Title: Neuromedin U: a candidate biomarker and therapeutic target to predict and overcome resistance to HER kinase inhibitors

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Abstract

Intrinsic and acquired resistance to HER-targeting drugs occurs in a significant proportion of HER2-overexpressing breast cancers. Thus, there remains a need to identify predictive biomarkers that could improve patient selection and circumvent these types of drug resistance. Here we report the identification of neuromedin U (NmU) as an extracellular biomarker in cells resistant to HER-targeted drugs. NmU overexpression occurred in cells with acquired or innate resistance to lapatinib, trastuzumab, neratinib, afatinib, all of which displayed a similar trend and suggested NmU induction may be an early response. An analysis of 3489 cases of breast cancer showed NmU to be associated with poor patient outcomes, particularly those with HER2-overexpressing tumours independent of established prognostic indicators. Ectopic overexpression of NmU in drug-sensitive cells conferred resistance to all HER-targeting drugs, whereas RNAi-mediated attenuation sensitized cells exhibiting acquired or innate drug resistance. Mechanistic investigations suggested that NmU acted through HSP27 as partner protein to stabilize HER2 protein levels. We also obtained evidence of functional NmU receptors on HER2-overexpressing cells, with the addition of exogenous NmU eliciting an elevation in HER2 and EGFR expression along with drug resistance. Lastly, we found that NmU appeared to function in cell motility, invasion and anoikis resistance. In vivo studies revealed that NmU attenuation impaired tumour growth and metastasis. Taken together, our results defined NmU as a candidate drug response biomarker for HER2-overexpressing cancers, and a candidate therapeutic target to limit metastatic progression and improve the efficacy of HER-targeted drugs.

Precis: Findings define an extracellular protein that stabilises levels of the breast cancer oncoprotein HER2 as a candidate biomarker for HER2-targeting drugs, as well as a candidate therapeutic target to limit metastatic progression and improve the efficacy of these drugs.
Introduction

HER2-overexpression occurs in approximately 25% of breast cancer and is associated with poor prognosis (1, 2). Targeting HER2 with trastuzumab (Herceptin, Genentech), a monoclonal antibody directed against the extracellular domain of the growth factor receptor, has shown activity as a single agent and in combination with classical chemotherapy in HER2-overexpressing breast cancer (3-5). HER2 has also been successfully targeted with lapatinib (Tykerb, GlaxoSmithKline), a reversible dual kinase inhibitor directed at the intracellular ATP binding domain of the receptor and its dimerisation partner epidermal growth factor receptor (EGFR/HER1), rendering it kinase inactive (8, 9). Unfortunately, not all HER2-positive patients respond to trastuzumab (6,7) or lapatinib (10-12) and those that initially respond may acquire resistance. More recently developed neratinib (Pfizer/Puma Biotechnology), is an irreversible small molecule tyrosine kinase inhibitor (TKI) of EGFR/HER1, HER2 and HER4 (13). In advanced HER2-overexpressing breast cancers, including trastuzumab-refractory and trastuzumab-naïve patients, single-agent neratinib demonstrated some clinical benefits in both cohorts (14) and Phase III clinical trials are underway. Our pre-clinical studies show that HER2-overexpressing cell lines acquire resistance to neratinib, as they did to trastuzumab and lapatinib; although it is too early to know if this will occur in the clinic. Afatinib (BIBW 2992, Boehringer Ingelheim), another irreversible oral small molecule TKI of EGFR/HER1, HER2 and HER4 has shown single-agent activity in a Phase II trial including trastuzumab-refractory HER2-positive metastatic breast cancers (15).

Targeting HER2 and other HER family members is, undoubtedly, providing significant clinical benefit in patients with HER2-overexpressing breast cancers. However, innate/primary- and acquired- resistance represent substantial clinical challenges, in addition to the lack of means to predict patients’ response. Predictive biomarkers for HER-targeting drugs (ideally extracellular, minimally-invasive) are needed for improved patient selection and thus enhanced patient outcome, as are novel therapeutic strategies to circumvent resistance. Our previous studies indicate that analysing extracellular RNAs in medium conditioned by cells can be a useful starting point to identifying potential biomarkers, some proving to have functional relevance within the cell (16-20).

Neuromedin U (NmU) is a secreted neuropeptide and a member of neuromedin peptide family originally isolated from porcine spinal cord (21). NmU, originally named due to its potent uterine contraction-inducing activity, is synthesised as a 174 amino acid precursor and cleaved to a 25 amino acid biologically-active peptide. Two G-coupled receptors for NmU have been identified, NmU-R1 (predominantly in the periphery, especially GI tract) and NmU-R2 (predominantly in central nervous system). Evidence suggests a role for NmU in pain, stress, immune-mediated...
inflammatory diseases, and feeding regulation (see reviews: (22, 23)). A limited number of studies to date have associated NmU with cancer; with conflicting observations depending on the cancer type. While NmU has never previously been associated with breast cancer or, indeed, with any HER2-overexpressing cancers, our studies point to a role for NmU as a biomarker for HER-targeted drugs. In addition, NmU may serve as a new therapeutic target to help block tumour growth and metastasis, circumvent primary or acquired resistance and so add value to HER-targeted therapies.

**Materials and Method:** (Detailed methods in *SI Materials & Methods.*)

**Cell culture and treatments:** SKBR3, HCC1954, MDA-MB-361, T47D cells, obtained from ATCC, were cultured in RPMI-1640 (Sigma-Aldrich) with 10% FCS (PAA) and 1% L-glutamine. Trastuzumab-conditioned (resistant) SKBR3 (SKBR3-TR) cells, and both SKBR3 and HCC1954 -lapatinib-resistant cells (SKBR3-LR, HCC1954-LR), and -neratinib-resistant cells (SKBR3-NR, HCC1954-NR) resulted from continuously exposing cells to the respective drug for over 6 months. To assess if functional NmU receptors are expressed on cells, SKBR3 and HCC1954 (1x10^6 cells) were seeded in 25cm^2 flask, allowed to attach overnight and subsequently treated with 1μM of NmU-25 (Bachem, Switzerland) for 24-48 hours. Cell lines were authenticated by STR profiling; were tested for *Mycoplasma* quarterly and were impact tested prior to introducing into murine models. Most recently tested in Dec. 2013/Jan 2014. Trastuzumab was obtained from St. Vincent’s Hospital. Lapatinib, neratinib and afatinib were purchased from Sequoia Chemicals Ltd. (Pangbourne, UK).

**Short-term drug exposure assays:** SKBR3 and HCC1954 cells were seeded (5x10^5 cells, 25cm^2 flasks) and allowed to grow to 80% confluency before being exposed, for 48 hours, to lapatinib (1μM), trastuzumab (12.5μg/ml), neratinib (0.5μM), or afatinib (0.5μM).

**NmU knock-down and over-expression:** Two NmU-specific siRNAs (designated NmU-1 and NmU-2 (s225456 and s21351, Ambion, UK)), and scrambled siRNA (Scr) (negative control; AM4611, Ambion, UK) (30nM) was used. Transient transfections were harvested 72 hours post-transfection for RNA and protein extraction. NmU full-length cDNA and mock controls (plasmid lacking NmU cDNA) used were of pcDNA3.1(+). Lipofectamine 2000 (Invitrogen, Ireland) was used for transfection following the manufacturer’s instructions. Stable transfectants were established by selecting with zeocin (50μg/ml and 300μg/ml for HCC1954 and SKBR3 transfected cells, respectively (Invitrogen, Ireland). For *in vivo* studies HCC1954-LR-LUC+ (*i.e.* acquired resistance cells) cells stably expressing the luciferase gene (HCC1954-LR-LUC+) were transfected.
with shRNAs for NmU cloned in the pLKO.1 vector (shRNA #1-#5, Open biosystems, Cat. #: RHS4080_NM-006681). After verifying successful NmU protein knock-down and conferred drug sensitivity in vitro, one (shRNA #1) variant was selected for in vivo analysis. HCC1954-LR-LUC+ cells with no NmU knock-down were used as a control.

**Proliferation assays:** Cells with acquired-resistance compared to aged-parent cells and NmU cDNA- versus mock-transfected cells (HCC1954 variants, 2x10³ cells/well; SKBR3 variants, 5x10³ cells/well) were seeded in 96-well plates for 24 hours prior to drug additions. Subsequently, a range of concentrations for lapatinib, neratinib, trastuzumab or afatinib was added to the cells. Five days later, proliferation was assessed using the acid phosphatase method.

**Assessing effects of NmU siRNAs with HER-targeted drugs:** Forty-eight hours following transfection with NmU-1, NmU-2 siRNA or Scr sequences, cells were exposed to their approximate IC₅₀ concentrations of lapatinib. A fixed concentration of trastuzumab (15µg/ml), neratinib (1µM) and afatinib (0.5µM) was assessed for all 4 cell line variants.

**qPCR:** Total RNA was isolated from cell lines and CM using TriReagent (Sigma-Aldrich), and treated with DNase enzymes. cDNA was prepared from 500ng cell-derived and 4μl CM-derived total RNA, respectively. NmU (Hs00183624_m1, ABI, UK) was quantified using the threshold cycle (CT) adjusting to the levels of β-actin (4352933E, ABI, UK) as endogenous control.

**Immunoprecipitation:** Primary antibody was added (2µg/100µl of cell extract) to total cell protein (1µg/µl; total volume of 100µl), mixed and incubated for 120min at 4°C. 20µl of 50% protein A agarose beads (Sigma-Aldrich) was added to the cell extract mix and incubated for 60min at 4°C. This mixture was centrifuged at 5000rpm for 5min. The beads were washed 5 times with the cold Lysis buffer and centrifuged 5000rpm for 2min. The beads were resuspended in 20µl of Laemmli’s sample buffer, boiled for 5 min, and centrifuged for 5min. 18μl of the samples were loaded per lane in a SDS-polyacrylamide gel for electrophoresis.

**Immunoblotting and enzyme-linked immunosorbent assay:** Total cellular proteins (30-40µg, depending on the specific protein’s abundance; but constant for any given protein) were resolved on 6-10% SDS-PAGE and transferred to PVDF membranes (Millipore, Ireland). Primary antibodies used included EGFR (Neomarker); HER2 (Calbiochem); HSP27 (Enzo Life Sciences); NmU-R1 (Sigma-Aldrich); NmU-R2 (LifeSpan Biosciences); β-actin (Sigma-Aldrich). Membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Cell
Signalling) and proteins were visualized by chemiluminescence (Millipore). Detection was performed with a Chemidoc exposure system (Bio-Rad Laboratories). ELISA kit for NmU (Peninsula Laboratories, US), HER2 (Calbiochem, US), EGFR, p-EGFR (R&D Systems, US) were used according to the manufacturer’s instructions.

**Wound-healing assay:** HCC1954-mock and NmU, HCC1954-LR associated Scr- or siRNA-transfected cell variants (5x10⁵ cells/well) and SKBR3-mock and NmU variants (1x10⁶ cells/well) were seeded on 6-well plates and cultured for 48 hours to confluence. Monolayer was scratched with a pipette tip and the resulting wounded areas were monitored by phase contrast microscopy and determined using NIH Image J software.

**Migration and invasion assay:** Migration assays were performed using 8μm pore size 24-well transwell chambers (BD Biosciences, Oxford, UK). For invasion assessment, the inserts were pre-coated with ECM (Sigma-Aldrich). siRNA- or Scr-transfected cells; and NmU cDNA- versus mock-transfected cells (HCC1954 variants, 1x10⁵/insert; SKBR3 variants, 1x10⁴/insert) were seeded in the upper compartment and allowed to migrate for 48 and 72 hours, respectively. Cells in the upper chamber were removed and migrated/invaded cells were stained with crystal violet. Staining was solubilised in 10% acetic acid, and read at 595nm.

**Anoikis assay:** siRNA- or Scr-transfected cells; and NmU cDNA- versus mock-transfected cells (HCC1954 variants, 1x10⁵ cells/well; SKBR3 variants, 1x10⁴ cells/well) were seeded onto a 24-well plates coated with Poly(hydroxyethyl methacrylic) acid (Sigma-Aldrich) or 95% ethanol and cultured for 24 and 48 hours, respectively. Alamar blue dye (100μl; Serotec, UK) was added/well and absorbance read at 570nm; reference wavelength, 600nm.

**Assessing potential clinical relevance of NmU in breast cancer**
NmU expression was evaluated in microarray datasets representing 3,489 breast tumours, including luminal A (n=1,521), luminal B (n=676), HER2 (n=476) and basal (n=454) molecular subtypes. Method used for obtaining and collating the data are detailed in SI Materials & Methods and Table S1. All calculations were performed in R (http://cran.r-project.org/).

**In vivo analysis:** All in vivo work reported was carried out at University College Dublin (UCD), approved by the UCD Animal research ethics committee (AREC) and performed according to local guidelines. Balb C nu nu mice (Charles River) were implanted in the mammary fat pad with 10x10⁶ HCC1954-LR-LUC control (n=10) or HCC1954-LR-LUC-shRNA NmU cells (n=20). Tumour
growth was monitored by calliper measurement and bioluminescence imaging (IVIS Spectrum, Perkin Elmar). Treatment was due to start once tumour volume reached 200mm². When mice reached endpoint they were sacrificed and *ex vivo* imaging performed to detect any metastatic spread.

**Statistical Analyses:** Statistical analysis on cell line- and CM-derived data was performed in Excel. P values were generated using Student’s T-tests, with p<0.05 considered as statistically significant. GraphPad Prism 5.0 was used for graph generation. (Graph Pad Software Inc, La Jolla, USA). All statistical analysis performed is detailed in SI Materials & Methods.

**Results**

**Intracellular & extracellular NmU mRNA levels are associated with acquired resistance to lapatinib.**

To identify extracellular -as well as intracellular- mRNAs that may be associated with resistance to HER-targeted agents, our initial analysis included HER2-overexpressing cell line models, SKBR3 and HCC1954, and lapatinib-resistant (LR) variant compared to their aged parent populations. Comparing the concentration of lapatinib that inhibits 50% of proliferation (IC₅₀) for SKBR3 that had acquired resistance (SKBR3-LR) (IC₅₀=3µM) in relation to SKBR3 aged (-Ag) cells (IC₅₀=0.09µM) as controls (and so termed SKBR3-Ag), a 29.8±2.2 fold resistance to lapatinib was observed. For HCC1954 cells, a similar trend was observed with a 19.1±2.8 fold resistance to lapatinib in HCC1954-LR (IC₅₀=5µM) compared to its age-matched population, HCC1954-Ag (IC₅₀=0.3µM) (Fig. 1A).

Evaluating mRNAs in medium conditioned by SKBR3-LR and HCC1954-LR cells, compared to conditioned medium (CM) from their age-matched control cell lines, indicated significantly higher levels of extracellular NmU to be associated with lapatinib resistance. This observation was validated by qPCR in CM from these cells (Fig. 1B(i)&(ii)). The trend of increased NmU mRNA levels observed in CM from the resistant cells compared to the sensitive cell lines was subsequently found to reflect that in the corresponding cells (Fig. 1B(iii)&(iv)).

Investigating if the induced expression of NmU may be an early response to drug exposure, we treated cells with lapatinib (1µM) for 48 hours and assessed NmU levels by qPCR. Here we found that the levels of NmU mRNA detectable in CM from the lapatinib-exposed SKBR3 and HCC1954 cells (Fig. 1C(i)&(ii)) were significantly higher than in the corresponding untreated control CM,
even after this relatively short-term exposure to drug. As expected, a similar trend was found in the corresponding cells (Fig. 1C(iii)&(iv)). Considering a broader range of HER-targeted drugs, treating cells (HCC1954 as example) for 48 hours with trastuzumab (12.5μg/ml) resulted in induced NmU mRNA levels in CM (3.9±1 fold; p=0.04) and corresponding cells (3.9±1.15 fold; p=0.06) (Fig. 1D(i)&(ii)). In relation to neratinib (0.5μM), the resulting induced NmU mRNA was 3.6±1 fold (p=0.07) and 3.6±0.54 fold (p=0.009) for CM and cells, respectively (Fig. 1D(iii)&(iv)). The levels of induced NmU mRNA for afatinib (0.5μM), was 3.3±0.49 fold (p=0.01) in CM and 3±0.38 fold (p=0.006) in cells (Fig. 1D(v)&(vi)).

**Induced NmU protein expression occurs in cells with acquired resistance to other HER-targeted agents and is not restricted to lapatinib.**

We questioned if the changes at the mRNA level in acquired-resistant cells translated to NmU protein. In agreement with our mRNA observations, NmU protein levels were significantly higher in lapatinib-conditioned cells (SKBR3-LR, HCC1954-LR) compared to their age-matched control cells (SKBR3-Ag, HCC1954-Ag) (Fig. 2A(i)&2A(ii)). Interestingly, this observation was not limited to lapatinib, but was also found in relation to other HER-targeted drugs where acquired-resistance populations were available. Specifically, a similar trend (*i.e.* significantly increased NmU protein levels) was observed when we compared our trastuzumab-resistant (SKBR3-TR or -TR) and neratinib-resistant (SKBR3-NR or -NR) cells with their age-matched control cells (SKBR3-Ag) (Fig. 2A(i)) and our neratinib-resistant HCC1954 (HCC1954-NR or -NR) cells with their age-matched cells (HCC1954-Ag) (Fig. 2A(ii)).

**Endogenous NmU protein levels may be associated with innate sensitivity/resistance to HER-targeting drugs.**

SKBR3 cells are sensitive to both lapatinib and trastuzumab; HCC1954 cells are sensitive to lapatinib but resistant to trastuzumab; conversely, MDA-MB-361 is resistant to lapatinib, but sensitive to trastuzumab (24). Although T47D are described as having normal HER2 expression (25), they are also unresponsive to both drugs (26, 27) and so we elected to include these in our analysis. As shown in Fig. 2B, endogenous levels of NmU protein appeared to correlate with the innate sensitivity versus resistance profile of these 4 cell lines.

**NmU expression is prognostic for poor outcome in breast cancer, particularly for HER2-positive and luminal A subtypes**
To determine if NmU has relevance in human breast cancer rather than being solely a cell line/CM-related observation, we collated and mined microarray data relating to 3,489 breast tumours. Kaplan-Meier estimates of survival (Fig. 3A) indicated high levels of NmU expression to be associated with poor outcome for breast cancer patients (p<1e-14). Considering each of the breast cancer molecular subtypes within this general population of breast tumours, we found NmU expression to be particularly associated with poor outcome for those patients who had HER2-positive tumours (Fig. 3B; p<5e-6) and luminal A tumours (Fig. 3C; p<8e-6). These associations were not significant with luminal B (Fig. 3D; p=0.081) or basal-like (Fig. 3E; p=0.456) tumours.

Although complete clinicopathological information was unavailable for all 3,489 patients, multivariate analysis correcting for tumour size, grade, ER status, lymph node status and age of patient, where this information was available, confirmed NmU as an independent prognostic biomarker rather than it being simply a surrogate for an already-established parameter (Fig. 3F). Specifically, considering all tumour types where information on these five parameters was available (n=966), high levels of NmU expression was associated with poor outcome (p=0.007; hazard-ratio=1.4). Considering the HER2-overexpressing subtype, which is particularly relevant to this study, detailed clinical information was available for only ninety-five patients. Following correction, high levels of NmU in HER2-overexpressing tumours tended towards statistical significance (p=0.07; hazard-ratio=2.1). As information on tumour grade and lymph node status was available for a substantial number of the HER2-overexpressing tumours (n=360 specimens), we evaluated NmU in this cohort and found it to be independently associated with poor outcome for HER2-overexpressing patients (p=0.004; hazard ratio=1.8).

NmU affects sensitivity to lapatinib, trastuzumab, neratinib and afatinib

To assess if NmU might be functionally involved in resistance to HER-targeted drugs, we stably transfected NmU cDNA into SKBR3 and HCC1954 parent cells and established successful over-expression of NmU compared to levels in mock-transfected cells, using both qPCR and ELISA (Fig. 4A(i)&(ii)). For all drugs tested i.e. lapatinib (Fig. 4B(i)), trastuzumab (Fig. 4B(ii)), neratinib (Fig. 4B(iii)) and afatinib (Fig. 4B(iv)), the anti-proliferative effects resulting were significantly compromised in the NmU-transfected cells compared to the mock-transfected cells. The exception to this being the response of HCC1954-NmU to trastuzumab (Fig. 4B(ii)).

To further explore a functional role for NmU in resistance to HER-targeted drugs, NmU was subsequently knocked-down in both our acquired resistant cell lines (SKBR3-LR, HCC1954-LR)
and innately resistant/unresponsive cells (MDA-MB-361, T47D). Again, qPCR and ELISAs established significant knock-down of NmU mRNA as shown in Fig. 5(i) and protein Fig. 5(ii), respectively, compared to levels in scrambled (Scr) control cells. In relation to effects on response to drug, while some variation was observed between cell lines and siRNAs, NmU knock-down (compared to scrambled control, Scr) was found to increase the anti-proliferative effects achieved in response to all of the 4 HER2 drugs tested (Table 1(A&B); also, Table S2(A &B)).

Proposed mechanism-of-action

We next investigated how NmU knock-down may be enhancing the effects of this range of HER-targeted drugs. As HER2 is a target for all 4 drugs and EGFR is also a target of lapatinib, neratinib and afatinib, we assessed the levels of these specific targets –their total amounts and their phosphorylated forms– using ELISAs and immunoblotting. Of great interest, with both SKBR3-LR and HCC1954-LR cells, NmU knock-down was associated with significantly reduced levels of total HER2 protein (Fig. 6A(i)&(ii)).

By mining the literature, we found that HSP27 has previously been reported as highly expressed in HER2-positive breast cancer (28) and, importantly, has been recognised as reducing cellular trastuzumab susceptibility by directly binding to HER2 and thus increasing its stability (29). We hypothesised, therefore, that HSP27 may be the linking molecule in this chain of events facilitating the mechanism by which NmU contributes resistance to HER-targeted drugs. To investigate this, NmU was immunoprecipitated and assessed by immunoblotting for HSP27 binding. NmU-immunoprecipitation did, indeed, pull HSP27 out and further increased levels of the NmU:HSP27 complex were observed in NmU over-expressing (HER-drug resistant) cell lines variants compared to their sensitive mock transfected- parent lines (Fig. 6B (i–ii)). Subsequently, knock-down of NmU in lapatinib-resistant SKBR3-LR and HCC1954-LR (Fig. 6C (i-ii)) demonstrated relative decreased levels of HSP27. A proposed model of this mechanism of action is outlined (Fig. 6D (i-ii)).

Upon NmU silencing, the total amounts of EGFR present was not significantly affected in both cell lines (Fig. S1 A(i)&(ii)), but phosphorylation of the EGFR that was present was significantly reduced (Fig. S1B).

To further explore the functional role of NmU, after establishing that both NmU receptors (NmU-R1, NmU-R2) are expressed by SKBR3 and HCC1954 cells, we observed that treating these cells with exogenous NmU (NmU-25) induced expression of both HER2 and EGFR proteins (Fig. S2A). This suggests that either or both of NmU-R1 and NmU-R2 are functionally active on these cells. Interestingly, exposure to exogenous NmU also induced a low, but significant, level of resistance to
lapatinib (SKBR3, p=0.004; HCC1954, p=0.00004), trastuzumab (SKBR3, p=0.003; HCC1954, p=0.000002), neratinib (SKBR3, p=0.005; HCC1954, p=0.01) and afatinib (SKBR3, p=0.002; HCC1954, p=0.0017) in both SKBR3 and HCC1954 cell lines (Fig. S2B).

NmU expression is also associated with other phenotypic characteristics including cell motility, invasion and resistance to anoikis

To assess what other functional role(s) NmU may have, NmU-overexpressing and NmU knock-down cells were further evaluated. Events associated with more “aggressive” cancers include the ability of the cells to move (migration assays), digest and migrate through extracellular matrix during intravasation and extravasation (invasion assays) and to survive in suspension, as necessary to survive in the peripheral circulation en route to metastasise (anoikis assays). NmU cDNA overexpression in HCC1954 compared to HCC1954-mock was associated with increased motility as evaluated by wound-healing (Fig. S3A(i)), increased migration through transwell (Fig. S3B(i)), increased invasion through extracellular matrix-coated transwells (Fig. S3C(i)), and resistance to anoikis (Fig. S3D(i)). Conversely, NmU knock-down in HCC1954-LR was associated with opposite effects i.e. decreased cellular motility (Fig. S3A(ii)), decreased migration (Fig. S3B(ii)), decreased invasion (Fig. S3C(ii)), and increased sensitivity to cell death by anoikis (Fig. S3D(ii)). (Representative NmU cDNA transfected in SKBR3 results are summarised in Fig. S4).

In vivo, NmU knock-down reduced tumour growth and metastasis

In order to establish the relevance of NmU in the orthotopic in vivo setting, HER-drug resistant HCC1954-LR cells that expressed luciferase underwent NmU knock-down in vitro and were subsequently injected into the mammary fat pad of immunocompromised animals. As indicated in Fig. 7A, orthotopic growth of HCC1954-LR shRNA NmU knock-down (NmU-) tumours was significantly impaired relative to control (p=0.0056). This is indicated by representative bioluminescence images taken at Day 56 (Fig. 7B). Within the timeframe of the current study [65 days], tumours from 7/10 of the control animals had reached endpoint [15mm diameter], and animals sacrificed. Metastatic lesions were found in the lymph nodes, liver and lungs (Fig. 7C&D). At this time [Day 65], only 2/20 animals bearing HCC1954-LR NmU- reached endpoint. In these animals at time of sacrifice, metastatic lesions were found in the liver and lungs, but not in the lymph nodes. Tumours in the remaining 18/20 animals were significantly below endpoint diameter with no evidence of metastases. A significant survival benefit was, thus, observed (p≤0.0001) (Fig 7E).

Discussion
Neuromedin U (NmU) has previously been associated with cancer, but an association between NmU and breast cancer or with HER2-overexpression in any cancer type has not previously been established. Specifically, in typically small studies of AML (30), ovarian (31), pancreatic (32), lung (33), bladder (34) and renal cancer (35), NmU has been associated with oncogenic characteristics. Conversely, preliminary studies of oesophageal (36) and oral (37) cancers suggest tumour suppressor activities. Here we report that increased NmU mRNA expression is significantly associated with poor outcome in breast cancer; particularly for those patients with HER2 or luminal A tumours. Our data also suggests that NmU expression has independence as a prognostic factor for poor outcome and is not simply a surrogate for an already established prognostic biomarker.

Our initial observation of NmU being associated with resistance to HER-targeting drugs was made through analysis of medium conditioned by cells that had acquired lapatinib-resistance compared to CM from their respective aged parent populations. This observation reflected that of the cells themselves. This is in keeping with our previous observations from a range of cell lines (16, 17, 19) and pilot study analysis of serum and corresponding tumour tissue from breast cancer patients (18). These findings indicate that full-length mRNAs can be selectively expelled from cells. Similar observations –studying different mRNAs– have been reported by others (38). To further explore whether or not the response of increased intracellular and extracellular NmU mRNA is an early event, sensitive cells (SKBR3 and HCC1954 parent cells) were exposed to lapatinib short-term, showing some increased NmU mRNA transcription and expulsion from cells within this time-frame. To establish if the increase in NmU levels (intracellular and extracellular) is specific to lapatinib or if NmU mRNA may have relevance as a biomarker of response to other HER-targeting drugs, our analysis was expanded to also investigate the response to trastuzumab, neratinib and afatinib. In all cases, increased transcription and expulsion of NmU mRNA resulted. The lower fold changes observed in CM compared to cells may suggest that this is not a dynamic event; that cells limit the amount of mRNAs they select to send out into the extracellular environment; and/or that some of extracellular mRNA was degraded. However, the NmU mRNA levels in CM from resistant cells were always significantly higher than that in CM from sensitive cells and evidence suggests that this effect is partly an early event; albeit the levels detected in short-term treated cells were not as enhanced as that in acquired-resistance cells. This observation of NmU as a potential extracellular, as well as intracellular, predictive biomarker (while not the main focus on this manuscript) warrants further investigation.

For a more comprehensive understanding, we also elected to expand these studies to evaluate NmU protein, in addition to mRNA. Considering innately sensitive and resistant cells, here we found NmU protein levels to correlate with innate resistance versus sensitivity to HER-targeting drugs i.e.
with the low levels in SKBR3 (sensitive to trastuzumab and lapatinib) progressive increasing in HCC1954 and MDA-MB-361 (resistant to one or other of these drugs) and at highest levels in T47D (unresponsive to both HER-targeted drugs considered here) cells. Furthermore, in relation to acquired resistance, NmU proteins level were always found to be significantly higher in cells with acquired resistance to lapatinib, trastuzumab and neratinib compared to their aged control populations. This suggests that NmU protein, as well as its mRNA, potentially has broad application as a predictive biomarker for response to HER-targeted drugs.

Investigating a functional role of NmU in relation to HER-targeting, our over-expression (in sensitive cells) and knock-down (in both acquired-resistant and innately-resistant cells) studies showed NmU levels to inversely correlate with cellular response to all 4 HER-targeting drugs tested. Specifically, NmU over-expression generally conferred resistance to the anti-proliferative effects of all drugs tested. (The exception to this was the HCC1954 cells, where NmU over-expression did not affect response to trastuzumab. This is likely to be due to the fact that HCC1954 are innately resistant to trastuzumab (24)). Our NmU immunoprecipitation and associated investigations to identify the direct mechanism by which NmU is acting and thus conferring resistance to these drugs indicated NmU to directly partner with HSP27. By directly binding to HER2, HSP27 has previously been shown to increase HER2 stability and thus reduce cellular sensitivity to this drug (29). This is also in keeping with our observation of knock-on effects of NmU expression increasing HER2 protein levels.

Conversely, while there were some differences between cell lines and effectiveness of the two NmU siRNAs used, NmU knock-down added substantially to the anti-proliferative effects of lapatinib, trastuzumab, neratinib and afatinib in both innately- and acquired- resistant cell lines studied. Interestingly, this knock-down in NmU was directly associated with subsequent reduced expression of its identified partner HSP27, HER2 protein and the phosphorylated/activated form of EGFR, the latter possibly being a consequence of a reduced transactivation by HER2. Of note, NmU knock-down also resulted in increased sensitivity of T47D cells to all 4 drugs despite the fact that T47D cells express normal levels of HER2 i.e. T47D is not a HER2-overexpressing cell line (25). As both targets of these drugs (HER2 for all 4 drugs; also EGFR for lapatinib, neratinib and afatinib) were directly affected by the relatively modest NmU knock-down (up to 40% achieved with siRNA), these suggests that co-targeting NmU, while targeting HER2/EGFR, may help circumvent resistance to these drugs.

In keeping with our hypothesis that NmU is involved in regulating response to HER-targeted drugs, treating sensitive HER2-overexpressing cells (SKBR3 and HCC1954) with exogenous NmU was
found to slightly, but significantly, reduce the anti-proliferative effects of lapatinib, trastuzumab, neratinib and afatinib, in association with increased expression of HER2 and EGFR receptors.

The potential benefits of targeting/co-targeting NmU in HER2 breast cancer is further supported by our observations that NmU knock-down significantly decreases migration/motility, invasion and resistance to anoikis, while its over-expression has the opposite effects. Importantly, in vivo studies implementing an orthotopic model of HER2-positive tumours resistant to lapatinib (and to other HER-targeted drugs) further supports this, as NmU stable knock-down dramatically impaired tumour growth and metastatic spread. These observations of NmU being associated with more “aggressive” phenotypic characteristics of cancer cells concur with the reports of NmU being associated with oncogenic characteristics in other cancer types including AML, pancreatic, ovarian, lung and bladder cancers (30-32, 35).

In conclusion, here we provide evidence that NmU is associated with poor outcome from breast cancer, particularly being prognostic of unfavourable outcome for patients with HER2-overexpressing tumours; that it has potential as a predictive biomarker of response to a broad range of HER-targeting drugs; and that it is mechanistically involved in conferring this resistance by working in concert with its partner protein HSP27. Our results provide a strong rationale for more extensive analysis of NmU as a companion biomarker for HER-targeted drugs and as a co-target to help circumvent resistance and thus add value to these drugs.

Acknowledgements: Science Foundation Ireland’s funding of Molecular Therapeutics for Cancer, Ireland 08/SRC/B1410 (JC, AT, LOD); HEA PRTLI Cycle 5 funding of TBSI (LOD); Irish Cancer Society’s support of Breast-Predict [CCRC13GAL]; (AT, LOD) and the Health Research Board of Ireland [HRA_POR/2013/342] (LOD).
References


Table 1A. NmU knock-down partly restores sensitivity in cells with either acquired or innate resistance to HER-targeting (calculations based on when non-transfected (NT) is set to 100%)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug conc.</th>
<th>Cell Line</th>
<th>Drug + SRC (% proliferating cells)</th>
<th>(+) Anti-proliferative benefit with NmU-1</th>
<th>P</th>
<th>(+) Anti-proliferative benefit with NmU-2</th>
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Table 1B. NmU knock-down partly restores sensitivity in cells with either acquired or innate resistance to HER-targeting (calculations based on when Scr+drug is set to 100%)

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**Figure legends**

**LEGENDS HAVE BEEN MODIFIED IN VIEW OF REVIEWERS #2’S COMMENT.**

**Fig. 1.** Increased levels of NmU mRNA in CM and corresponding cells with acquired lapatinib-resistance with induced NmU also observed after short-term drug exposure.  
A, HER2-overexpressing cells that were conditioned with lapatinib (SKBR3-LR and HCC1954-LR) compared to their aged-parent control populations (SKBR3-Ag and HCC1954-Ag) demonstrated increased resistance to lapatinib.  
B, qPCR analysis of NmU mRNA in CM from (i) SKBR3-LR and (ii) HCC1954-LR compared to CM from their aged-parent cells. Corresponding cellular NmU mRNA demonstrated similar trend ((iii)&(iv)).  
C, qPCR analysis of NmU mRNA following short-term (48 hours) exposure of the parent cell populations SKBR3 and HCC1954 to lapatinib in their respective CM ((i)&(ii) and in the corresponding cells (iii)&(iv)).  
D, qPCR analysis of NmU mRNA following short-term exposure of the parent cell populations (HCC1954, as example), to (i) trastuzumab, (iii) neratinib, and (v) afatinib showing and NmU mRNA levels in the CM ((i), (iii)&(v)) and their corresponding cells ((ii), (iv) & (vi)). Data are presented as fold change assigning control an arbitrary 1. All results represent biological repeats n=3±SEM, where *=p<0.05, **=p<0.01, ***=p<0.001.

**Fig. 2.** Increased NmU protein expression in cells with acquired-resistance to lapatinib, trastuzumab and neratinib; also in cells with innate resistance to HER-targeting drugs  
A(i), Higher levels of NmU protein, analysed by ELISA, were also found in (i) lapatinib-resistant (SKBR3-LR), trastuzumab-resistant (SKBR3-TR) and neratinib-resistant (SKBR3-NR) cells compared to the corresponding SKBR3-Ag cells.  
A(ii), for HCC1954, the same trend was found with lapatinib-resistant (HCC1954-LR) and neratinib-resistant (HCC1954-NR) cells compared to their control HCC1954-Ag cells. Data are presented as NmU protein quantity relative to the amount in the aged control (100%) population.  
B, Increased levels of NmU protein were also associated with innate resistance to HER-targeted drugs. Results represent biological repeats n=3±SEM, where *=p<0.05, **=p<0.01, ***=p<0.001.

**Fig. 3.** NmU expression is prognostic for poor outcome for breast cancer patients, particularly within the HER2-positive and luminal A molecular subtypes.  
A, Kaplan-Meier estimates indicate that high levels of NmU are associated with poor prognosis in breast cancer (n=3,489). The association of NmU expression with patients outcome in relation to each breast cancer molecular subtype was subsequently investigated and shown to be significant for  
B, HER2-positive (n=476) and  
C, luminal A (n=1,521) tumours but not  
D, luminal B (n=676) and  
E, basal-like (n=454).
multivariate analysis reporting on NmU following adjustment for a range of established clinicopathological parameters indicated its independence as a poor prognostic biomarker.

**Fig. 4. NmU over-expression reduces sensitivity to HER-targeted agents.** Following NmU cDNA over-expression **A**, levels of NmU mRNA (qPCR) and protein (ELISA) were confirmed in (i) SKBR3 and (ii) HCC1954 cells compared to mock-transfected populations. **B**, Comparative influences on proliferation of SKBR3-NmU and HCC1954-NmU cells compared to the mock-transfected control cells in the presence of (i) lapatinib, (ii) trastuzumab, (iii) neratinib and (iv) afatinib. Results represent n=3±SEM, where *=p<0.05, **=p<0.01, ***=p<0.001.

**Fig. 5. Confirmation of NmU knockdown by siRNAs.** Following transfection with two NmU-siRNA (NmU-1 or NmU-2) or scrambled sequence (Scr), levels of (i) NmU mRNA (qPCR) and (ii) protein (ELISA) showed partial knock-down of NmU expression in both the acquired lapatinib-resistant SKBR3-LR, HCC1954-LR; innately unresponsive T47D and MDA-MB-361 cells. Results represent n=3±SEM, where *=p<0.05, **=p<0.01, ***=p<0.001.

**Fig. 6. NmU’s mechanism-of-action involves complexing with its partner protein HSP27, increasing HER2 stability thus inducing resistance.**

NmU siRNA knock-down in both SKBR3-LR and HCC1954-LR cells was associated with **A**, significantly reduced levels of HER2 protein, as shown by (i) ELISA and (ii) immunoblotting. Immunoprecipitation of NmU in NmU-overexpressing cells, compared to mock-transfected cells, was associated with **B**, significantly increased levels of HSP27 as shown by (i) immunoblotting and (ii) corresponding densitometry. NmU siRNA knock-down in both SKBR3-LR and HCC1954-LR cells was associated with **C**, significantly reduced levels of HSP27 protein, as shown by (i) immunoblotting and (ii) corresponding densitometry. **D**, Our proposed mechanism-of-action by which (i) increased NmU results in increased NmU:HSP27 complexity, subsequent increased HER2 stability and thus resistance and (ii) how our NmU knock-down is, therefore, re-sensitising the resistant cells to these drugs.

**Fig. 7. In vivo, NmU knock-down dramatically impairs tumour growth & metastatic spread.**

10 million cells were implanted orthotopically into the mammary fat pad of Balb c nude mice (HCC1954-LR, n=10; HCC1954-LR NmU-, n=20) and allowed to grow. Animals were sacrificed once they reached end-point [15mm tumour diameter] **A**, Tumour growth curve comparison of HCC1954-LR NmU− and HCC1954-LR cell lines. **B**, Representative bioluminescence images of HCC1954-LR NmU− and HCC1954-LR cell lines taken 56 days post implantation. *Ex vivo* imaging was performed for assessment of metastatic burden. Metastasis was observed in the liver (7/7...
mice), lungs (4/7 mice) and lymph nodes (2/7 mice) in mice bearing the HCC1954-LR cell lines. In the HCC1954-LR NmU- cell line, 2 mice reached end-point and were sacrificed on Day 64. Metastatic deposits were observed in liver and lungs only. **D.** Quantification of metastatic burden in HCC1954-LR and HCC1954-LR NmU- cell lines at time of sacrifice. **E.** Survival comparison of HCC1954-LR and HCC1954-LR NmU- cell lines. By day 64, 70% of mice bearing HCC1954-LR tumour had reached endpoint compared to 10% of those with HCC1954-LR NmU- tumour. This was a significant difference (p≤0.0001, Mantel-Cox test).

**Table 1: NmU knock-down partly restores sensitivity in cells with either acquired or innate resistance to HER-targeting**

NmU knock-down in these cells enhanced the effectiveness of lapatinib, trastuzumab, neratinib and afatinib at decreasing proliferation compared to that observed in corresponding Scr-transfected cells.
Figure 1
Figure 2
All breast cancer subtypes

HER2-overexpressing

Luminal A

Luminal B

Basal-like

Figure 3
Figure 4
Figure 5
Figure 6

**HER2**

**HSP27**

**Stability**

**Lapatinib**

**Trastuzumab**

**Neratinib**

**Afatinib**

**RESISTANCE**

**RE-SENSITISING**
Comparison of tumour growth between HCC1954-LR and HCC1954-LR NmU knockdown cells

A

P=0.0056

B

HCC1954-LR control

C

Location of metastatic lesions in HCC1954-LR NmU- and HCC1954-LR model

D

Average Radiance (p/s/cm²/sr)

E

Survival Comparison HCC1954-LR v HCC1954-LR Nmu-

Figure 7
Neuromedin U: a candidate biomarker and therapeutic target to predict and overcome resistance to HER kinase inhibitors

Sweta Rani, Claire Corcoran, Liam Shiels, et al.

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