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Lapatinib dysregulates HER2 signaling and impairs the viability of human uveal melanoma cells

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Running Title: Lapatinib inhibits tumourigenesis in human uveal melanoma

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Abbreviations:
AKT: Protein kinase B; BAX: Bcl-2-associated X Protein; BCL-xL: B-cell lymphoma-extra large; BRAF: v-raf murine sarcoma viral oncogene homolog B1; DMEM, Dulbecco's Modified Eagle Medium; EGFR: Epidermal Growth Factor Receptor; ERK: extracellular-signal-regulated kinase; FBS, Fetal Bovine Serum; GCT, Giant cell tumour; HER: Human Epidermal Growth Factor Receptor; ITS, Insulin-Transferrin-Selenium; JAK: janus kinase; KIT: tyrosine-protein kinase; MAPK: Mitogen-Activated Protein Kinase; MEK: Mitogen-activated protein kinase kinase; MTT, thiazolyl blue tetrazolium bromide; mTOR: mammalian target of rapamycin; NRAS: neuroblastoma RAS viral oncogene homolog; PBS, phosphate-buffered saline; PI, propidium iodide;
Abstract

Although uveal melanoma (UM) is rare, it is the principal type of intraocular malignancy. Up to 50% of UM patients develop metastatic disease with low chances of survival beyond 18 months. At present there are no drugs that are effective in the treatment of primary or metastatic disease. We recently tested the hypothesis that the ErbB receptor family member HER2 may be a novel drug target in UM. We found that afatinib, which targets several ErbB receptors, including HER2, has potent anti-cancer and anti-metastatic actions in UM. The present study was undertaken to further evaluate the potential value of HER2 targeting in UM using the multi-kinase inhibitor lapatinib, that is currently approved for the treatment of HER2-positive cancers in patients.

The anti-UM actions of lapatinib were assessed using cell viability, cell death and cell cycle analysis, and its anti-metastatic actions were evaluated using wound healing and colony formation assays. Immunoblotting was used to substantiate the actions of lapatinib on apoptotic and HER2 signaling. The anti-UM actions of lapatinib were further evaluated in vivo in a UM xenograft mouse model.

Lapatinib decreased the viability of four UM cell lines (IC_{50} values in MTT reduction assays were 3.67-6.53 µM). The antiproliferative activity of lapatinib was corroborated in UM cells isolated from patient tumors. In UM cell lines lapatinib promoted apoptosis and cell cycle arrest, and strongly inhibited cell migration and reproductive cell growth. Lapatinib dysregulated HER2-AKT/ERK/PI3K signalling in UM cell lines, as indicated by increased STAT1 expression, decreased expression of BCL-xL and cyclin D1, and decreased Bcl-xL:Bax ratio, which is consistent with enhanced apoptosis. Importantly, lapatinib and suppressed tumourigenesis in vivo in mice carrying UM cell xenografts.

Together the present findings are consistent with the assertion that HER2 is a viable therapeutic target in UM. Lapatinib was active in primary and metastatic UM and is a clinically approved HER2 inhibitor. The activity of lapatinib in UM patients could now be evaluated directly in clinical trials.

Keywords: Uveal melanoma, lapatinib, anti-cancer, anti-metastatic, HER2 inhibition
Introduction

Uveal melanoma (UM) is a rare cancer that is very different from its cutaneous counterpart. As the primary intraocular malignancy UM accounts for over 85% of ocular tumours and is also the second most common type of melanoma (~5% of all cases). The incidence of UM is similar in males and females and affects both eyes equally; however, it is more frequently identified in Caucasians and in adults aged over 40.

Although the survival rate is around 84% for early-stage UM (AJCC stages I and II), patients often experience treatment delays due to the difficulty in distinguishing tumours from benign tissues. UM tumours are often asymptomatic until they reach a significant size. Not surprisingly, the mortality rate increases dramatically in late stage UM. Up to 50% of patients develop metastases, particularly in liver, prior to diagnosis. The median survival rate of UM patients with liver metastases is 4-6 months and those whose disease is advanced may survive <3 months.

Clinically, enucleation has been widely used in the treatment of primary UM tumours, but this may lead to irreversible eye damage. More recently, other treatment options such as brachytherapy, proton beam therapy and phototherapy, have been used to treat primary UM tumours with the aim of preserving vision. Nevertheless, such non-pharmacological approaches are unable to prevent metastatic lesions developing in distant tissues. Even though hepatic chemoembolization, isolated hepatic perfusion, intra-arterial chemotherapy, radiotherapy, and surgical resection are effective in the treatment of patients with liver cancer, they are generally ineffective in UM tumours that have metastasized to the liver.

Both cutaneous melanoma and UM are derived from melanocytes. However, unlike cutaneous melanoma, environmental factors such as UV radiation and latitude, are not associated with the development of UM. Furthermore, cutaneous melanoma is characterised by mutations that activate the v-raf murine sarcoma viral oncogene homolog B1 (BRAF), neuroblastoma RAS viral oncogene homolog (NRAS), tyrosine-protein kinase (KIT) and phosphatidylinositol 3-Kinase (PI3K) pathways, whereas UM is unrelated to these mutations. UM is characterised by low tumour mutational burden compared to cutaneous melanoma where mutagenic effect of UV light is apparent. However, UM displays a distinct genetic profile that may be associated with its development and prognosis. Notably, the most common mutations in UM occur in the tumour suppressor gene and the guanine nucleotide binding protein Gaq/Ga11 (GNAQ/11) gene, which accounts for over 40% of genetic mutations in UM, followed by BRCA1 associated protein 1 (BAP1). Mutations within these two regulatory genes result in increased cell growth, proliferation and metastasis leading to poor prognosis in UM. Other molecular changes including monosomy of chromosome 3, amplification or gain of chromosome 8q all appear to contribute to the grim prognosis of UM patients. Drugs that are used clinically for the treatment of cutaneous melanoma are ineffective in the treatment of UM.
The epithelial growth factor receptor (EGFR) family consists of 4 members: ErbB1–4. ErbB receptors are transmembrane proteins that have a cytoplasmic binding domain, a transmembrane domain and an intracellular domain that interacts with downstream signalling pathways. Receptor activation causes hetero- or homo-dimerization, followed by autophosphorylation on tyrosine residues in the intracellular kinase domain. Activation of downstream pathways, such as the PI3K/protein kinase B (AKT), Ras/mitogen-activated protein kinase kinase (MEK)/extracellular-signal-regulated kinase (ERK), phospholipase C Gamma (PLCγ)/protein kinase C (PKC), and janus kinase (JAK)/signal transducer and activator of transcription (STAT) cascades, regulate cell survival, proliferation, differentiation, motility, apoptosis, survival, invasion, migration, adhesion, and angiogenesis. Therefore, ErbB isoforms have been widely studied as cancer drug targets.

With advances in gene profiling, multi-kinase inhibitors have been suggested to have potential value in the development of new treatment strategies in UM. We recently tested several multi-kinase inhibitors in UM cell lines. We found that afatinib, which is a potent inhibitor of multiple ErbB receptors, including EGFR, HER2 and HER4, induced cell death and prevented cell migration in UM cell lines. It is noteworthy that EGFR and HER4 are not commonly expressed in UM tumours. Therefore, neither of these receptors is likely to be a viable molecular target for UM drug development. The finding that afatinib dysregulated HER2 signaling to exert its anti-UM actions suggests that HER2 could be a novel therapeutic target in UM. To substantiate the potential clinical significance of HER2 in UM, we investigated the anti-UM actions of the HER2 inhibitor lapatinib in the present study. Lapatinib was selected because it is clinically approved for use in patients with HER2-positive breast cancers that are resistant to the front-line agent trastuzumab. Thus, lapatinib has the potential advantage that it may be more rapidly translated to clinical application in UM.

Results

Lapatinib decreased the viability of UM cells

The anti-UM actions of lapatinib were evaluated in C918, 92.1 and Mel202 cells that were derived from primary UM tumours and in OMM-1 cells that were isolated from a subcutaneous metastasis. All four cell lines were treated with lapatinib over the concentration range 0 to 50 µM. Cell viability was then estimated using MTT reduction assays. As shown in Fig. 1, the IC50 values of lapatinib ranged from 3.67 uM to 6.53 µM across the four UM cell lines.
Fig 1. Lapatinib decreases the viability of UM cell lines. Mel202 (A), C918 (B), 92.1 (C) and OMM1 (D) cells were treated with lapatinib (0-50 µM) at 37°C for 24 h. Cell viability was assessed in MTT reduction assays. IC$_{50}$s of lapatinib in UM cell lines were estimated by non-linear regression (GraphPad Prism 7.0; San Diego, CA).

**Lapatinib induced apoptosis and cell cycle arrest in UM cell lines**

The capacity of lapatinib to promote UM cell death was evaluated using Annexin-V/PI staining and flow cytometry. Apoptosis was found to be the principal cell death mechanism in all four UM cell lines after treatment with lapatinib (5 µM, 24 h; Fig. 2). Thus, lapatinib increased the proportion of apoptotic cells to 2.73-6.40-fold of control (p<0.001, Fig. 2B, 2D, 2F, 2H). In accord with these findings, lapatinib (5 µM) also decreased viability and activated apoptosis in three tumour-derived cell lines from UM patients (Fig. 3).
Fig 2. Lapatinib activates apoptosis in UM cell lines. Mel202 (A & B), C918 (C & D), 92.1 (E & F) and OMM1 (G & H) cells were treated with lapatinib (5 µM) at 37°C for 24 h, stained with Annexin V-FITC/PI and subjected to flow cytometry. Representative cell death profiles are shown for Mel202 (A), C918 (C), 92.1 (E) and OMM1 (G) cells. The percentages of viable, necrotic and apoptotic cells are indicated as mean ± SD for Mel202 (B), C918 (D), 92.1 (F) and OMM1 (H) cells. Experiments were performed on
3 independent occasions and each experiment included three repeats. Control treatments consisted of vehicle (DMSO) alone. ***p < 0.001 vs. control by One-way ANOVA and Dunnett’s post-hoc test.
Fig 3. Lapatinib decreases viability and activates apoptosis in primary UM-tumour derived cell lines. UM tumour-derived cell lines were treated with lapatinib 5 µM for 24 h at 37°C. The viability of each primary cell line was assessed using MTT reduction (A, C and E). After treatment, cells were stained with Annexin V-FITC/PI and subjected to flow cytometry. The percentages of viable, necrotic and apoptotic cells are indicated as mean ± SD for each primary cell line in B, D and F. Experiments were performed on 3 independent occasions and each experiment included three repeats; control treatments consisted of vehicle (DMSO) alone. **p < 0.01; ***p < 0.001 vs. control by One-way ANOVA and Dunnett’s post-hoc test.

To further evaluate the impact of lapatinib on viability, UM cells were stained with PI and subjected to cell cycle analysis by flow cytometry. The proportion of cells in G0/G1 phase was increased by lapatinib (5 µM, 24 h; P<0.001), while the proportion of cells in G2/M phase was decreased (P<0.001) and cells in S phase were unchanged (Fig. 4). Taken together, these findings indicate that lapatinib is highly effective in inducing apoptosis and cell cycle arrest in UM cell lines.

Fig 4. Lapatinib induces cell cycle arrest in UM cell lines. Mel202 (A), C918 (B), 92.1 (C) and OMM1 (D) cells were treated with lapatinib (5 µM) for 24 h at 37°C. Cells were stained with PI and subjected to flow cytometry. The percentages of cells in G0/G1, G2/M and S phases are shown as mean ± SD. Experiments were performed on 3 independent occasions and each experiment included three repeats; control treatments
consisted of vehicle (DMSO) alone. ***p < 0.001 vs. control by One-way ANOVA and Dunnett’s post-hoc test.

**Lapatinib inhibited UM cell migration and suppressed reproductive growth**

The impact of lapatinib on UM cell migration was examined in scratch-wound healing assays. As shown in Fig 5A-F, lapatinib decreased rates of migration in the Mel202, C918 and 92.1 cell lines that were derived from primary UM tumours (to 0.26–0.36-fold of control; P < 0.001).

Colony formation assays were also performed to assess reproductive cell growth upon lapatinib treatment (5 µM, 24 h). As shown in Fig. 5G, lapatinib significantly decreased the number of viable colonies post treatment in all three cell lines (P<0.001).

These findings suggest that lapatinib has anti-metastatic actions in UM.
Fig 5. Lapatinib is anti-metastatic in primary tumour-derived UM cell lines. The anti-migratory actions of lapatinib were assessed in scratch-wound assays. UM cell lines were treated with lapatinib 5 µM at 37°C for 24 h. Cell images were captured at 0 and 24 h; representative images are shown for Mel202 (A), C918 (C) and 92.1 (E) cells. The rate of cell migration was estimated as the means of each repeat and are indicated as fold of control (means ± SD) for each cell line (B, D and F). Reproductive cell growth after lapatinib treatment was evaluated in colony formation assays. (G) Colony number is indicated as the percentage of control (mean ± SD). Experiments were performed on 3 independent occasions and each experiment included four repeats; control treatments consisted of vehicle (DMSO) alone. ***p < 0.001 vs. control by One-way ANOVA and Dunnett’s post-hoc test.

**Lapatinib modulates STAT1 and apoptotic signaling in UM cells**

STAT1 is an important regulator of apoptosis. In the present study, the capacity of lapatinib to modulate the expression of STAT1 and its downstream signaling was examined. Treatment with lapatinib substantially increased STAT1 expression to 1.4 - 4.9-fold of control across all four UM cell lines (Fig. 6A, 6C, 6D, 6F, 6G, 6I, 6J, 6L). Further, lapatinib decreased the expression of the anti-apoptotic Bcl-XL and increased the pro-apoptotic BAX in all four UM cell lines (Fig. 6). Consistent with the activation of apoptosis Bcl-xL:BAX ratios were markedly decreased by lapatinib (5 µM, 24 h), as shown in Fig. 6B, 6E, 6H and 6K.

Because lapatinib induced cell cycle arrest in UM cell lines (Fig. 4) we assessed the expression of cyclin D1 - a key cell cycle mediator that is also downstream from STAT1. Treatment with lapatinib (5 µM, 24 h) decreased cyclin D1 expression in UM cells to 0.24–0.47 fold of control (Fig. 6C, 6F, 6I and 6L).

In summary, lapatinib induced cell death was associated with dysregulated expression of STAT1 and its downstream targets cyclin D1, BAX and Bcl-xL.
Fig 6. The lapatinib-mediated activation of apoptosis is associated with the modulation of STAT1, Bcl-xL and cyclin D1 expression in UM cell lines. Expression of Bcl-xL,
BAX, STAT1 and cyclin D1 was determined by Western blotting with β-actin as the loading control. Cells were treated with lapatinib (5 µM) at 37°C for 24 h, then harvested, lysed, denatured and subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis. Representative images of proteins of interest are shown for Mel202 (A), 92.1 (D), C918 (G) and OMM-1 (J) cells. Densitometry analysis for protein quantification was conducted using Image J. Bcl-xL:BAX expression ratios are shown for Mel202 (B), C918 (E), 92.1 (H) and OMM-1 (K) cells. The expression of STAT1, Bcl-xL and cyclin D1 relative to β-actin is indicated as fold of control (mean ± SD) for Mel202 (C), 92.1 (F), C918 (I) and OMM-1 (L) cells; control treatments consisted of vehicle (DMSO) alone. Experiments were performed on three separate occasions. *p < 0.05; **p < 0.01; ***p < 0.001 vs. control by unpaired t-test.

**Lapatinib exerts its anti-UM actions by inhibiting HER2 signalling**

Lapatinib is an established inhibitor of HER2 and is used clinically in the treatment of HER2-positive cancers, including breast cancers that are resistant to the first-choice agent trastuzumab. Unlike other ErbB receptor isoforms, it has been found previously that HER2 is uniformly expressed in UM cells.

We assessed the impact of lapatinib on the expression of HER2 and its phosphorylated isoform in the four UM cell lines. In these experiments cells were initially cultured in serum-free medium and were then treated with 20% FBS for 10 min immediately prior to treatment with lapatinib (L+; Fig. 7) or vehicle (C+; Fig. 7). This rapidly activated HER2 phosphorylation that was attenuated by lapatinib (Fig. 7; compare the values for lapatinib and control in the Tables at right). Important downstream targets of HER2 include ERK, PI3K and AKT. Inclusion of lapatinib also prevented the activation of these pathways after serum addition (Fig. 7; compare L+ versus L- relative to C+ versus C-).

Overall, these findings indicate that lapatinib inhibits HER2 and its downstream signaling and suggests that these may be early events in its anti-UM actions.
Fig 7. The lapatinib-mediated activation of cell death is associated with inhibition of HER2 and its downstream signaling cascades in UM cell lines. In each experiment, four sets of cells were cultured in serum-free medium for 24 h. Two of the four sets of cells
were then treated for 10 min with medium containing 20% FBS while the other two sets of cells remained serum-free. In the next step, one each of the sets of serum-treated and serum-free cells was treated with lapatinib (5 μM) at 37°C for 1 h, while the others were treated with vehicle alone (DMSO), and lysates were prepared. This produced a four-way design that evaluated the effect of serum addition and lapatinib addition on the signalling pathways (Key: C-: vehicle control without serum stimulation; C+: vehicle control with serum stimulation; L-: lapatinib treatment without serum stimulation; L+: lapatinib treatment with serum stimulation). Expression of HER2, AKT, ERK and PI3K and their phosphorylated isoforms was evaluated by Western blotting and densitometry analysis. Representative images of p-HER2, HER2, p-AKT, AKT, p-PI3K, PI3K, p-ERK and ERK are shown for Mel202 (A), 92.1 (B), C918 (C) and OMM-1 (D) cells. Densitometry analysis was conducted using ImageJ and the ratios (L+/L-) and (C+/C-) were calculated for the effects on lapatinib and DMSO respectively on the expression of phosphorylated and total forms of the proteins. These data as fold of corresponding control (no serum stimulation) in the tables at right (mean ± SD) are shown in the Tables beside panels A-D. Experiments were repeated on three occasions. #p < 0.05; ##p < 0.01; ###p < 0.001 vs. control by Two-way ANOVA.

**Lapatinib has potent anti-tumour activity in a UM xenograft mouse model**

The anti-UM actions of lapatinib were examined further in a xenograft model. Lapatinib was administered to nude mice that carried UM cell xenografts at a dose of 25mg/kg for 14 d; tumour growth was suppressed (Fig. 8A and 8B). From PET scan analysis, the final tumour sizes in the lapatinib-treated mice were smaller than those in controls (Fig. 8C).

Confirmatory immunohistochemical staining was undertaken in tumour samples that were collected at the end of the experimental treatments. From H&E staining the tumour architecture was improved by lapatinib treatment (Fig. 8D). Staining with the cell proliferation marker Ki67 was decreased by lapatinib and apoptosis, as reflected by TUNEL staining, was increased (Fig. 8D).

Overall, these data indicate that lapatinib inhibits tumour growth, suppresses cell proliferation, and activates tumour cell apoptosis in vivo in mice carrying UM-cell xenografts.
Fig 8. Lapatinib inhibited tumour growth in UM xenografted mice. BALB/c nude mice were inoculated with C918 cells. After 14 d, mice received either lapatinib (25 mg/kg per day, n = 10) or vehicle (n = 12) on day 10 by intraperitoneal injection; treatments were continued for a further 14 d. Tumour volumes and body weight of mice were
measured every 2 d. At the end of the experiment, mice were either sacrificed to harvest tumour samples or were subjected to whole body PET scan (n = 5 for lapatinib and 6 for vehicle). Tumour size vs treatment time is indicated in (A) and representative tumour images at the end of experiment are shown in (B). Data are presented as tumour volumes at each time point (mean ± SD; n = 5 for lapatinib and 6 for vehicle); p < 0.05 vs. control by unpaired t-test. Representative PET scans are shown in (C). Harvested tumours were embedded in paraffin and sections were prepared for staining. Representative images of hematoxylin and eosin (H&E) staining of tumour sections are shown in the panels at left, TUNEL staining is indicated in the central panels and Ki67 staining is shown in the right panels (D).

Discussion

ErbB receptors regulate cellular homeostasis. Dysregulation of the receptors leads to impairment of proliferative and pro-survival mechanisms in cells and may contribute to disease progression 48-50. Intracellular signaling cascades downstream from ErbB receptors are regulated by phosphorylation events that are mediated by kinase intermediates. The development of small molecule inhibitors of ErbB receptor-linked kinases has revolutionised the treatment of a number of cancers 51. The ErbB receptor member EGFR was initially suggested to be a potential drug target in UM. However, EGFR inhibitors like gefitinib have been disappointing in clinical trials that have been conducted in UM patients 25,37,38,52. Despite these outcomes, small molecules that target other members of the ErbB family have not been widely considered as alternative agents for use in patients with UM.

We found previously studies that the EGFR, HER2 and HER4 inhibitor afatinib, was an effective anti-cancer and anti-metastatic agent in UM 34. Because EGFR inhibition appears to be of limited value in UM 34,36,53, and the expression of EGFR and HER4 is restricted 34 these receptors are unlikely targets for afatinib; in contrast, HER2 is expressed in UM tumours 34,45,54. Thus, it is now important to evaluate in greater detail the potential clinical value of HER2 targeting in the treatment of UM.

Lapatinib is a high affinity HER2 inhibitor (Table 1) 55, and is currently approved in combination with cytotoxic agents such as capecitabine for HER2-positive breast cancers 56-58. Lapatinib is a reversible inhibitor of the kinase binding site of HER2, and blocks downstream proliferative and pro-survival signaling 56. Lapatinib has advantages of receptor targeting specificity over afatinib. Afatinib is 28-fold more potent against EGFR than HER2, and is also effective against common mutant EGFRs, whereas the relative activity of lapatinib against HER2 is greater (Table 1) 59. Thus, off-target effects at EGFR in multiple tissues are expected to be less likely with lapatinib. In the present study, we investigated the anti-cancer and anti-metastatic actions of lapatinib in a range of UM models. Lapatinib decreased UM cell viability by inhibiting cell proliferation and by promoting apoptosis and cell cycle arrest. Lapatinib also decreased tumourigenesis in vivo in mice that carried UM cell xenografts. Afatinib and lapatinib have different efficacies against other cancer types 60. Comapred to afatinib 34,
Lapatinib was more effective in inhibiting UM cell migration and reproductive cell growth, which suggests that it may have utility in the suppression of UM metastasis. In contrast, afatinib was slightly more effective in the induction of cell apoptosis and cell cycle arrest (Table 1). Taken together, these findings suggest that afatinib may be considered for the treatment of primary UM tumours, while lapatinib may be used as an adjuvant therapy in the prevention of UM metastasis.

Table 1. The comparison of anti-UM effect of afatinib and lapatinib

<table>
<thead>
<tr>
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<th>Afatinib</th>
<th>Lapatinib</th>
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<tbody>
<tr>
<td>Molecular target</td>
<td>Irreversible inhibitor of EGFR, HER2 and HER4 (^{59})</td>
<td>Reversible inhibitor of EGFR and HER2 (^{55})</td>
</tr>
<tr>
<td>IC(_{50}) in inhibiting HER2 (nM)</td>
<td>14.59 (^{59})</td>
<td>9.2 (^{55})</td>
</tr>
<tr>
<td>IC(_{50}) in reducing cell viability in UM cell lines (µM)</td>
<td>Mel202: 5.29 ± 1.21 (^{34})</td>
<td>5.69 ± 1.21 (^{34})</td>
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<tr>
<td></td>
<td>Mel92.1: 4.52 ± 1.41 (^{34})</td>
<td>4.89 ± 0.71 (^{34})</td>
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<td></td>
<td>C918: 3.43 ± 0.82 (^{34})</td>
<td>6.53 ± 1.30 (^{34})</td>
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<tr>
<td></td>
<td>OMM-1: 4.47 ± 1.16 (^{34})</td>
<td>3.67 ± 0.58 (^{34})</td>
</tr>
<tr>
<td>Cell death mechanism in UM cell lines (5 µM treatment for 18 and 24 h, respectively)</td>
<td>Apoptosis (5.79-10.20-fold of control) (^{34})</td>
<td>Apoptosis (2.73-6.40-fold of control)</td>
</tr>
<tr>
<td>Inhibition of UM cell migration (5 µM treatment for 24 h)</td>
<td>0.23–0.73-fold of control (^{34})</td>
<td>0.26–0.36-fold of control</td>
</tr>
<tr>
<td>Inhibition of UM reproductive cell growth (5 µM inhibitor for 24 h)</td>
<td>0.02–0.29-fold of control (^{34})</td>
<td>0.01–0.04-fold of control</td>
</tr>
<tr>
<td>UM cell cycle arrest (5 µM treatment for 18 and 24 h, respectively)</td>
<td>G0/G1: 1.33–1.53-fold of control (^{34})</td>
<td>1.19–1.75-fold of control</td>
</tr>
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<td></td>
<td>G2/M: 0.34–0.46-fold of control (^{34})</td>
<td>0.26–0.74-fold of control</td>
</tr>
<tr>
<td>Tumour growth inhibition in xenograft model</td>
<td>0.30-fold of control group (15 mg/kg per day for 16 days) (^{34})</td>
<td>0.23-fold of control (25 mg/kg per day for 14 days)</td>
</tr>
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</table>

HER2 is not activated by ligand binding but is instead a signal transducer that heterodimerises with other ErbB receptors that are ligand activated \(^{61,62}\). HER2 is an important driver of tumourigenesis in several cancers, including HER2-positive breast cancers where its expression is amplified \(^{63}\). HER2 overexpression or activation in breast cancer is often accompanied by poor prognosis due to more aggressive and invasive behaviour \(^{64,65}\). HER2 expression was inversely correlated with outcomes from breast cancer treatment \(^{66-68}\). HER2 activation is also associated with increased tumour...
size and invasiveness 69,70. In a large study (n=1,012), ~37% of patients with HER2 positive breast cancer reportedly had brain metastases 71.

HER2 is linked to the activation of multiple downstream signalling pathways that regulate tumourigenesis, including STAT1-regulated cascades 72-74. STAT1 regulates cell cycle progression and the inhibition of HER2 was found to increase STAT1 expression and promote cell cycle arrest by downregulation of cyclin D1 72,75 which suppresses tumourigenesis 76,77. STAT1 also regulates the transcription of Bcl-2 genes that modulate apoptosis. Thus, the activation of STAT1 upregulates pro-apoptotic BAX and downregulates the anti-apoptotic Bcl-2 and Bcl-xL 78. In the present study, lapatinib promoted cell cycle arrest and apoptosis by decreasing the expression of cyclin D1 and the anti-apoptotic Bcl-xL.

HER2 also regulates the AKT, ERK and PI3K-linked signaling pathways that modulate cell proliferation, migration, and death 79 and that contribute to tumourigenesis in multiple cancer types 80,81. The overarching consensus is that the activation of HER2-AKT/ERK/PI3K cascades increases cell proliferation, survival and migration 82-84. The present findings that lapatinib impairs PI3K, Akt and ERK signalling downstream from HER2 are consistent with its antiproliferative and antimigratory actions in UM cells.

Lapatinib has additional advantages that could facilitate its clinical translation. The anti-cancer activity of lapatinib due to HER2 inhibition has been established in studies of HER2-positive breast cancer, including advanced metastatic disease 85-87. Lapatinib is currently administered orally in a once daily dosage regimen (dose range 100 to 1500 mg per day) and produces $C_{\text{min}}$ values in the range 0.29–0.77 µM and $C_{\text{max}}$ values in the range 0.70–3.39 µM 88,89. These plasma concentrations likely fall within the range of those required for effective anti-UM actions (Fig. 1). Lapatinib also crosses the blood brain barrier, because it has been shown that brain metastases were decreased to 50%-53% of control in xenografted mice with metastatic breast cancer 85.

The present study found that lapatinib decreased tumour cell migration and reproductive growth, which suggests that the drug may be an effective adjuvant in the prevention of UM metastases. The finding that the anti-cancer actions of lapatinib are consistent with inhibition of HER2 and its downstream targets supports the potential utility of lapatinib in UM (Fig. 9). Clinical trials to test this directly in UM patients may now be warranted.
Fig 9. The proposed mode of action of lapatinib in UM cell lines. Lapatinib inhibits HER2 and its downstream signaling along PI3K/AKT and Ras/MEK/ERK pathways. UM apoptosis is activated by upregulation of BAX and STAT1 as well as a downregulation of Bcl-xL and cyclin D1.

Key: intracellular p, phosphorylated residues in receptors; AKT: Protein kinase B; BAX: Bcl-2-associated X Protein; BCL-xL: B-cell lymphoma-extra large; ERK: extracellular-signal-regulated kinase; MEK: Mitogen-activated protein kinase kinase; mTOR: mammalian target of rapamycin; RAS: RAS viral oncogene homolog; PI3K:
Phosphatidyinositol 3-Kinase; RAF: rapidly activated fibrosarcoma; STAT1, signal transducer and activator of transcription-1.

This principal findings from the present study are that lapatinib is a potential candidate for the treatment of UM, based on its anticancer and anti-metastatic activities in in vitro, ex vivo and in vivo models. Importantly, the present study supports the assertion that HER2 is a promising therapeutic target in UM. Taken together, lapatinib is a model HER2 inhibitor that is already approved for the treatment of HER2-positive breast cancer that could now be evaluated further in clinical trials in UM patients.

Material and Methods

Reagents and biochemicals

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Insulin-Transferrin-Selenium (ITS), L-Glutamine, Penicillin-Streptomycin (P/S) and Roswell Park Memorial Institute Medium (RPMI-1640) were purchased from Thermo Scientific (Lidcombe, NSW, Australia). Giant cell tumour (GCT) conditioned medium was obtained from United Biosciences (Carindale, QLD, Australia). The β-actin antibody, dimethyl sulfoxide (DMSO) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Lapatinib was from Selleck Chemicals (Houston, Texas, USA), dissolved in DMSO and stored at -20°C. Antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA): Akt (pan, Cat. #: 4685), Bcl2-associated X protein (Bax; D2E11, Cat. #: 5023), Bcl-xL (54H6, Cat. #: 2764), cyclin D1 (Cat. #: 55506), GAPDH (D16H11, Cat. #: 5174), HER2/ErbB2 (Cat. #: 4290), ERK (Erk, Cat. #: 4695), PI3K p85 (19H8, Cat. #: 4257), phospho-Akt (Ser473, Cat. #: 4060), phospho-HER2/ErbB2 (Yr1196, Cat. #: 6942), phospho-PI3K p85 (Tyr458)/p55 (Tyr199) (E3U1H, Cat. #: 17366), phospho-ERK (Thr202/Tyr204, Cat. #: 4370) and STAT1 (D1K9Y, Cat. #: 14994). The FITC Annexin V Apoptosis Detection Kit II was purchased from BD Bioscience (North Ryde, NSW, Australia). Goat anti-mouse and anti-rabbit IgGs that were conjugated with horseradish peroxidase were obtained from Bio-strategy delivery technology (Tullamarine, VIC, Australia). PVDF membranes were from Merck Millipore (Bayswater, VIC, Australia).

UM cell lines

UM cell lines used in this study were obtained as indicated previously. All cell lines were regularly checked for mycoplasma with MycoAlert Mycoplasma Detection kit (Lonza, Mount Waverley, VIC Australia) to ensure optimal viability. RPMI-1640 was used to culture C918, Mel202 and 92.1 cells and DMEM was used to maintain OMM-1 cells. All culture media was supplemented with 10% heat-inactivated FBS (v/v), 1% L-Glutamine and 1% P/S (Thermo Scientific, Lidcombe, NSW, Australia). Cells were incubated in a humidified incubator (5% CO₂) at 37°C.

Cell viability assay
Assays of MTT reduction were used to determine cell viability after lapatinib treatment. UM cells were cultured in 96 well plates (2x10⁴ cell/well). Cells were treated with lapatinib (10 µM) in RPMI-1640 or DMEM containing 1% FBS. MTT (0.5 mg/mL) was added 24 h later and, after incubation in the dark for 2 h, cells were washed with phosphate-buffered saline (PBS; 0.154 M NaCl, 0.001 M KH₂PO₄, 0.003 M Na₂HPO₄; pH 7.4), DMSO was added, and plates were shaken for 10 min at room temperature. Absorbance was measured at 550 nm in a microplate reader (Model 680, Bio-Rad, Gladesville, NSW, Australia). IC₅₀ values were calculated by non-linear regression of MTT inhibition as a function of drug concentration (GraphPad Prism 7.0; San Diego, CA).⁹⁰

Annexin V/propidium iodide flow cytometry assay

UM cells were seeded and treated with lapatinib (5 µM) in medium containing 1% FBS. Cells were collected 24 h after treatment, washed with PBS, suspended, and stained with annexin V and propidium iodide (PI) for 20 min at room temperature. Samples were subjected to flow cytometry (Guava easy®cyte; Merck Millipore, Bayswater, VIC, Australia) and apoptotic and necrotic cells were quantified as described previously.⁹¹

Cell cycle analysis

Cells were seeded and treated with lapatinib (5 µM) for 24 h, then harvested and washed twice in PBS before fixing overnight in cold 70% ethanol at -20 °C. The ethanol was removed, samples were washed with PBS and then stained in the dark with PI for 30 min at 37 °C, after which they were analysed by flow cytometry (Guava easy®cyte).

Scratch-wound cell migration assays

Cells were cultured on 24-well microplates (5 x 10⁴ cells/well). After 24 h scratches were made with a Wound Maker™ instrument (Sartorius, Dandenong South, VIC, Australia). Cells were washed with PBS and incubated in medium containing 1% FBS (v/v) and lapatinib (5 µM) for 24 h. Cells were incubated at 37°C and photos were taken at 2 h intervals with an Essen IncuCyte S3² instrument (10X magnification; Sartorius). Cell migration rates were determined using Image J software (National Institutes of Health, USA) with the Colony Counter Plugin. Migration rate was calculated as:

\[ Migration \ Rate \% = \left( \frac{\text{Area}(\text{initial}) - \text{Area}(\text{final})}{\text{Area}(\text{initial})} \right) \times 100 \]

Area (initial) is the area of the scratch measured immediately after wounding (t = 0 h). Area (final) is the area of the wound measured 24 h after the scratch was applied.

Colony formation assay

Cells were treated with lapatinib (5 µM) in 12 well plates and then aliquoted into 24-well plates (200 cells/well) for 6-8 days. Methanol (100%) was used to fix cells for staining with crystal violet. Colony growth was defined microscopically as a cluster of at least 50 cells. Photos were taken in an Essen IncuCyte S3², using whole-well scan
mode at 4 X magnification. Image J software was used to identify the leading edge of the cell population.

**Western blot**

Cells were treated with lapatinib and incubated for 24 h before they were harvested with lysis buffer containing NP-40 (1% IGEPAL, 150 mM NaCl and 50 mM Tris, pH 7.8) containing protease inhibitors. Lysates were then centrifuged at 15,000 rpm (10 min, 4°C) to separate protein-containing supernatants and cell remnants; supernatant fractions were denatured on a heat block.

Proteins in supernatant fractions were separated by electrophoresis, transferred to a PVDF membrane and incubated in 5% non-fat milk dissolved in PBS containing Triton 0.05% X-100 (PBST) at room temperature for 30 min. The membranes were incubated overnight with a primary antibody at 4°C. Membranes were washed three times with PBST and were then incubated at room temperature with a secondary antibody for 1 h. Signals were detected using chemiluminescence (SuperSignal West Pico, Thermo Scientific, Lidcombe, NSW, Australia) and were visualized with ImageQuant LAS500 (GE Health Care, Silverwater, NSW, Australia).

**Primary UM tumour derived cell lines**

Human UM tumour samples were obtained with approval from St. Vincent’s Hospital Sydney Human Ethics Committee (HREC/17/SVH/346) and experiments were strictly conducted as per the relevant guidelines and regulations. Tumour tissues were surgically removed, cut into segments, treated with trypsin-EDTA and then washed three times with PBS (pH 7.4). Individual cells were collected and incubated at 37°C in RPMI-1640 medium containing 20% FBS (v/v), 1% L-glutamine, 1% P/S, 1% ITS and 2% GCT under a 5% CO₂ atmosphere. All experiments were conducted in cells at passage 2 to 5.

**UM xenograft mouse model**

Animal ethics approval was obtained from the Laboratory Animal Ethics Committee of Jiangsu Institute of Nuclear Medicine (Wuxi, China). Animal experiments were conducted in accordance with approved protocols and regulations. The study Results were reported according to ARRIVE guidelines. C918 cells were mixed in a 2:1 ratio with Matrigel and injected subcutaneously in BALB/c nude mice (5 weeks old; male; Chang Zhou Cavens Laboratory Animal Co., Ltd, Changzhou, China). Tumour volume was measured with callipers every 3 d until they reached ~100 mm³ in size. Tumour volumes were calculated as \( \frac{a \times b^2}{2} \), where a and b are the length and width of the tumours, respectively. Once tumours reached the desired volume (around day 10), mice were randomly assigned to two groups to receive either lapatinib (25 mg/kg; \( n=7 \)) or vehicle (\( n=7 \)) once daily by intraperitoneal injection. Body weights and tumour sizes were measured every 2 d for 14 d. When treatments were complete, the mice were anesthetised with 1% pentobarbital sodium (5 mL/kg) by
intraperitoneal injection. Tumours were excised, weighed, photographed, and fixed in 4% paraformaldehyde for subsequent analysis.

**Positron emission tomography (PET) scanning**

$^{68}$Ga Activity was eluted from a $^{68}$Ge/$^{68}$Ga generator and used to prepare $[^{68}$Ga] Ga-NOTA-PRGD2 tracer, as described previously (25). On the day of scanning, the mice received ~3.7MBq of $^{68}$Ga NOTA-PRGD2 under anaesthesia via tail vein injection. Dynamic imaging acquisition was conducted for 60 min after tracer administration using an Inveon microPET scanner (Siemens Medical Solutions, Erlangen, Germany). Vendor software (ASI Pro 5.2.4.0) was used to detect regions of interest using decay-corrected whole-body coronal images.

**Histology and immunohistochemistry**

Tumour tissues fixed in paraffin blocks were cut into 8 µ sections and were stained with hematoxylin and eosin (Beyotime Institute of Biotechnology, Jiangsu, China). Sections were incubated (4°C) with an anti-Ki67 antibody (Cat. #: ab15580, Abcam, Shanghai, China), followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Immunohistochemical staining was conducted with a DAB substrate kit (Shanghai Bio-Platform Technology Company, Shanghai, China) and light microscopy (Olympus; Tokyo, Japan).

**TUNEL assay**

TUNEL assay was used to detect cell death in paraffin-embedded tumour sections. Briefly, sections were placed on slides and stained with the TUNEL assay kit (Beyotime Institute of Biotechnology, Jiangsu, China), as described previously; nuclei were counter-stained with hematoxylin. Images were analysed using a KF-PRO-120 slide scanner (Konfoong Bioinformation Tech, Ningbo, China).

**Statistics**

Data are presented as mean ± standard deviation (SD) with significance defined as p<0.05. Observers were blinded in in vivo studies. Statistical analysis was conducted using one-way ANOVA and Dunnett’s post-hoc test to compare multiple independent groups in GraphPad Prism 7.0.

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90 (!!! INVALID CITATION !!!).


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Author contribution
FZ conceived the idea and designed the study; WS, JZW, XZ, KW, SC, RMC, MCM, LL performed the experiments; WS, JZW, XZ, KW, HZ, LZ, MM and FZ analysed the data; JZW drafted the manuscript; KW, XZ, LZ, MM and FZ edited the manuscript.

Data availability statement
Raw data are available for a reasonable request to the corresponding authors.

Competing interests
The authors declare no conflict of interest.