GAACACAGTTGAGAGGAGAG	62	172	NM_001194977
NCOA1	NCOA1	GGAACACGACGAAATAGCCATACC	TAATCGCCGTGCAATACAAATCA	67.1	189	XM_532891.5
NRG1	NRG1	CATCGCCCTGCTTGTGGTC	GTGGTGAGGCCCGTTTGCTATG	67	144	XM_853054.3
PGR	PR	CAATGGAAGGGCAGCATAAC	CAGCATTTCTAAGGCGACA	57	102	NM_001003074.1
PLCB4	PLCB4	CCAGTCAGGAGTATTACTAGAACG	AAAGATCCTCCTCTATATCTGTCCGAG	64.5	184	XM_854626.3
CDH3	PCAD	GCCCCGCCCTATGACTCCCTATT	GAAGCGGCTGCCCCACTCG	67	137	XM_005620790.2
RAC1	Rac1	TCCCTTATCCTATCCGCAAA	ATGATAGGGGTGTTGGGACA	58	128	NM_001003274.2
	Rac1b	TGGGATACAGCTGGACAAGA	CTTGTCTTTGCCCCTGGAG	58	108	JN_182651.1
SNAI2	SLUG	CTTCACTCCGACTCCAAACG	TGGATTTTGTGCTCTTGCAG	60	147	XM_005637933.1
SRC	cSRC	CATTGGGAAGGGGGAGTTTGGAGA	TGCCGAAGTTGCGTCATCACAGAG	66	134	XM_544774.5
TGFA	TGFα	CCGCCTTGGTGGTGGTCTCC	AGGGCGCTGGGCTTCTCTGT	61	83	NM_001003244.4
Reference genes						
HNRNPH2	HNRPH	CTCACTATGATCCACCACG	TAGCCTCCATAACCTCCAC	61.2	151	XM_538576
RPL13	RPL13	GCCGGAAGGTTGTAGTCGT	GGAGGAAGGCCAGGTAATTC	61	87	XM_003432726
RPS5	RPS5	TCACTGGTGAGAACCCCCT	CCTGATTCACACGGCGTAG	62.5	141	XM_533568
RPS19	RPS19	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	62	95	XM_533657
SDHA	SDHA	GCCTTGGATCTCTTGATGGA	TTCTTGGCTCTTATGCGATG	61	92	DQ402985
TBP	TBP	CTATTTCTTGGTGTGCATGAGG	CCTCGGCATTCAGTCTTTTC	57	96	XM_849432
YWHAZ	YWHAZ	CGAAGTTGCTGCTGGTGA	TTGCATTTCCTTTTTGCTGA	58	96	XM_843951

Abbreviations: APC, adenomatous polyposis coli; Axin 2, axis inhibitor 2; Bcl-2, B-cell leukaemia 2 oncogene; CCND1, cyclin D1; CDH, cadherin; cMET, tyrosine-protein kinase Met (or HGF) proto-oncogene; cSRC, rous sarcoma proto-oncogene; Cyclin D1, cyclin D1; EGFR, epidermal growth factor receptor; ERBB, human epidermal growth factor receptor, EGFR, ERBB1; ER, oestrogen receptor; FYN, FYN oncogene related to SRC, FGR, YES; HER1, human epidermal growth factor receptor; HER2, human epidermal growth factor 2; HER3, human epidermal growth factor 3; HNRPH, hetrogenous nucleiar ribo protein H; HRP, horseradish peroxidase; HSP90, heat shock protein 90; ID1/2, inhibitor of DNA binding 1/2; LEF1, lymphoid enhancer-binding factor 1; LGR5, low-density-lipoprotein related protein; MUC1, mucin 1; NCOA3, nuclear receptor coactivator; NRG1, neuregulin, heregulin-alpha; PR, progesterone receptor; PLCB4, phospholipase C beta-4; RAC1, ras-like protein; RPL13, ribosomal protein L43; SDHA, succinate dehydrogenase complex subunit A; SLUG, SNAIL; TBP, TATA-box binding protein; TCF, T-cell-specific transcription factor.

TABLE 2 IC-50 values

Inhibitor	Target	CMT-U27 (μM)	CIPm (μM)
Afatinib	EGFR + HER2	22	69
Aurora A	Aurora A	1	13
AZD1480	JAK2	28	9
AZD7762	CHK1 + CHK2	301	1
Barasertib	Aurora A + B	77	822
Dasatinib	SRC	17	23
OSI-906	IGF-1R	18	54
PP2	LCK + FYN	41	70

Abbreviations: CHK1/2, cell cycle checkpoint kinase 1/2; CMT, canine mammary tumour cell line; IGF-1, Rinsulin-like growth factor 1 (receptor); JAK2, Janus kinase 2; LCK, lymphocyte specific protein-tyrosine kinase; PP2, inhibitor for cSRC-family kinases.
IC-50 values for proliferation inhibition of CMT-U27 and CIPm cells analysed by MTT assays using various TKIs. For dose response curves, see Figure S1.

2.5 | TCF reporter assay

Cells were seeded in a 24-well Primaria plate (Corning) at a density of 100 000 CMT-U27 cells/well and allowed to reach 80% confluency 24 hours before transfection. Transfection was performed in FBS-free medium using 3 μL Lipofectamine 2000 (Invitrogen), 800 ng pTOPFLASH (TOP) or pFOPFLASH (FOP) (a gift from Prof. Dr. Hans Clevers, Hubrecht Institute, The Netherlands) and 0.5 ng human ß-actin promoter Renilla construct[44] as an internal control. The half-maximum inhibitory concentration (IC50) of the inhibitors was analysed by an MTT assay, and the cells were treated with the IC50 concentration of the inhibitors (Table 2). All compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in medium. A final concentration of 0.2% DMSO was used as a control. The firefly and Renilla luciferase activities were analysed using a Dual-Luciferase Assay System (Promega, Leiden, The Netherlands) in a Centro LB 960 luminometer (Berthold Technologies, Vilvoorde, Belgium).

2.6 | Cell viability

Cell viability was analysed by a colorimetric 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, Zwijndrecht, The Netherlands). Briefly, cells were seeded in 96-well Primaria plates (Corning) at a density of 15 000 cells/well (for CMT-U27 cells) and 10 000 cells/well (for CIPm cells) in DMEM/F12 with 10% FBS. After 24 hours of incubation cell adhesion and spreading, the cells were treated with the following eight inhibitors: afatinib, Aurora A inhibitor I, AZD1480, AZD7762, barasertib, dasatinib, OSI-906 and PP2 (inhibitor for cSRC-family kinases) (all from Selleck Chemicals, Munich, Germany). The inhibitors had different susceptibilities to different kinase pathways and were dissolved in DMSO to a final stock concentration of 10 mM. The inhibitors were added in a concentration range from 1 nM to 1 mM diluted in medium to a final concentration of 0.2% DMSO and incubated for 48 hours. The absorbance was measured at a wavelength of 595 nm by an Anthos Multimode Detector spectrophotometer (Anthos Mikrosystem GmbH, Krefeld, Germany). IC50 curves were plotted with Sigma Plot version 12.5.

2.7 | Cell migration

Cells were seeded in 6-well Primaria plates (Corning) at a density of 1 000 000 cells/well (for CMT-U27 cells) and 500 000 cells/well (for CIPm cells). After 24 hours, the monolayer of cells was scraped with a 200 μL pipette tip to create a straight gap. The medium was removed, and the cells were washed with Hank's balanced salt solution (HBSS) (Invitrogen). The cells were treated with 100 nM everolimus (Selleck Chemicals), 50 nM BEZ235 (Selleck Chemicals), 20 μM SRC-I1 (Enzo, Lausen, Switzerland) or 10 μM PP2 (Selleck Chemicals). At 0, 24 and 48 hours, cells migrating into the scratched area were imaged with an Olympus microscope (Olympus, Tokyo, Japan) and analysed with ImageJ software version 1.4.7. The closure of the gap was calculated as follows: percentage of scratch closure = $a–b/a$, where a is the distance of the gap at t = 0, and b is the distance of the gap at t = 24 or 48 hours.

2.8 | Cell Invasion

A 3D chemotaxis μ-slide (Ibidi GmbH, Munich, Germany) and DMEM/F12 medium (Invitrogen) were pre-equilibrated for 24 hours at 37°C before use. CMT-U27 and CIPm cells were resuspended at 300000 cells/100 μL DMEM/F12 with 1 mg/mL collagen-I gel solution (rat tail collagen, Sigma-Aldrich). Six microliters of the collagen gel containing cell suspension was quickly seeded into the central chamber of the 3D chemotaxis μ-slide. The slides were incubated upside down for 1 hour at 37°C in an incubator. Then, the reservoirs were filled with 70 μL DMEM/F12 (left side of the collagen cell suspension) or with DMEM/F12 with FCS as a chemoattractant and with or without dasatinib as an inhibitor (10 μM final concentration, Selleck Chemicals) (right side). The slides were incubated for 22 hours in a humidified Petri dish at 37°C. The cells were then stained with 2.5 μg/mL calcein AM (Invitrogen) dissolved in DMSO and diluted 1000× in Hank's balanced salt solution (Invitrogen) for 1 hour. After 3 washes with Hank's balanced salt solution, the fluorescence of the cells was analysed with a Leica SPE-II microscope.

2.9 | Xenograft

To investigate the effect of inhibition of cSRC or PI3K signalling on the metastasis of canine mammary cells, a xenograft model in zebrafish was used that has been validated previously[35,45] and has been used in our laboratory to investigate the metastasis of canine insulinoma cells.[36] The transgenic zebrafish line Tg(fli1:GFP)[46] (a generous gift from Brant M. Weinstein, PhD, National Institutes of Health) was used for the xenograft experiments. The zebrafish were raised and maintained according to standard procedures.[47]

Dechorionized 2-day-post-fertilization zebrafish embryos were anaesthetised with 0.003% tricaine (Sigma-Aldrich) and positioned on a 10-cm Petri dish coated with 1% agarose. Single cell suspensions of CMT-U27-RED (pHAGE2-EF1a-DsRed-PuroR)[48] cells were resuspended in 2% polyvinylpyrrolidone (Sigma-Aldrich) in Hank's balanced salt solution (200 cells/nL) and were implanted within 1 hour, as reported previously.[36] The zebrafish embryos were either injected with 2 nL CMT-U27 RED cell suspension or with 2 nL CMT-U27 RED cell suspension containing 10 μM dasatinib or 10 μM everolimus. To investigate the compound in the zebrafish, dasatinib was added to E3 medium (0.1 μM). Three and 5 days post-injection (dpi), the embryos were analysed with confocal fluorescence imaging techniques. CMT-U27 RED cells were injected in the yolk sac[49] or in the duct of Cuvier.[50] Cells injected into the yolk sac have to migrate to the blood circulation before they may form distant metastases, whereas direct injection into the bloodstream using the duct of Cuvier does not require intravasation and can form distant metastases more easily.[51,52]

The embryos were directly inspected for proper injection and were fixed on day 3 or 5 (dpi) and imaged under a confocal microscope. The experiments were performed in triplicate with 30 to40 well-injected embryos for dasatinib and 10 embryos for the everolimus experiments. The survival rate at 1 dpi was >90% in each experiment. The localization of tumour cells and the percentages of embryos with metastasis were manually scored.

2.10 | Statistics

Statistical analysis was conducted using IBM SPSS Statistics version 22 and 24 software (SPSS Benelux, Gorinchem, The Netherlands). The data were normally distributed; therefore, one-way analysis of variance and post hoc Bonferroni tests were used. Compared to the DMSO control group, $P < 0.05$ was considered significant, indicated with an asterisk (*) for the Wnt activity (n = 3*4) and for the scratch assay results. The gene expression results from the siRNA experiments were analysed using a nonparametric test in SPSS (version 24) against the MOCK group (n = 6); $*P < 0.01$. The number of zebrafish embryos was scored for cell survival and distant metastases. The results were statistically analysed with Fisher's exact test (Microsoft

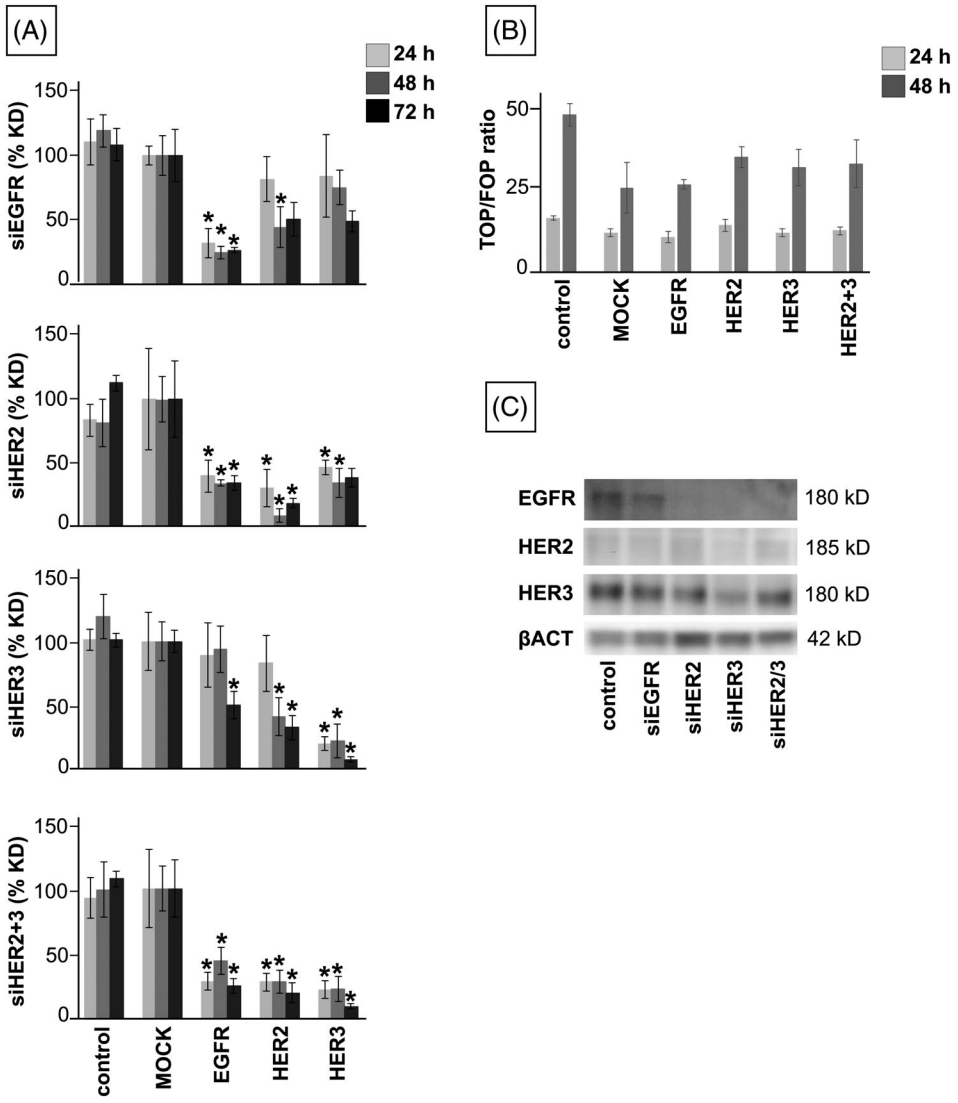


FIGURE 1 The effect of human epidermal growth factor (HER) messenger RNA (mRNA) silencing on wingless-type MMTV integration site family (Wnt) activity. **A,** epidermal growth factor receptor (EGFR), HER2, HER3 and HER2/3 mRNA expression relative to MOCK-treated cells analysed by quantitative polymerase chain reaction in canine mammary tumor cell line (CMT)-U27 cells that were nont-ransfected (Control C), transfected with the MOCK control (MOCK), or small interfering RNA against EGFR, HER2, HER3 or both HER2 and HER3 (HER2/3). B, The effects on Wnt activity presented as TOP/FOP reporter activity relative to the MOCK control. C, Western blot analysis of HER proteins after siRNA treatment for 72 hours. $*P < 0.01$ compared to the MOCK control

Excel version 2010), and $P < 0.01$ was considered to be significant (n = 10-40 embryos for each condition).

3 | RESULTS

3.1 | Silencing of HER mRNAs

Three out of 12 canine mammary cell lines had highly elevated ligand-independent basal Wnt activity. These cell lines were also shown to have relatively high expression levels of EGFR, HER2 and HER3 mRNA compared with the other cell lines. The question is whether this is only an association or whether overexpression of HER proteins is a direct cause of Wnt pathway activation. We used the well-described CMT-U27 cell line for the studies. Treatment with various siRNAs resulted in a time-dependent decrease in the mRNA expression levels of EGFR, HER2 and HER3 (Figure 1A). Knockdown with EGFR siRNA resulted not only in significant downregulation of EGFR mRNA expression but also in lower levels of HER2 mRNA expression after 24 hours. The siRNA against HER2 resulted in fast downregulation of all three mRNAs, whereas the siRNA against HER3 was observed to be selective at 24 hours; but after longer incubation times, the mRNA expression of EGFR and HER2 were also significantly inhibited (Figure 1A). The combination of siRNA against HER2 and HER3 resulted in substantially decreased mRNA expression levels for all three receptors. However, despite the strongly decreased mRNA expression levels, no inhibition of basal Wnt activity, which was indicated by the TOP/FOP-luciferase reporter activity, was observed after 48 or 72 hours compared with the mock incubations (Figure 1(B)). The lack of the effect was not caused by stable protein expression, because Western blot analysis after 72 hours also showed decreased expression levels in the protein bands (Figure 1C). Additional analysis of various Wnt target genes even showed limited increases in the mRNA expression levels of axis inhibitor 2 (*AXIN2*), *LEF1*, low-density-lipoprotein related protein (*LGR5*) and *SRC* after 72 hours (Figure S2 siRNA qPCR).

3.2 | Cell proliferation, Wnt activity, migration and invasion in vitro

Previous research has shown that the high Wnt activity in three cell lines is sensitive to inhibition by the TKI Src-I1, leading to the hypothesis that increased HER-mediated cSRC activity causes elevated Wnt activity. Therefore, we investigated the effect of EGFR and the HER2 TKI afatinib with a variety of related TKI inhibitors on cell proliferation, Wnt activity and migration of CMT-U27 cells with high Wnt activity and CIPm cells with low Wnt activity. All TKIs inhibited cell proliferation in a dose-dependent manner (Figure S1 IC50) with IC50 values in the μM range (Table 2). No differences in cell viability were observed between CMT-U27 and CIPm cells in the 0.2% DMSO control compared with medium. In general, CMT-U27 cells were slightly more sensitive to inhibition than CIPm cells.

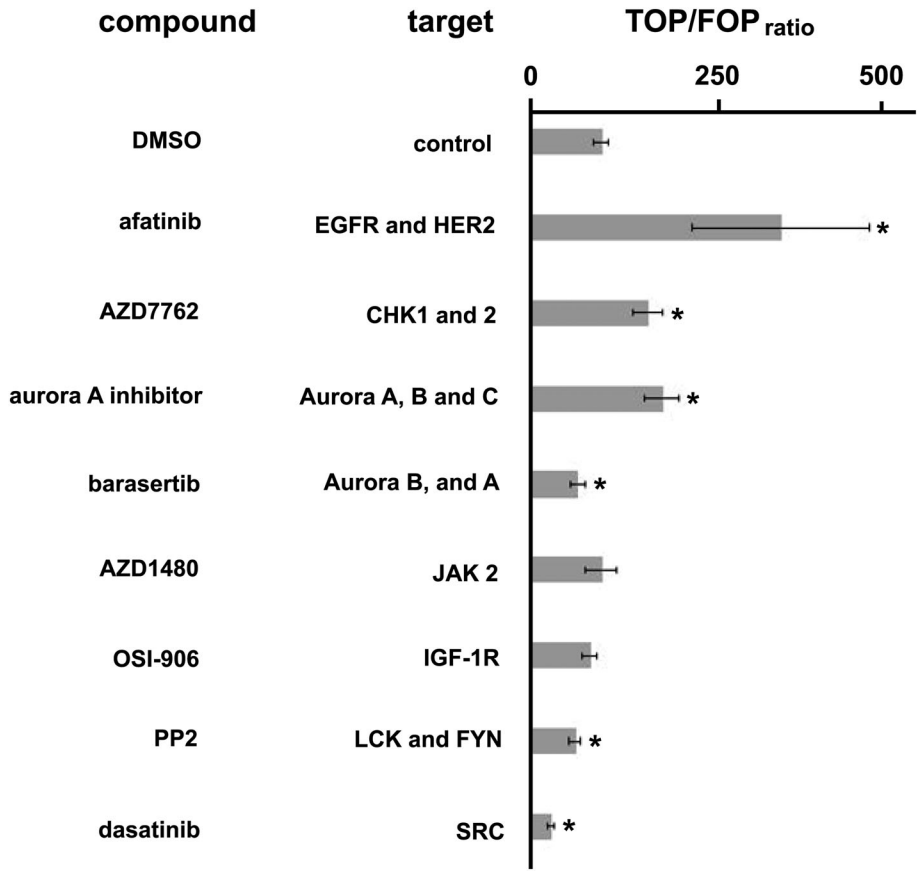
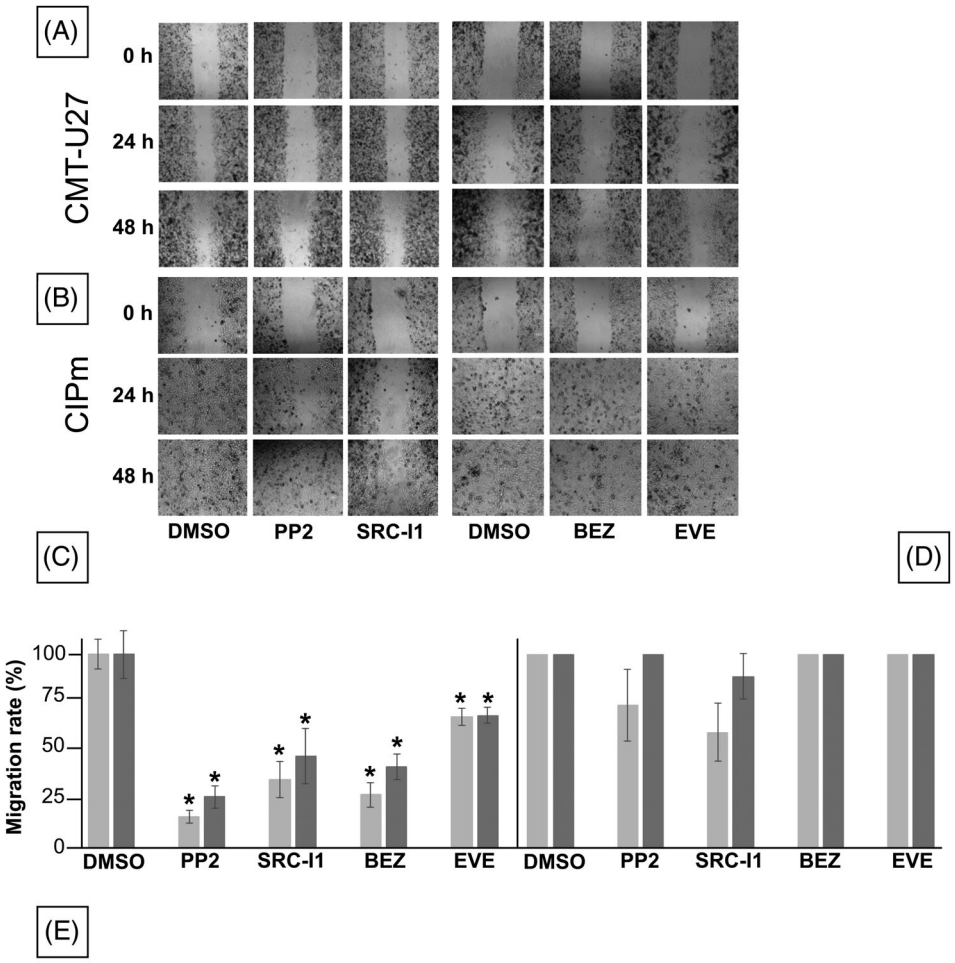


FIGURE 2 The effect of tyrosine kinase inhibitor (TKIs) on wingless-type MMTV integration site family (Wnt) activity. The effects of canine mammary tumor cell line (CMT)-U27 cells after incubation for 48 hours with tyrosine kinase inhibitors on wingless-type MMTV integration site family (Wnt) signalling analysed by TOP/FOP luciferase reporter activity. The epidermal growth factor receptor (EGFR) inhibitor afatinib stimulated Wnt activity 3.5-times. The Aurora B and A inhibitor barasertib, the lymphocyte specific protein-tyrosine kinase (LCK) and FYN inhibitor PP2 (inhibitor for rous sarcoma proto-oncogene [cSRC]-family kinases), and the cSRC inhibitor dasatinib significantly downregulated Wnt reporter activity. The mean ratio of three independent experiments is shown. *$P < 0.05$ vs the control



(E)

		CMT-U27		CIPm	
	conc.	24 h	48 h	24 h	48 h
DMSO	0.2%	46%	70%	100	100
PP2	10 µm	8% (*)	19% (*)	74	100
SRC-I1	20 µm	17% (*)	35% (*)	62	91
DMSO	0.2%	32%	86%	100	100
BEZ	50 nM	9% (*)	42% (*)	100	100
EVE	100 nM	22% (*)	56% (*)	100	100

FIGURE 3 The effect of tyrosine kinase inhibitors (TKIs) on cell migration. The migration rate of canine mammary tumor cell line (CMT)-U27 cells (high basal wingless-type MMTV integration site family [Wnt] activity) or CIPm (low basal Wnt activity) after treatment with various TKIs for 24 to 48 hours. Representative images and quantification of scratch closure are shown for control cells (0.2% dimethyl sulfoxide) and cells after incubation with PP2 (inhibitor for rous sarcoma proto-oncogene [cSRC]-family kinases) (10 µM), Src-I1 (20 µM), BEZ (50 nM) and everolimus (100 nM) in CMT-U27 (A,C) and CIPm (B, D) cells. Results, which are expressed as the percentage of scratch closure, were calculated as follows: percentage of scratch closure = $a - b/a$, where a is the distance of the gap at t = 0 h, and b is the distance of the gap at t = 24 or 48 hours. The mean ± SEM of two independent experiments is presented with six data points for each experiment. *P < 0.05 vs the DMSO control. For the fastest scratch closure of CIPm cells, the migration rate is shown as a percentage relative to the control (E)

Remarkably, the high basal Wnt activity in the HER2/3-overexpressing CMT-U27 cells was further stimulated by the EGFR and HER2 inhibitor afatinib (Figure 2). Consistent with previous results, significant inhibition of Wnt activity was observed with the Src family of tyrosine kinase (SFK) inhibitors, including PP2 and dasatinib.

The inhibitors PP2 and Src-I1 also showed strong inhibition of CMT-U27 cell migration in a scratch assay together with inhibitors of the PI3K pathway (Figure 3 A, C). None of the investigated inhibitors affected the migration of the control CIPm cells (Figure 3 B, D). The scratched gaps were filled completely within 48 hours, and there was no significant difference in the migration rate. Both cell lines moved through the collagen towards the chemoattractant in the invasion assay (Figure 4A), where CMT-U27 cells were further from the border of the matrix than the CIPm cells (Figure 4B, (D)). Dasatinib completely inhibited the invasion of CMT-U27 cells and partially inhibited the invasion of CIPm cells (Figure 4(C), (E)).

3.3 | The effect on metastasis in vivo

Finally, the effects of PI3K/mTOR or cSRC inhibition on the metastatic properties of the CMT-U27 cell line were investigated in a zebrafish embryo xenograft model that was previously validated in our laboratory[36] and used in this study because of the 3R principle (reduction, refinement and replacement) of animal use in biomedical research.[53] Because the PI3K/mTOR inhibitor everolimus[54] and the cSRC inhibitor dasatinib[55] are used in clinical trials for humans with advanced breast cancer, they were used in this study.

Most of the zebrafish embryos survived the injection with DsRed-labelled CMT-U27 cells (CMT RED), with 76% survival after yolk sac injection and 80% survival after injection into the duct of Cuvier. Pre-treatment of CMT-U27 cells with 1 µM dasatinib before xenografting greatly reduced both cell survival and the number of larvae with clearly visible metastases (Figure 5) at 3 and 5 days post injection

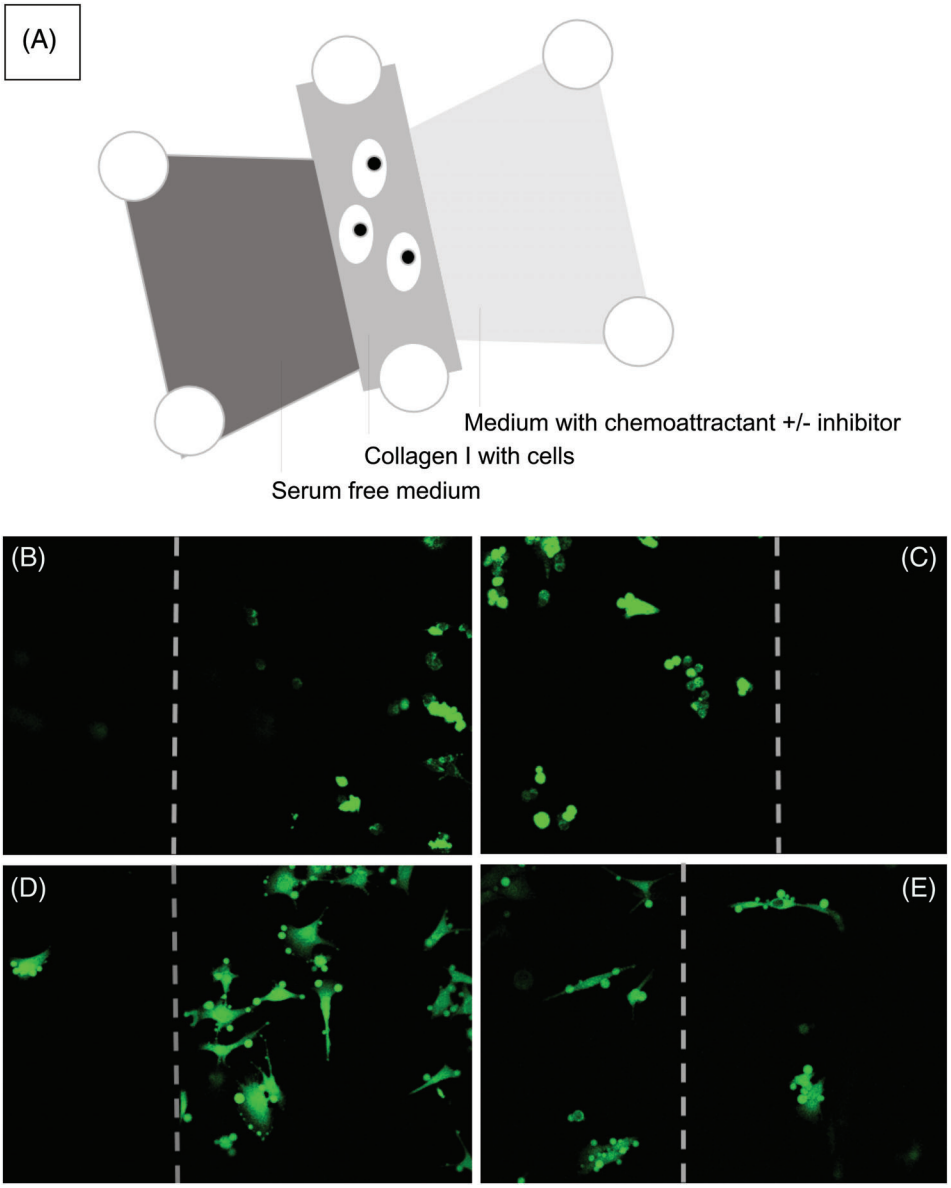


FIGURE 4 The effect of the rous sarcoma proto-oncogene (cSRC) inhibitor dasatinib on invasion. The invasion ability of canine mammary tumor cell line (CMT-U27) (high basal wingless-type MMTV integration site family [Wnt] activity) and CIPm (low basal Wnt activity) cells after treatment with a chemoattractant and with or without dasatinib (10 μM). The chemotaxis μ-slide (A) had a collagen I suspension loaded with cells, chemoattractant free medium and chemoattractant ± dasatinib. The dashed lines represent the border between the collagen I matrix and the chemoattractant compartment. CMT-U27 (B) and CIPm (D) cells migrated from the matrix to the chemoattractant. Dasatinib completely inhibited the invasion of CMT-U27 cells (C), whereas CIPm cell invasion (E) was partially inhibited [Colour figure can be viewed at wileyonlinelibrary.com]

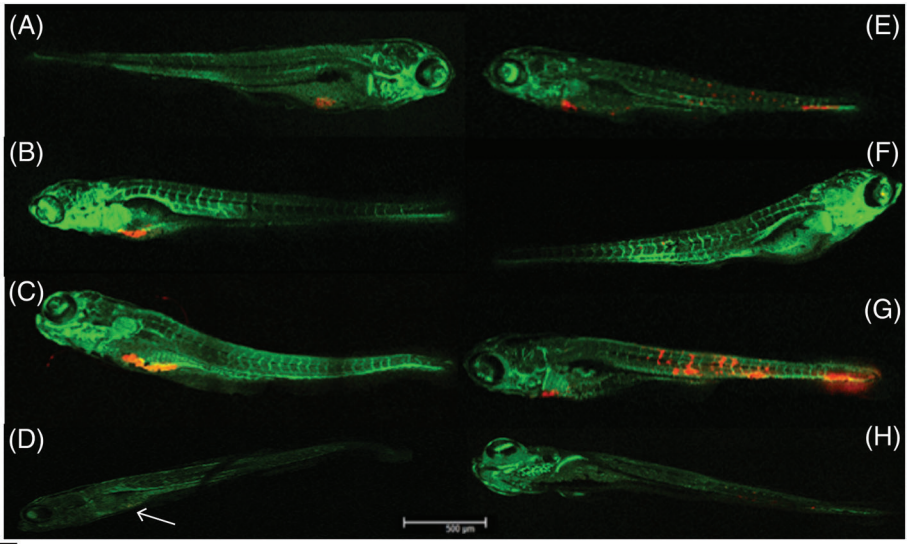
(dpi). This was observed after injection in the yolk sac and after direct injection in the blood stream, where more metastases were observed without preincubation. Even when dasatinib was added to the egg medium, no metastases were observed (not shown).

When CMT RED cells were injected into the yolk sac in the presence of everolimus, the number of metastases increased (Figure 6). More clumps of tumour cells were observed when CMT RED cells were injected in the duct of Cuvier, extravasation of the CMT RED cells was observed and everolimus slightly increased the amount of metastases.

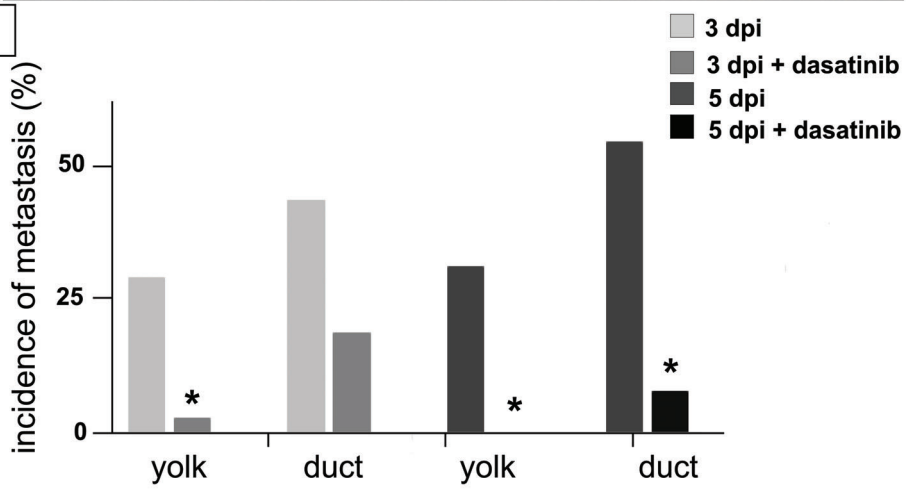
4 | DISCUSSION

In the present study, we investigated our hypothesis that overexpression of HER2/3 is related to the ligand-independent high basal Wnt activity of canine mammary cancer cells through the activation of cSRC (Figure 7).

Activation of Wnt signalling is related to the stemness and migration of cells, suggesting that inhibition of Wnt activity could play a central role in reducing or even preventing breast cancer metastasis in some cases. Therefore, we investigated the underlying cause of the high basal Wnt activity in the CMT-U27 metastatic canine mammary cell line, which was selected from a panel of canine mammary cell lines where 3 out of 12 cell lines were characterized by high ligand-independent Wnt signalling.[24] Additional studies showed that these cell lines had *HER2*/3 overexpression, absent *PTEN* mRNA, and sensitivity to Src-I1, indicating a possible relationship between HER signalling and Wnt activity.[20] However, in the present study, we were unable to show a direct relation by silencing HER protein expression with siRNAs. The knockdown percentages were greater than 50% for all tested siRNAs. *EGFR*, *HER2* and *HER3* and the combination of



(I)



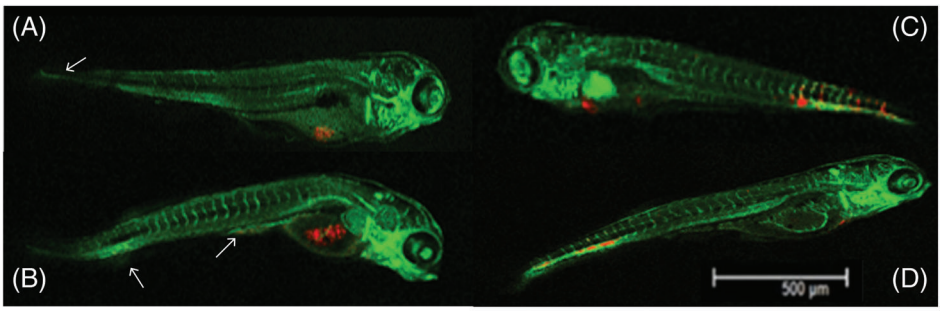
(J)

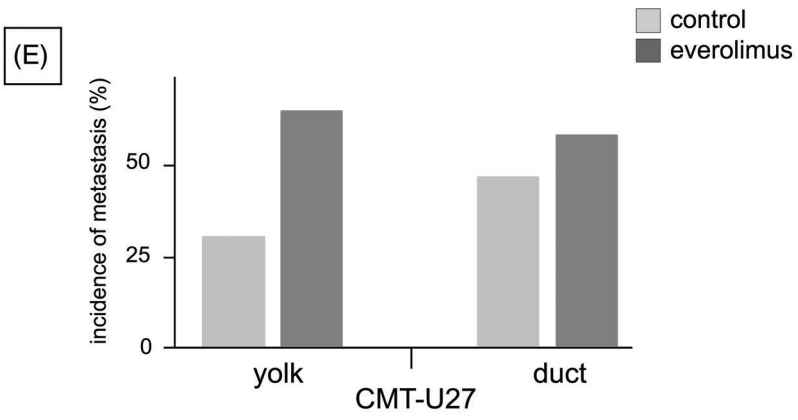
dpi	dasatinib	yolk sac metastasis	yolk sac cell survival	duct of Cuvier metastasis	duct of Cuvier cell survival
3	-	26.7%	76.7%	40.0%	73.3%
3	+	2.5% (*)	55.0%	17.1%	42.9% (*)
5	-	28.6%	75.0%	50.0%	84.4%
5	+	0.0% (*)	2.7% (*)	7.1% (*)	17.9% (*)

FIGURE 5 The effect of the rous sarcoma proto-oncogene (cSRC) inhibitor dasatinib in zebrafish embryos. The effect of the cSRC inhibitor dasatinib on xenografted DsRed-labelled canine mammary tumor cell line (CMT)-U27 cells in zebrafish embryos. Three days post-injection (3 dpi) in the yolk sac of untreated cells (A) cell proliferation in the yolk sac and cells in the tail veins were observed, whereas 10 μM dasatinib-treated cells (B) remained in the yolk sac. At 5 dpi for the control cells (C), further yolk sac proliferation and clear extravasation in the tail veins were observed, whereas after dasatinib pretreatment, only limited survival in the yolk sac was observed (D). When the control cells were directly injected in the duct of Cuvier at 3 dpi (E) tumorous masses were observed to have formed in the tail veins, and extravasation was observed near the heart, which is an effect that was not observed in dasatinib-treated cells (F). At 5 dpi, tumorous masses of control cells were extended (G), whereas only some of the dasatinib-treated cells (H) ended in the tail veins but did not form tumorous masses. Statistical analysis was performed (I, J) using Fisher's exact test. *$P < 0.05$ was considered significant [Colour figure can be viewed at wileyonlinelibrary.com]

HER2 + 3 had decreased mRNA expression levels at 24 hours and decreased protein expression levels after 72 hours for all tested siRNAs. Nevertheless, these decreased expression levels were not accompanied by decreased Wnt activity, as indicated by a TCF reporter assay of target genes. There was no knockdown effect of EGFR or HER2/3 on WNT target genes, such as β-catenin, AXIN2 and cyclin D1.[56] Even when the *β-catenin* mRNA expression level decreased after *HER3* silencing, there was no inhibition of Wnt activity. Enhanced mRNA expression of *LGR5* and *LEF1* was observed after knockdown of the HER proteins, although this did not result in an increased TOP/FOP ratio or *cyclin D1* expression. Rather, the slight upregulation of *AXIN2* mRNA expression indicated limited stimulation of the Wnt pathway.[57] Resistance to inhibition with the HER2 monoclonal antibody trastuzumab in $HER2^+$ human breast cancer is related to HER3 expression and activation,[58,59] implicating a key role for HER3. However, according to our experiments, this did not seem to be related to active Wnt signalling. Recently, we showed that HER3 protein expression could be inhibited with the TKI Src-I1 in CMT-U27 cells,[20] together with inhibition of elevated Wnt activity. From our siRNA experiments in this study, we concluded that the reduction in Wnt activity is not caused by the decreased HER3 expression level.

The TKI Src-I1, which is an inhibitor of cSRC, also decreased cell proliferation but only at a rather high concentration (20 μM) of Src-I1.[20] According to Bain et al, the IC50 value of Src-I1 is approximately





(F)

		control			Injected with everolimus		
Cell line	Injection	Metastasis	Cell survival	Total	Metastasis	Cell survival	Total
CMT-U27	yolk	8	23	30	5	7	9
	duct	12	22	30	4	5	8

FIGURE 6 The effect of the phosphatidyl-3-kinase (PI3K) inhibitor everolimus in zebrafish embryos. The effect of the mammalian target of rapamycin (mTOR) inhibitor everolimus on xenografted DsRed-labelled canine mammary tumor cell line (CMT)-U27 cells in zebrafish embryos. Three days post-injection (3 dpi) of untreated cells in the yolk sac (A) cell proliferation in the yolk sac and cells in the tail veins were observed, whereas after pre-treatment with 10 µM everolimus (B), even more CMT RED cells were observed, indicating that the cells survived, proliferated and intravasated (arrows). Additionally, extravasation was more pronounced when CMT RED cells were directly injected into the circulation through the duct of Cuvier **(C)**, showing tumour cells that formed a clump halfway through the tail, and the cells with everolimus resulted in tail vein tumours and extravasation (D). Statistical analysis was performed using (E, F) Fisher's exact test. *$P < 0.05$ was considered significant [Colour figure can be viewed at wileyonlinelibrary.com]

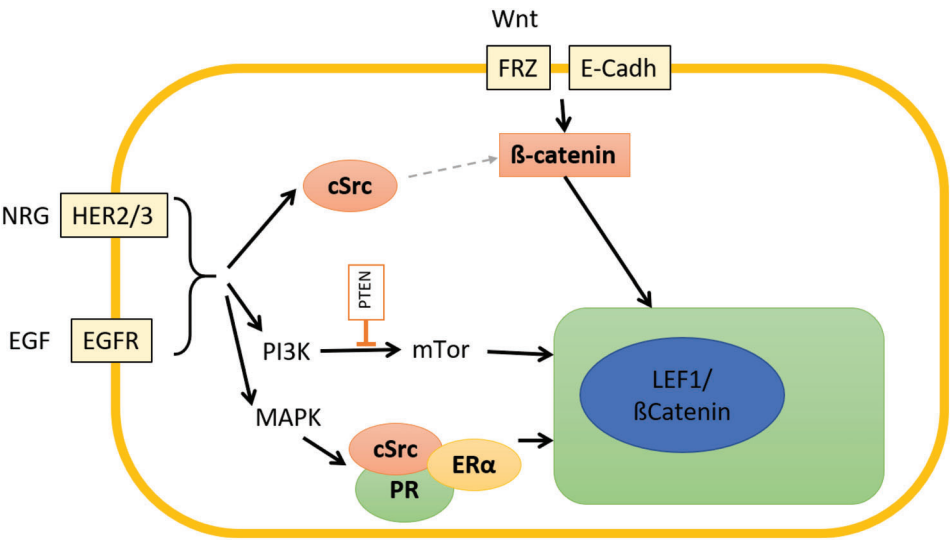


FIGURE 7 Interacting pathway scheme. The cell lines with high basal wingless-type MMTV integration site family (Wnt) activity are also characterized by human epidermal growth factor 2/3 (*HER2/3*) overexpression, high *E-cadherin* expression levels and loss of phosphatase and tensin homolog (*PTEN*) expression. The question is whether stimulation of the phosphatidyl-3-kinase (PI3K) pathway is involved in enhancing Wnt signalling through rous sarcoma proto-oncogene (cSRC) activation [Colour figure can be viewed at wileyonlinelibrary.com]

0.18 µM, which is far below the concentration that we used.[20,60] Therefore, we investigated a panel of related TKIs to find a connection between cSRC, Wnt and EGFR signalling. Afatinib, a small molecule inhibitor of HER signalling,[61] upregulated Wnt activity, most likely through inhibition of PI3K/mTOR activity, and we have also observed this with the mTOR inhibitor everolimus in the same cell line.[20] Therefore, it is highly unlikely that HER signalling plays a role in the activation of the Wnt pathway.

The cSRC inhibitors dasatinib and PP2 decreased Wnt activity in CMT-U27 cells. Both dasatinib and PP2 target members of the SRC family of protein kinases (SFK), including SRC, LYN and FYN. Activated SRC can stimulate the canonical Wnt pathway by phosphorylating Dishevelled 2,[62,63] phosphorylating β-catenin,[64] enhancing the synthesis of β-catenin,[65] or interacting with β-catenin.[66] Increased Wnt/β-catenin activity leads to elevated SLUG (SNAIL) expression that promotes EMT.[67] As expected, inhibition of Wnt activity resulted in the inhibition of the invasive and migratory ability of CMT-U27 cells. However, PI3K/mTOR inhibition also resulted in decreased migratory rates, indicating that both pathways were related to inhibition of the migratory ability of these cells. To investigate whether this also had consequences for the metastatic properties, a zebrafish xenograft experiment was performed.

For these experiments, we used dasatinib as a cSRC inhibitor and everolimus as a PI3K/mTOR inhibitor because these compounds have also been used in human clinical trials.[30,68] Cell viability and incidence of metastases were strongly reduced after pre-treatment of CMT-U27 cells with dasatinib followed by injection in the yolk sac or in the duct of Cuvier of zebrafish larvae. When cells were pre-treated with 10 μM everolimus and then injected into the yolk or duct of Cuvier larvae, survival was good, but in contrast with dasatinib, it resulted in an increased incidence of metastasis. The fact that no decrease in metastasis but rather a limited increase was observed was consistent with our findings that everolimus further upregulated the already high basal Wnt activity.[20]

It is concluded that the highly activated Wnt signalling in the CMT-U27 canine mammary cell line, which is similar to human HER2-overexpressing luminal cell lines, is not related to HER2/3 signalling. Additionally, no relation was observed between HER proteins and cSRC activation. Inhibition of PI3K/mTOR activity due to HER signalling and loss of PTEN expression was associated with enhanced metastasis in a zebrafish xenograft model.

The cSRC inhibitor dasatinib decreased Wnt signalling, migration and metastasis. The fact that the cSRC inhibitor dasatinib was effective in decreasing metastasis of our $HER2^+$ and $PTEN^-$ CMT-U27 cells makes it worthwhile to further evaluate this drug for the treatment of patients with this type of breast cancer because PTEN loss has been associated with a worse outcome in HER2-amplified human breast cancer patients.[69] Because human breast cancer patients have already been treated with dasatinib in a phase II study,[70,71] it may be of interest to relate the clinical outcome to data on HER and Wnt signalling.

ACKNOWLEDGEMENTS

We greatly acknowledge the help of Richard Wubbolts and Heiko Henning from the Utrecht University Center for Cell Imaging for technical assistance with imaging.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Timmermans-Sprang EPM, Mestemaker HM, Steenlage RR, Mol JA. Dasatinib inhibition of cSRC prevents the migration and metastasis of canine mammary cancer cells with enhanced Wnt and HER signalling. *Vet Comp Oncol*. 2019;17:413–426. https://doi.org/10.1111/vco.12490