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Growth inhibition by tyrosine kinase inhibitors in mesothelioma cell lines ☆

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ABSTRACT

Clinical outcome following chemotherapy for malignant pleural mesothelioma is poor and improvements are needed. This preclinical study investigates the effect of five tyrosine kinase inhibitors (PTK787, ZD6474, ZD1839, SU6668 and SU11248) on the growth of three mesothelioma cell lines (NCI H226, NCI H28 and MSTO 211H), the presence of growth factor receptors and inhibition of their downstream signalling pathways. GI_{50} values were determined: ZD6474 and SU11248, mainly VEGFR2 inhibitors, gave the lowest GI_{50} across all cell lines (3.5–6.9 μM) whereas ZD1839 gave a GI_{50} in this range only in H28 cells. All cell lines were positive for EGFR, but only H226 cells were positive for VEGFR2 by Western blotting. ZD6474 and ZD1839 inhibited EGF-induced phosphorylation of EGFR, AKT and ERK, whereas VEGF-induced phosphorylation of VEGFR2 was completely inhibited with 0.1 μM SU11248. VEGFR2 was detected in tumour samples by immunohistochemistry. VEGFR2 tyrosine kinase inhibitors warrant further investigation in mesothelioma.

1. Introduction

Malignant mesothelioma (MM) is an aggressive tumour of serosal surfaces, such as pleura, peritoneum and pericardium, and usually has a poor prognosis. The incidence of malignant pleural mesothelioma (MPM) is increasing as a result of widespread exposure to asbestos in the past and is expected to peak around 2015.[1] The disease affects more men than women in a ratio of 5:1, and tends to have a long latency period of 20–40 years following asbestos exposure, affecting people in the fifth to seventh decade of life. Approximately 80% of MPM is attributed to exposure to asbestos fibres, although some tumours are 'spontaneous' with no evidence of asbestos exposure. There are approximately 2000 new cases of MPM diagnosed per annum and it accounts for 1% of all cancer related mortality in the United Kingdom. Following diagnosis, median survival of patients is approximately 6–12 months. Most patients are unsuitable for radical surgery. Radiation therapy has been shown to alleviate pain in the majority of patients treated, but the duration of symptom control is short-lived, so chemotherapy is generally the treatment of choice. Recent trials have shown a partial response rate of 32% using a combination of pemetrexed and carboplatin.[2] A phase III trial using pemetrexed with cisplatin[3] significantly improved response rates, time to progression, overall survival and quality of life compared to single agent cisplatin and suggested this to be used as front line chemotherapy in MPM patients. Response rate in this study was 41%, but relapse rates remain high and long-term survival is poor, so improved treatment for mesothelioma is still required.

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Small molecule receptor tyrosine kinase inhibitors have been developed against targets such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptors (VEGFRs) and platelet derived growth factor receptors (PDGFRs). Although EGFR has been identified in the majority of mesothelioma tumours,[4,5] gefitinib (an EGFR tyrosine kinase inhibitor) has not been found to be active in malignant mesothelioma.[6] This report describes the effect of a range of VEGFR2 and EGFR tyrosine kinase inhibitors on the growth of mesothelioma cell lines and their effect on signalling pathways downstream of VEGFR2 and EGFR.

2. Materials and methods

2.1. Cell culture

Three human mesothelioma cell lines were used in this study. MSTO-211H (from a biphasic mesothelioma) and NCI-H28 were obtained from ATCC, and NCI-H226 was obtained from Cancer Research UK. H28 and H226 cells were derived from epithelial tumours. Cells were grown in RPMI 1640 (R8758, Sigma–Aldrich) containing 2 mM glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate and 10% foetal calf serum (v/v) (FCS)(Sigma–Aldrich). All cell lines were subcultured weekly and were negative for mycoplasma.

2.2. Growth inhibition studies

Growth inhibition studies using the tyrosine kinase inhibitors detailed in Table 1 and Fig. 1 were carried out using the SulphurRhodamine B (SRB)[7] assay in 96 well tissue culture plates as previously described.[8] Cells were seeded at 5×10^3 cells per well and treated for 72 h with 0–30 μM of the inhibitors. The concentration of tyrosine kinase inhibitor giving 50% growth inhibition (GI_{50}) with each cell line was calculated using GraphPad Prism software.

Table 1 – Tyrosine kinase inhibitors.

Drug	Name	Target
PTK 787[11]	Vatalinib	VEGFR2, VEGFR1, PDGFRβ
ZD6474[12]	Vandetanib	VEGFR2, VEGFR3, EGFR
SU6668[13]		VEGFR2, FGFR1, PDGFRβ
SU11248[14]	Sunitinib	VEGFR2, PDGFRβ
ZD1839[15]	Gefitinib	EGFR

2.3. Western blotting

Treatment of cells for cell lysates required for Western blotting was carried out in 60 mm dishes seeded with 1×10^6 cells. After 24 h to allow for cell attachment, cells were washed with PBS. Fresh medium, with or without serum, was added as required. Following a further incubation of 24 h, the medium was replaced with test medium containing human recombinant EGF (hEGF), $hVEGF_{165}$ (Sigma–Aldrich, UK), tyrosine kinase inhibitors or serum free or serum rich control medium. Short incubations were stopped by washing cells with ice-cold PBS and adding lysis buffer (62.5 mM Tris/HCl pH 6.8, 2% sodium dodecyl sulphate (w/v), 10% glycerol (v/v)) to prepare cell lysates for protein estimation and western blotting.

Western blotting was performed to determine the effects of treatment on the expression and activation of proteins. Cell lysates were prepared and 50 μg aliquots of protein (calculated from quantification using the Pierce BCA protein assay) from each sample were loaded on a 4–20% gradient polyacrylamide gel (Invitrogen). Following SDS–PAGE and electroblotting,[9] membranes were treated to block non-specific binding and incubated with primary antibodies as indicated in Table 2. Secondary HRP-conjugated antibodies (Goat anti-rabbit or goat anti-mouse, Dako) were used to detect the primary antibodies and antibody-labelled protein bands were visualised by enhanced chemiluminescent detection (ECL) (GE Healthcare, Little Chalfont, UK). Equal loading of protein was verified using anti-actin antibody (Sigma).

2.4. RNA preparation and RT-PCR for VEGFR2

RNA samples were prepared from cells in the exponential growth phase using the RNeasy minikit (QIAGEN) and were

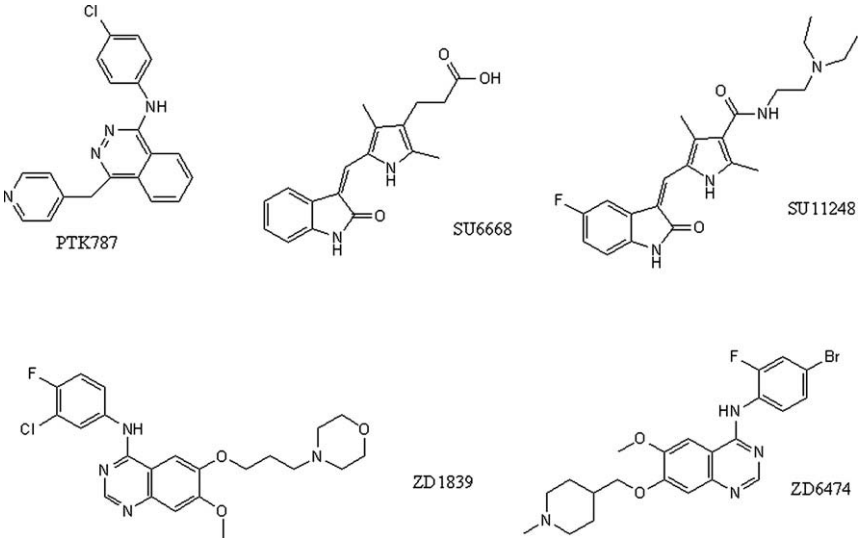

Fig. 1 – Structures of tyrosine kinase inhibitors used in the study.

Table 2 – Primary antibodies used in Western blot analysis.

Target	Source	Dilution	TBS tween with	Incubation	Species
EGFR (NCL-EGFR)	Novocastra	1:100	5% Milk	1 h RT	Mouse
Phospho-EGFR (Tyr 1068)	Cell-signalling	1:1000	5% BSA	ON 4 °C	Rabbit
VEGFR2	Cell-signalling	1:1000	5% BSA	ON 4 °C	Rabbit
P-VEGFR2 (Tyr1175)	Cell-signalling	1:1000	5% BSA	ON 4 °C	Rabbit
P-VEGFR2 (Tyr951)	Cell-signalling	1:1000	5% Milk	ON 4 °C	Mouse
MAP Kinase (ERK 1/2)	Santa Cruz Biotech.	1:1000	5% Milk/5% BSA	ON 4 °C	Rabbit
Phospho-MAP kinase (ERK 1/2)	Santa Cruz Biotech.	1:1000	5% Milk/5% BSA	1 h RT	Mouse
AKT	Cell-signalling	1:1000	5% BSA	ON 4 °C	Rabbit
Phospho-AKT (Ser 473)	Cell-signalling	1:1000	5% BSA	ON 4 °C	Rabbit
Actin AC40	Sigma Aldrich	1:1000	5% Milk	1 h RT	Mouse

BSA – Bovine Serum Albumin; RT – Room Temperature; ON – Overnight.
% is w/v.

reverse transcribed using the Superscript First strand synthesis system (Invitrogen). Reverse transcription PCR amplification was performed for VEGFR2 using primers GAG GGC CAC TCA TGG TGA TTG and TGC CAG CAG TCC AGC ATG GTC TG[10] and 35 cycles of 94 °C 40 s, 57 °C 30 s, 72 °C 50 s. Actin primers used were CAA CTC CAT CAT GAA GTG TGA and GCC ATG CCA ATC TCA TCT TG. The RT-PCR products were analysed by agarose gel electrophoresis with ethidium bromide staining to visualise and record the RT-PCR DNA bands.

2.5. *DNA preparation, PCR amplification and sequencing of EGFR*

DNA was extracted from cells of each mesothelioma cell line using the QIAamp DNA mini kit (QIAGEN Ltd., Crawley, UK) and eluting DNA from the columns in water.

Exons 18–21 of *EGFR* were amplified in a final 50 μL volume reaction using 1.5 mM $MgCl_2$, 200 nM dNTPs (Invitrogen), 1 U of Platinum Taq polymerase (Invitrogen) and 200 μM of each primer. Sequences of the forward and reverse primers, respectively, used for amplification and sequencing of EGFR exons were: exon 18 AGGGCTGAGGTGACCCTTGT, TCCCCACCAGACCATGAGAG; exon 19 ACCATCTCACAATTGCCAGTTAAC, GAGGTTCAGAGCCATGGACC; exon 20 ATGCGAAGCCACACTGACGTG, GGCCTGGCTTGCTTACCTTGT; exon 21 TCACAGCAGGGTCTTCTCTGTTT, ATGCTGGCTGACCTAAAGCC. PCR amplification was performed using the following conditions: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min.

For the sequencing reaction, 20 μL of the PCR products was purified using Ampure reagent (Agencourt, Beckman-Coulter) following manufacturer's instructions, sequenced with each forward and reverse primers using BigDyev3.1 terminators (Applied Biosystems) and run on a 3130xl Genetic Analyser (Applied Biosystems).

2.6. *Immunohistochemistry*

Immunohistochemistry was performed on paraffin-embedded tissue sections from tumour biopsies from 17 patients with pleural mesothelioma. Antigen retrieval was performed by boiling sections for 10 min in citrate buffer. Sections were stained with VEGF Receptor 2 or Phospho-VEGF receptor 2 antibody (Cell Signalling technology) in dilutions of 1:125 and 1:300, respectively. A biotinylated swine anti-rabbit secondary antibody was used, followed by peroxidise-conjugated streptavidin/biotin complex (Dako) and visualised using DAB.

3. Results

The growth inhibition curves obtained from SRB assays with the five tyrosine kinase inhibitors for three human mesothelioma cell lines are shown in Fig. 2. The GI_{50} values obtained are shown in Table 3. The values ranged from 3.5 μM to >30 μM and the compounds generally showed similar growth inhibitory activities across the three cell lines, apart from ZD18139 (gefitinib), which inhibited the growth of the H28 cells (GI_{50} = 5.0 ± 3.6 μM) at a significantly lower concentration than for the MSTO (GI_{50} = 18.3 ± 0.7 μM) or H226 cells (GI_{50} = 20.5 ± 2.9). The two compounds showing the most potent growth inhibitory effects in all three cell lines were ZD6474 (GI_{50} range 5.0–6.7 μM) and SU11248 (GI_{50} range 3.5–6.9 μM). These compounds are mainly active against VEGFR2 and were further investigated using Western blotting.

The presence of EGFR and VEGFR2 in the cell lines was investigated and all cell lines were found to express similarly high levels of EGFR as shown in Fig. 3, confirmed by densitometry (data not shown). Activating mutations of *EGFR* have been reported to confer resistance to TK inhibitors in some tumours,[16] however no mutations were detected in exons 18,19, 20 or 21 of *EGFR* in any of the cell lines studied.

Using RT-PCR, both the H226 and MSTO cells showed the presence of mRNA for VEGFR2. However, using Western blotting the VEGFR2 protein was found only in the H226, as shown in Fig. 3. No VEGFR2 was detected in the H28 cells by either RT-PCR or Western blotting. The beta actin mRNA and protein were used as an internal reference for RT-PCR to verify RNA integrity and protein loading in Western blots respectively.

The growth response of H226 cells to 10 or 30 ng/ml VEGF was measured over 7 days using the SRB assay. The cells survived in serum free medium over this time period. No increase in growth was found with VEGF compared with either serum free or serum rich medium (data not shown).

The effect of VEGF in serum free medium on the activation of the VEGFR2 in H226 cells was investigated and the results of VEGFR2 and downstream AKT phosphorylation by Western blotting are shown in Fig. 4. Phosphorylation of VEGFR2 was

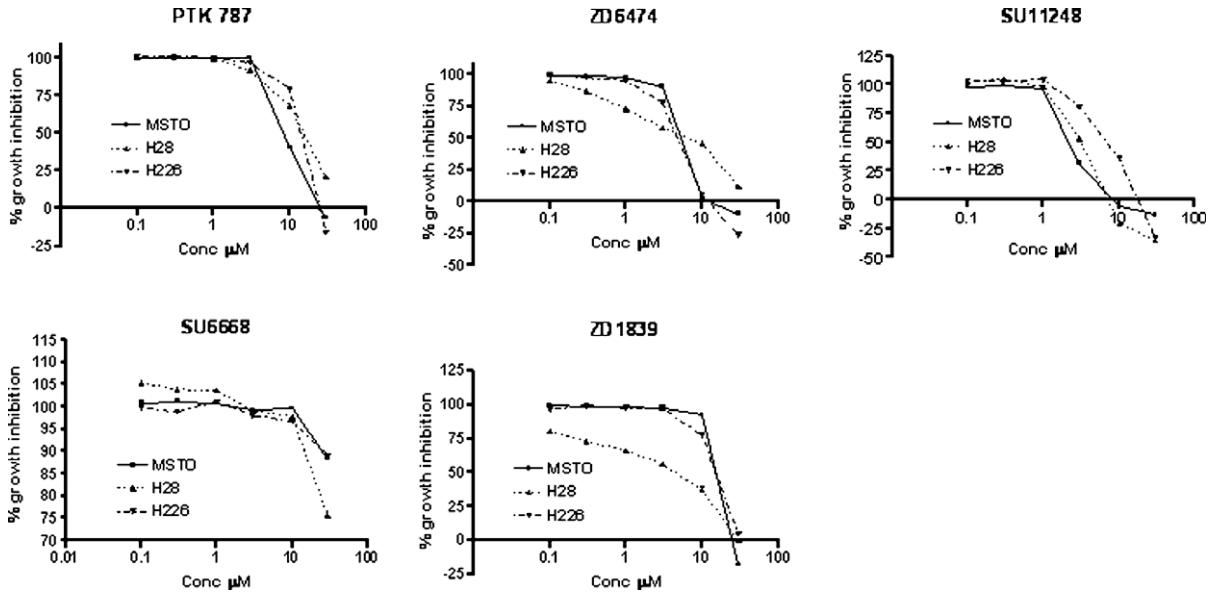

Fig. 2 – Growth inhibition curves of tyrosine kinase inhibitors in three mesothelioma cell lines. The results are from individual representative experiments.

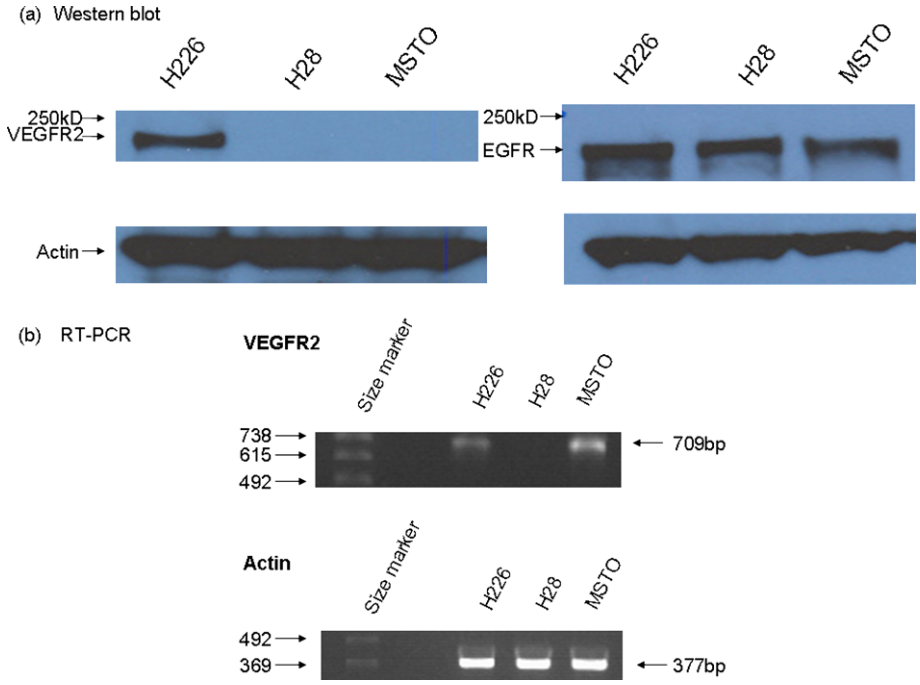

Fig. 3 – (a) VEGFR2 and EGFR in mesothelioma cell lines detected by Western blotting. (b) VEGFR2 detected by RT-PCR in mesothelioma cell lines. Actin was used to check for equal loading in both methods.

Table 3 – GI_{50} values (μM ± SD, n = 3) for tyrosine kinase inhibitors in mesothelioma cell lines.

	MSTO	H28	H226
PTK 787	10.8 ± 3.4	17.8 ± 2.4	16.3 ± 0.7
ZD 6474	6.7 ± 0.6	5.0 ± 3.6	6.3 ± 1.3
SU 6668	>30	>30	>30
SU 11248	3.6 ± 1.0	3.5 ± 0.4	6.9 ± 1.2
ZD 1839	18.3 ± 0.71	5.7 ± 1.72	20.5 ± 2.91

detected using the Tyr1175 antibody, but not the Tyr951 antibody. Maximum phosphorylation of the receptor was seen after 10 min and this had rapidly decreased by 30 min. However downstream phosphorylation of AKT was minimal at 5 min, and increased up to 30 min. Since both VEGFR2 and AKT activation were seen at the ten minute time point, this was used in subsequent studies of pathway activation.

A dose-dependent activation of the EGFR was seen in all cell lines following incubation with EGF in serum free medium for 10 min (Fig. 5). Phosphorylated ERK 1/2 was also seen following treatment with EGF. However, no change in the activation of ERK was seen in any cell line following 10 min treatment with VEGF. As expected, VEGF had no effect on the activation of EGFR.

H226 cells were treated with EGF or VEGF and the tyrosine kinase inhibitors in serum free medium for 10 min to investigate downstream signalling pathways and the results are shown in Fig. 6. With EGF, the receptor was phosphorylated in serum free medium and this activation was inhibited in a dose-dependent response with ZD6474 and ZD1839. SU11248 had little effect apart from at the highest dose used (10 μM) and also had no effect on activation of AKT and ERK1/2. Downstream activation of AKT and ERK1/2 was inhibited in a dose-dependent manner by ZD6474 and ZD1839. Constitutive phosphorylation of AKT and ERK1/2 was seen without

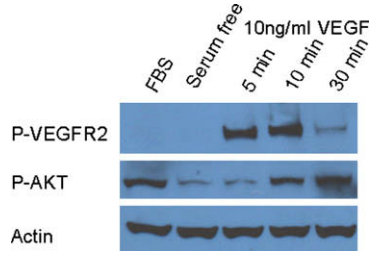

Fig. 4 – Time course of VEGFR2 and AKT phosphorylation induced by VEGF treatment of H226 cells, detected by Western blotting. P-VEGFR2 was detected using the phosphorylated Tyr 1175 antibody.

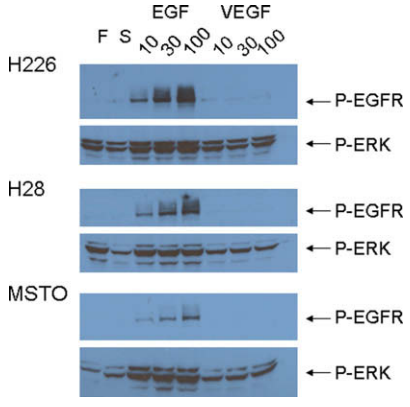

Fig. 5 – Phosphorylation of EGFR and ERK1/2 after 10 min treatment with 10–100 ng/ml EGF or VEGF in serum free medium shown by Western blotting. Controls were F: medium with 10% FBS; S: serum free medium.

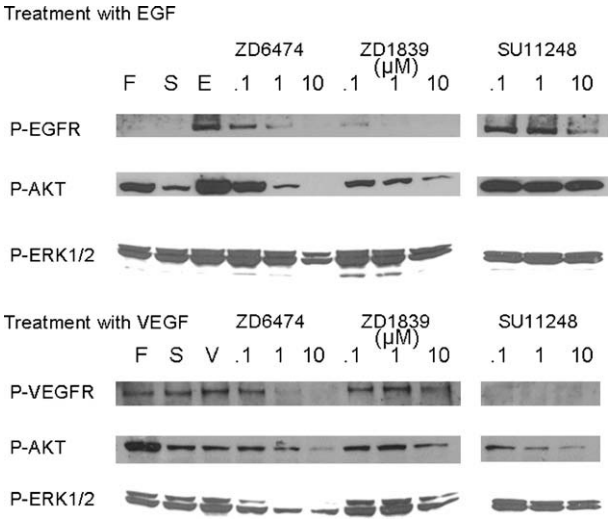

Fig. 6 – Western blotting for p-EGFR or p-VEGFR2, p-AKT and p-ERK1/2 after treatment of H226 cells with EGF (E) or VEGF (V) alone or with increasing concentrations of tyrosine kinase inhibitors for 10 min. Controls were F: medium with 10% FBS; S: serum free medium.

EGF stimulation, although p-AKT was reduced in serum free medium.

VEGFR phosphorylation induced by VEGF was inhibited by both ZD6474 and SU11248, with 0.1 μM SU11248 abolishing all phosphorylation. P-AKT was also reduced with increasing doses of both ZD6474 and SU11248, whereas there was little effect of SU11248 on ERK1/2 activation. ZD1839 had virtually no effect on VEGF stimulation of H226 cells.

Representative results from staining paraffin-embedded mesothelioma tissue samples for VEGFR2 and p-VEGFR2 are shown in Fig. 7. VEGFR2 antibody showed positive staining in all tissue sections, with either cytoplasmic staining or both membranous and cytoplasmic staining. Intense staining was seen in 12 of 17 samples, and membranous staining was seen in the same percentage of samples. No p-VEGFR2 staining could be detected in any tissue sample with the p-VEGFR2 Tyr 1175 antibody.

4. Discussion

Malignant mesothelioma represents a great challenge to both clinicians and cancer researchers due to its poor prognosis and marked resistance to current therapies. Tumours may be of epithelial, sarcomatous or mixed type in origin and those of epithelial type may have slightly better prognosis. Although there have been some improvements in treatment over the past few years, better understanding of the molecular basis of the disease and improved treatment is required. The tyrosine kinase inhibitors have potential in the treatment of mesothelioma due to the presence of growth factor receptors in tumours, and at present some of these molecules, including PTK787 and SU11248, are in clinical trials. This paper has investigated some of the effects of growth factor receptor tyrosine kinase inhibitors on mesothelioma cell lines and their downstream effects in relation to activity of the MAPK and AKT.

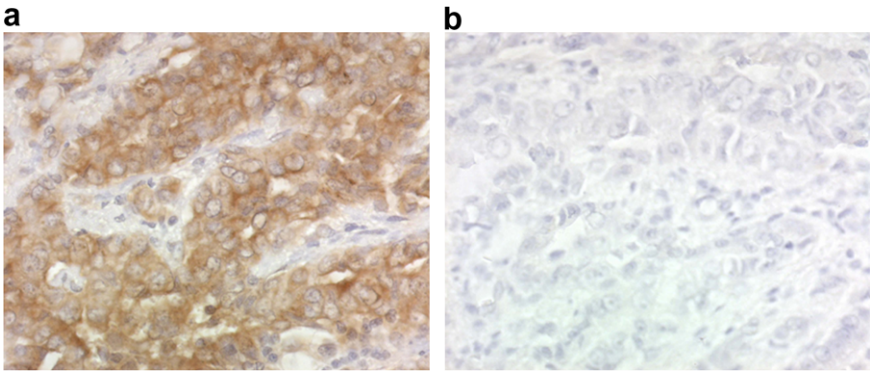

Fig. 7 – Immunohistochemistry of mesothelioma samples stained for (a) VEGFR2 and (b) p-VEGFR2.

The receptor tyrosine kinase inhibitors with the lowest GI_{50} values in all cell lines were ZD6474 and SU11248, both of which are inhibitors of VEGFR2. However, no evidence of these receptors was found in the NCI-H28 cell line by either Western blotting or RT-PCR (Fig. 2), and therefore the inhibitors must be exerting their effects by some other means. All the cell lines used expressed EGFR (Fig. 2), which may be the main target for ZD6474 in the H28 and MSTO cells, both of which show no evidence of VEGFR2. However, the growth inhibition curves showed the H28 cells to be sensitive to lower doses (0.3 and 1 μM) of ZD6474 than the other cell lines, which showed growth inhibition starting at 3 μM, suggesting a less specific pathway of inhibition in the H28 cells.

Mesothelioma cell lines are reported to express PDGF-β receptors but normal mesothelial cells express PDGF-α receptors,[17] and some effect of SU11248 and PTK787 could be due to inhibition of these receptors. This remains to be investigated in the cell lines studied. Imatinib, an inhibitor of PDGFβ, has been used in a study of mesothelioma cell lines,[18] and induced cytotoxicity and apoptosis selectively on PDGFRβ positive mesothelioma cells. Combination of imatinib with gemcitabine or pemetrexed showed a synergistic effect in their study.

It has also been shown that the increased rate of synthesis of proteoglycans in malignant mesothelioma cell lines stimulated by EGF, PDGF and insulin-like growth factor in serum free medium is blocked by genistein, suggesting that protein kinase activation is the main pathway for growth factor stimulation of matrix synthesis.[19] Exposure to EGF also induced a fibroblast-like morphology in STAV epithelial mesothelioma cells, with a suppression of cell surface proteoglycan synthesis but an increase in soluble (shed) syndecan 1, suggesting autoregulatory loops attenuating the effects of growth factors.[20] The use of tyrosine kinase inhibitors in mesothelioma may therefore also have an inhibitory effect on tumour matrix synthesis.

A study of mesothelioma cell lines with gefitinib[21] showed that pre-treatment of cells with 1 μM of the drug for 30 min significantly inhibited the EGF-induced phosphorylation of EGFR, AKT and ERK1/2 and that sensitivity to gefitinib was not related to the degree of inhibition of EGFR in MPM cell lines. A phase II trial of gefitinib in patients with malignant mesothelioma[6] concluded that although 97% of patients had EGFR overexpression, gefitinib was not active in these patients. The presence of mutations in EGFR have been correlated with the activity of gefitinib in NSCLC[22] but no EGFR mutation was detected in exons 18–21 in any of the mesothelioma cell lines used in our study or in a study of 66 mesothelioma samples.[23] However, other recent reports on EGFR in mesothelioma[4,5] showed EGFR immunoreactivity in only 56% and 44% of tumours, respectively. The latter report also showed EGFR expression was associated with a favourable prognosis, but may be due to other indicators of prognosis such as cell type, since EGFR positivity was more common in the epithelial subtype of mesothelioma.[24]

Both VEGF and its receptors have been identified in mesotheliomas by immunohistochemistry[25,26] and RT-PCR,[27] with VEGFR2 expression varying from 43% to 69%, suggesting an active autocrine loop. Molecules which target VEGFR2 inhibition may therefore be important in the treatment of mesothelioma. VEGF and VEGFC autocrine loops were also suggested in a study of mesothelioma cell lines.[28] However, in this paper, VEGFR2 was reported to be present in both H28 and MSTO-211H cells, in contrast to our results where VEGFR2 was detected at the protein level only in H226 cells. Although VEGFR2 was detected in tumour samples, no phosphorylated receptor was detected using the p-VEGFR2 antibody (tyr 1175). A recent publication[29] has tested several VEGFR2 phosphorylated antibodies and showed differences in detection of the antigen by immunohistochemistry. The lack of detection of the p-VEGFR2 in our study may not therefore be due to lack of phosphorylation. The AKT pathway is frequently activated in MM and inhibition of this pathway inhibits cell growth and increases sensitivity to conventional chemotherapeutic agents.[30] It has also been shown that inhibition of EGFR resulted in apoptotic cell death and AKT hypophosphorylation in mesothelioma cell lines.[31] The lack of inhibition by SU11248 of P-AKT and P-ERK1/2 after stimulation with EGF seen in the H226 cells, expressing VEGFR2 and EGFR, suggests that in these cells the main stimulation of these pathways is via the EGFR. Some inhibition of activation is seen when the cells are treated with VEGF, although not as pronounced as with ZD6474 which again may be exerting some of its effect via the EGFR, although little inhibition is seen with the EGFR inhibitor gefitinib. VEGF was seen to have no effect on P-ERK in any cell line, irrespective of the presence of VEGFR2. Inhibition of the activated receptors was seen at much lower concentrations of the inhibitors than required to show growth

inhibition of any of the cell lines, as indicated in Figs. 2 and 6. The main effect of the tyrosine kinase inhibitors found in this study indicates that it is the presence of VEGFR2 rather than EGFR that is important in the mesothelioma cell lines. The difference in effects of the inhibitors in the cell lines studied indicates the need for assessment of receptors in tumour samples. A review of the implications for combining anti-VEGF and anti-EGFR agents has recently been published.[32] This review suggests the potential of this approach to anti-cancer therapy will be elucidated by large, ongoing clinical trials. The possibility of using the tyrosine kinase inhibitors in mesothelioma alone or with the established combination of cisplatin with pemetrexed warrants further investigation to improve the response rate and survival of patients with this disease.

Conflict of interest statement

None declared.

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