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Dual targeting of EGFR and ERBB2 pathways produces a synergistic effect on cancer cell proliferation and migration *in vitro*

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Abstract

Members of the epidermal growth factor receptor (EGFR/ERBB) gene family are frequently dysregulated in a range of human cancers, and therapeutics targeting these proteins are in clinical use. We hypothesized that similar pathways are involved in feline and canine tumours and that the same drugs may be of clinical use in veterinary patients. We investigated EGFR and ERBB2 targeting using a panel of feline and canine cell lines. EGFR and ERBB2 were targeted with siRNAs or tyrosine kinase inhibitors (TKIs) and their effect on cellular proliferation, colony formation and migration was investigated *in vitro*. Here we report that EGFR and ERBB2 combined siRNA targeting produced synergistic effects in feline and canine cell lines similar to that reported in human cell lines. We conclude that dual EGFR and ERBB2 targeting using TKIs should be further evaluated as a potential new therapeutic strategy in feline head and neck and mammary tumours and canine mammary tumours.

Introduction

In humans, amplification or overexpression of the oncogenes epidermal growth factor receptors 1 and 2 (EGFR and ERBB2/HER2) is associated with the development and progression of certain types of aggressive breast cancer, head and neck cancer (HNSCC), ovarian, stomach and uterine cancer. They are receptor tyrosine kinases and members of the erythroblastic leukaemia viral oncogene homolog (*ERBB/EGFR*) gene family. This family consists of four members: EGFR/ERBB1, ERBB2, ERBB3 and ERBB4,[1] and are involved in a range of normal cellular processes including migration, survival, proliferation, and cell cycle progression.[2] The receptors are expressed in a wide range of epithelial and neuronal tissues.[3] Gene knockout studies have demonstrated that the gene family is crucial during development, as homozygous null mice die during early to mid-gestation due to multiple defects in epithelial organ development.[3,4]

EGFR and ERBB2 have been reported to be amplified in a number of human, canine and feline cancers.[5] EGFR has been reported to be overexpressed in feline mammary carcinomas (FMC)[6,7] and oral squamous cell carcinomas (FOSCC).[8,9] We have previously shown that knockdown of EGFR in FOSCC has an additive effect when used in combination with radiation.[10] EGFR has been reported to be both a positive[9] and negative[8,11] prognostic factor in FOSCC and increased expression was associated with decreased overall survival in FMC.[12] Feline mammary carcinomas have also been reported to have an increase in tumourigenicity with increased EGFR expression.[6]

In canine cancers EGFR overexpression has been reported in canine mammary tumours (CMT),[13–15] transitional cell carcinomas,[16,17] medullary thyroid cancer,[18] and gastric epithelial tumours.[19] Gama and colleagues[20] reported EGFR expression to be significantly associated with malignancy but could only report a trend towards reduced disease-free and overall survival. A more recent paper using enzyme-linked immunosorbent assay (ELISA) to detect EGFR levels in canine mammary tumours reported a statistically significant association between high EGFR levels and decreased disease-free and overall survival times.[21] Epidermal growth factor (EGF, the principal ligand of EGFR) was reported to stimulate proliferation, migration, angiogenesis, and survival in canine mammary carcinoma cell lines,[22] while a correlation was reported between EGFR and microvessel density in samples from malignant CMT[23] suggesting the EGFR pathways might be involved in stimulation of angiogenesis. Kennedy *et al.*[22] demonstrated that vascular endothelial growth factor (VEGF) production by a CMT cell line was stimulated by the addition of EGF and blocked by vandetanib (ZD6474), a tyrosine kinase inhibitor (TKI) that targets VEGF receptor 2 (VEGFR-2), EGFR and rearranged during transfection (RET) tyrosine kinases.

Investigations into ERBB2 expression levels have mainly been focused around FMC[24–26] and CMT.[27,28] A wide range of expression percentages have been reported following the use of multiple techniques and different interpretations. ERBB2 plays a role in normal development of mammary tissue,[29] with its overexpression reported to increase the tumour metastatic potential in human breast cancer (HBC),[30] as well as predicting response to HER2 targeting drug trastuzumab. In humans, targeting of EGFR in HNSCC and HER2 in HBC is well established. Drugs such as small molecule tyrosine kinase inhibitors (TKIs) that block the ATP binding pocket of the receptor[31] or monoclonal antibodies (mAbs) that bind directly to the receptors on the cancer cell surface blocking ligand binding and also potentially mediate an antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cell mediated phagocytosis (ADCP) effects[32] have been of value in a subgroup of patients where the tumours are dependent on the EGFR pathway. The use of these drug classes are still in their infancy in veterinary medicine.

The overall aim of this study was to investigate the potential for EGFR and ERBB2 targeting in veterinary medicine using a panel of feline and canine cell lines. EGFR and ERBB2 were targeted with siRNAs or TKIs, and their effect on cellular proliferation, colony formation and migration was investigated. Potential synergistic effects of dual targeting of the receptors was investigated in accordance to the Bliss Additivism model.[33] Here we report that EGFR and ERBB2 combined targeting by siRNAs produced a synergistic effect. We conclude that dual targeting of EGFR and ERBB2 should be further evaluated as a potential new therapeutic strategy in feline and canine head and neck and mammary tumours.

Materials and methods

Cell culture and reagents

Cell culture reagents were obtained from Gibco Thermo Fischer Scientific (Paisley, UK) unless otherwise specified. The SCCF1 cell line is a previously characterised feline cell line derived from a laryngeal squamous cell carcinoma and was a gift from Professor T.J. Rosol, Ohio State University, USA.[34] The feline SCCF-SMG cell line was isolated from a bone-invasive FOSCC, and has been characterised in our laboratory (unpublished data). Both were grown in William's E Medium with GlutaMAX supplemented with 10% fetal bovine serum (FBS), 0.05 mg/mL gentamicin and 10 ng/mL EGF.

The CatMC[35] (feline mammary adenocarcinoma) and REM134[36] (REM, canine mammary adenocarcinoma) cell lines were previously characterised and generously gifted by Professor R. Else, R(D)SVS, The University of Edinburgh, UK. The LILLY cell line (also from a canine mammary tumour) was generously provided by Dr R. De Maria, Department of Animal Pathology, University of Torino, Italy and was derived from a grade three simple carcinoma from a 13-year-old mixed breed entire female. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with added GlutaMAX, supplemented with 10% FBS and 5 mL of 100 μl/mL penicillin and

streptomycin. The media was changed and the cells washed with phosphate buffered saline (PBS) every 48 h until confluent. Once confluent, cells were re-seeded by washing with PBS and adding 3 mL of Trypsin-EDTA 0.25% to T75 flasks and incubated until detachment. The human breast cancer cell lines SK-BR-3 and BT474 cell lines were both purchased from ATCC, USA and cultured as above and according to manufacturer's recommendations.

RNA interference

A previously published and validated siRNA against feline *EGFR* (GenBank accession numbers HQ185236.1 and KR811314.1)[10] was validated for the use in dog cells together with new siR-NAs designed against a feline *ERBB2* sequence we previously obtained and published on GenBank (accession number: KC710349.1). Multiple siRNA sequences against *ERBB2* were designed using a combination of online design tools including the siDESIGN Centre (Thermo Scientific, 2013) and i-Score designer (i-score web service programme, 2009). Potential siRNAs were evaluated for GC content and Reynolds scores were obtained.[37] BLAST searches were then performed for selected siRNAs against the feline whole genome sequence (wgs) and nucleotide collection.[38] The selected siRNAs were constructed using Ambion Silencer® siRNA Construction Kit (Life Technologies™, Thermo Scientific) with DNA oligonucleotide templates purchased from eurofins MWG Operon (eurofinsdna.com, Ebersberg, Germany). Commercially available negative (Silencer® Negative control number 1, Life Technologies) and positive [glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA ON-TARGET™ *plus* GAPDH Control Pool, Life Technologies, Thermo Scientific] control siRNAs were purchased. The positive and negative controls had been previously validated for use in the dog and cat (data not shown).

Transfections

Cells were seeded into 96 or 6 well plates at 1.5×10^3 or $1–2 \times 10^5$ cells per well, respectively in their respective media containing no antibiotics. The cells were incubated overnight to reach approximately 30–50% confluence the following day when

they were transfected according to manufacturer's protocol using Lipofectamine RNAiMAX™ and OptiMEM® I Reduced-Serum media (both from Life Technologies, Thermo Scientific) using individual concentrations of siRNAs of 60 nM. When a siRNA cocktail was used in the dual transfection experiments, each siRNA was used at an individual concentration of 20 nM. These concentrations were selected following multiple optimisation steps using quantitative polymerase chain reaction (qPCR) and western blot analysis.

Protein detection

Cells were lysed in urea lysis buffer (7 M urea, 0.1 M DTT, 0.05% Triton X-100, 25 mM NaCl, and 20 mM Hepes pH 7.5). Equal amounts of protein were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and transferred to Amersham Hybond-C nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) and hybridised to an appropriate primary antibody and HRP-conjugated secondary antibody for subsequent detection by enhanced chemiluminescence (ECL). Nitrocellulose membranes were ink stained in Ponceau S solution for visualisation of protein bands and conformation of equal loading. Non-specific antibody binding was blocked by incubating the membranes in PBS Tween (PBST) with 5% milk (PBST/5% milk) for 1 h at room temperature. When probing for phosphorylated proteins the blocking solution had 1% 1 M β-glycerophosphate added to it. Primary antibodies against pEGFR (mouse phospho ab24918), pERBB2 (rabbit phospho Y877) and β-actin (mouse ab6276) were purchased from Abcam® (Cambridge, UK), EGFR (mouse ab-12 cocktail R19/48) from Thermo Fisher Scientific, GAPDH (mouse 6C5, CB1001) from Merck Millipore (Hertfordshire, UK), and c-erbB2 (rabbit A0485) from DakoCytomation (Glostrup, Denmark). Secondary HRP-conjugated antibodies were rabbit anti-mouse IgG and swine anti-rabbit IgG obtained from DakoCytomation (Glostrup, Denmark). The antibody dilutions, incubation times and temperatures for the different antibodies are shown in Table 1. Membranes were stripped for reprobing using Restore™ Plus Western Blot Stripping

Table 1. Summary of antibody dilutions, incubation times and conditions used for western blot analysis

Antibody	Raised in species	Dilution	Incubation time
Anti-EGFR	Mouse	1:50	Overnight at 4 °C
Anti-EGFR (phospho)	Mouse	1:200	4–5 h at room temperature or overnight at 4 °C
Anti-ERBB2	Rabbit	1:1000	3–4 h at room temperature or overnight at 4 °C
Anti-ERBB2 (phospho)	Rabbit	1:500	3–4 h at room temperature or overnight at 4 °C
Anti-GAPDH	Mouse	1:1000	1 h at room temperature
Anti-β actin	Mouse	1:10,000	1 h at room temperature
Anti-mouse	Rabbit	1:1000	1 h at room temperature
Anti-rabbit	Swine	1:1000	1 h at room temperature

Buffer (Thermo Fisher Scientific) according to manufacturer's recommendations.

Immunocytochemistry

Cells were seeded into chamber slides at a density of $3–5 \times 10^4$ cells per well in a total of 200 μL media and incubated at 37 °C/5% CO_2 for 24 h. Cells were fixed in cold acetone and incubated with an appropriate primary antibody (anti-EGFR and anti-ERBB2 antibodies as listed in Table 1) for subsequent detection. Secondary antibodies goat anti-mouse AlexaFluro© 488 IgG, IgA, IgM (A11011) and goat anti-rabbit AlexaFluro 568 IgG (A10667) both from Life Technologies were incubated at room temperature for 1 h in a dark, humid chamber. Cover slips were removed, slides were washed and mounted using anti-fade mounting media containing DAPI (Vectorshield®, Vector Laboratories Inc, Burlingame, CA, USA) and visualized with a Leica DMLB2 microscope fitted with a digital ORCA-ER digital camera and Lumencor Spectraz LED light engine®.

Real-time PCR (qPCR)

All qPCR reactions were performed on the Roche LightCycler® 480 machine following manufacturer's instructions. The most stably expressed reference genes hypoxanthine-guanine phosphoribosyltransferase (HPRT) and GAPDH were selected using geNorm™ Reference Gene Selection Kit (Primerdesign) with SYBR® green. Hydrolysis probes and primers were individually designed, tested and supplied by Primerdesign based on previously published sequences (NCBI references EGFR GenBank accession numbers: HQ185236.1

and KR811314.1, and ERBB2 GenBank accession number KC710349.1). RNA was extracted using RNeasy Mini Kit QIAshredder according to manufacturer's protocol and first strand cDNA synthesis was performed using Qiagen Omniscript® Reverse Transcription Kit (both from Qiagen, Manchester, UK).

The qPCR reactions were performed using PerfectProbe™ detection chemistry (Primerdesign) on triplicate replicates from untreated, scrambled, EGFR and ERBB2 transfected cells according to manufacturer's instructions. Primers used were EGFR sense 5′ ACTGTACCTACGGCTGTTCT 3′ and antisense 5′ CCACCACCACCACCAAGA 3′. ERBB2 sense 5′ ACAGCACTTTCTACCGTTCA 3′ and antisense 5′ AGGGTCTGGGCAGAAGAA 3′. Cycling conditions are provided in Table 2. Analysis of relative gene expression levels were performed using crossing point (Cp) values obtained for each target and reference gene using qbase[plus] (Biogazelle). The delta Cp value for each sample was determined and the relative expression level of the target gene was calculated according to previously described methods.[39]

Drug treatment of cells

Three commercially available TKIs were used: gefitinib (EGFR specific), AG825 (ERBB2/HER2 specific) and GW583340 (dual EGFR and ERBB2) all supplied by Tocris Biosciences, Bristol, UK. TKIs were supplied in powdered form and each drug was separately dissolved in dimethyl sulphoxide (DMSO) to create stock solutions of 10 mM. Aliquots were made and stored at −20 °C. The cell lines were treated with each TKI at a range of concentrations (0.1-150 μM) as indicated.

Table 2. Summary of qPCR cycling conditions

Programme name	Target temperature (°C)	Ramp rate (°C s^{-1})	Hold	Number of cycles
Pre-incubation	95	4.4	10 min	1
Amplification	95	4.4	15 s	40
	50	2.2	30 s	
	72	4.4	15 s	
Cooling	40	2.2	10 s	1

Cell proliferation assays

Cells were grown in the media as stipulated previously during all experiments, except for when their basal proliferation rate in response to EGF was determined. The cells were grown in their respective media with no serum added, and either a range of EGF (5, 10 or 50 ng/mL) was added to the 96-well plates for 24 h, or 50 ng/ml was added and the cells were cultured for up to 48 h. Cells were seeded at 5×10^3 cells per well and incubated at 37 °C/5% CO_2. Cell proliferation was assessed at 24 and 48 h as measured by proxy by reading absorbance at 490 nm using Wallac 1420 Manager programme (Perkin Elmer) following treatment with CellTitre 96® AQueous One Solution (Promega, Southampton, UK) according to manufacturer's protocol.

For all other experiments, cells were seeded into 96-well plates at a density of 1.5×10^3 cells per well and incubated at 37 °C/5% CO_2 for 24 h in their respective media with serum. Cells were then treated with TKIs at the indicated concentrations or were transfected with the indicated concentrations of siRNA. Cell proliferation was assessed at specified time points up to 96 h following drug exposure or transfection as described above.

Colony formation assays

Twenty-four hours following transfection or drug treatment cells were seeded into 10 cm plates at a density of 300–500 cells per plate and incubated at 37 °C/5% CO_2. Plates were checked for colony formation every 2 days. When visible colonies had formed (approximately 5–10 days) the cells were fixed with methanol and stained with 10% Giemsa stain for counting. The colonies were electronically counted and photographed using the CCD digital camera (Bio-Rad, Hemel Hempstead, UK) (Fig. 2E).

Cellular migration assay

Cells were seeded into 6-well plates at a density of 2.0×10^6 cells per well and incubated at 37 °C/5% CO_2 to achieve 100% confluence the following day. A scratch assay was performed as previously described.[40] Briefly, cells were treated with indicated concentrations of TKIs or 24–48 h following siRNA transfections, as previously described, and a scratch was made using a pipette tip. At regular time intervals phase contrast images of the cell monolayer were captured with a monochrome digital Axiocam camera fitted to a Zeiss Axiovert40 CFL (Carl Zeiss Ltd, Cambridge, UK) microscope until the gap had been filled. The gap width was measured at 10 different points for each image and the mean calculated for each well at each time point and expressed as the relative migratory distance.

Statistical analysis

One-way ANOVA or the nonparametric equivalent Kruskal–Wallis were used to compare differences between more than two samples. Two sample t-tests or the nonparametric equivalent Mann–Whitney U test were used to compare differences between two samples. Results were considered significant when $P < 0.05$. All statistical analysis was performed using Minitab® 17 Statistical software (Minitab Ltd.) and all graphs were generated using Microsoft® Office Excel 2013 software.

Results

All tumour cell lines expressed EGFR and ERBB2

Both the dog and cat cell lines showed an increase in cell proliferation in response to increasing EGF concentrations when grown in serum-free media with EGF only. They also showed sustained proliferation over a 48-h period when maintained

in serum free media containing 50 ng/mL EGF (data not show). All five cell lines expressed the two proteins at various levels as seen using western blot analysis (Fig. 1A). The two canine mammary tumour cell lines (REM and LILLY) only had faint bands present in the western blots, and when normalized to the β-actin controls (Fig. 1B) the LILLY cell line (both proteins) and REM cell line (EGFR only) had comparatively the lowest expression levels. To confirm protein expression immunocytochemistry was performed and compared with the feline SCCF1 cell line which has been previously extensively validated.[9] Immunocytochemistry revealed that the proteins were primarily localized to the cell membrane in the SCCF1 cell line (Fig. S1, Supporting Information), while in the REM and LILLY cell lines the protein expression was weaker and predominantly throughout the cytoplasm of the cells. To confirm expression in these cell lines RT-PCR was performed using previously validated PCR primers[10] (GenBank accession number HQ185236.1 and KC710349.1). These produced bands of the expected sizes as visualized by agarose gel electrophoresis (data not shown).

The siRNAs successfully reduced mRNA and protein levels of their targets

A range of siRNAs against both targets were produced and tested in the SCCF1 cell line. They produced variable levels of mRNA knockdown when validated by qPCR at different siRNA concentrations (20–100 nM). The siRNAs produced dose dependent effects in the cell line (from 20% to up to 75% reduction). The optimum concentration for individual siRNA transfections was determined to be 60 nM (data not shown) at 72 h following transfections. The siRNAs that produced the greatest reduction in mRNA levels of their specific targets are shown in Fig. 1C and Table 3. Reduction of EGFR and ERBB2 protein levels were confirmed using western blot analysis in both cat (Fig. 1D–F) and dog cells (Fig. 1E).

Targeting EGFR caused anti-proliferative effects, reduced colony formation and migratory ability *in vitro*

To initially validate the effect of *EGFR* and *ERBB2* silencing, we used three of the cell lines (SCCF1,

CatMC and REM). Silencing of *EGFR* significantly reduced cellular proliferation in all three cell lines ($P < 0.001$ all three cells lines, Fig. 2A,C,D). *ERBB2* silencing caused a variable response with some reduction in cellular proliferation observed in the CatMC cell line ($P < 0.001$) but no effect could be demonstrated in the SCCF1 or REM cell lines (Fig. 2B-D). Similarly, *ERBB2* targeting produced some reduction in colony formation ability in the SCCF1 cell line only ($P > 0.001$) and no effect in the other two cell lines, but all three cell lines responded to *EGFR* targeting (Fig. 2F,G, $P < 0.001$, Fig. 2H, $P = 0.002$). Evidence of reduced migratory ability following *EGFR*, but not *ERBB2* targeting was also observed in the SCCF1 cell line (Fig. 2I–J). It was not possible to perform scratch assays post-transfection in the two mammary cell lines due to their significantly reduced migration rate compared to the SCCF1 cell line (data not shown).

Low dose dual siRNA targeting caused synergistic effect *in vitro*

We then tested the effect of targeting the two receptors simultaneously. The siRNA against *EGFR* was so effective at the optimal dose of 60 nM that it completely blocked the cell proliferation in the cell lines (Fig. 2), so in order to show an effect of the addition of *ERBB2* we had to reduce the concentration of siRNAs to a third of their optimum siRNA concentrations. During the optimisation we had shown that the siRNAs were effective at reducing the mRNA levels in a dose responsive manner, with 20 nM concentrations causing approximately 20% reduction in mRNA levels after 24 h (data not shown). Transfecting the cell with these low siRNA concentrations also ensured that the total siRNA concentration used was not increased over what had been used during optimisations and transfections with individual siRNAs to avoid potential off target effects due to an overall higher dose of RNA. Dual targeting of both *EGFR* and *ERBB2* using low doses of siRNA produced a greater fractional inhibition of cellular proliferation than individual targeting of each receptor (Fig. 3A, $P < 0.001$) when compared with scrambled controls. The predicted effect of combined targeting was calculated according to the Bliss additivism model[41] and indicated that the
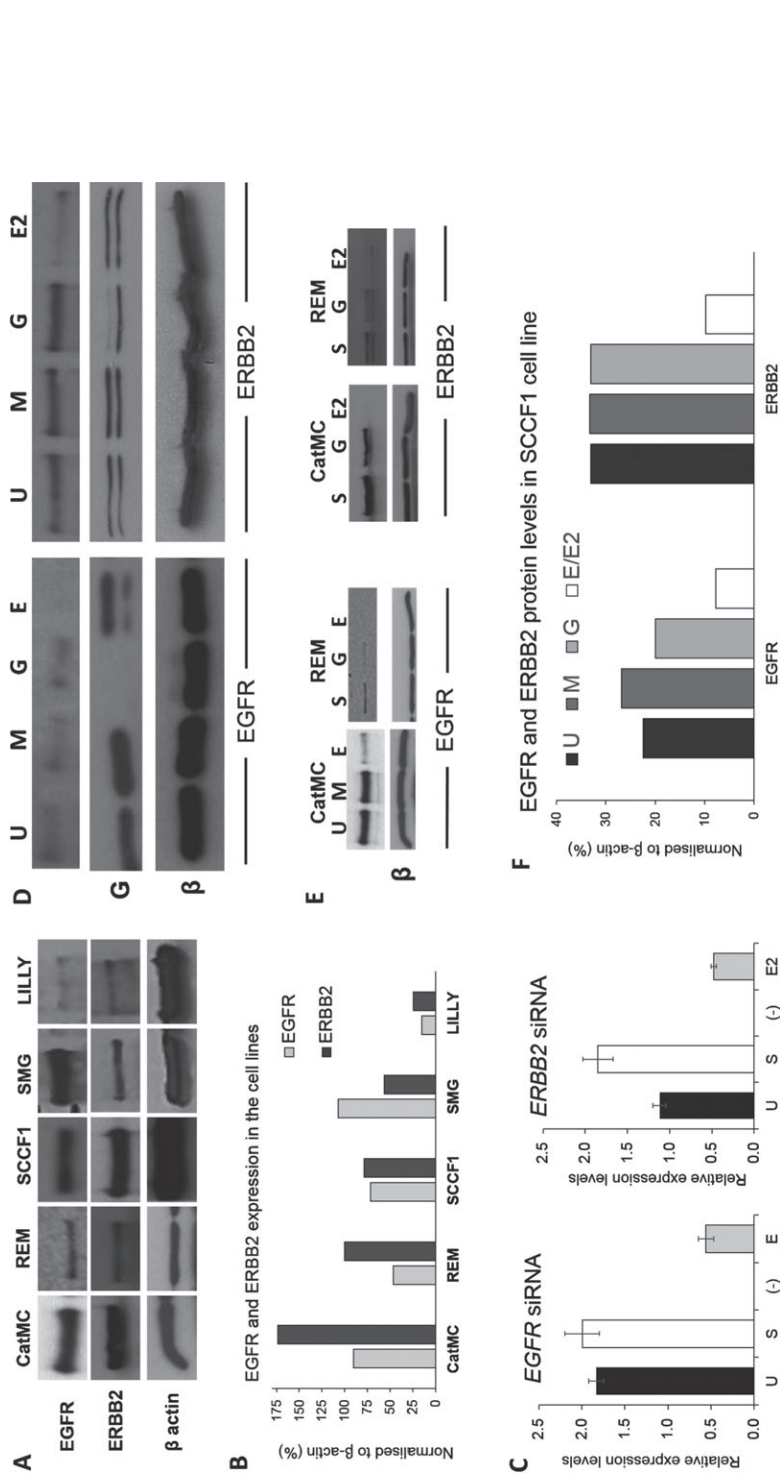
Figure 1. Confirmation of EGFR and ERBB2 expression in the cell lines and validation of siRNAs. (A) Western blot analysis of cell lysates from all cell lines showing expression of EGFR (170 kDa) and ERBB2 (180 kDa) proteins. β-actin (42 kDa) is shown as loading control. (B) Image J analysis of protein bands from the western blots in (A) normalized to their loading controls show relative protein expression between the cell lines. (C) Relative expression levels of EGFR (left) and ERBB2 (right) mRNA levels 72 h following siRNA transfections. The best siRNA against EGFR (E) reduced the mRNA levels by approximately 75% compared with scrambled control transfected (S) and untreated (U) cells. The best siRNA against ERBB2 (E2) produced approximately 75 and 50% reduction in mRNA levels when compared with scrambled (S) transfected and untreated (U) cells, respectively. Examples shown is from the SCCF1 cell line, (–) shows negative controls, bars show standard errors of the mean. (D) Western blot analysis of protein levels of EGFR (left) and ERBB2 (right) 72 h following transfection in the SCCF1 cell line, (E) in the CatMC cell line (blots to the left) and REM canine cell line (blots to the right). U, untreated cells; M, mock transfected cells; S, scrambled control transfected cells; G, GAPDH positive control siRNA transfected cells; and E, EGFR, E2, ERBB2 transfected cells. Top row shows EGFR or ERBB2 as indicated, rows marked G show positive control GAPDH (37 kDa), and rows marked β show β-actin loading controls. (F) Image J analysis of western blots seen in (D) showing the reduction in EGFR and ERBB2 protein levels when normalized to the loading control.
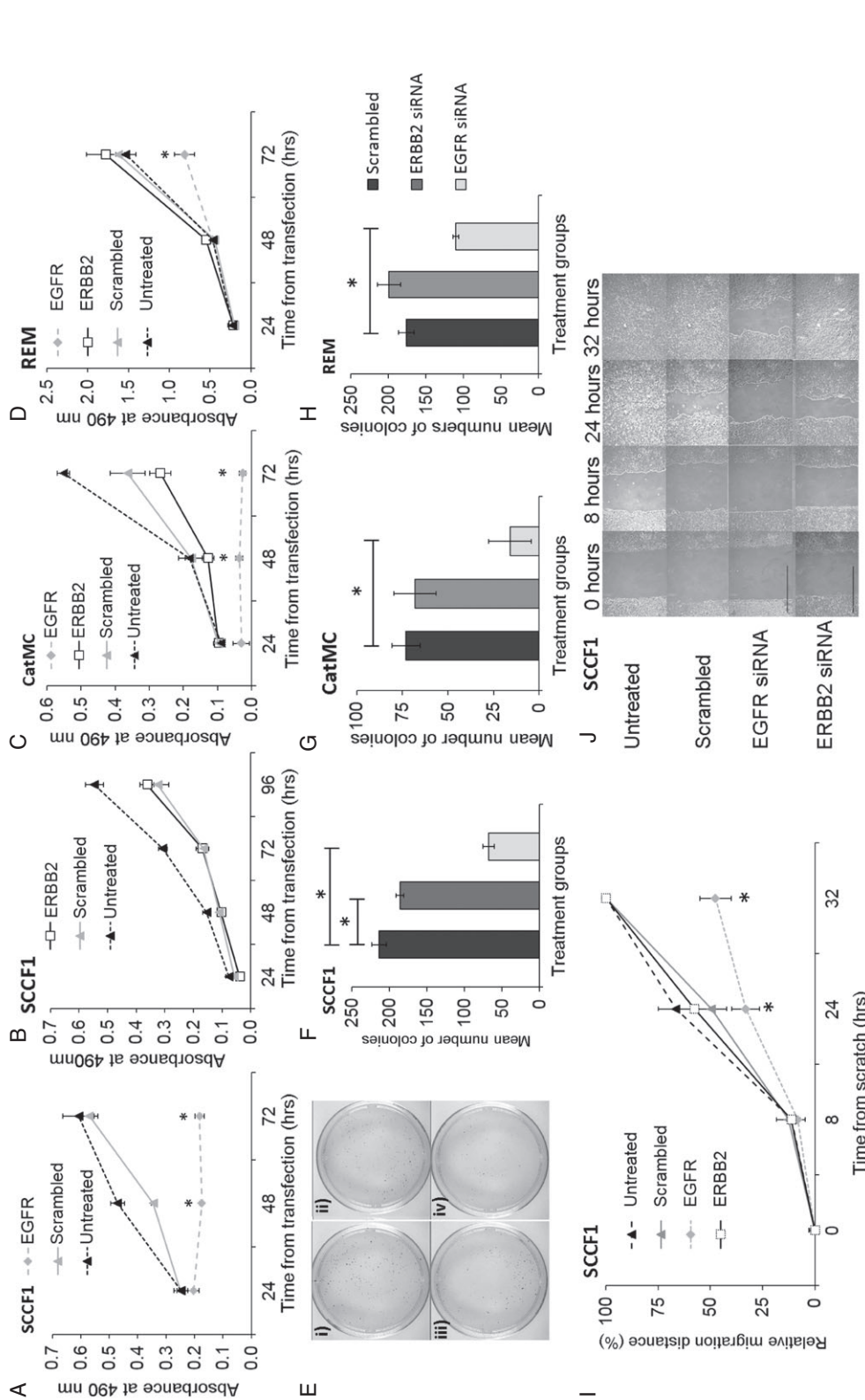
Figure 2. Effect of EGFR and ERBB2 silencing on the cell lines. (A–D) EGFR silencing significantly reduced proliferation in all three cell lines, while ERBB2 silencing had a marginal effect in the CatMC cell line only. (A) and (B) SCCF1 cell line *P < 0.001 by one-way ANOVA at 48 and 72 h following transfections. (C) CatMC cell line, *P < 0.001 by one-way ANOVA at 48 and 72 h following transfections. (D) REM cell line, *P < 0.001 by one-way ANOVA at 72 h following transfections. (E) Representative images showing colonies formed in the SCCF1 cell line following no treatment (i), scrambled transfected (ii), ERBB2 (iii) and EGFR (iv) siRNA transfections. (F–H) Colony formation assays of the three cell lines: EGFR knockdown significantly reduced the colony formation ability of the cell lines, while ERBB2 knockdown only had a marginal effect in the SCCF1 cell line, and no detectable effect in the mammary cell lines. (F) *P < 0.001, (G) *P < 0.001, (H) *P = 0.002, by two sample *t*-test. (I) Migration assays in the SCCF1 cell line following siRNA transfections. EGFR silencing reduced the relative migration distance whereas ERBB2 targeting had no effect on migration. *P < 0.001 by one-way ANOVA (24 h) and Kruskal–Wallis (32 h). (J) Representative images from SCCF1 migration assay. All transfections performed with siRNA concentrations of 60 nM. Error bars show standard deviations.

Table 3. Sequences of siRNA designed against the *ERBB2* and *EGFR* genes

siRNA	siRNA sequence antisense strand 5′–3′	Target	GC (%)	BLAST homology (nt collection)	BLAST homology (whole genome)
ERBB2 2	UUGACAACCCCAUUCUUGCUU	ERBB2	43	14/21	18/21
EGFR 8	AGCUUCAUCAAGGAUUUCCUU	EGFR	38	17/21	17/21
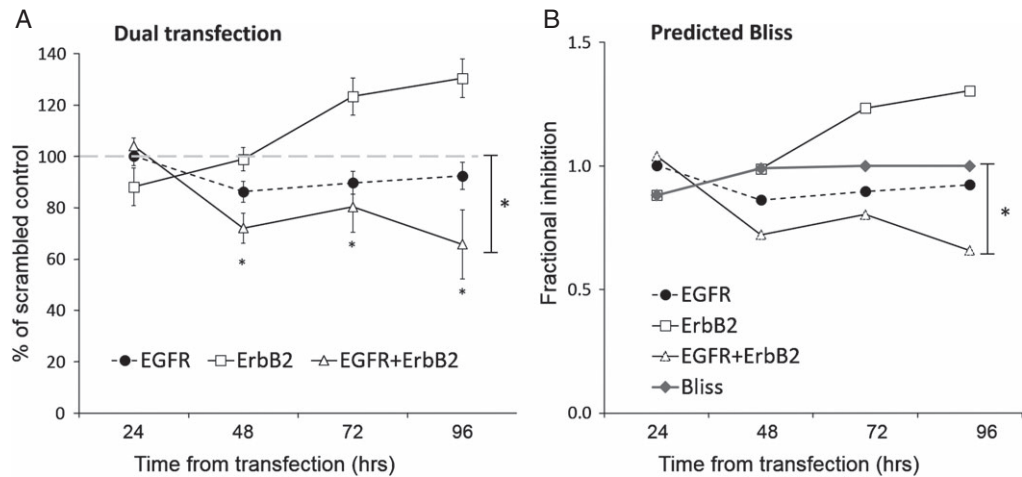
Figure 3. Effect on cellular proliferation when both receptors are targeted simultaneously in the SCCF1 cell line. (A) When the cells were transfected with siRNAs at the lower concentration of 20 nM individually the effect on cell proliferation was absent (ERBB2) or negligible (EGFR). When the two siRNAs (at 20 nM concentration each) were combined a greater reduction in cellular proliferation was observed, $*P < 0.001$ by one-way ANOVA. (B) The Bliss graph shows the predicted effect on fractional inhibition based on the fractional response to each siRNA individually. A fractional inhibition equal to Bliss indicates additive effects and a greater fractional inhibition as demonstrated here indicates synergistic effects of dual targeting (*).

combined targeting of *EGFR* and *ERBB2* caused a synergistic effect in the SCCF1 cell line (Fig. 3B).

Human TKIs blocked EGFR and ERBB2 phosphorylation *in vitro*

To investigate if the same effects could be achieved by using readily available drugs we selected three TKIs developed to block the ATP binding pockets of the equivalent human proteins: gefitinib (specific EGFR inhibitor), AG825 (specific HER2/ERBB2 inhibitor), and GW583340 (dual inhibitor of EGFR and HER2/ERBB2). These were validated for use in feline cells by performing phosphorylation assays following serum starvation. The cells were treated with EGF/serum with or without the TKI drugs, and the levels of phosphorylation of the receptors were determined using western blot analysis (Fig. 4). The receptors from the feline cells became phosphorylated in response to EGF and serum,

but if the equivalent TKI was added no change in phosphorylation status were observed. This is consistent with the respective TKIs blocking phosphorylation of the human equivalent receptors in the feline cells. This was expected, as the ATP binding pocket of the cat and dog receptors share a 100% amino acid sequence homology with the human amino acid sequences and a 99% amino acid sequence homology with the entire tyrosine kinase domain of the receptors [GenBank Accession numbers HQ185236.1 and AY527212.1 (EGFR), NM_001048163.1 and NM_001003217.1 (ERBB2), respectively]. We therefore extrapolated that the TKIs would be effective in dog cell lines as well.

Human TKIs blocked proliferation and caused a reduction in colony formation and migratory ability *in vitro* in a panel of cell lines

Drug assays were performed in the three cell lines to determine the relative effect of the drugs on
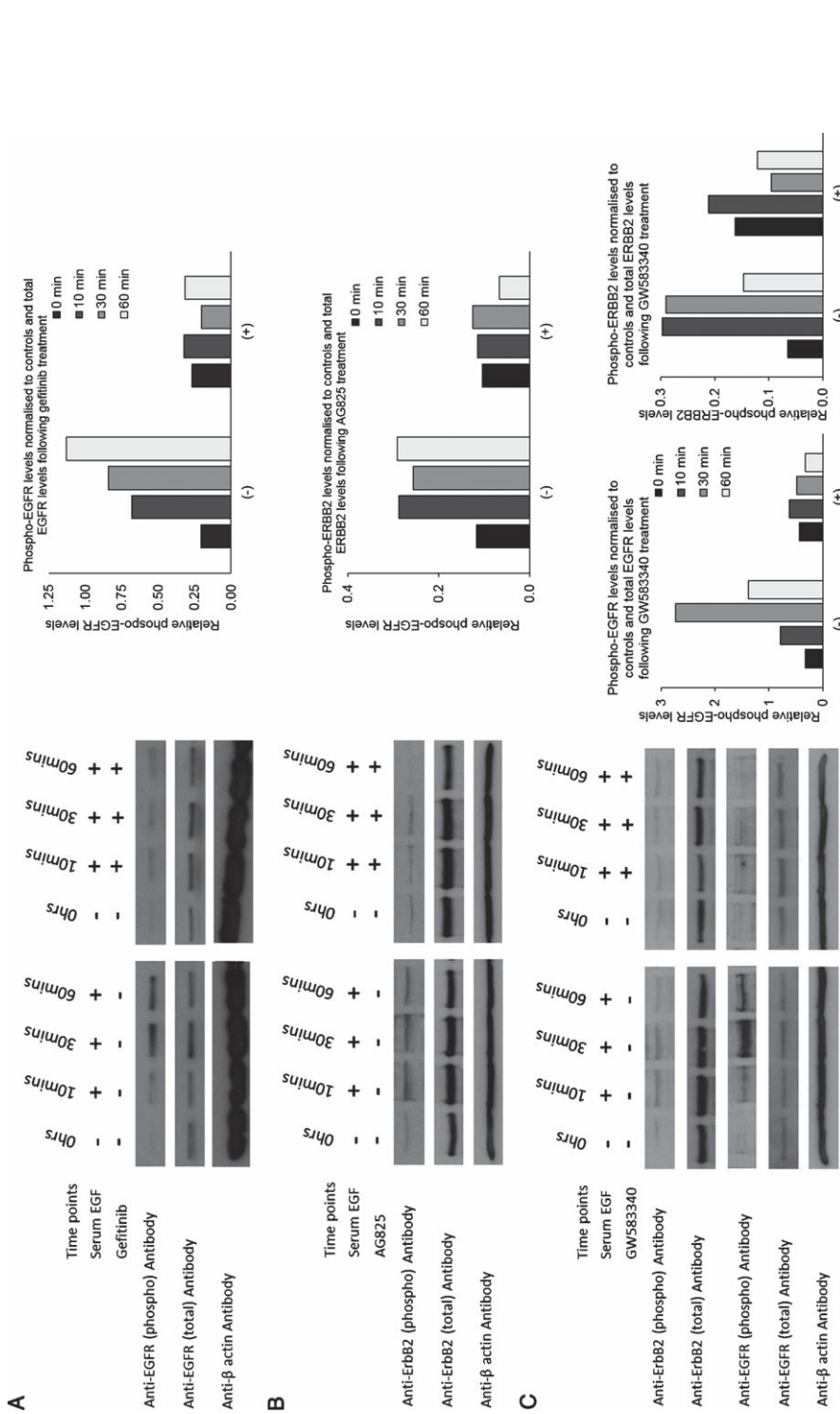
Figure 4. TKIs effectively block receptor phosphorylation. Left panels (A–C) show western blots showing the effect on receptor phosphorylation when serum starved SCCF1 cells were treated with EGF/serum. The addition of EGF/serum increased phosphorylation levels of both receptors. Total receptor levels remained relatively constant. When the cells were simultaneously treated with serum/EGF and the indicated TKIs (right panels A–C) no increase in phosphorylation levels were apparent. The β-actin bands show equal loading. On the right Image J analysis of each western blot is shown. All bands (both total and phospho bands for each receptor) were normalized to the β-actin loading controls, and then the normalized values of the phospho bands were divided by the normalized values of the total receptor bands to give the relative phospho levels for each receptor. (+) EGF/serum and drug treated cells, (−) EGF/serum only treated cells, 0 min represents the serum starved cells. All TKIs were used at 10 μM.
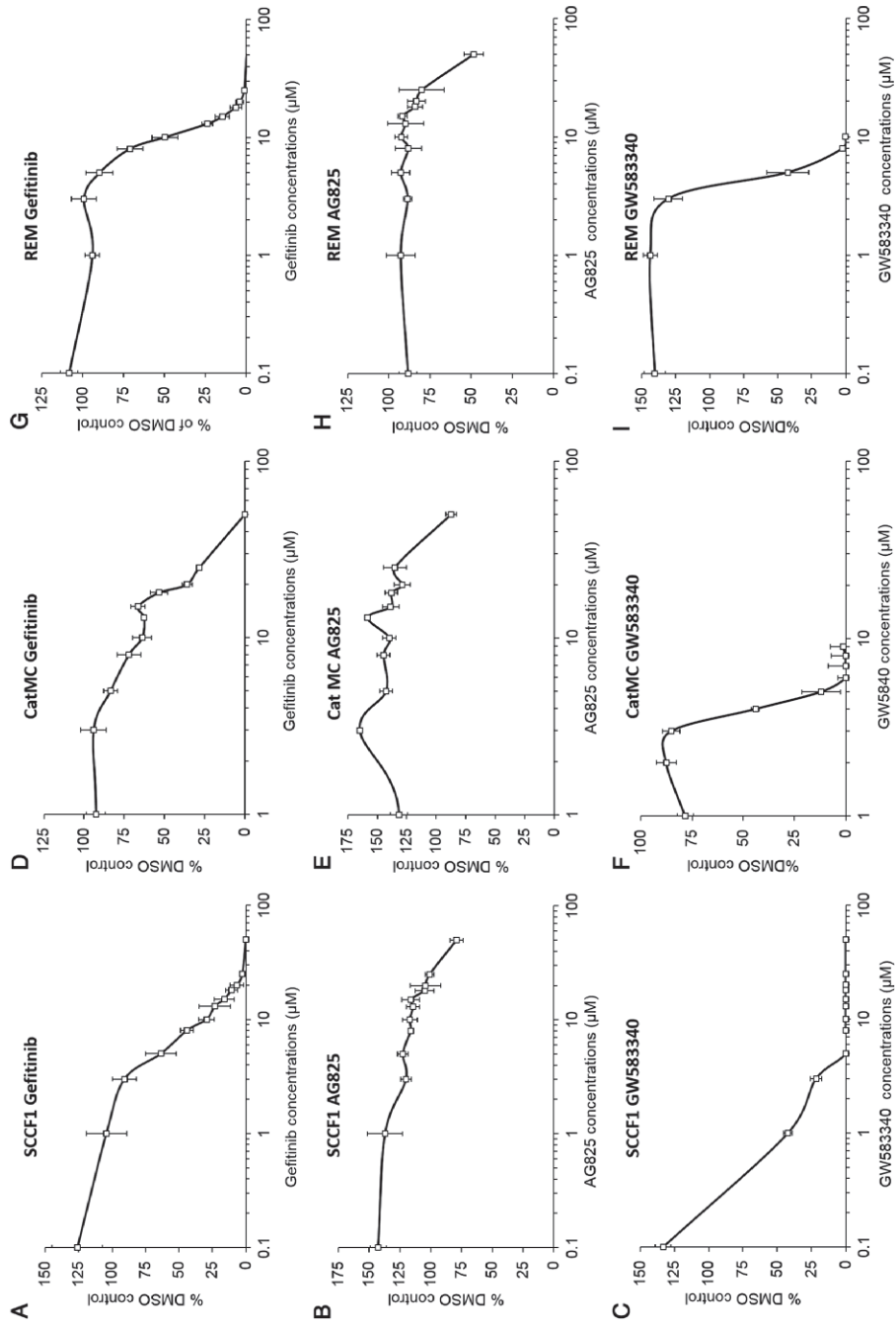
Figure 5. Dose response curves to the TKIs based on cellular proliferation assays: SCCF1 (A–C), CatMC (D–F) and REM (G–I) following 72 h of treatment with gefitinib (A, D, G), AG825 (B, E, H), and GW583340 (C, F, I). Drug concentrations are given in log scale on the x-axis and the y-axis represents percentage proliferation as compared with DMSO control treated cells. Error bars show standard deviations. Graphs show representative results from one of a minimum of three independent experiments.

Table 4. Approximate IC50 estimations for the cell line panel based on cell proliferation assays performed using free online IC50 toolkit (http://www.ic50.tk/)

	Cell lines				
Drugs	SCCF1	SCCF-SMG	CatMC	REM	LILLY
Gefitinib	5.1 µM	27.7 µM	18.3 µM	28.3 µM	22.8 µM
AG825	_ [a]	_ [a]	_ [a]	_ [a]	_ [a]
GW583340	0.6 µM	6.8 µM	1.1 µM	8.4 µM	7.5 µM

[a]IC50 doses for AG825 could not be determined as we could not achieve complete response to the drug at the concentration range tested. Testing values above this range was deemed irrelevant (i.e. >50–100 µM) as such high doses equivalents would never be achievable *in vivo*.

cellular proliferation (Fig. 5). Similar to what was observed when using the siRNAs, the cell lines were relatively more sensitive to EGFR inhibition than ERBB2 inhibition when targeting the receptors individually. The dual inhibitor, however, reduced cellular proliferation most effectively, with the lowest IC$_{50}$ estimated for all three cell lines (Table 4). As these are unrelated compounds, the difference in IC$_{50}$ could also be due to difference in target affinity between the compounds. The results do however exactly mirror what was observed following siRNA transfections targeting the receptors independently and combined.

For comparison, two human breast cancer cell lines with known EGFR/ERBB2 status were also treated with the same drugs, and a similar pattern was observed with minimal response to ERBB2 inhibition on its own at the concentrations 0.01-10 µM, moderate reduction in cellular proliferation with EGFR inhibition while dual receptor inhibition produced the most profound effect on cellular proliferation of all (Fig. 6).

Colony formation assays following drug treatment of the cell lines showed the same pattern as observed following siRNA transfections (Fig. 7). EGFR blockage was effective in reducing the colony formation ability of the cell lines whereas ERBB2 had no effect.

The migratory abilities of the cell lines were highly variable. The two mammary carcinoma cell lines exhibited a relatively low propensity towards migration, even when untreated (data not shown). After 35–48 h both untreated and DMSO control treated cells (as shown in Fig. 8) would only on average have migrated a third to approximately half of the distance compared with the squamous cell carcinoma cell line which was highly mobile and would

close the 'wound' in less than 24 h. The reduction in migratory distance was therefore much easier to assess in the latter. At 8 h the DMSO and AG825 treated SCCF1 cells would have migrated on average a quarter of the distance whereas the gefitinib and GW583340 treated cells would have migrated less than one tenth of the distance. After 24 h the DMSO and AG825 treated cells had closed the 'wound' while the cells treated with the EGFR and dual inhibitors would only have covered a quarter of the distance (Fig. 8). A similar response was observed in the CatMC cell line, but it was not dose dependent. At 24 h the EGFR and dual inhibitor treated cells had only migrated a third of the distance of the DMSO-treated cells. The REM cell line was the least migratory of all which made assessment difficult as in 35 h the DMSO-treated cells would only have migrated approximately a third of the distance. A slight response to ERBB2 and dual inhibition was observed ($P < 0.001$), and although this was statistically significant this is unlikely to be biologically significant as the overall migration of this cell line was minimal. Overall, dual targeting produced similar effects as EGFR targeting alone, and did not appear to confer any further benefits on cellular migration in the cell line. We then tested the effects of the TKIs on the complete panel of cell lines (Fig. 9). Consistently, the cell lines were more sensitive to EGFR targeting (singly or in combination) and produced response curves to the left of the ERBB2 targeting TKI. Although the different target affinity of these compounds cannot be dismissed as a potential contributing factor in the effect observed, this pattern exactly mirrored what was observed when targeting the receptors individually and combined using the siRNAs.
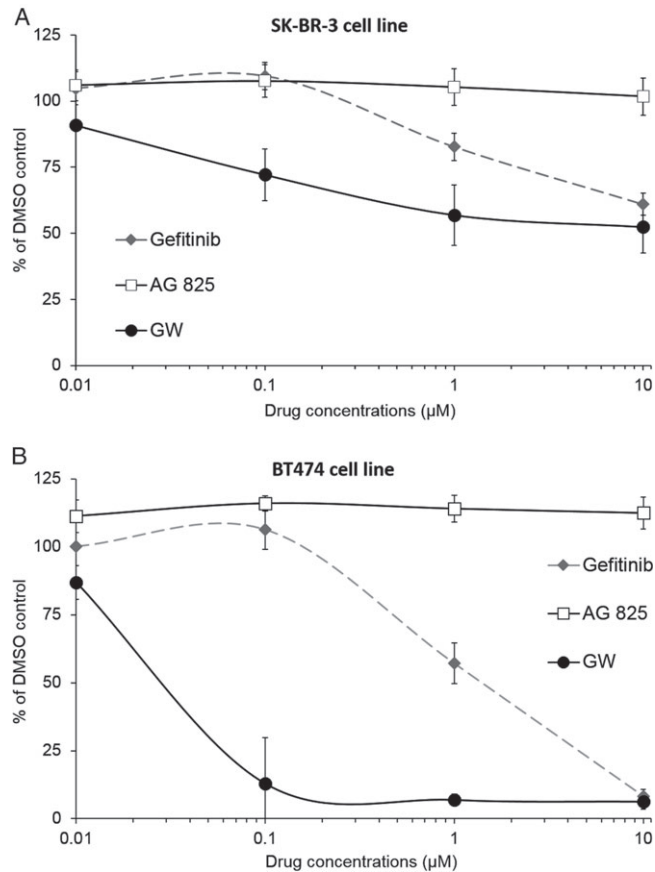
Figure 6. Dose response curves to the TKIs for the human breast cancer cell lines. (A) SK-BR-3 and (B) BT474 60 h following treatment with gefitinib, AG825, or GW583340. Drug concentrations are given on the *x*-axis and the *y*-axis represents percentage proliferation as compared with DMSO control treated cells. Error bars show standard deviations. Graphs show representative results from one of a minimum of three independent experiments.

Discussion

Both the feline and the canine cell lines had the ability to proliferate for up to 48 h when grown in serum-free media containing EGF, and an increase in proliferation rate was also observed in a dose dependent manner to the addition of increasing concentrations of EGF to the media, indicating the cells harboured a functional EGFR pathway. All five cell lines we tested expressed EGFR and ERBB2 but to varying degrees. Both mammary carcinomas and oral squamous cell carcinomas are tumours with reported overexpression of EGFR and ERBB2, albeit with widely variable levels of overexpression as assessed by immunohistochemistry (IHC). The use of a standardised human test for the assessment of both ERBB2 and EGFR levels (Hercept Test™, DAKO, Glostrup, Denmark) for mammary

tumours is widely reported, although the interpretation of expression levels differ. A recent paper[42] sets out guidelines for how the test should be used in canine mammary tumours when assessing ERBB2 levels with only 3+ positive tumours classed as positive. This is more stringent than previously reported studies where commonly 2+ and 3+ were classed as positive. Frequently these studies would report ERBB2 overexpression in around 30% of canine[28] and 30–60% of feline[12,43] mammary tumours. Scrutinizing the data reported by Ressel et al. (2013), a more severe interpretation using 3+ as the cut off would reduce the positive tumours to only 3 of 35 (8.6%). In FMC Ordás *et al.*[44] investigated the link between the ERBB2 positive tumours and increased gene copy numbers. In HBC, there is a link between HER2 increased gene copy number
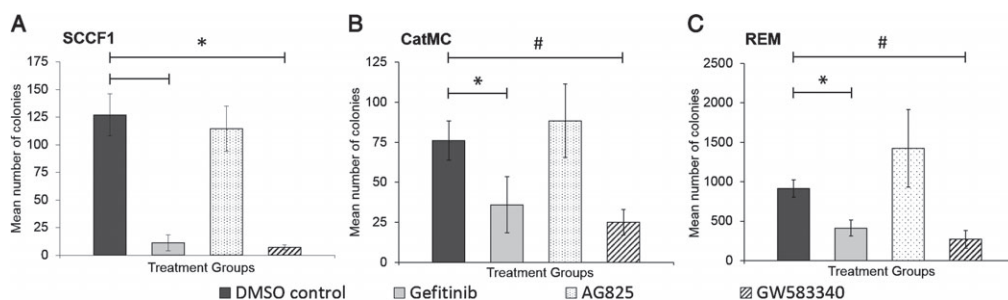
Figure 7. Treatment with TKIs reduced the colony forming ability of the cell lines when compared to DMSO controls. Colony formation assays of the three cell lines (A) SCCF1, (B) CatMC and (C) REM: gefitinib (at 10 μM) and GW583340 (at 4 μM) significantly reduced the colony formation ability of the cell lines when compared to DMSO controls. AG825 (at 10 μM) had no effect in any of the cell lines, (*) and (#) indicates statistically significant results: (A) *$P < 0.001$, (B) *$P = 0.047$ and #$P = 0.009$, (C) *$P = 0.01$ and #$P = 0.006$ by two sample *t*-test.

and response to trastuzumab therapy with 85–90% of HER2 positive cases having gene amplification.[42] In the Ordás' study only 2 of 12 (16.7%) ERBB2 positive tumours showed gene amplification, and when testing four FMC cell lines De Maria *et al.*[24] found no gene amplification in any of them. This may suggest that some of the reported ERBB2 positivity are either false positives or FMC ERBB2 overexpression rely on different pathways to HBC.

An even lower percentage was reported[25] (5.5%) in a study where they optimized a number of IHC protocols despite including both 2+ and 3+ as ERBB2 positive tumours. This study demonstrated how much variability can be associated with processing and interpretation. Peña *et al.*[42] goes some way in trying to alleviate this problem, and one of their suggestions with respect to the Hercept Test™ is to use commercially available control slides containing HBC cells of each HER2 reactivity for internal validation. As targeted therapies are starting to become available to the veterinary patient,[45] it is of paramount importance that a robust, reliable, and repeatable system is available for the evaluation of expression levels. If this can be achieved, it would prove a valuable tool for the veterinary oncologist when choosing potential targeting therapies.

With respect to EGFR expression in mammary tumours, less is known. It has been suggested that FMC is a good model of hormone negative HBC,[7,24] as a significant proportion of hormone negative [oestrogen receptor (ER) and progesterone receptor (PR)] and triple negative (ER, PR and HER2 negative) tumours express EGFR. These triple negative HBC are associated with a very poor prognosis.

In CMT EGFR overexpression has been reported in around 40% of malignant tumours.[20,27] A functional study[46] treating CMT and HBC cell lines with humanized mAbs against EGFR and HER2 revealed that both antibodies blocked tumour cell proliferation by inducing growth arrest in G_0/G_1 phase, but at a lower efficiency than what the same mAbs produced in the HBC cell lines. The authors suggested that this might be due to the CMT cell lines expressing the molecules at a 2-log lower expression levels compared with the human cell lines.

EGFR overexpression has been reported in FOSCC,[8] and its prognostic potential has been investigated. A small study of 22 FOSCC samples showed an inverse relationship between EGFR expression levels and survival,[11] but a larger study of 67 tumour samples could not verify this.[9] We have previously confirmed ERBB2 expression in the feline cell lines by PCR and sequencing (GenBank accession number KC710349.1), ICC, and western blot analysis. To our knowledge, no ERBB2 IHC study of FOSCC biopsy samples has been reported.

The link between protein expression detected using western blotting from a cell line and the levels observed by IHC in associated tumour biopsy samples are unknown for the cell lines used in this study, but the fact that they all seemed more sensitive to EGFR rather than ERBB2 inhibition is interesting. The use of receptor-specific siRNAs first confirmed that the effect observed in the cell lines were due to the specific knockdown of their respective receptors. Despite observing a clear reduction in ERBB2 mRNA and protein levels following transfections,
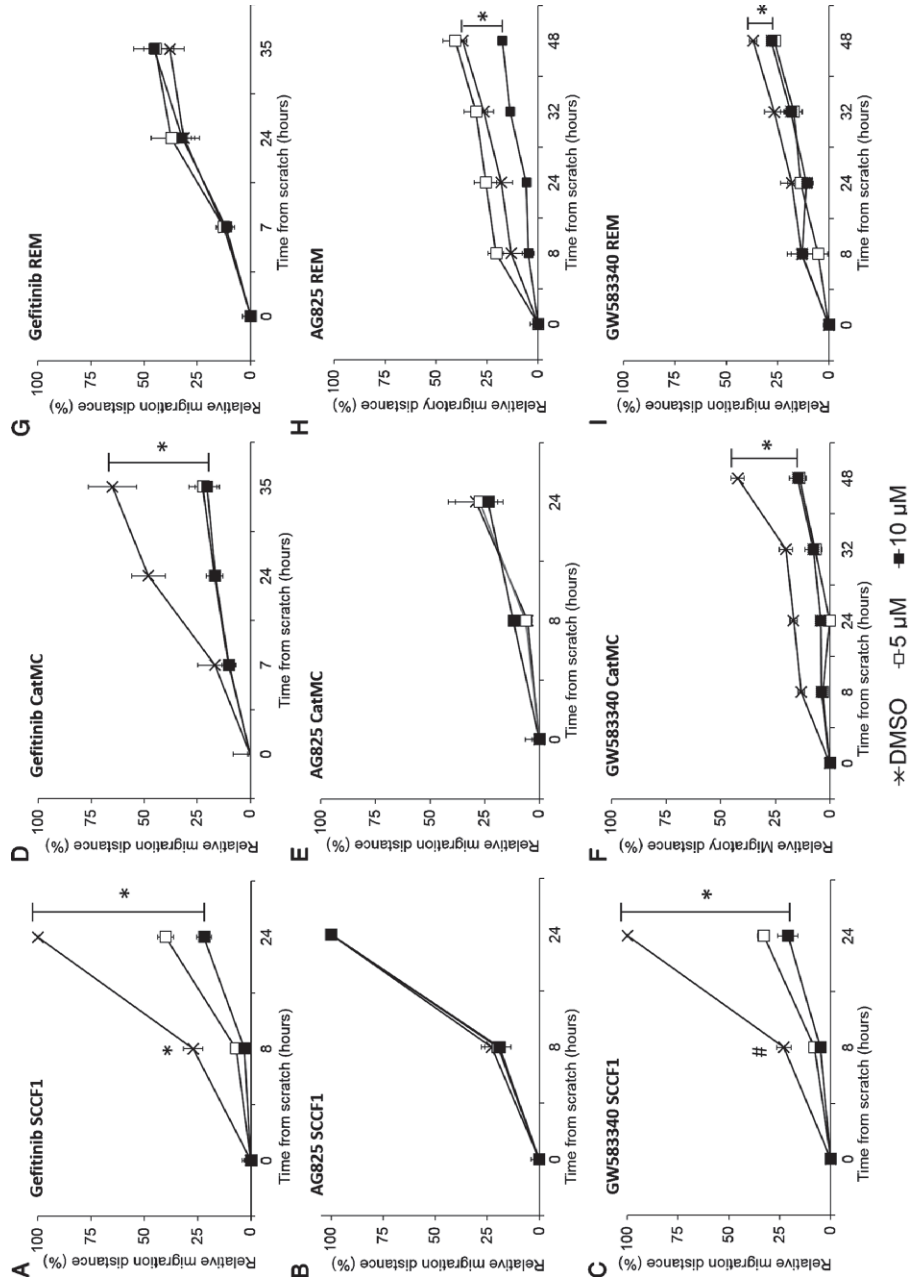
Figure 8. The relative migration distance of the three cell lines were reduced following TKI treatments. (A–C) The SCCF1 cell line showed a dose dependent reduction in their relative migration distance in response to gefitinib and dual inhibition (GW) (*P < 0.001). (D–F) The CatMC cell line showed a reduction in relative migratory distance when treated with the gefitinib (*P < 0.001) and GW583340 (*P = 0.003). (G–I) The REM cell line was minimally migratory but showed some reduction in relative migratory distance after AG825 and GW583340 treatments (*P < 0.001).
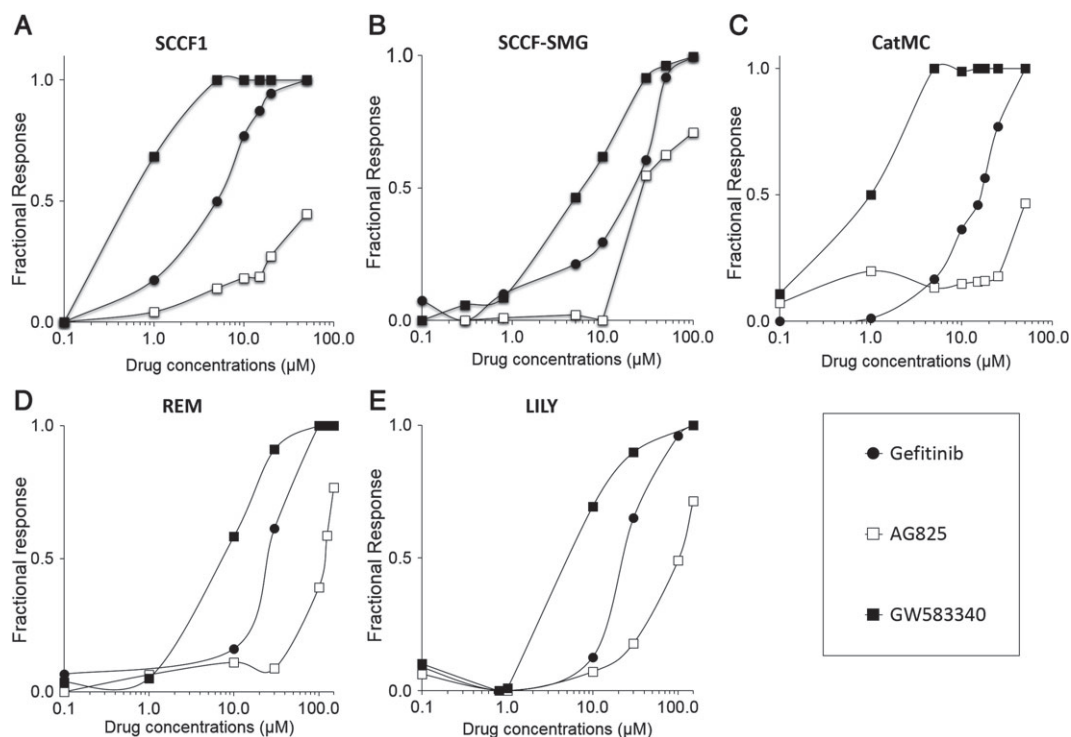
Figure 9. Dose response curves for the complete panel of cell lines. All cell lines tested were more sensitive to EGFR compared to ERBB2 targeting. The feline cell lines were overall more sensitive to the TKIs than the canine cell lines were. Graphs shown are representative results from one of triplicate experiments.

no measureable effect was observed in any of the cell lines. ERBB2 is different to the remaining receptors in its gene family in that it is incapable of ligand binding, and hence relies on its heterodimer partner for ligand activation.[47] It is the preferred dimerisation partner for all family members,[47] a possible explanation for why dual siRNA targeting caused a synergistic effect despite minimal effect of ERBB2 monotherapy.

EGFR silencing on the other hand consistently produced reduced cellular proliferation, colony formation and migratory ability. The effect of dual siRNA targeting when compared with individual targeting suggested that a synergistic action might be achieved when targeting the receptors simultaneously. A synergistic action is highly beneficial as drugs can be given at lower doses and achieve the same efficiency. In effect a drug that previously was only effective at toxic doses separately may yield the same results at subtoxic doses.[33]

The use of small molecule inhibitors in veterinary medicine is an attractive option. The use of toceranib[48] and masitinib[49] in the treatment of mast cell tumours is well established. The feline, canine and human mRNA and amino acid sequences show high homology for the *EGFR* and *ERBB2* genes, and modelling have shown great structural homology between the canine and human proteins,[46] suggesting that human TKIs should be effective in the feline and canine cells. Here we confirm using western blot analysis that treatment of cells with TKIs does reduce phosphorylated levels of the proteins following treatment, supporting the possible use of human TKIs in veterinary medicine. When used in humans, tumours that initially responded to a specific TKI were reported to develop acquired resistance over time, and this is a great disadvantage of TKIs. First generation TKIs gefitinib and erlotinib produced overall response rates of up to 75% in patients with non-small-cell lung cancer (NSCLC) who carried activating EGFR mutations. Unfortunately, the median progression free survival was less than 1 year due to a secondary mutation being selected for over time. The mutation

responsible for 50% of these cases is the T790M mutation in exon 20 of the human EGFR gene, but the mechanisms for the remaining 50% of cases are largely unproven.[50] ERBB2 amplification have been implicated as one of the mechanisms that confer acquired resistance in NSCLC,[51] which provides a rationale to target ERBB2 in combination with EGFR.

In human medicine, monoclonal antibodies rather than TKIs have been used in the treatment of HBC and HNSCC (trastuzumab against HER2 and cetuximab against EGFR, respectively). The use of humanized mAbs in veterinary patients would not be possible as the humanization would render them useless because of patient immune responses and at worst could cause adverse reactions and anaphylaxis.[45] Singer *et al.* reported the caninization of a mAb against canine EGFR, and showed its efficiency *in vitro* in two canine mammary cell lines.[45] This shows how personalized veterinary oncology is becoming a distinct possibility.

The doses required in dogs and cats *in vivo* is hard to extrapolate from *in vitro* studies, but some comparisons to human drug doses can be made. An *in vitro* dose of 1 μM gefitinib is equivalent to a clinical dose of 250 mg per day used in NSCLC.[52] In NSCLC *in vitro* studies doses of gefitinib above 2 μM would class the cell line as insensitive to the drug.[52] Early studies on HNSCC cell lines used much higher doses than this[53] while more recent studies all have used doses below 2 μM gefitinib.[54,55] The cell lines assessed in this study would therefore all be classed as relatively insensitive to gefitinib requiring relatively high doses (IC_{50} doses 5–28 μM). The dual inhibitor GW583340 required a lower dose (SCCF1 and CatMC IC_{50} doses of 0.6 and 1.1 μM, respectively), suggesting a dual inhibitor of some form (monoclonal antibodies, RNAi or TKIs) might be a better therapeutic option.

In summary, we have assessed the *in vitro* effect of targeting the EGFR family in feline and canine tumour cell lines from two tumour types (oral squamous cell carcinomas and mammary tumours) known to benefit from EGFR family targeting in humans using RNA interference and currently available TKIs. The benefit of TKIs over mAbs is that they are cheaper, can be given orally, and the human form of the drug can be utilized

without the problems associated with humanized mAbs. It is also currently easier to achieve dual targeting with TKIs, with several dual targeting TKIs readily available. A caninized version of cetuximab, however, has now been produced[45] and shown to stimulate ADCP as well as blocking the extracellular region of the receptor. TKIs cannot stimulate the immune response, as they act solely by blocking the ATP-binding pocket of the receptors inhibiting their phosphorylation. In addition, acquired resistance to therapy is a commonly reported problem encountered in human medicine in the clinical use of TKIs.

Veterinary medicine is moving towards the possibility of offering more personalized cancer treatments, and both mAbs and TKIs are likely to play a role in this in the future. The next step requires drugs to be identified that can be taken into controlled clinical trials for further assessment of their potential. Two things we can learn from the story so far of targeted therapies in the human field is that firstly, without effective patient selection the benefits of targeted therapies are difficult to achieve and secondly, that targeted therapies are rarely effective as sole agents and should be incorporated into multimodal treatment options, for example in combination with surgery, radiotherapy and established chemotherapy protocols. Therefore it will be crucial to establish good protocols for expression profiling that can be used repeatedly and reliably in the veterinary species.

Acknowledgements

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. ICC: Since LILY and REM only produced weak bands on the Western blots the protein expression was confirmed with ICC: weak positive staining (compared to e.g. SCCF1) – results consistent with Western blot results. Scale bars: SCCF1 20 μm, REM 100 μm, LILY 100 μm.

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