Neratinib-resistance and cross-resistance to other HER2-targeted drugs due to increased activity of metabolism enzyme cytochrome P4503A4

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Running title: Understanding neratinib-resistance

Background: Neratinib is in Phase 3 clinical trials but, unfortunately, the development of resistance is inevitable. Here, we investigated effects of acquired neratinib-resistance on cellular phenotype and the potential mechanism of this resistance.

Methods: Neratinib-resistant variants of HER2-positive breast cancer cells were developed and their cross-resistance investigated using cytotoxicity assays. Similarly, sensitivity of trastuzumab-resistant and lapatinib-resistant cells to neratinib was assessed. Cellular phenotype changes were evaluated using migration, invasion and *anoikis* assays. Immunoblotting for HER-family members and drug efflux pumps, as well as enzyme activity assays were performed.

Results: Neratinib-resistance conferred cross-resistant to trastuzumab, lapatinib and afatinib. Furthermore, the efficacy of neratinib was reduced in trastuzumab- and lapatinib-resistant cells. Neratinib-resistant cells were more aggressive than their drug-sensitive counterparts, with increased CYP3A4 activity identified as a novel mechanism of neratinib-resistance.

Conclusion: The potential of increased CYP3A4 activity as a biomarker and/or target to add value to neratinib warrants investigation.

Introduction

Targeted therapies has substantially improved treatment of HER2-overexpressing cancers. These include trastuzumab (Vogel et al., 2002), pertuzumab (Agus et al., 2005), and lapatinib (Burris et al., 2005), with neratinib, afatinib and T-DM1 currently under-going clinical trials. Neratinib is an irreversible EGFR, HER2 and HER4 inhibitor (Bose et al., 2009). A Phase 1 trial of neratinib showed an acceptable safety profile with anti-tumour activity observed for advanced solid tumours (Wong et al., 2009). Phase 2 data showed substantial clinical activity in terms of progression-free survival and reduced tumour burden (Burstein et al., 2010). Swaby *et al*. (2009) indicates that dual administration of neratinib with trastuzumab is well-tolerated and produces a clinical response. Other trials investigating neratinib efficacy are also underway.

Innate- and acquired-resistance and cross-resistance to anti-cancer medication dominates as the main reason that anti-cancer drugs fail in the clinic (Gottesman et al., 2002; Germano and O'Driscoll, 2009; Raguz et al., 2008; Tan et al., 2010). Continued efforts to decipher the mechanism(s) of resistance are necessary to predict and circumvent this problem.

An understanding of the ability of cancer cells to acquire neratinib-resistance and the associated effects on cell behaviour is lacking. After developing novel neratinib-resistant cell variants of HER2-positive breast cancer cell lines, here we investigated if neratinib-resistant cells are cross-resistant to other drugs typically used for breast cancer; explored phenotypic changes that may have developed with neratinib-resistance; and elucidated mechanisms that may be responsible for neratinib-resistance.

Materials and methods

Cell culture

HCC1954 and EFM192A HER2-positive breast cancer cell lines (termed HCC1954 Par and EFM192A par) were obtained from ATCC and Leibniz-Institut DSMZ, respectively, and were cultured in RPMI-1640 (Sigma-Aldrich) with 10% FBS (Gibco) and 1% L-glutamine (Sigma-Aldrich). HCC1954 neratinib-resistant (HCC1954-NR) and EFM192A neratinib-resistant (EFM192A-NR) variants were established by continuous exposure to increasing concentrations of neratinib (5nM-250nM for HCC1954-NR cells, 5nM-80nM for EFM192A-NR cells) for several months. Lapatinib-resistant HCC1954 and SKBR3 cells (SKBR3-LR, HCC1954-LR) and trastuzumab-resistant SKBR3 (SKBR3-TR) cells were established as we previously described (Rani et al., 2014). Anti-cancer drugs were obtained from Sequoia Research (Pangbourne UK) and St. James's Hospital. Ketoconazole was purchased obtained from Sigma-Aldrich.

Toxicity assays

The IC_{50} values of neratinib, afatinib, lapatinib, trastuzumab and docetaxel for all cell variants were determined. Using 96-well plates (Sigma), HCC1954, HCC1954-NR and HCC1954-LR were seeded at $3x10^3$cells/well; EFM192A and EFM192A-NR, at $5x10^3$cells/well; and SKBR3-LR and SKBR3-TR cells at $1x10^4$ cells/well. After 24 hours, cells were exposed to relevant drugs (see Supplementary Material) for 5 days. The effect on cells was assessed using acid phosphatase assay. (Breslin and O'Driscoll, 2016).

Migration assays

HCC1954-Par and HCC1954-NR were seeded at $1x10^5$ cells in RPMI (with 1% FBS) in cell culture insert (BD Sciences, UK) in 24-well plates (Sigma-Aldrich). 400μl of complete RPMI

was placed under inserts. Cells were allowed to migrate for 24 hours before evaluating as previously described (O'Brien et al., 2015). As EFM192A variants did not migrate through inserts, as an alternative cells were seeded at 5×10^5 cells/well in 24-well plates and wound-scratch assays performed as previously (O'Brien et al., 2015). For the equivalent wound healing assay with the HCC1954-Par and HCC1954-NR cell variants, cells were seeded at 2×10^4 cells/well in 24-well plates.

Invasion assays

Performed as for migration assays, but inserts were first coated with extracellular matrix (Sigma-Aldrich) (Corcoran et al., 2012) and cells were allowed to invade over 48 hours.

Anoikis **assays**

All cell variants were seeded at 5×10^4 cells/ml into poly-HEMA-coated (Sigma-Aldrich) 24-well plates; with 95% ethanol-coated wells as controls. After 48 hours, 100µl of alamar blue (Serotec) was added for 5 hours before reading at 570nm.

Immunoblots

Cell pellets were lysed using cell extraction buffer (Invitrogen) supplemented with protease inhibitor cocktail (Roche). Protein was quantified using BioRad protein assay (BioRad). 30-50µg protein was separated using SDS gels and transferred onto PVDF (Bio-Rad). Blots were incubated overnight with primary antibodies at 4°C: HER2 (Calbiochem); EGFR, HER3, HER4 (Cell Signalling); PGP, BCRP, CYP3A4 (Santa Cruz) and β-actin (Sigma-Aldrich). Appropriate secondary antibodies were applied for 1 hour at room temperature before developing using Immobilon Western Chemiluminescent HRP substrate (Millipore).

CYP3A4 activity

HCC1954-Par and HCC1954-NR were seeded at $3x10^3$ cells/well and EFM192A-Par and EFM192A-NR were seeded at $5x10^3$ cells/well in a 96-well plates. 48 hours post-seeding, P450-Glo CYP3A4 (Promega) assay was performed by removing medium, adding Luciferin-IPA solution, and incubated at 37°C for 1 hour before adding 50μL of luciferin detection reagent. After 20 minutes at room temperature, solution from each well was transferred to a white opaque 96-well plate and read using a Mithras LB-940 (Berthold Technologies). To block CYP3A4 activity, HCC1954-NR cells were seeded at $3x10^3$ cells/well and EFM192A-NR cells were seeded at $5x10^3$ cells/well in 96-well plates. After 24 hours, cells were exposed to 40nM ketoconazole alone or in combination with neratinib (325nM for HCC1954-NR cells; 46.7nM for EFM192A-NR cells) treatments for 5 days. The effect on cells was assessed using acid phosphatase assay.

Results

Neratinib-resistant variants

Once established as neratinib-resistant, HCC1954-NR and EFM192A-NR cells were 6.5-7 fold increase in resistance, respectively, compared to their age-matched controls. The age-matched control cells are HCC1954-Par and EFM192A-Par cells which were routinely cultured in parallel with their counterparts that were developing neratinib-resistance (Table 1).

Cross-resistance

Both HCC1954-NR and EFM192A-NR cells showed substantial cross-resistance to afatinib and lapatinib, compared to their age-matched controls (Table 1). EFM192A-Par and

EFM192A-NR are insensitive to trastuzumab *i.e.* both variants maintained >80% viability in 500µg/mL trastuzumab. Cross-resistance to docetaxel did not occur.

Considering cross-resistance to neratinib of lapatinib-resistant and trastuzumab-resistant cells (Table 2), HCC1954-LR and SKBR3-LR cells demonstrated approximately 2-fold and 15-fold cross-resistance to neratinib compared to respective parent cells, while SKBR3-TR cells exhibited 3.3-fold increase in neratinib-resistance.

Increased cell aggression

HCC1954-NR cells were significantly more migratory than HCC1954-Par by 1.3-fold (when assessed using transwell assays). Using the wound healing assay, HCC1954-NR cells demonstrated 21% increased wound closure when compared to HCC1954-Par ((Figure 1(i)), while EFM192A-NR demonstrated 17% increased wound closure compared to EFM192A Par cells (Figure 1(i)). HCC1954-NR cells were also 1.3-fold more invasive than HCC1954-Par cells (Figure 1(ii)). Furthermore, HCC1954-NR cells were more resistant to *anoikis* with only 2.8±0.2% of HCC1954-NR cell death, compared to 12±1.7% of HCC1954-Par cells. Similarly for EFM192A-NR cells, where only 24.7±1.1% of EFM192A-NR cells died compared to 33.7±0.8% of EFM192A-Par cells (Figure 1(iii)).

Effects on drug targets and transporters

Drug targets and drug transporters are often altered in drug-resistant cells. As indicated in Figure 2, expression of the entire EGFR-family was significantly reduced in both neratinib-resistant variants compared to drug-sensitive parents; if expressed at all. Based on the densitometry analysis of n=3 independent immunoblots for each protein and where total expression in parental cells was always set at an arbitrary value of 1, EGFR was decreased in

HCC1954-NR cells to 0.66±0.05, while EGFR is undetected in EFM192A cells. HER2 expression was decreased to 0.45±0.07 in HCC1954-NR cells and 0.59±0.14 in EFM192A-NR cells. HER3 was decreased to 0.75±0.04 in HCC1954-NR cells and 0.71±0.03 in EFM192A-NR cells. Finally, HER4 was undetectable in HCC1954 variants and was decreased to 0.52±0.08 in EFM192A-NR cells compared to controls.

P-glycoprotein (PGP) expression was significantly decreased in HCC1954-NR and EFM192A-NR compared to control cells; by 0.36±0.07 and 0.42±0.04, respectively. Breast cancer resistant protein (BCRP) expression was also decreased in HCC1954-NR and EFM192A-NR cells to 0.72±0.05 and 0.74±0.04, respectively.

CYP3A4 activity

While CYP3A4 protein expression levels did not change significantly between the drug resistant and drug sensitive counterparts (Supplementary Figure 1), CYP3A4 activity was significantly increased in both the neratinib-resistant cell line variants (Figure 3A). Specifically, the HCC1954-NR cells had 2.9±0.3-fold increased CYP3A4 activity when compared to HCC1954-Par cells. Similarly, EFM192A-NR cells displayed 1.5±0.2-fold increased CYP3A4 activity compared to EFM192A-Par control cells. Efforts to block CYP3A4 activity –using ketoconazole at a final concentration of 40nM– to establish if this could help to at least particularly restore neratinib sensitivity. Ketoconazole alone had no effects on cell viability but, when added with neratinib, ketoconazole induced a small (4.4% for HCC1954-NR; 17.4% for EFM192A-NR, respectively) but significant restoration of neratinib sensitivity (Figure 3B).

Discussion

It is estimated that ~70% of breast cancer patients with HER2-overexpressing tumour are either innately-resistant or acquire resistance to HER2-targeted drugs (Arribas et al.., 2011). So, although data from clinical trials of neratinib indicates that many patients are gaining initial benefit, there is a gap in available information on the effects of neratinib-resistance in cells; when that, inevitably, develops. For this reason, studies performed over the past number of years have been trying to understand the mechanism(s) of neratinib-resistance, in order that it may be predicted, prevented or reversed. In 2012, using SKBR3 as cell line model and what they described as a genome-wide functional RNAi screen, Seyhan et al. reported on multiple genes whose inhibition was associated with neratinib resistance. In 2014, from pre-clinical *in vitro* and *in vivo* studies and analysis of ~3,500 patients' specimens, we identified Neuromedin U as associated with poor outcome for patients with HER2-overexpresing tumours and our pre-clinical indicated over-expression of NmU to be significantly associated with resistance to a range of HER2-targeted drugs including neratinib (Rani et al., 2014). In efforts to establish how Neuromedin U expression might be controlled, we found loss of miR-630 (predicted to control Neuromedin U expression) to be associated with increased expression of Neuromedin U and with resistance to the HER2-targeted drugs including neratinib (Corcoran et al., 2014). In an effort to further elucidate other mechanism of neratinib-resistance, the studies described here were performed.

Neratinib-resistant cells developed here were found to be cross-resistant to all other HER2-targeting drugs investigated. Interestingly, this cross-resistance is bi-directional, as both lapatinib- and trastuzumab-resistant cells are also cross-resistant to neratinib. Trends observed here have also been observed in clinical trials as Burstein *et al*. (2010) found that drug-naïve patients responded better to neratinib than patients previously treated with trastuzumab. Similarly, Awada *et al*. (2013) reported that patients with prior exposure to

lapatinib did not respond to neratinib as well as those who had no prior lapatinib exposure. Therefore, based on this novel cell line data it could be hypothesised that, unfortunately, in the case of neratinib-resistance, if a patient were to suffer disease progression while being treated with neratinib, the efficacy of other HER2-drugs is compromised by the previous exposure to neratinib.

In addition to cross-resistance to HER2-targeting drugs, HCC1954-NR and EFM192A-NR cells also developed a more aggressive phenotype, being more migratory and invasive, with increased ability to avoid *anoikis*. Such increased cell aggression has also been observed by ourselves and others in association with drug-resistance in other cancer types and is associated with a poor prognosis for patients (Kleer et al., 2003; Lee et al., 2012); but never previously associated with neratinib.

To elucidate the mechanism that facilitates neratinib-resistance, we first explored associations between neratinib-resistance and neratinib's targets. All EGFR family members targeted by neratinib were reduced in the drug-resistant variants. Additionally, HER3 (not targeted by neratinib but heterodimerises with HER2 (Fichter et al., 2014)) was also reduced. These observations are contrary to typical HER2-drug resistance, where EGFR and HER2 are increased in association with lapatinib- (McDermott et al., 2014; Corcoran et al., 2014) and trastuzumab-resistance (Browne et al., 2011). The decreases expression of drug targets is potentially due to the irreversible binding nature of neratinib. This theory is supported by findings of Azuma *et al*. (2014) whose afatinib-resistant PC9 lung cancer cells had down-regulated EGFR, HER2 and HER3 in comparison to drug-naïve control cells.

Additionally, we assessed drug efflux pumps, PGP and BCRP. PGP is one of the most common mediators of drug-resistance (Gottesman, 2002; Germano and O'Driscoll, 2009). In contrast to classical drug-resistance, neratinib-resistant cells show down-regulation of PGP. This may be because neratinib inhibits PGP activity and can reverse multidrug-resistance in PGP over-expressing MCF7 and KBv200 cells (Zhao et al., 2012). This trend has also been observed with lapatinib (Dai et al., 2008), which was also found to reduce PGP activity; but never previously with neratinib. Overexpression of BCRP is associated with breast cancer drug-resistance (Doyle and Ross, 2003). However, similarly to PGP, BCRP's reduced expression here suggests that it does not have a functional role in neratinib-resistance.

CYP3A4, a cytochrome P450 metabolising enzyme, plays a role in the metabolism of ~half of all drugs (Guengerich, 1999), including neratinib (Abbas et al., 2011). Abbas *et al*. (2011) demonstrated that co-administration, to healthy adults, of ketoconazole (CYP3A4 inhibitor) with neratinib increased neratinib's peak plasma concentrations. While this was not investigated in associated with cancer, it suggested to us that alterations of CYP3A4 activity may also alter the availability of neratinib in drug-resistance. Supporting this hypothesis, we observed increases CYP3A4 activity with neratinib resistance, suggesting that the increased CYP3A4 activity enhances neratinib metabolism and thereby drives neratinib-resistance. Furthermore, initial efforts to blocks this increased CYP3A4 activity with ketoconazole in the neratinib-resistant variants showed a significant, *albeit* limited, restoration of neratinib sensitivity. Future studies optimising the ketoconazole concentration and sequencing time of ketoconazole prior to neratinib may achieve a more marked re-sensitisation to neratinib.

This first study of neratinib-resistance shows that its development confers resistance to a range of HER2-drugs. PGP and BCRP apparently do not play functional roles in this

resistance. The reduced/loss of expression of HER2-family members –thus their reduced availability for drug targeting– may be a contributing factor. Of particular interest, however, is the increased CYP3A4 activity. Further investigations of CYP3A4 activity as a possible predictive biomarker of response and as a target to circumvent resistance (and thus add value to neratinib) are now warranted.

Legends

Figure 1. Acquired neratinib-resistance results in phenotypic changes associated with increased cellular aggression. (i). Neratinib-resistant cells are more migratory than parent cells as shown for HCC1954-NR compared to HCC1954-Par cells by both transwell assays and wound-healing assay. EFM192A-Par and EFM192A-NR cells do not transverse through transwells, regardless of cell seeding density, but EFM192A-NR were shown to be more migration than EFM192A-Par cells when suing the wound healing assay; (ii). HCC1954-NR cells are more invasive than HCC1954-Par cells. (iii). Neratinib-resistant cells are more resistant to *anoikis* than parent cells. $n=3\pm SEM$, $*p<0.05$, $**p<0.01$, $***p<0.001$.

Figure 2. Associations between neratinib-resistance and EGFR family members and drug transporter. (A). Association between neratinib-resistance and expression of the EGFR family and drug efflux pumps PGP and BCRP. SKBR3 (and HCC1954) cells served as positive control in EGFR blot for EFM192A cell variants; (B). Densitometry for HCC1954-Par and HCC1954-NR immunoblots (i) EGFR; (ii) HER2; (iii) HER3; (iv) HER4; (v) PGP, and (vi) BCRP; (C). Densitometry for EFM192A-Par and EFM192A-NR immunoblots (i) EGFR; (ii) HER2; (iii) HER3; (iv) HER4; (v) PGP, and (vi) BCRP. $n=3\pm SEM$, $*p<0.05$, $**p<0.01$, $***p<0.001$.

Figure 3. CYP3A4 activity in neratinib-resistance compared to parent cells and partial restoration of neratinib sensitivity to former neratinib-resistance cells by reducing CYP3A4 activity with ketoconazole. (A) CYP3A4 activity is substantially and significantly higher in both (i) HCC1954-NR and (ii) EFM192A-NR cells in comparison to their respective control cells. (B). Co-treatment with ketoconazole and neratinib significantly decreased cell viability in both (i) HCC1954-NR and (ii) EFM192A-NR cells when compared to neratinib treatment alone. $n=3\pm SEM$, $*p<0.05$, $**p<0.01$.

Supplementary Figure 1. CYP3A4 protein expression in neratinib-resistance compared to parent cells. (A) Representative immunoblots showing CYP3A4 protein expression in (i) HCC1954-Par and HCC1954-NR cells and (ii) EFM192A-Par and EFM192A-NR cells. (B). Densitometry from (i) HCC1954-Par and HCC1954-NR immunoblots and (ii) EFM192A-Par and EFM192A-NR immunoblots, representing $n=3\pm SEM$ for each.

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Legends

Figure 1. Acquired neratinib-resistance results in phenotypic changes associated with increased cellular aggression. (i). Neratinib-resistant cells are more migratory than parent cells as shown for HCC1954-NR compared to HCC1954-Par cells by both transwell assays and wound-healing assay. EFM192A-Par and EFM192A-NR cells do not transverse through transwells, regardless of cell seeding density, but EFM192A-NR were shown to be more migration than EFM192A-Par cells when suing the wound healing assay; (ii). HCC1954-NR cells are more invasive than HCC1954-Par cells. (iii). Neratinib-resistant cells are more resistant to *anoikis* than parent cells. n=3±SEM, *p<0.05, **p<0.01, ***p<0.001.

Figure 2. Associations between neratinib-resistance and EGFR family members and drug transporter. (A). Association between neratinib-resistance and expression of the EGFR family and drug efflux pumps PGP and BCRP. SKBR3 (and HCC1954) cells served as positive control in EGFR blot for EFM192A cell variants; (B). Densitometry for HCC1954-Par and HCC1954-NR immunoblots (i) EGFR; (ii) HER2; (iii) HER3; (iv) HER4; (v) PGP, and (vi) BCRP; (C). Densitometry for EFM192A-Par and EFM192A-NR immunoblots (i) EGFR; (ii) HER2; (iii) HER3; (iv) HER4; (v) PGP, and (vi) BCRP. n=3±SEM, *p<0.05, **p<0.01, ***p<0.001.

Figure 3. CYP3A4 activity in neratinib-resistance compared to parent cells and partial restoration of neratinib sensitivity to former neratinib-resistance cells by reducing CYP3A4 activity with ketoconazole. (A) CYP3A4 activity is substantially and significantly higher in both (i) HCC1954-NR and (ii) EFM192A-NR cells in comparison to their respective control cells. (B). Co-treatment with ketoconazole and neratinib significantly decreased cell viability

in both (i) HCC1954-NR and (ii) EFM192A-NR cells when compared to neratinib treatment alone. n=3±SEM, *p<0.05, **p<0.01.

Supplementary Figure 1. CYP3A4 protein expression in neratinib-resistance compared to parent cells. (A) Representative immunoblots showing CYP3A4 protein expression in (i) HCC1954-Par and HCC1954-NR cells and (ii) EFM192A-Par and EFM192A-NR cells. (B). Densitometry from (i) HCC1954-Par and HCC1954-NR immunoblots and (ii) EFM192A-Par and EFM192A-NR immunoblots, representing n=3±SEM for each.

Table 1. IC_{50} values for HER2-targeted drugs and docetaxel in HCC1954-Par, HCC1954-NR, EFM192A-Par and EFM192A-NR cells with corresponding fold-difference, showing that neratinib-resistant cells are also cross-resistant to other HER2-targeted drugs, but not docetaxel.

IC_{50} and fold difference for HCC1954 and EFM192A parent and neratinib-resistance cell variants				
Drug	**HCC1954-Par**	**HCC1954-NR**	**Fold Difference**	**p**
Neratinib	49 nM	325 nM	6.5 ± 0.4	1.08×10^{-4}
Afatinib	103 nM	3.6 µM	37 ± 7.2	0.008
Lapatinib	273 nM	2.7 µM	10 ± 0.8	3.7×10^{-7}
Trastuzumab	Innate resistance	Innate resistance	N/A	N/A
Docetaxel	0.6 nM	0.65 nM	1.08 ± 0.04	0.15
Drug	**EFM192A-Par**	**EFM192A-NR**	**Fold Difference**	**p**
Neratinib	6.8 nM	46.7 nM	6.8 ± 0.3	5.1×10^{-5}
Afatinib	151.3 nM	5.1 µM	34.6 ± 4.4	0.002
Lapatinib	50 nM	7.97 µM	162.9 ± 22	4×10^{-6}
Trastuzumab	>500µg/mL	>500µg/mL	N/A	N/A
Docetaxel	2.6 nM	2.7 nM	1.03 ± 0.07	0.692

N/A = not applicable, as both the parent and neratinib-resistant variants were insensitive to trastuzumab.

Table 2. IC_{50} values for neratinib in HCC1954-Par and -LR, SKBR3-Par, -LR and -TR cells with corresponding fold-difference values, showing that cells with acquired-resistance to lapatinib and trastuzumab are also cross-resistant to neratinib.

Sensitivity of lapatinib and trastuzumab-resistant cells to neratinib			
Neratinib (IC_{50})		**Fold difference**	**p**
HCC1954-Par	HCC1954-LR		
53 nM	91 nM	1.7±0.1	0.001
SKBR3-Par	SKBR3-LR		
4.7 nM	69.3 nM	15.3±3.2	0.002
SKBR3-Par	SKBR3-TR		
4 nM	15 nM	3.3±0.2	2.4×10^{-4}

Supplemental Methods

Drug concentrations used in assays

Neratinib (0-100nM for HCC1954-Par; 0-900nM for HCC1954-NR; 0-125nM for EFM192A-Par and 0-1μM for EFM192A-NR), afatinib (0-900nM for HCC1954-Par; 0-7μM for HCC1954-NR; 0-5μM for EFM192A-Par and 0-10μM for EFM192A-NR), lapatini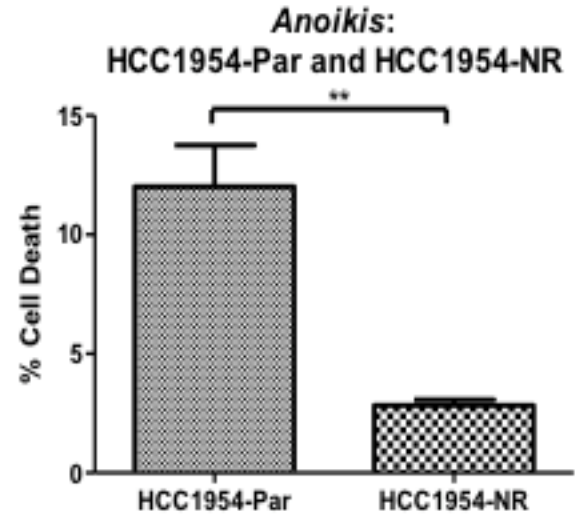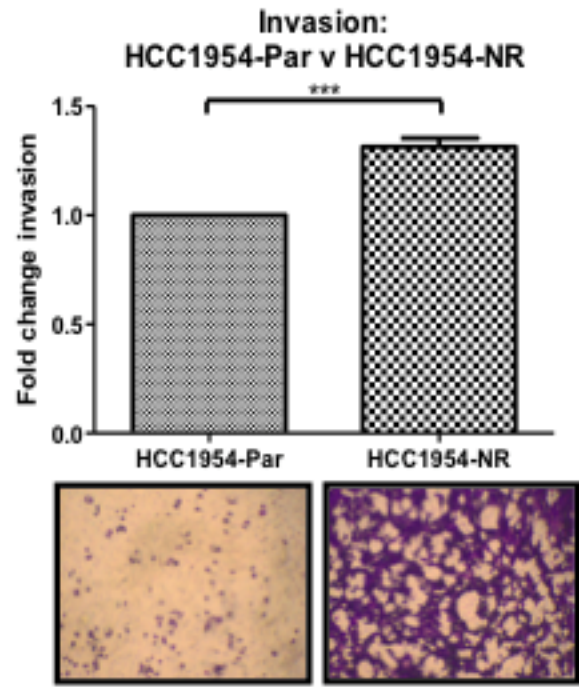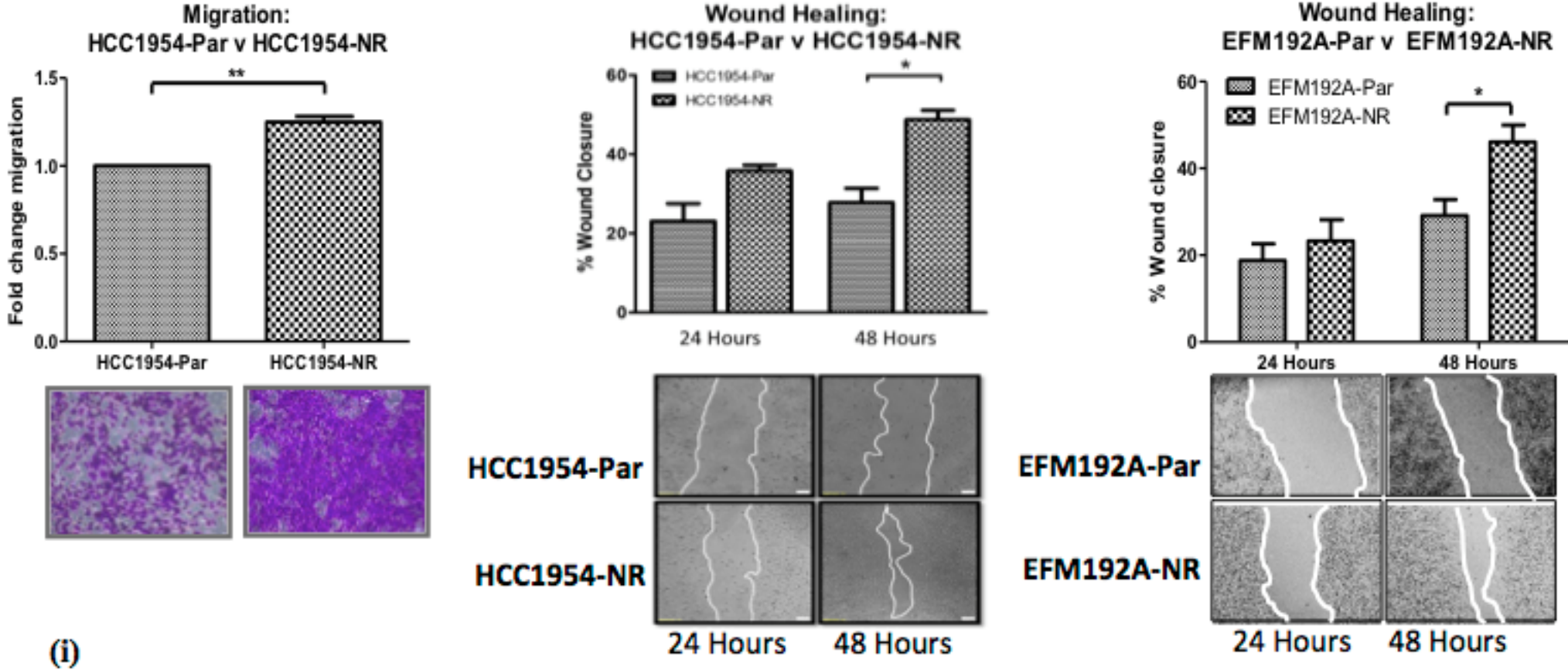b (0-1.4μM for HCC1954-Par; 0-10μM for HCC1954-NR; 0-2.5μM for EFM192A-Par and 0-10μM for EFM192A-NR), trastuzumab (0-500μg/mL for EFM192A-Par and 0-500μg/mL for EFM192A-NR), docetaxel (0-7nM for HCC1954-Par; 0-7nM for HCC1954-NR; 0-125nM for EFM192A-Par and 0-125nM for EFM192A-NR).

Migration:
HCC1954-Par v HCC1954-NR

Wound Healing:
HCC1954-Par v HCC1954-NR

Wound Healing:
EFM192A-Par v EFM192A-NR

(i)

HCC1954-Par

HCC1954-NR

24 Hours   48 Hours

EFM192A-Par

EFM192A-NR

24 Hours   48 Hours

Invasion:
HCC1954-Par v HCC1954-NR

(ii)

Anoikis:
HCC1954-Par and HCC1954-NR

Anoikis:
EFM192A-Par and EFM192A-NR

(iii)
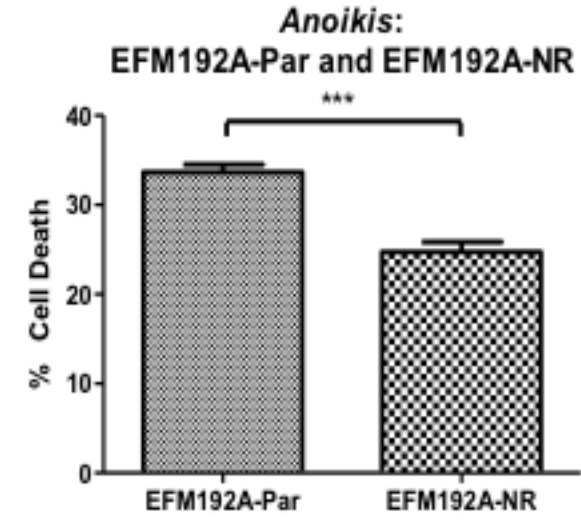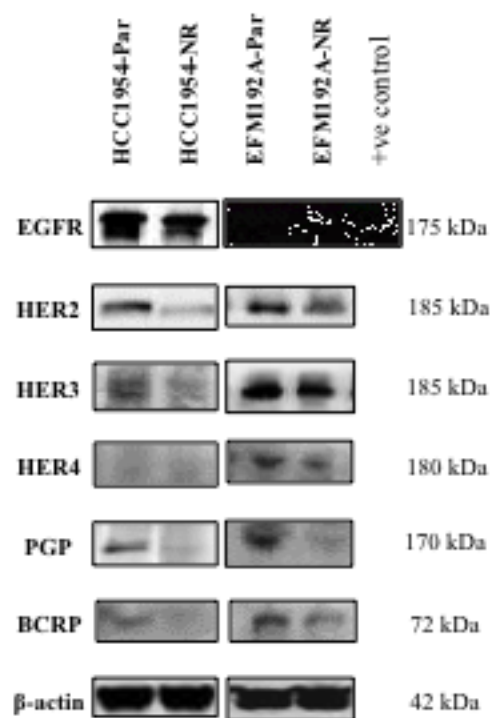
Figure 1

Figure 2A

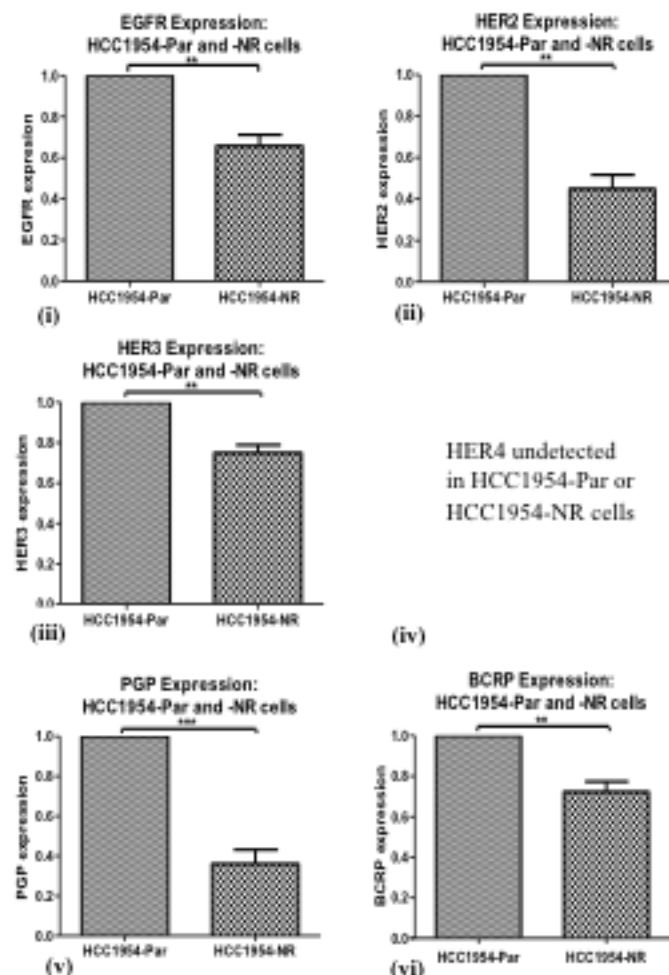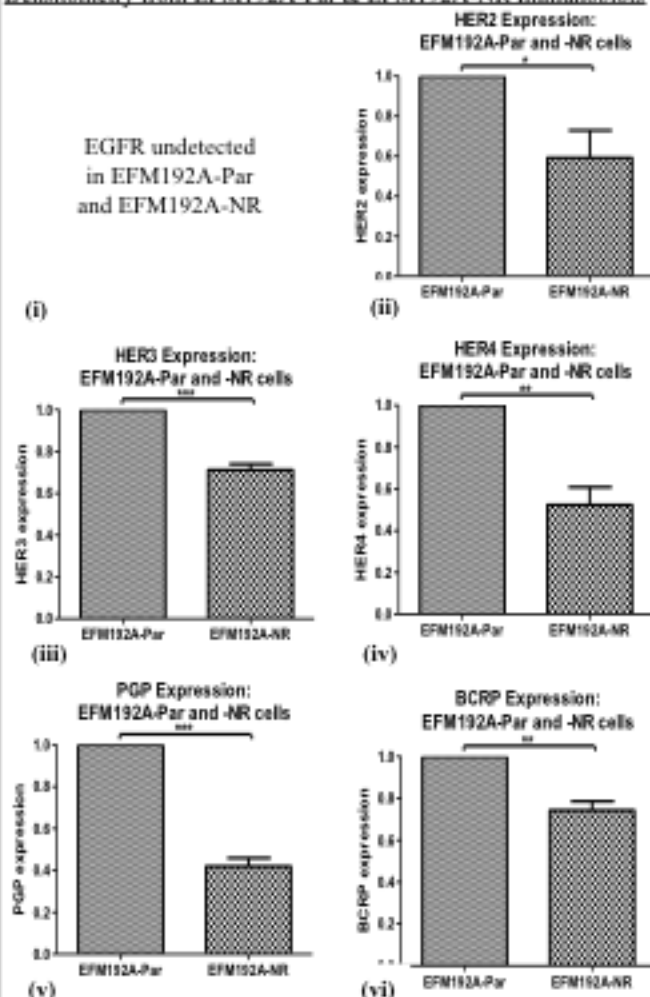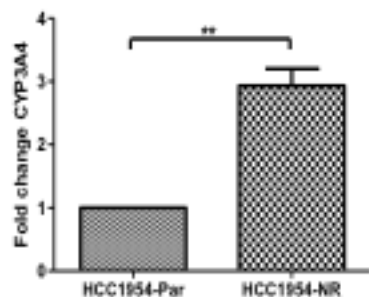**Figure 2B**

Densitometry from HCC1954-Par & HCC1954-NR Immunoblots

EGFR Expression: HCC1954-Par and -NR cells (i)

HER2 Expression: HCC1954-Par and -NR cells (ii)

HER3 Expression: HCC1954-Par and -NR cells (iii)

HER4 undetected in HCC1954-Par or HCC1954-NR cells (iv)

PGP Expression: HCC1954-Par and -NR cells (v)

BCRP Expression: HCC1954-Par and -NR cells (vi)
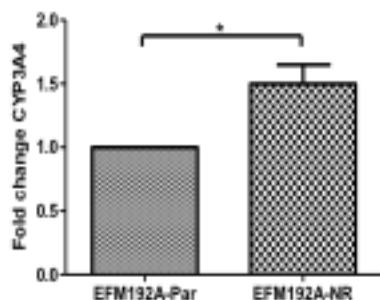
Figure 2C

Densitometry from EFM192A-Par & EFM192A-NR Immunoblots

EGFR undetected in EFM192A-Par and EFM192A-NR (i)

HER2 Expression: EFM192A-Par and -NR cells (ii)

HER3 Expression: EFM192A-Par and -NR cells (iii)

HER4 Expression: EFM192A-Par and -NR cells (iv)

PGP Expression: EFM192A-Par and -NR cells (v)

BCRP Expression: EFM192A-Par and -NR cells (vi)

CYP3A4 activity: HCC1954-Par v HCC1954-NR

CYP3A4 activity: EFM192A-Par v EFM192A-NR
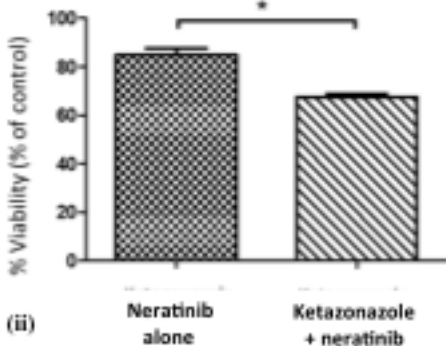
A. (i)

(ii)

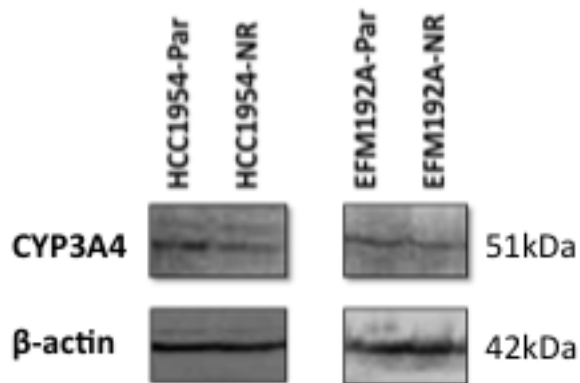**Ketoconazole and neratinib treatment: HCC1954-NR cells**
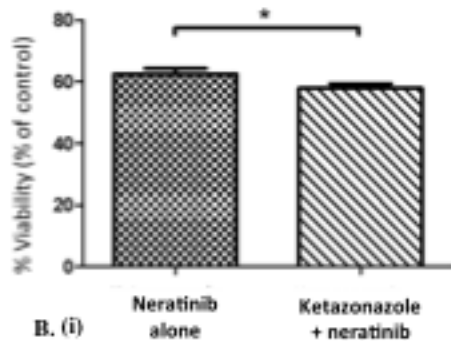
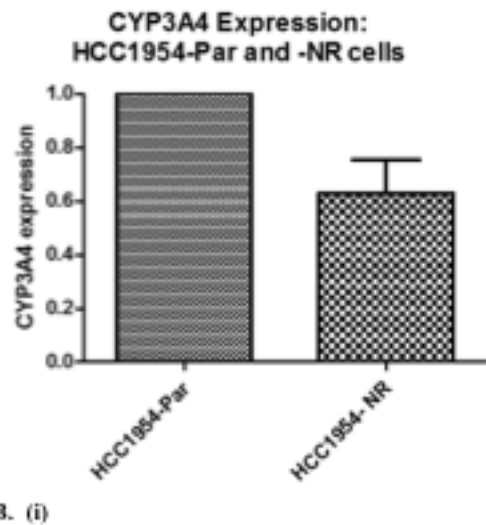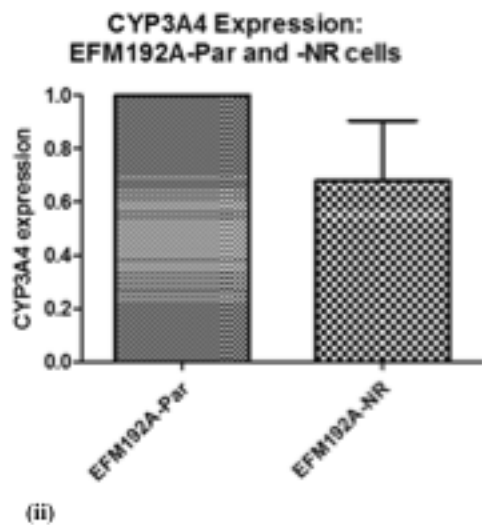**Ketoconazole and neratinib treatment: EFM192A-NR cells**

B. (i)

(ii)

Figure 3

A. (i) (ii)

B. (i) (ii)

Supplementary Figure 1.