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# MAPK and PI3K signalling differentially regulate angiogenic and lymphangiogenic cytokine secretion in squamous cell carcinoma of the head and neck

*Sutima Luangdilok* [a,d], *Carol Box* [a], *Kevin Harrington* [b], *Peter Rhŷs-Evans* [c], *Suzanne Eccles* [a,*]

[a] *Tumour Biology and Metastasis Team, Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, McElwain Laboratories, Cotswold Rd., Belmont, Sutton, Surrey SM2 5NG, United Kingdom*

[b] *Targeted Therapy Team, Section of Cell and Molecular Biology, Chester Beatty Laboratories, Fulham Rd., London SW3 6JB, United Kingdom*

[c] *Head and Neck Unit, Royal Marsden Hospital, Fulham Rd., London SW3 6JJ, United Kingdom*

[d] *Siriraj Hospital Medical School, Mahidol University, Bangkok 10700, Thailand*

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ABSTRACT

Vascular endothelial growth factors (VEGF-C and VEGF-A) play important roles in tumour-induced lymphangiogenesis and angiogenesis respectively, key processes implicated in promoting tumour growth and metastatic spread. Previous work from our laboratory has shown that EGFR overexpression in squamous carcinomas of the head and neck (SCCHN) is linked to high levels of VEGF-A and VEGF-C (but low levels of VEGF-D) and is associated with poor prognosis. The present study explored the signalling pathways regulating the induction of VEGF-C and VEGF-A in the SCCHN cell lines CAL 27 and Detroit 562. The addition of exogenous EGF induced the expression of VEGF-C and VEGF-A in a concentration-dependent manner and this was blocked by a selective EGFR inhibitor, gefitinib. In both cell lines stimulated with endogenous or exogenous ligand, inhibition of MEK1/2 (with U0126 or PD98059) or PI3K (with PI-103 or LY294002) resulted in a marked reduction of EGFR-induced VEGF-A expression, whereas exogenous EGF-induced VEGF-C upregulation was blocked by inhibitors of MEK but not PI3K. Inhibition of p38 MAPK suppressed EGF-induced VEGF-C upregulation in CAL 27 cells, but inhibited EGF-induced VEGF-A upregulation in Detroit 562. Taken together, our evidence suggests that both endogenous and exogenous EGFR activation induces VEGF-A expression requiring both PI3K and MAPK signalling whereas VEGF-C expression is dependent on MAPK, but not the PI3K or mTOR pathways in SCCHN cell lines. p38 MAPK appears to be differentially linked to either VEGF-A or VEGF-C regulation in different cellular contexts.



* Corresponding author: Address: McElwain Laboratories, Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, 15 Cotswold Rd., Belmont, Sutton, Surrey SM2 5NG, United Kingdom. Tel.: +44 020 8722 4210; fax: +44 020 8722 4134.
E-mail address: Sue.Eccles@icr.ac.uk (S. Eccles).

*Abbreviations*: BTC, betacellulin; EGF(R), epidermal growth factor (receptor); ERK, extracellular signal-regulated kinase; HRG, heregulin; IGF, insulin-like growth factor; IL, interleukin; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PLCγ, phospholipase Cγ; SCCHN, squamous cell carcinoma of the head and neck; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor

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## 1. Introduction

Squamous cell carcinomas of the head and neck (SCCHN) develop in the upper aerodigestive tract, primarily the oral cavity, oropharynx, hypopharynx and larynx. SCCHN accounts for about 6% of all cancers worldwide and the incidence is rising, with tobacco, alcohol consumption and human papilloma virus infection contributing significantly to its epidemiology.[1,2] Despite improvements in management, the 5 year survival rate is still around 50%. Metastasis, rather than the primary tumour, is the underlying cause of death in most cancer patients. In SCCHN, spread is predominantly by direct invasion of adjacent tissues and dissemination to local lymphatics, with distant metastasis a late event. The incidence of distant metastasis is increasing as local control improves; nevertheless, the extent of lymph node metastasis is generally considered the most reliable adverse prognostic factor in SCCHN.[3,4] The epidermal growth factor receptor (EGFR) is a major driver of oncogenesis in SCCHN.[2,5] It is expressed in up to 90% of tumours and levels correlate with nodal metastases and advanced pathological stage.[6] High EGFR expression levels are a second independent adverse prognostic factor in SCCHN.[7]

The process of metastasis is complex and consists of multiple steps: detachment of tumour cells and access to blood or lymphatic vessels, their survival in the circulation, arrest in the microvasculature of target organs, exit from the vessels and growth and invasion into organs to form a metastatic focus. Tumour cells can escape from the primary site via existing or newly formed vessels through the process of angiogenesis or lymphangiogenesis. Vascular endothelial growth factors (VEGFs) are produced by tumour cells and directly or indirectly stimulate angiogenesis and lymphangiogenesis via binding to their tyrosine kinase receptors.[8] VEGF-A is the main angiogenic cytokine, expressed by a variety of solid human tumours, and signals primarily through VEGFR-2/KDR/FLK1 on vascular endothelial cells. VEGF-C is largely lymphangiogenic and acts via VEGFR-3/FLT4 on lymphatic endothelial cells.[9–11] However, both ligands and receptors can heterodimerise, and it is now clear that ligand-receptor interactions are more complicated than first described, contributing to their diversity of function.[12,13] For example, VEGF-A binds both VEGFR-1/FLT1 and VEGFR-2 and proteolytically processed VEGF-C can activate VEGFR-2.[14] The role of VEGF-B is less well defined: it binds only to VEGFR-1 and has little angiogenic activity. Recent studies suggest that it acts as a survival factor for endothelial cells rather than a mitogen, but there are few if any reported links to cancer progression.[15,16] VEGF-D binds to and activates VEGFR-3 but its role in lymphangiogenesis and lymphatic metastasis is less clear.[9,17,18]

VEGF-D and VEGF-C expression has been detected in a wide range of human tumours and previous work from our laboratory has highlighted complexities in both SCCHN and colorectal cancer where high levels of VEGF-C (but low levels of VEGF-D) were associated with poor prognosis.[19,20] VEGF-C production is induced by many ligands including platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and pro-inflammatory cytokines such as interleukin (IL)-1α and tumour necrosis factor (TNF). In breast cancer, heregulin-β1 potently upregulates VEGF-C expression via an ERB-B2-p38 mitogen-activated protein kinase (MAPK)-nuclear factor (NF)κB signalling pathway.[21] However, mechanisms underlying the regulation of VEGF-C in SCCHN have not been explored in detail.

VEGF-A is considered to be one of the most potent angiogenic cytokines and its overexpression has been shown to correlate with reduced overall survival of patients with SCCHN.[22] Although an association between VEGF-A and lymph node status in SCCHN was reported in some studies, meta-analysis indicated that this was not statistically significant.[22] In addition, VEGF-A has also been implicated in lymphangiogenesis.[23,24] In a model of chemical skin carcinogenesis in VEGF-A transgenic mice, VEGF-A induced tumour and sentinel lymph node lymphangiogenesis (via VEGFR-2-expressing lymphatic vessels) and promoted lymphatic metastasis.[25] Although the possibility that VEGF-A directly promotes lymphatic metastasis (and if so the underlying mechanism) is controversial, this possibility has to be considered when seeking molecular mechanisms of tumour lymphatic dissemination.

Previous work from our laboratory has shown that stimulation of SCCHN *in vitro* with ERB-B ligands [betacellulin, EGF, heregulin (HRG)] leads to upregulation of VEGF-C and VEGF-A which is blocked by treatment with an anti-EGFR antibody (ICR62).[26] The present study was undertaken to explore the signalling pathways involved in EGF-induced VEGF-C upregulation in SCCHN cell lines. The signalling pathways required for VEGF-A upregulation were also determined in the same cell lines thereby allowing direct comparison of these regulatory elements.

## 2. Materials and methods

### 2.1. Cell culture and reagents

The EGFR overexpressing SCCHN cell lines CAL 27 and Detroit 562 were from ATCC (Rockville, MD, USA). Both cell lines harbour wild-type *EGFR* and *PTEN* genes, mutated *CDKN2A* and *TP53* genes whilst Detroit 562 harbours the *PIK3CA* H1047R mutation (http://www.sanger.ac.uk/genetics/CGP/CellLines/). LICR-LON-HN3, -HN4 and -HN6 were from The Ludwig Institute for Cancer Research (London, UK). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS). For serum-free culture, 0.1% (w/v) BSA was added to the medium. Recombinant human EGF was purchased from Sigma (Poole, Dorset, UK). The signalling inhibitors used were: LY294002 (Calbiochem/Merck Biosciences, Nottingham, UK), PI-103 (PIramed, Slough, Berkshire, UK), U0126, PD98059, SB202190, SB203580 and rapamycin (Tocris Bioscience, Ellisville, Missouri, USA) and gefitinib (Biaffin GmbH & Co KG, Kassel, Germany). The antibodies used (1:1000 dilution) were: phospho-Tyr1068-EGFR (44-788G, Biosource/Invitrogen, Carlsbad, CS, USA); EGFR (clone F4, Sigma); p38MAPK (clone A12, Santa Cruz Biotechnology, CA, USA) and phospho-p38 MAPK (clone D8, Santa Cruz) and AKT (#9272), phospho-Ser473AKT (#4058), ERK1/2 (#9102), phospho-ERK1/2 (#9101), mTOR (#2972) and phospho-mTOR

(#2971) all from Cell Signalling Technology (Danvers, MA, USA).

### 2.2. Reverse transcription-polymerase chain reaction (RT–PCR)

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and converted to cDNA using Omniscript reverse transcription kit (Qiagen) according to manufacturer's instructions. cDNA was amplified by PCR using a Thermohybaid thermocycler. The master mix for one sample comprised RedTaq polymerase (Sigma), 1xTaq buffer, 0.25 mM dNTPs, 0.2 μM each forward and backward primer and double-deionised water. Double-deionised water served as a negative control. The PCR cycle consisted of 95 °C for 2 min followed by cycles of 95 °C for 1 min, annealing temperature (dependent on each pair of primers) for 1 min and extension at 72 °C for 1 min then 72 °C for 10 min. The VEGF-A primer set has been described previously.[26] The VEGF-C primers were designed to span two introns to reduce the possibility of DNA amplification. The sequences of the primers used are:

VEGF-C forward, 5′-ACGAGCTACCTCAGCAAGAC-3′
VEGF-C reverse, 5′-CATCTCCAGCATCCGAGGAA-3′
VEGF-A forward, 5′-CTCTACCTCCACCATGCCAAGT-3′
VEGF-A reverse, 5′-ATCTGGTTCCCGAAACCCTGAG-3′
β-actin forward, 5′-TCGACAACGGCTCCGGCAT-3′
β-actin reverse, 5′-AAGGTGTGGTGCCAGATTTTC-3′

#### 2.2.1. Western blotting

Cells were lysed with lysis buffer [150 mM NaCl, 1 mM EDTA, 50 mM Tris, 1% (v/v) Triton X-100, 1 mM NaF, 1 mM $Na_3VO_4$, 10 μg/ml Nα-tosyl-lys-chloromethylketone, 1 mM dithiothreitol, 5 μM fenvalerate, 5 μM potassium bisperoxo (1,10-phenanthroline) oxovanadate (V), 1 mM phenylmethylsulphonyl fluoride, protease (P8340) and phosphatase inhibitor cocktails I and II (P2850 and P5726, Sigma)]. Cell lysates were incubated on ice for 15 min and centrifuged at 12,000 rpm for 10 min. Protein concentration was estimated using a DC protein assay kit (Bio-Rad) according to manufacturer's instructions. Proteins were electrophoresed on NuPAGE Novex 4-12% Bis–Tris gels (Invitrogen) then transferred to PVDF membranes (Invitrogen) according to manufacturer's instructions.

### 2.3. Enzyme-linked immunosorbent assay (ELISA)

Cells in 6-well plates were serum-starved for 48 h to reduce, as far as possible, the levels of endogenous growth factor secretion enabling assessment of acute, exogenous EGF-driven responses. Cells were then pre-treated with inhibitors for 1 h before stimulating with 60 ng/ml EGF for 20-24 h. Supernatants of cell cultures were collected and stored at –20 °C until required for ELISA. The concentrations of VEGF-C and VEGF-A were quantified by human VEGF-C ELISA kit (IBL, Gunma, Japan) and human VEGF-A ELISA kit (R&D Systems, Abingdon, UK) according to manufacturers' instructions. To normalise samples, corresponding cell lysates were collected to estimate total protein concentration of each sample.

### 2.4. Statistical analysis

Statistical analysis was performed using Prism 2.01 (GraphPad Software Inc., San Diego, CA, USA). For evaluation of statistical differences, ANOVA test followed by Bonferroni's multiple comparison tests was employed when comparing more than two groups. A $p$ value (two-tailed) of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. SCCHN cell lines secrete varying levels of VEGF-C and VEGF-A

An ELISA that detects the mature form of VEGF-C, reflecting its biological function, was used to determine the levels of endogenous secreted VEGF-C in five widely available SCCHN cell lines (Fig. 1a). Two cell lines (Detroit 562 and CAL 27) expressed high levels of secreted VEGF-C whereas the other three cell lines (HN3, HN4 and HN6) expressed low levels. The secretion of VEGF-A was also determined by ELISA (Fig. 1b) and the results showed moderate VEGF-A levels in supernatants of all cell lines with HN4 cells producing the

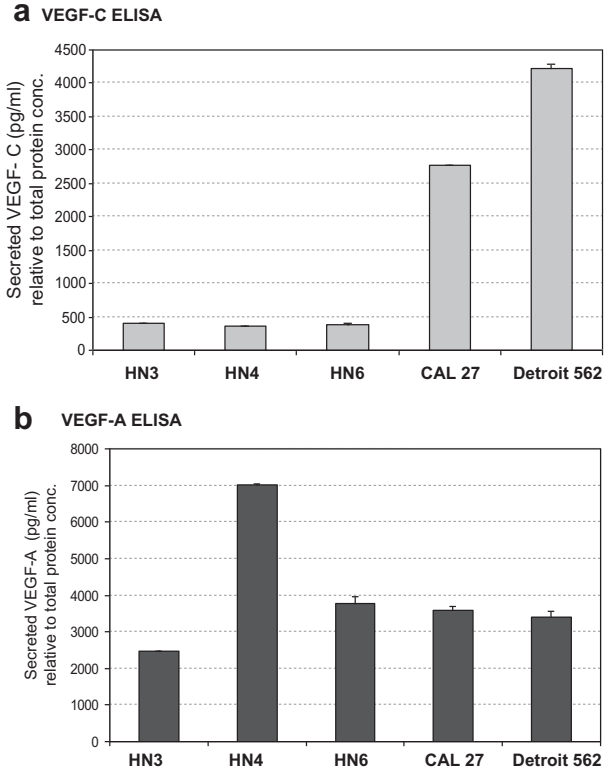


**Fig. 1 – Secreted VEGF-C and VEGF-A proteins in supernatants of SCCHN cells. Cells were grown in 6-well plates in DMEM supplemented with 2% (v/v) FCS. Cell supernatants were collected and subjected to VEGF-C (a) and VEGF-A (b) ELISA assays. Cell lysates were also collected and measured for the total protein concentration in order to perform normalisation between samples. Data are representative of two independent experiments shown as mean ± SD.**

highest concentration of VEGF-A. CAL 27 and Detroit 562 were selected for further studies as they expressed significant levels of both VEGF-C and VEGF-A.

### 3.2. *Exogenous EGF induces VEGF-C and VEGF-A expression and secretion in SCCHN cells*

CAL 27 cells were serum-starved for 48 h then stimulated with a range of EGF concentrations (2–60 ng/ml) for 20 h. Total RNA was prepared from cells and RT–PCR was performed to assess the expression of VEGF-C and VEGF-A (Fig. 2a). Increased VEGF-C mRNA expression was observed when cells were stimulated with 5 ng/ml EGF and the levels increased in a concentration-dependent manner up to 60 ng/ml EGF. VEGF-A levels also increased in response to EGF with the highest levels seen at 60 ng/ml. Levels of secreted VEGF-C and VEGF-A in cell supernatants measured by ELISA also showed a concentration-dependent increase when stimulated with exogenous EGF (Fig. 2a) and the data were consistent with those obtained by RT–PCR.

A similar study of VEGF-A and VEGF-C regulation was also performed in Detroit 562 (Fig. 2b). The results derived from RT–PCR and ELISA assays consistently showed VEGF-C and VEGF-A induction when cells were stimulated with 2 ng/ml EGF and the levels increased with higher concentrations. A concentration of 60 ng/ml EGF was therefore selected for induction of VEGF-C and VEGF-A expression in subsequent exogenous 'EGF-induced' experiments.

To investigate the effect of EGFR inhibition on EGF-induced VEGF-C and VEGF-A upregulation, CAL 27 and Detroit 562 cells were serum-starved then stimulated with 60 ng/ml EGF for 20 h in the presence of a selective EGFR tyrosine kinase inhibitor, gefitinib. Cell culture supernatants were collected and analysed using VEGF-C and VEGF-A ELISAs. The results showed that basal (unstimulated) levels of VEGF-C and VEGF-A were greater in Detroit 562 than in CAL 27 supernatants whilst a clinically achievable concentration of gefitinib (1 μM) was sufficient to reduce VEGF-C and VEGF-A levels to basal in each case (Fig. 3a and b). Western blots confirmed that EGF stimulation led to a marked increase in the levels

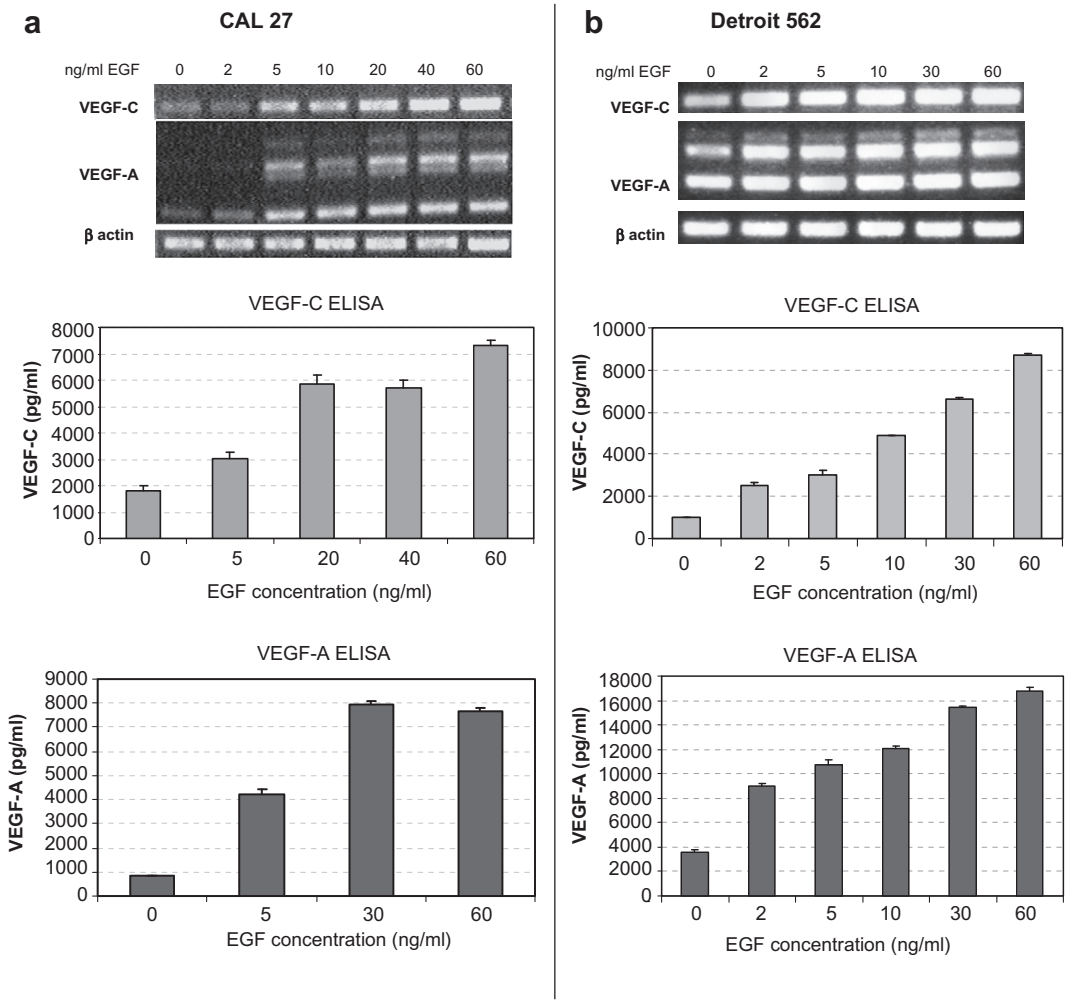


**Fig. 2 – EGF upregulates VEGF-C and VEGF-A in a concentration-dependent manner in SCCHN cell lines. CAL 27 (a) and Detroit 562 (b) cells were serum-starved in 0.1% (w/v) BSA for 48 h and then stimulated with the indicated EGF concentrations for 20 h. Total RNA was extracted, treated with DNase and converted to cDNA. RT–PCR was then performed to detect the expression of VEGF-C and VEGF-A. The quantity of secreted VEGF-C and VEGF-A in cell supernatants was determined by ELISA. Data are representative of two independent experiments shown as mean ± SD.**

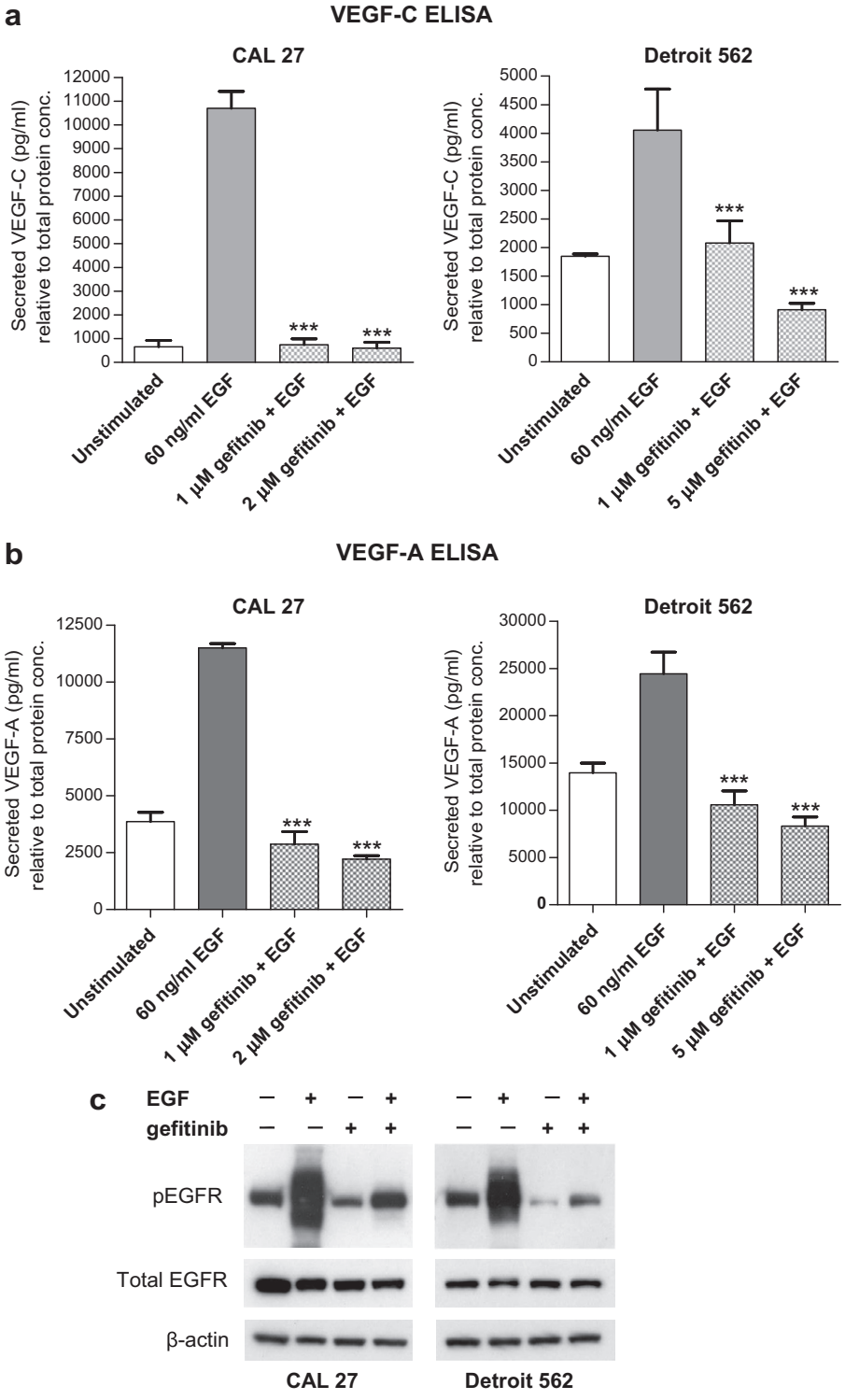


**Fig. 3 – EGFR inhibition reduces EGF-mediated VEGF-A and VEGF-C upregulation in SCCHN cell lines. CAL 27 and Detroit 562 cells were serum-starved, pre-treated with the EGFR inhibitor gefitinib at indicated concentrations and then stimulated with 60 ng/ml EGF for 20 h. Supernatants were collected and subjected to ELISA to measure concentrations of secreted VEGF-C (a) and VEGF-A (b). Data are representative of two independent experiments shown as mean ± SEM. ANOVA test $p<0.0001$, followed by Bonferroni's multiple comparison test for pairwise comparisons with EGF stimulated sample, $^{***}p < 0.001$ were performed. (c) Cells were serum-starved, pre-treated with gefitinib (1 μM for CAL 27 and 5 μM for Detroit 562) then stimulated with 60 ng/ml EGF for 30 min. Cell lysates were collected and subjected to Western blot analysis using a phospho-specific (Y1068) EGFR antibody.**

of phosphorylated EGFR, whereas this was inhibited by gefitinib in both cell lines (Fig. 3c).

### 3.3. Inhibition of PI3K, MEK1/2, p38MAPK or mTOR can reduce VEGF-C and VEGF-A expression and secretion in SCCHN cells

To further explore the signalling pathways regulating EGF-mediated VEGF-C expression, compounds targeting major downstream elements including PI3K, MEK/ERK and p38 MAPK were tested. The dose responses of CAL 27 to inhibitors of PI3K (PI-103[27,28] or LY294002), MEK1/2 (U0126 or PD98059), p38 MAPK (SB202190 or SB203580) or mTOR (rapamycin) were first determined using the methylene blue growth inhibition assay. The concentrations of the inhibitors used in subsequent studies took into account information obtained from both literature sources[29] and from $GI_{50}$ values determined in this study (data not shown). To confirm that the inhibitory effects of the pharmacological agents on VEGF-C/-A upregulation resulted from inhibition of their targets, Western blot analysis was performed to assess levels of total and phosphorylated proteins in CAL 27 cells. Briefly, CAL 27 cells were serum-starved and stimulated with 60 ng/ml EGF in the presence of signalling inhibitors for 24 h then subjected to Western blot analysis. The cellular levels of inhibitor target proteins following 48 h serum-starvation were comparable to the more physiological levels observed in non-starved cells (supplementary Fig. S1). In addition, all inhibitors were shown to block the phosphorylation of the desired target proteins at the concentrations and exposures used (supplementary Fig. S2). 500 nM PI-103 and 10 μM LY294002 strongly inhibited EGF-induced phosphorylation of AKT at 24 h. SB203580 (10 μM) was found to block p38 MAPK phosphorylation better than 10 μM SB202190. Rapamycin (100 nM) was also found to effectively inhibit mTOR phosphorylation.

CAL 27 cells were serum-starved and pre-treated for 1 h with the above inhibitors. Following stimulation with EGF for 20 h, the supernatants were harvested and RNA isolated from the cells. RT–PCR (Fig. 4) consistently showed that VEGF-C upregulation was inhibited by MEK1/2 inhibitors (U0126 and PD98059) and p38 MAPK inhibitors (SB202190 and SB203580), but not by PI3K or mTOR inhibitors. In contrast, the induction of VEGF-A mRNA expression was shown to be inhibited by MEK1/2 inhibitors and by a PI3K inhibitor (LY294002). Similarly ELISA results (Fig. 5a) demonstrate that VEGF-C secretion is suppressed by MEK and p38 MAPK inhibitors but not by PI3K/mTOR inhibition. VEGF-A upregulation was blocked by PI-103, LY294002, U0126 and PD98059 and to a lesser extent by an mTOR inhibitor (rapamycin), but not by a p38 MAPK inhibitor (SB202190) (Fig. 5b).

A similar experiment performed with Detroit 562 cells and ELISAs (Fig. 5c and d) showed that, consistent with the results in CAL 27, PI3K inhibitors blocked VEGF-A upregulation, but not VEGF-C upregulation and MEK inhibitors reduced both VEGF-C and VEGF-A induction. In contrast to CAL 27, however, p38 inhibitors blocked VEGF-A upregulation but not VEGF-C upregulation and an mTOR inhibitor did not inhibit the induction of VEGF-A or VEGF-C in Detroit 562 (Fig. 5d). Taken together, the results from both cell lines indicate that MEK, but not PI3K or mTOR, is required for EGF-mediated VEGF-C upregulation whereas both PI3K and MEK were necessary for VEGF-A upregulation.

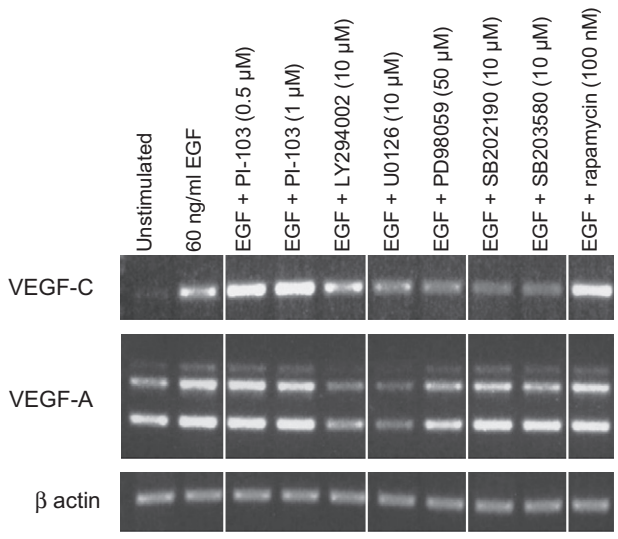


**Fig. 4 – PI3K and MAPK inhibitors but not an mTOR inhibitor block EGF-mediated upregulation of VEGF-A and VEGF-C mRNA in CAL 27 cells. CAL 27 cells were serum-starved and pre-treated for 1 h with the indicated concentrations of inhibitors to PI3K (PI-103 or LY294002), MEK (U0126 or PD98059), p38 MAPK (SB202190 or SB203580) or mTOR (rapamycin) and then stimulated with 60 ng/ml EGF for 20 h. RT–PCR was used to assess mRNA expression and samples for each primer pair were electrophoresed together on one gel. Data are representative of two independent experiments.**

### 3.4. Inhibitors of EGFR (gefitinib), PI3K (PI-103) and MEK1/2 (U0126) downregulate endogenous VEGF-C and VEGF-A

Detroit 562 cells express relatively high levels of VEGF-A and VEGF-C in the absence of exogenous EGF stimulation (Fig. 2b), possibly because they endogenously express multiple EGFR/ERB-B ligands.[30] Detroit 562 cells were grown in DMEM/2% (v/v) FCS with or without inhibitors of EGFR (gefitinib), PI3K (PI-103) or MEK1/2 (U0126) for 48 h. Cell culture media were collected and VEGF-C and VEGF-A measured by ELISA. VEGF-C levels were significantly reduced by gefitinib (5 μM; approximately 0.5× the 96 h $GI_{50}$[5] or by U0126 (10 μM), but not by PI-103. On the other hand, VEGF-A levels were markedly decreased by gefitinib (0.5 μM and above), PI-103 (500 nM) and U0126 (5 and 10 μM) (Fig. 6), mirroring the effects seen in cells stimulated with exogenous EGF. These results suggest that activation of EGFR signalling pathways with either exogenous or endogenous ligand can evoke secretion of angiogenic and lymphangiogenic cytokines in SCCHN and that the same signalling pathways are implicated, albeit with different degrees of sensitivity to selective inhibitors.

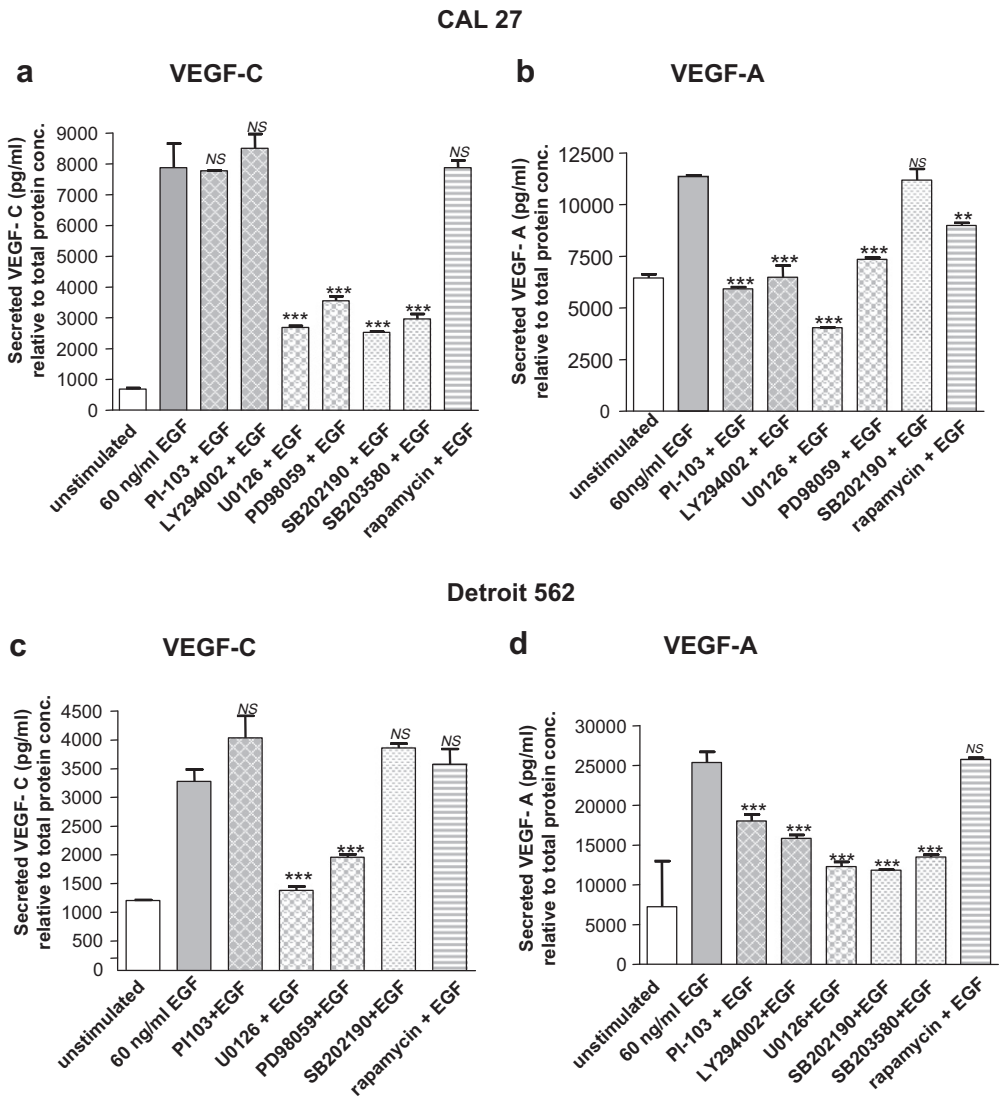


**Fig. 5 – PI3K and/or MAPK are required for EGFR-mediated VEGF-A and VEGF-C secretion in CAL 27 or Detroit 562 cells. Cells were serum-starved and pre-treated for 1 h with inhibitors of PI3K (0.5 μM PI-103 or 10 μM LY294002), MEK (10 μM U0126 or 50 μM PD98059), p38 MAPK (10 μM SB202190 or 10 μM SB203580) or mTOR (100 nM rapamycin) and then stimulated with 60 ng/ml EGF for 20 h. Supernatants were collected and subjected to ELISA to measure concentrations of secreted VEGF-C (a) and (b) or VEGF-A (c) and (d). Data are representative of two independent experiments shown as mean ± SEM. ANOVA test $p < 0.0001$, followed by Bonferroni's multiple comparison test for pairwise comparisons with EGF stimulated sample was carried out where $^{***}p < 0.001$; $^{**}p < 0.01$; NS, not significant ($p > 0.05$).**

## 4. Discussion

The extent of lymphatic spread is an important prognostic factor in SCCHN and VEGF-A and VEGF-C upregulation (but interestingly, VEGF-D downregulation) has been linked with poor survival.[20,31] EGFR activation is an important pathway linked to tumour secretion of VEGF-C and VEGF-A. The present study explored the downstream signalling events controlling EGFR-mediated VEGF-C and VEGF-A transcription and secretion in two SCCHN cell lines, CAL 27 and Detroit 562. Pharmacological approaches were used to target the major elements of the EGFR signalling pathway: PI3K, mTOR, MEK/ERK and p38 MAPKs. Changes in mRNA expression of VEGF-A and -C were not always apparent when changes at the protein level were clear, possibly because the compounds may also affect protein stability or secretion. Nevertheless, protein levels are the more important measure of functional potential. Selective inhibition of MEK1/2 (by U0126/PD98059) or PI3K (with PI-103/LY294002) led to a significant reduction in EGF-induced VEGF-A upregulation in both SCCHN cell lines tested (CAL 27 and Detroit 562). This finding is supported by studies showing that both MEK-ERK1/2 and PI3K-AKT signalling were required for HGF- or EGF-mediated VEGF-A upregulation in SCCHN[32,33]; and that the PI3K pathway regulates insulin-like growth factor (IGF-1)-induced VEGF-A secretion via the transcription factor hypoxia-inducible factor-1α (HIF1α) in a squamous tongue carcinoma cell line.[34]

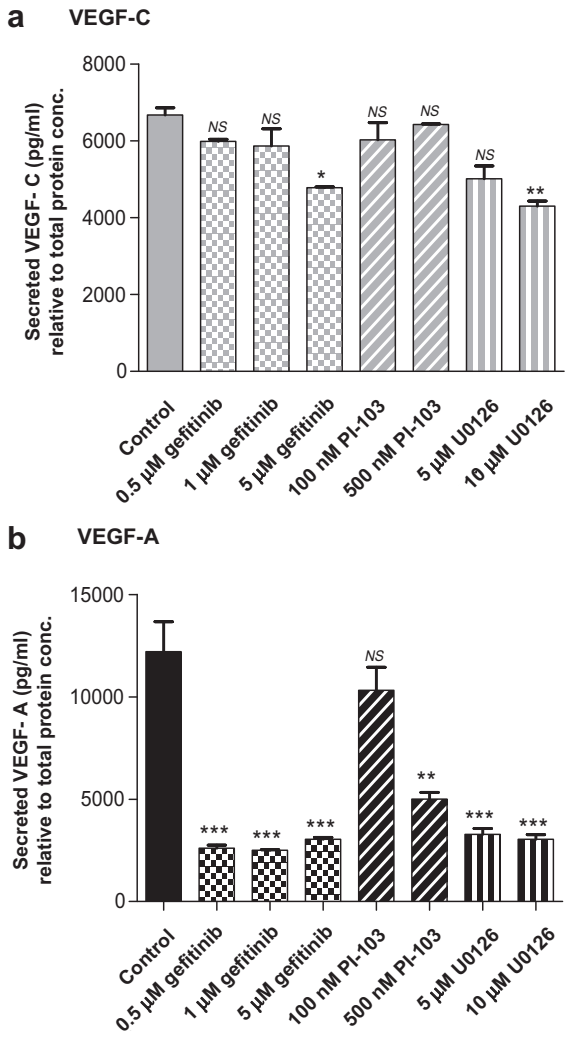


**Fig. 6 – Inhibitors of EGFR, PI3K and MEK significantly inhibit constitutive secretion of VEGF-A but not VEGF-C in Detroit 562 cells. Detroit 562 cells were cultured in DMEM supplemented with 2% (v/v) FCS with or without inhibitors of EGFR (gefitinib), PI3K (PI-103) or MEK (U0126) for 48 h. Cell supernatants were collected and subjected to VEGF-C (a) and VEGF-A ELISAs (b). Data are representative of two independent experiments shown as mean ± SEM. ANOVA test, followed by Bonferroni's multiple comparison test for pairwise comparisons with control sample were performed where $^{***}p < 0.001$; $^{**}p < 0.01$; NS, not significant ($p > 0.05$).**

Furthermore, the present study shows that p38 MAPK was required for EGF-mediated VEGF-A upregulation in one SCCHN cell line (Detroit 562), but not in a second (CAL 27). The former finding is consistent with a study by Akagi and colleagues in gastric cancer cells where blockage of p38 MAPK, ERK1/2 or PI3K pathways suppressed EGF induction of VEGF-A.[35] In addition, p38 MAPK was shown to be required for VEGF-A upregulation mediated by other cytokine-receptor systems including HRG-β1[36] and protease-activated receptor 2 (PAR-2)[37] in breast cancer and IL-1β-induced VEGF-A upregulation in human vascular smooth muscle cells.[38] In SCCHN cell lines, treatment with SB203580 showed a requirement for p38 MAPK in the upregulation of VEGF-A following exposure to hypoxia.[39]

We also showed that EGF-mediated VEGF-C upregulation was blocked by the selective inhibition of MEK-ERK signalling (with U0126 or PD98059), but not through PI3K inhibition (with PI-103 or LY294002) in both SCCHN cell lines (CAL 27 and Detroit 562). However, inhibition of p38 MAPK (with SB203580) significantly reduced VEGF-C induction by EGF in CAL 27, but not in Detroit 562 suggesting that regulation of VEGF-C expression is dependent on cellular context rather than simply on histogenic origin. The regulation of VEGF-C expression remains a relatively underexplored area. In B13LM pancreatic tumour cells, inhibitors of mTOR, p38 and JNK but not a MEK1/2 inhibitor suppressed VEGF-C expression[40] whilst in lung cancers, IGF-1-stimulated VEGF-C induction was mediated through PI3K and to a lesser extent by ERK1/2.[41] In MCF-7 breast cancer cells VEGF-C upregulation via HRG-β1 was dependent on p38 MAPK, but not ERK1/2 or PI3K.[21] Pro-inflammatory cytokines such as IL-1β and TNF-α can induce VEGF-C expression in fibroblasts and breast cancer cells.[21,42] It is therefore evident that regulation of VEGF-C can vary in different cellular contexts (depending on the initiating stimulus) or in different pathological processes.[43]

Rapamycin treatment had no effect on the levels of secreted VEGF-C and caused a small reduction in VEGF-A in CAL 27 but not in Detroit 562 cells. It is well established that VEGF-A, but not VEGF-C expression is regulated by the transcription factor HIF1α[44] due to the presence of a hypoxia response element in the promoter of the *VEGF A* gene. Since mTOR can regulate levels of HIF1α,[45] this could explain why the mTOR inhibitor rapamycin reduced VEGF-A, but not VEGF-C protein levels.

In addition to exogenous EGF stimulation, the present study also investigated the effect of selective inhibition of EGFR, PI3K and MAPKs on endogenously-stimulated VEGF-C and VEGF-A. SCCHN cells produce several ERB-B ligands that may enhance proliferation and also secretion of angiogenic cytokines and proteases[26,30] through endogenous signalling pathways. Selective inhibition of EGFR by gefitinib or MEK/ERK1/2 by U0126 blocked the production of both VEGF-A and VEGF-C in unstimulated Detroit 562 cells. Inhibition of VEGF-A was observed at a gefitinib concentration of 0.5 μM, however, 10-fold higher concentrations were required to inhibit VEGF-C production. This suggests that regulation of VEGF-A is almost entirely dependent upon EGFR activity whilst VEGF-C is regulated in part by EGFR but also other pathways. Detroit 562 cells express varying levels of all four EGFR/ERB-B receptors and several endogenous ligands including the pan-activating ligand BTC. In SCCHN, EGFR and MAPK regulate both endogenous and exogenous EGF-induced production of VEGF-C and VEGF-A. PI3K, however, was required for the expression of VEGF-A, but not VEGF-C under both endogenous and exogenous EGF-stimulated conditions. This finding is supported by the recent report of Tsutsui and colleagues[46] who used immunohistochemistry to demonstrate that AKT expression correlates more strongly with VEGF-A expression than VEGF-C expression in breast cancers.

Some of the earliest investigations of gefitinib reported that this inhibitor reduced levels of secreted VEGF-A and IL-8 *in vivo*.[47] Targeting EGFR or MAPKs would, theoretically,

not only limit angiogenesis but also lymphangiogenesis in SCCHN as pharmacological inhibition of these targets blocked both VEGF-C and VEGF-A upregulation. However, there are other cytokines (*e.g.* PDGFs and angiopoietins) that contribute to angiogenesis and lymphangiogenesis[48] and these need to be taken into account in developing therapeutic applications to prevent tumour spread in this disease.

## Conflict of interest statement

Dr. Suzanne Eccles is an employee of The Institute of Cancer Research which has a commercial interest in the development of the PI3K inhibitor GDC-0941, an optimised derivative of PI-103. There was a commercial collaboration with PIramed and intellectual property arising from the program has been licensed to Genentech.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.10.009.

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