An Engineered Bivalent Neuregulin Protects Against Doxorubicin-Induced Cardiotoxicity With Reduced Proneoplastic Potential

Steven M. Jay, PhD; Ashwin C. Murthy, MD; Jessica F. Hawkins, BS; Joshua R. Wortzel, AB; Matthew L. Steinhauser, MD; Luis M. Alvarez, PhD; Joseph Gannon; Calum A. Macrae, MD, PhD; Linda G. Griffith, PhD; Richard T. Lee, MD

Background—Doxorubicin (DOXO) is an effective anthracycline chemotherapeutic, but its use is limited by cumulative dose-dependent cardiotoxicity. Neuregulin-1β is an ErbB receptor family ligand that is effective against DOXO-induced cardiomyopathy in experimental models but is also proneoplastic. We previously showed that an engineered bivalent neuregulin-1β (NN) has reduced proneoplastic potential in comparison with the epidermal growth factor–like domain of neuregulin-1β (NRG), an effect mediated by receptor biasing toward ErbB3 homotypic interactions uncommonly formed by native neuregulin-1β. Here, we hypothesized that a newly formulated, covalent NN would be cardioprotective with reduced proneoplastic effects in comparison with NRG.

Methods and Results—NN was expressed as a maltose-binding protein fusion in Escherichia coli. As established previously, NN stimulated antineoplastic or cytostatic signaling and phenotype in cancer cells, whereas NRG stimulated proneoplastic signaling and phenotype. In neonatal rat cardiomyocytes, NN and NRG induced similar downstream signaling. NN, like NRG, attenuated the double-stranded DNA breaks associated with DOXO exposure in neonatal rat cardiomyocytes and human cardiomyocytes derived from induced pluripotent stem cells. NN treatment significantly attenuated DOXO-induced decrease in fractional shortening as measured by blinded echocardiography in mice in a chronic cardiomyopathy model (57.7±0.6% versus 50.9±2.6%, P=0.004), whereas native NRG had no significant effect (49.4±3.7% versus 50.9±2.6%, P=0.813).

Conclusions—NN is a cardioprotective agent that promotes cardiomyocyte survival and improves cardiac function in DOXO-induced cardiotoxicity. Given the reduced proneoplastic potential of NN versus NRG, NN has translational potential for cardioprotection in patients with cancer receiving anthracyclines.

Key words: anthracyclines ■ translational cancer chemotherapy protocols ■ protein engineering

The anthracycline doxorubicin (DOXO) is a widely used chemotherapeutic that is particularly efficacious against the subset of breast cancers that overexpress the ErbB2 (HER2) receptor.1 Limiting its clinical use, however, is a cumulative dose-related cardiotoxicity characterized by left ventricular dysfunction that can ultimately culminate in congestive heart failure.2 Oncologists now limit the dosage of DOXO, and thus DOXO-induced cardiomyopathy is no longer a widespread clinical problem. However, this dose limitation is potentially deleterious for patients with cancer for whom DOXO is the most effective therapy. Therefore, approaches to mitigate the cardiotoxic effects of DOXO to enable increased dosing to combat malignancies remain an unmet clinical need.

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leads to improved survival in animal models of ischemic, viral, and dilated cardiomyopathy11 and has demonstrated cardioprotective efficacy in clinical trials.12

However, despite its efficacy, NRG is not clinically relevant as a therapeutic for DOXO-induced cardiomyopathy because of its well-established role in proneoplastic signaling.13–16 We have previously designed an engineered bivalent neuregulin-1β (NN) that demonstrates reduced proneoplastic potential in comparison with NRG.17 Whereas NRG is widely reported to participate in malignant signaling pathways via the induction of ErbB2/3 heterotypic interactions,13–16 NN biases signaling away from these and toward ErbB3 homotypic interactions in high ErbB3- and low ErbB4-expressing cancer cells.17 Because ErbB3 is a very weak18 or inactive19 kinase, ErbB3 homotypic interactions can result in a cytostatic or antineoplastic phenotype.17

If NN retains similar cardioprotective properties in comparison with NRG without the proneoplastic properties of NRG, then NN could represent a more translationally relevant therapy for DOXO-induced cardiomyopathy. We hypothesized that NN would promote cardioprotection in a manner similar to NRG, provided that NN-induced biasing to exclude ErbB2 participation in cardiomyocyte signaling was not extreme. Although the role of ErbB2 in chemotherapy-related cardiotoxicity remains unclear, substantial inhibition of ErbB2 signaling may impair cardiac function, as observed in patients receiving concurrent treatment with anthracyclines and the ErbB2 antibody trastuzumab.20 We tested the stated hypothesis in vitro in rat and human cardiomyocytes and in vivo in randomized and blinded mouse models of acute and chronic DOXO-induced cardiomyopathy. Our results indicate that NN induces antineoplastic or cytostatic responses in cancer cells that are stimulated toward known malignant signaling pathways and phenotypes by NRG, endowing NN with a reduced proneoplastic profile in comparison with NRG, and that NN is cardioprotective, working through similar mechanisms as NRG, and holds promise as a novel therapeutic for patients with cancer who are receiving anthracyclines.

**Methods**

**Protein Design and Purification**

The amino acid sequence for NN comprises 2 NRG domains separated by a hydrophilic, protease-resistant spacer (Figure 1A). The full sequence of NN is as follows: SHLVKCAEK EKETFCVNNGECFVMKDLNSPRSYLCKCNPNTFGDRCQNY VMASFYKHLGIEFMEAESAGGSSEGGSEGSGAGGSEGGS EGGTSGATASAGGSEGSEGGSEGSGAGGSEGSGAGG SEGTSAGATASAGG SEGSGAGGSEGSEG USESTGATGSGSHELVCA EKETFCVNNGECFVMKDLNSPRSYLCKCNPNTFGDRCQNY VMASFYKHLGIEFMEAESAGGSSEGGSEGSGAGGSEGGS EGGTSGATASAGGSEGSGAGGSEGSGAGGSEGSGAGG SEGTSAGATGSGSHELVCA EKETFCVNNGECFVMKDLNSPRSYLCKCNPNTFGDRCQNY VMASFYKHLGIEFMEAESAGGSSEGGSEGSGAGGSEGGS EGGTSGATASAGGSEGSGAGGSEGSGAGGSEGSGAGG SEGTSAGATGSGSHELVCA EKETFCVNNGECFVMKDLNSPRSYLCKCNPNTFGDRCQNY VMASFYKHLGIEFMEAESAGGSSEGGSEGSGAGGSEGGS EGGTSGATASAGGSEGSGAGGSEGSGAGGSEGSGAGG SEGTSAGATGSGSHELVCA EKETFCVNNGECFVMKDLNSPRSYLCKCNPNTFGDRCQNY VMASFYKHLGIEFMEA. Additional information is available in the Online-only Data Supplement.

**Immunoblot and proteomic array analyses, cell culture procedures, and in vitro functional assays are described in the Online-only Data Supplement. Molecules used for stimulation included DOXO (doxorubicin hydrochloride, Sigma-Aldrich), solubilized in sterile saline), NRG (epidermal growth factor–like domain, Peprotech), or NN. In general, NN was used at one-half the molar dose of NRG because NN contains 2 NRG domains; therefore, this dosing scheme should result in approximately equivalent doses.**

**In Vivo Studies**

Two different randomized and blinded mouse models of DOXO-induced cardiomyopathy, acute and chronic, were used. For the acute model, 8- to 12-week-old male C57BL/6 mice (total of 33, Charles River) were administered a single DOXO injection (20 mg/kg IP; Sigma-Aldrich). Selections of DOXO dose and route of administration were based on previous studies.21 NRG (100 μg/kg IP) or NN (100 μg/kg IP) was administered daily starting 3 days before and for 4 days after DOXO administration. The timing and dosage of treatment injections were based on a previous study.7 Controls were treated with vehicle (0.2% bovine serum albumin in phosphate-buffered saline) only. Baseline echocardiography was performed on all animals 2 days before initial treatment injection, and animals were randomly assigned into groups based on fractional shortening (FS) values. Blinded echocardiography was performed after final treatment injection. Mice were anesthetized with pentobarbital (30-70 mg/kg). The left ventricle (LV) was imaged in the short-axis view at the midpapillary muscle, and 2-dimensional measurements of LV end-diastolic diameter and LV end-systolic diameter were recorded. FS

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**Figure 1.** Design of covalently linked bivalent neuregulin-1β (NN) and validation of its reduced proneoplastic potential in comparison with neuregulin-1β (NRG). A, NN was produced via covalent linkage of 2 NRG domains separated by a flexible, protease-resistant spacer. Purity was indicated by Coomassie-stained gel. B, Schematic of expected differential ErbB receptor complexation induced by NRG, NN, and doxorubicin-sensitive human mammary ductal carcinoma cell line MDA-MB-175VII stimulated for 15 minutes by NRG or NN. GAPDH expression was measured as a loading control. Data are representative of 3 independent experiments. C, Cell number was assessed by measurement of DNA content via fluorescence (CyQUANT assay, ex=485 nm; em=530 nm) after stimulation of MDA-MB-175VII cells by control media (indicated by dashed line) or the indicated doses of NRG (left of slash) or NN (right of slash; n=6). Probability values were calculated by using a Wilcoxon rank-sum test. em indicates emission; ex, excitation; and RFU, relative fluorescence units.
was then calculated (FS=[100×(LV end-diastolic diameter−LV end-systolic diameter)/LV end-diastolic diameter]). Heart rates were determined by using M-mode images. Animals were euthanized following the final echocardiogram, and their hearts were excised and flash frozen before thawing and homogenization in lysis buffer for immunoblot analysis.

For the chronic model, 8- to 12-week-old female C57BL/6 mice (total of 44, Charles River) were administered serial DOXO injections (4 mg/kg IP; Sigma-Aldrich). Mice were injected once weekly for 5 consecutive weeks at the initiation of the study (weeks 0–4) and then again for 5 weeks beginning 16 weeks after the initial injection (weeks 16–20). Selection of DOXO dose and route of administration was based on a previous study.\textsuperscript{22} NRG (50 μg/kg IP) or NN (50 μg/kg IP) was administered daily for 1 week starting on the day of the first DOXO injection (week 0) and again daily for 1 week during week 16. Vehicle-only treated animals were used as controls (0.2% bovine serum albumin in phosphate-buffered saline). Animals were randomly assigned as in the acute model. Blinded echocardiography was performed 6, 12, 22, and 24 weeks after the initial DOXO injection. Anesthesia and analysis were identical to those used in the acute model. Animals were euthanized following the final echocardiogram, and their hearts were excised and fixed in 4% paraformaldehyde overnight before dehydration, rehydration, and fixation in paraffin by standard methods. Five-micrometer sections were cut and stained with hematoxylin and eosin or Masson trichrome. All animal protocols were approved by the Harvard Institutional Animal Care and Use Committee and performed in an Association for Assessment and Accreditation of Laboratory Animal Care–certified facility.

Statistical Analysis
All data are shown as mean±standard error of the mean unless stated. Statistical significance was calculated by using nonparametric analyses as indicated in the figure legends. Additional information is available in the Online-only Data Supplement.

Results
Design of Covalently Linked NN and Validation of Its Reduced Proneoplastic Potential in Comparison With NRG
We previously reported the development of bivalent ligands consisting of a noncovalent linkage of 2 monomeric receptor-binding domains separated by a flexible, protease-resistant spacer and a high-affinity coiled-coil domain.\textsuperscript{17} To reduce the possibility for dissociation of ligands and thereby promoting the potential for clinical translation, a new covalently linked NN was engineered without a coiled-coil domain and with a corresponding increase in the length of the spacer (Figure 1A). Purity of the final product was verified by observation of a single band on a Coomassie-stained gel (Figure 1A).

Binding of NRG1B to ErbB3 and the subsequent formation of ErbB2/3 heterotypic interactions are associated with proneoplastic signaling in a wide range of cancers, including DOXO-sensitive breast cancers.\textsuperscript{14,16,23} We previously demonstrated that NN promotes sequestration of ErbB3 into weak/nonsignaling homotypic interactions, resulting in a cytostatic or antineoplastic phenotype\textsuperscript{17} (summarized schematically in Figure 1B). Here, we used the DOXO-sensitive, human mammary ductal carcinoma cell line MDA-MB-175VII,\textsuperscript{24} which is stimulated by a neuregulin-1 autocrine loop whereby ErbB2 and ErbB3 are phosphorylated,\textsuperscript{25,26} to assess whether NN would exhibit reduced proneoplastic characteristics in comparison with native NRG. When stimulated for 15 minutes with NRG, MDA-MB-175VII cells exhibited dose-dependent phosphorylation of ErbB2 and ErbB3, as expected (Figure 1C). Stimulation with NN for 15 minutes, on the other hand, resulted in levels of phospho-ErbB2 and phospho-ErbB3 that were lower than or similar to control levels, even at the highest tested doses of NN (Figure 1C). Furthermore, phosphorylation of downstream effectors Akt and ERK1/2, known mediators of proliferation, migration, and survival in cancer cells, was also modestly increased on stimulation by NRG but unchanged or decreased by stimulation with NN in comparison with control levels (Figure 1C). Additionally, NRG stimulation promoted maintenance or growth of MDA-MB-175VII, whereas stimulation with an equivalent dosage of NN resulted in reduced cell viability in comparison with native NRG at dosages ≥50 nmol/L (Figure 1D). Thus, these data confirm the bioactivity of NN and reinforce its reduced proneoplastic potential in comparison with NRG in DOXO-sensitive breast cancer cells.\textsuperscript{17}

NN Stimulates ErbB Receptor Phosphorylation on Cardiomyocytes
Given the well-established roles for NRG in cardioprotection\textsuperscript{6,11,27–30} and as a potential therapy for DOXO-induced cardiomyopathy,\textsuperscript{3,8} we wanted to test whether NN, with its reduced proneoplastic potential in comparison with NRG, stimulated cardioprotective signaling in a manner similar to NRG. Postnatal cardiomyocytes express ErbB2 and ErbB4, but not ErbB3.\textsuperscript{31} ErbB4, unlike ErbB3, has a fully active kinase domain and can participate in signaling when complexed with either ErbB2 or ErbB4.\textsuperscript{4} NRG stimulation of cardiomyocytes results predominantly in the formation of ErbB2/4 heterotypic interactions,\textsuperscript{9} initiating downstream signaling (Figure 2A). We predicted that NN stimulation would induce biasing away from ErbB2/4 interactions toward ErbB4 homotypic interactions, which would result in active downstream signaling that may or may not differ from that induced by ErbB2/4 interactions (Figure 2A).

To assess the effect of NN on ErbB receptor phosphorylation on cardiomyocytes, neonatal rat cardiomyocytes (NRCMs) were stimulated for 15 minutes with either 10 nmol/L NRG or NN and lysates were analyzed by using phosphoreceptor tyrosine kinase arrays that enabled simultaneous evaluation of phosphorylation of all ErbB receptors. Stimulation with NN resulted in apparent increased ErbB4 phosphorylation and decreased ErbB2 phosphorylation in comparison with NRG, although ErbB2 phosphorylation still occurred with NN stimulation (Figure 2B). Thus, we established that, although NN stimulation results in ErbB receptor phosphorylation on cardiomyocytes consistent with the generation of increased ErbB4 homotypic interactions in comparison with stimulation with NRG, inhibition of ErbB2 phosphorylation is not extreme.

NN Induces Intracellular Signaling Similar to NRG and Phosphorylates Known Mediators of Cardioprotection
To assess whether ErbB receptor phosphorylation on cardiomyocytes associated with NN induced different downstream signaling in comparison with NRG, NRCM
Lysates prepared by using the same conditions as described for Figure 2 were analyzed with intracellular phosphokinase arrays (Figure 3). Array analysis revealed substantial phosphorylation of Akt (S473) and ERK1/2, known mediators of cardioprotective effects, at similar levels for both NRG and NN stimulation (Figure 3A). Overall, no significant difference between NRG-treated cells and NN-treated cells was observed (Figure 3B). The same analysis was performed on lysates made 24 hours following stimulation by proteins or control media in the presence of 1 μmol/L DOXO. A distinction between the signaling profiles of NRCM stimulated with control media with DOXO (DOXO) and without DOXO (Control) was evident (Figure 3C and 3D). NRCMs stimulated with DOXO and NRG (DOXO+NRG) or DOXO and NN

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**Figure 2.** Bivalent neuregulin-1β (NN) stimulates ErbB receptor phosphorylation on cardiomyocytes. A, Schematic of expected differential ErbB receptor complexation induced by neuregulin-1β (NRG) in comparison with NN on cardiomyocytes. NRG is predicted to predominantly promote formation of ErbB2/4 heterotypic interactions, whereas NN is predicted to induce increased ErbB4 homotypic interactions. B, Phospho-receptor tyrosine kinase array analysis of neonatal rat cardiomyocytes stimulated for 15 minutes by 10 nmol/L NRG or NN or serum-free media (Vehicle). Data are representative of 3 independent experiments. Legend indicates relative locations of positive control (+), negative control (−), phospho-ErbB2 (pErbB2), and phospho-ErbB4 (pErbB4) spots on array membrane.

**Figure 3.** Bivalent neuregulin-1β (NN) induces intracellular signaling similar to NRG and phosphorylates, known mediators of cardioprotection. A, Expression data from phosphokinase array of neonatal rat cardiomyocytes (NRCMs) stimulated for 15 minutes by 50 nmol/L NRG or 25 nmol/L NN, Vehicle=serum-free media (n=3). B, Pathway analysis of data from A demonstrates similar protein phosphorylation patterns induced by NN and NRG. Data were thresholded to y-axis=0.20 and displayed in order of relative expression in Vehicle group from low to high as indicated. C, Expression data from phospho-kinase array of NRCMs stimulated for 24 hours by 50 nmol/L NRG or 25 nmol/L NN concurrently exposed to 1 μmol/L doxorubicin (DOXO), Vehicle=serum-free media (n=3). D, Pathway analysis of data from C demonstrates differential protein phosphorylation stimulated by DOXO in comparison with Vehicle. NN+DOXO and NRG+DOXO induced similar patterns that indicate significant regulation of numerous factors from their DOXO-induced state toward the Vehicle state, indicative of cardioprotective signaling. Data were thresholded to y-axis=0.75 and displayed in order of relative expression in the Vehicle group from low to high as indicated.
(DOXO+NN) had profiles that were generally more similar to Control, indicative of cardioprotective signaling, and that did not vary from each other in a statistically significant way (Figure 3C and 3D). Additional analyses of Akt and ERK1/2 phosphorylation at different doses and time points revealed no significant differences between NRG- and NN-induced effects. Overall, these data support the conclusions that NN is capable of inducing canonical cardioprotective signaling in cardiomyocytes and that downstream signaling induced by NN is similar to that induced by NRG in vitro in cardiomyocytes.

NN Is Cardioprotective From DOXO In Vitro
Because stimulation with NRG or NN resulted in similar downstream signaling profiles, we hypothesized that NN would retain similar cardioprotective qualities to NRG in the setting of DOXO-induced toxicity. To test this, we assessed phenotype and signaling in response to DOXO exposure. Stimulation with 100 nmol/L NRG in the presence of 1 μmol/L DOXO resulted in a significant increase in NRCM viability in comparison with DOXO-only treated cells, as expected (Figure 4A, P=0.013). Surprisingly, stimulation with 50 nmol/L NN resulted in significantly greater preservation of NRCM viability than an approximately equivalent dosage of 100 nmol/L NRG (Figure 4A, P=0.038). Additionally, stimulation with 25 nmol/L NN significantly reduced NRCM apoptosis associated with DOXO and did so more effectively than stimulation with 50 nmol/L NRG (Figure 4B, P=0.002). Taken together, these data indicate that NN is cardioprotective from DOXO in vitro with comparable or enhanced potency in comparison with NRG.

One validated mechanism for the cardiotoxicity of DOXO is the generation of double-stranded DNA breaks, ultimately leading to cell death. Phosphorylation of the histone H2A.X, which yields γ-H2A.X, occurs in response to double-stranded DNA breaks and is a very early indicator of DNA damage. Exposure of NRCM to 1 μmol/L DOXO and of human induced pluripotent stem cell–derived cardiomyocytes to 10 μmol/L DOXO resulted in increased γ-H2A.X signal (Figure 4C and 4D). The increase in γ-H2A.X was similarly attenuated when stimulated with approximately equivalent concentrations of NRG (100 nmol/L) or NN (50 nmol/L) (Figure 4C and 4D). Thus, the cardioprotective effect of both NRG and NN may be explained by an attenuation of the double-stranded DNA breaks induced by DOXO.

NN Does Not Interfere With DOXO Activity on Anthracycline-Sensitive Cancer Cells
One possibility for the apparent effects of NN in counteracting DOXO-induced cardiomyocyte toxicity is nonspecific interference with DOXO by NN. To examine whether NN interfered with the cytotoxic action of DOXO, DOXO-sensitive mammary adenocarcinoma line SK-BR-3 cells and HeLa cells were concurrently exposed to increasing doses of DOXO and a static dose of either 50 nmol/L NRG or 25 nmol/L NN. NN did not interfere with the cytotoxic action of DOXO at the assessed doses, indicating that the protective effects of NN on cardiomyocytes were not the result of the inactivation of DOXO (Figure 5A and 5B). Additionally, γ-H2A.X expression was unchanged in HeLa cells concurrently exposed to DOXO and NRG or NN (Figure 5C), further supporting the conclusion that NN does not interfere with DOXO activity and, thus, that any effects observed in cardiomyocytes are likely the result of stimulation of ErbB receptors and not of nonspecific DOXO inhibition.

Figure 4. Bivalent neuregulin-1β (NN) has similar cardioprotective qualities to neuregulin-1β (NRG) in vitro. A, Neonatal rat cardiomyocytes (NRCMs) were exposed to 1 μmol/L doxorubicin (DOXO) and were stimulated with the indicated concentrations of NRG or NN in serum-free conditions for 24 hours. The control condition was NRCM in serum-free media (Vehicle). Cell viability was quantified via fluorescence (CyQUANT assay, ex=485 nm, em=530 nm; n=8). B, Presence of cleaved caspase-3/7 was assessed in NRCMs after exposure to 1 μmol/L DOXO and stimulation with NRG or NN for 24 hours via fluorescence (SensoLyte assay, ex=354 nm, em=442 nm; n=8). The control condition was NRCM in serum-free media. For A and B, probability values were determined by using a Wilcoxon rank-sum test with a Bonferroni correction after an initial Kruskal-Wallis test to determine statistical significance; data are representative of at least 2 independent experiments. RFU indicates relative fluorescence units. C, Immunoblot analysis of γ-H2A.X in NRCM stimulated with NRG (100 nmol/L) or NN (50 nmol/L) concurrently exposed to 1 μmol/L DOXO for 24 hours. NRCMs were incubated with treatments for 24 hours before DOXO addition (48 hours total treatment time). D, Immunoblot analysis of γ-H2A.X in human induced pluripotent stem cell–derived cardiomyocytes (iPSC-CM) stimulated with NRG (100 nmol/L) or NN (50 nmol/L) concurrently exposed to 10 μmol/L DOXO for 24 hours. iPSC-CM were incubated with treatments for 24 hours before DOXO addition (48 hours total treatment time). For C and D, GAPDH expression was assessed as a protein-loading control, data are representative of 3 independent experiments, and the control condition was NRCM in serum-free media (Vehicle). em indicates emission; and ex, excitation.
To assess the efficacy of NN in vivo, we performed a pilot study applying a previously described model of acute DOXO-induced cardiomyopathy.7,21 We observed a trend, indicative of impaired left ventricular function, toward a decrease in FS, as detected by blinded echocardiography, of mice treated with DOXO only (DOXO) in comparison with animals that did not receive DOXO (Vehicle) (Figure 6A, 59.4±1.0% versus 54.5±1.6%, P=0.026). Animals treated with NN displayed a trend toward attenuation of the decrease in FS associated with DOXO exposure (Figure 6A, 59.4±1.4% versus 54.5±1.6%, P=0.053). FS was not affected by treatment with NRG or NN alone. Additionally, the analysis of cross-sectional heart lysates from NN-treated study animals exhibited a decrease in γ-H2A.X expression in comparison with DOXO-treated animals (Figure 6B), suggesting that the beneficial effect of NN in DOXO-exposed animals could at least be partially attributable to reduced double-stranded DNA breaks in the heart.

The efficacy of NN as a cardioprotective agent in vivo was further assessed by using a randomized and blinded mouse model of chronic DOXO-induced toxicity. In comparison with the acute model, this model is more representative of the clinical administration profile of DOXO. A significant decrease in FS of DOXO-treated mice (DOXO) in comparison with vehicle control (Vehicle) was observed at 24 weeks following the initial DOXO injection (Table and Figure 6C; 58.0±1.2% versus 50.9±2.6%, P=0.011). A significant increase in end-systolic diameter and significantly lower body weight were also associated with DOXO treatment at 24 weeks (Table). Indicative of cardioprotection, animals treated with NN had a significant attenuation of the decrease in FS associated with DOXO exposure at 24 weeks (Table and Figure 6C; 57.7±0.6% versus 50.9±2.6%, P=0.004). In this model, NRG treatment had no effect on FS (49.4±3.7% versus 50.9±2.6%, P=0.813), and NN treatment led to a significantly higher FS than NRG (57.7±0.6% versus 49.4±3.7%, P=0.001). NN-treated mice also exhibited, in comparison with mice given DOXO only (DOXO), a trend toward increased posterior wall thickness and significantly decreased end-systolic diameter at 24 weeks (Table), similar to the Vehicle group values and indicative of a protective effect of NN on the heart. Histological analysis of tissue from study animals revealed pockets of necrotic tissue and fibrosis in DOXO-only animals, with these being less prevalent in NRG- and NN-treated animals (Figure I in the online-only Data Supplement). Additionally, all animals treated with DOXO showed similar weight loss in comparison with the Vehicle control group (Table), and thus the systemic effects of DOXO were not inhibited by NN even though cardiac function was improved, further evidence that NN is not an inhibitor of DOXO activity but rather a counteracting factor that works through a different mechanism, namely, ErbB receptor stimulation. This was further confirmed by detection of ErbB2 and ErbB4 phosphorylation in mouse hearts following injection of NN or NRG (Figure II in the online-only Data Supplement). Overall, these data support the conclusion that NN is cardioprotective from DOXO.

**Discussion**

NRG1B is a promising cardiovascular therapeutic, but has limited relevance for cardioprotection against anthracycline-induced cardiotoxicity because of its proneoplastic potential. We previously reported the development...
explored whether NN has a differential effect in comparison with NRG. It also remains to be determined whether differences in receptor trafficking attributable to the tethered Ig-like domain. This immunoglobulin-like domain may enhance the duration of effects of GGF2 versus NRG via interactions with matrix molecules that prolong tissue residence times. The differences between NRG and GGF2 may help to explain the apparent superior cardioprotection induced by NN. A difference in the phosphoinositide-3 kinase/Akt and mitogen-activated protein kinase/ERK1/2 signaling pathways have previously been demonstrated in native NRG. A difference in the cardiomyocyte-signaling profiles in comparison with NRG, GGF2 is a larger protein that contains an immunoglobulin-like domain. This immunoglobulin-like domain may enhance the duration of effects of GGF2 versus NRG via interactions with matrix molecules that prolong tissue residence times. Thus, the differences in potency between NN and NRG in our experiments may be the result of increased cell or tissue interactions with NN because of its larger size or differences in receptor trafficking attributable to the tethered nature of the NRG domains on NN. It also remains to be explored whether NN has a differential effect in comparison with NRG on putative vascular and cardiac progenitor cells, which are reported to be specifically adversely affected by anthracycline exposure. Although the role of these cells in cardiac repair and regeneration is still unclear, a distinct effect of NN on them could potentially account for the differences observed in cardioprotection between NN and NRG in this study. In addition, it is possible that NN could stimulate cardiac regeneration via the division of preexisting cardiomyocytes, as suggested for native NRG. A difference in the efficiency or efficacy of stimulation of regenerative pathways could lead to the differential protective effects observed in these studies. Finally, we cannot rule out a differential effect of NN in comparison with NRG owing to differences in the regulation of expression of topoisomerase-IIβ, which was recently identified as an essential mediator of DOXO-induced cardiotoxicity. Further exploration of differential gene expression associated with NN and NRG may yield data to support or refute the potential association of NN and NRG with topoisomerase-IIβ expression.

In general, these data are congruent with previous work demonstrating the cardioprotective potential of NRG. Both the phosphoinositide-3 kinase/Akt and mitogen-activated protein kinase/ERK1/2 signaling pathways have previously been identified as critical mediators of NRG effects on cardiomyocytes that are not completely dependent on ErbB2 participation in signaling. Thus, the ability of NN to activate these pathways (Figure 3), despite inducing relatively...
less ErbB2 phosphorylation than NRG (Figure 2), is not surprising. It is important to note, however, that the approach to manipulating ErbB receptor interactions with bivalent NN described here is fundamentally different from conventional receptor blocking or deletion approaches. It is well known that complete exclusion of ErbB2 from cardiac signaling, via either conditional knockout preclinical studies or clinically via the ErbB2-targeted antibody trastuzumab, is deleterious, especially so in conjunction with anthracycline exposure. NN induced partial biasing of ErbB receptor interactions and activation, but substantial ErbB2 phosphorylation was still observed (Figure 2 and Figure II in the online-only Data Supplement), and so the negative effects associated with ErbB2 blockade are avoided.

### Table. Echocardiographic Parameters After Serial Doses of Doxorubicin in Mice

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<th>Treatment</th>
<th>Baseline</th>
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<th>P (24 wk vs DOXO)</th>
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<td>0.054</td>
</tr>
<tr>
<td>End-systolic diameter, mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.08±0.06</td>
<td>1.20±0.05</td>
<td>0.002*</td>
</tr>
<tr>
<td>DOXO</td>
<td>1.05±0.11</td>
<td>1.49±0.26</td>
<td>—</td>
</tr>
<tr>
<td>DOXO+NRG</td>
<td>1.10±0.05</td>
<td>1.44±0.74</td>
<td>0.536</td>
</tr>
<tr>
<td>DOXO+NN</td>
<td>1.06±0.06</td>
<td>1.24±0.13</td>
<td>0.029</td>
</tr>
<tr>
<td>End-diastolic diameter, mm</td>
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<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>2.76±0.32</td>
<td>2.86±0.20</td>
<td>0.11</td>
</tr>
<tr>
<td>DOXO</td>
<td>2.66±0.23</td>
<td>3.04±0.17</td>
<td>—</td>
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<tr>
<td>DOXO+NRG</td>
<td>2.78±0.16</td>
<td>2.83±0.07</td>
<td>0.07</td>
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<tr>
<td>DOXO+NN</td>
<td>2.72±0.14</td>
<td>2.93±0.25</td>
<td>0.247</td>
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<tr>
<td>Fractional shortening, %</td>
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<td></td>
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<tr>
<td>Vehicle</td>
<td>60.53±4.26</td>
<td>57.99±3.21</td>
<td>0.011*</td>
</tr>
<tr>
<td>DOXO</td>
<td>60.28±2.21</td>
<td>50.94±6.88</td>
<td>—</td>
</tr>
<tr>
<td>DOXO+NRG</td>
<td>60.41±2.45</td>
<td>49.37±9.91</td>
<td>0.813</td>
</tr>
<tr>
<td>DOXO+NN</td>
<td>60.74±2.24</td>
<td>57.69±1.74</td>
<td>0.004*</td>
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<tr>
<td>Left ventricular mass, g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>93.74±15.78</td>
<td>112.26±11.19</td>
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<tr>
<td>DOXO</td>
<td>88.27±11.03</td>
<td>114.30±15.57</td>
<td>—</td>
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<tr>
<td>DOXO+NRG</td>
<td>94.10±10.44</td>
<td>104.99±14.94</td>
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<tr>
<td>DOXO+NN</td>
<td>93.32±8.01</td>
<td>114.44±17.78</td>
<td>0.867</td>
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<tr>
<td>Body weight, g</td>
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<tr>
<td>Vehicle</td>
<td>20.30±0.75</td>
<td>31.98±4.53</td>
<td>0.0006*</td>
</tr>
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<td>DOXO</td>
<td>20.21±0.92</td>
<td>23.46±2.53</td>
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<tr>
<td>DOXO+NRG</td>
<td>21.00±1.03</td>
<td>23.97±2.84</td>
<td>0.807</td>
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<td>DOXO+NN</td>
<td>21.74±0.77</td>
<td>23.88±2.14</td>
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<td>Heart rate, bpm</td>
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<td></td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>536±49</td>
<td>519±81</td>
<td>0.331</td>
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<td>515±70</td>
<td>561±54</td>
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<td>574±66</td>
<td>447±100</td>
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</tr>
<tr>
<td>DOXO+NN</td>
<td>548±45</td>
<td>484±84</td>
<td>0.072</td>
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</table>

Data are mean±standard deviation. Vehicle = vehicle injections (0.2% BSA in PBS, no DOXO); DOXO = 4 mg/kg DOXO IP injections weekly during weeks 0 to 4 and 16 to 20; DOXO+NRG = 4 mg/kg DOXO IP injections weekly during weeks 0 to 4 and 16 to 20 with concurrent daily 50 μg/kg IP injections of NRG during weeks 0 and 16; and DOXO+NN = 4 mg/kg doxorubicin IP injections weekly during weeks 0 to 4 and 16 to 20 with concurrent daily 50 μg/kg IP injections of NN during weeks 0 and 16. BSA indicates bovine serum albumin; DOXO, doxorubicin; IP, intraperitoneal; NN, an engineered bivalent neuregulin-1β; NRG, epidermal growth factor–like domain of neuregulin-1β; and PBS, phosphate-buffered saline.

*P<0.0125 was considered statistically significant after Wilcoxon rank-sum test with Bonferroni correction.

†One animal from the Vehicle group and 2 animals from the DOXO+NN group were euthanized during the first 8 weeks of the study because of wounds received from fighting.
In summary, the data show that NN is protective from DOXO-induced cardiotoxicity in vitro in rat and human cardiomyocytes and in vivo in a mouse model. Based on these results, NN may have therapeutic potential in other cardiac pathologies, including acute or chronic ischemic heart disease. Additional results from ongoing clinical trials with NRG and GGF2 will help clarify the translational potential of NN.

Acknowledgments

We thank Jun Yoshioka for advice and assistance with in vivo studies; Sachiko Kanki, Francesco Loffredo, Sam Senyo, Samuel Lee, Will Chutkow, Andrew Krueger, and Caitlin O’Meara for technical assistance and reagents; Jen Lin for critical review of the manuscript; Lei Cai and Cathy MacGillivray for preparing and staining histological sections, and Harvard Catalyst for biostatistical consultation.

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Disclosures

Brigham and Women’s Hospital (BWH) and the Massachusetts Institute of Technology have filed for patents pertaining to the described bivalent ligand technology, listing Drs Alvarez, Jay, Lee, and Griffith as inventors. Dr Lee is a cofounder and co-owner of Provasculon, Inc. Dr Lee is a paid consultant to the company and serves on its Board of Directors. Provasculon has interests in regenerative cell therapy, an area related to the research. Dr Lee’s interests were reviewed by BWH and Partners HealthCare. The other authors report no conflicts.

References


CLINICAL PERSPECTIVE

Doxorubicin (DOXO) is an anthracycline chemotherapeutic that is effective against particular subsets of various cancers; for example, ErbB2-overexpressing breast cancer. However, the use of DOXO is limited by a dose-related cumulative cardiotoxicity that can lead to congestive heart failure. The relationship between DOXO dose and cardiotoxicity is sufficiently well-defined such that oncologists now limit cumulative dosage, and thus DOXO-induced cardiomyopathy is rarely seen clinically. Yet this dose limitation of DOXO is deleterious for those patients for whom it is the best available therapy for their cancer. Therefore, we used protein engineering to exploit the cardioprotective effects of the ErbB receptor ligand neuregulin-1β (the epidermal growth factor-like domain of neuregulin-1β), which is a known cardioprotective protein that also has proneoplastic characteristics. We hypothesized that by taking advantage of known differences in ErbB receptor biology in cancer cells and cardiomyocytes, we could develop a protein that had the cardioprotective qualities of the epidermal growth factor-like domain of neuregulin-1β without the proneoplastic effects. The present study demonstrates that an engineered bivalent neuregulin-1β induces cytostatic and antineoplastic responses in cancer cells that are stimulated toward malignant phenotypes by the epidermal growth factor-like domain of neuregulin-1β while retaining efficacy as a cardioprotective agent in a chronic-administration mouse model of DOXO-induced cardiotoxicity. Based on these results, we believe that an engineered bivalent neuregulin-1β has translational potential as a novel cardioprotective therapeutic for patients with cancer who may need to receive DOXO beyond the current cumulative dose threshold.
An Engineered Bivalent Neuregulin Protects Against Doxorubicin-Induced Cardiotoxicity With Reduced Proneoplastic Potential


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An Engineered Bivalent Neuregulin Protects Against Doxorubicin-Induced Cardiotoxicity
With Reduced Pro-Neoplastic Potential

Steven M. Jay, PhD; Ashwin C. Murthy, MD; Jessica F. Hawkins, BS; Joshua R. Wortzel, AB; Matthew L. Steinhauser, MD; Luis M. Alvarez, PhD; Joseph Gannon, Calum A. Macrae, MD PhD; Linda G. Griffith, PhD; Richard T. Lee, MD

SUPPLEMENTAL MATERIAL
SUPPLEMENTAL METHODS
Protein Design and Purification – Coding DNA for NN was designed in silico (SerialCloner) and obtained as a whole gene product without codon bias. The protein was expressed as a maltose binding protein (MBP) fusion in E. coli strain Origami B(DE3)PlysS (EMD Biosciences). The protein sequence was inserted, using standard restriction enzyme digestion/ligation techniques, into a modified pMAL-c5x vector (New England Biolabs) containing a poly-histidine sequence inserted 3 base pairs to the C-terminal side of the start codon. A cleavage site for tobacco etch virus protease (TEVp) was incorporated directly N-terminal of the initial NRG domain. Protein expression was induced with 0.3mM IPTG for 4-24h and soluble protein was harvested following cell lysis with BugBuster MasterMix (Novagen) supplemented with PMSF and protease inhibitor cocktail (Sigma). Lysates were clarified by centrifugation at 4000 x g for 30min and the pellet discarded. Protein within the supernatant was purified on NiNTA resin (Invitrogen) according to the manufacturer’s protocol and then buffer exchanged into a redox refolding buffer (20mM Tris, 2mM GSH, 1mM GSSG, pH 8.5; all reagents from Sigma) via size exclusion high-performance liquid chromatography (HPLC). The purified fusion protein was then subjected to cleavage by TEVp for ~12h at 4°C. Cleaved protein was further purified by removal of MBP via amylose affinity chromatography (New England Biolabs) and isolation of non-aggregated protein by size exclusion HPLC, followed by an endotoxin removal step using an EndoTrap Red column (Profos).
Bioactivity of purified protein was assessed using an MCF-7 proliferation assay.1

Cell culture – Human mammary adenocarcinoma cell line SK-BR-3 and human mammary ductal carcinoma cell line MDA-MB-175VII were cultured at 37°C with 5%
CO$_2$ in DMEM + 10% fetal bovine serum (FBS, Aleken Biologicals). Both cell lines were purchased from the American Type Culture Collection.

Neonatal rat cardiomyocytes were isolated as follows. Ventricles of 1- to 2-day old Sprague Dawley rats (Charles River Laboratories) were excised and minced, followed by serial digestion in an enzyme solution of pancreatin (0.06mg/ml, Sigma-Aldrich) and collagenase type II (0.04mg/ml, Worthington) in 1X HBSS (-) (Invitrogen) with 7.5% sodium bicarbonate (0.18%, Invitrogen). Cells were washed and pre-plated into T-75 flasks in Dulbecco’s modified Eagle’s medium (DMEM) with low glucose (Invitrogen) containing 15% FBS, 1% penicillin/streptomycin supplement (Invitrogen), and 2.56% 1M HEPES (Invitrogen) to remove non-myocytes. After 2h, myocytes were plated at 2.5x10$^5$ cells per 3.8 cm$^2$ well for eventual immunoblot analysis, at 2x10$^6$ cells per 9.6 cm$^2$ for eventual proteomic array analysis, and at 50,000 cells per 0.3 cm$^2$ well for functional studies. After 18h, the medium was changed to serum-free DMEM (low glucose) and incubated for another 18h prior to stimulation. All cultures were kept at 37°C, 95% humidity, and 5% CO$_2$.

Human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) were obtained from Cellular Dynamics International (iCell® Cardiomyocytes) and were cultured according to the supplier’s instructions.

Immunoblot Analysis – Cells or tissue samples were lysed in Bio-Plex Cell Lysis Buffer with the protease and phosphatase inhibitor cocktails Bio-Plex Factor 1 and Bio-Plex Factor 2 (Bio-Rad). Proteins were quantified using the MicroBCA Protein Assay Kit.
(Pierce). Loading buffer and 0.5M 2-mercaptoethanol (Sigma-Aldrich) were added to protein samples, which were boiled for 5min and run on 4-12% Bis-Tris gels. Membranes were probed with primary antibodies (2241 pErbB2, 4791 pErbB3, 4060 pAkt, 4370 pERK1/2, 2577 γ-H2A.X, 2118 GAPDH; all from Cell Signaling Technologies) and HRP-conjugated secondary antibodies (Bio-Rad).

Proteomic Array Analysis – Phospho-Receptor Tyrosine Kinase (RTK) Array kits and Phospho-Kinase Array kits (R&D Systems) were used to determine relative levels of tyrosine phosphorylation of RTKs or intracellular kinases, respectively, in NRCM. Lysates and membranes were prepared according to the specifications of the product inserts.

In Vitro Functional Assays – For cell viability studies with NRCM, cells were seeded at 50,000 cells per well in 96-well plate for 36h in complete media. Medium was changed to serum-free medium containing the designated doses of DOXO and/or NRG or NN, and the cells were incubated for an additional 24h. Cell viability was assessed using CyQUANT-NF proliferation kit (Invitrogen). For cell viability studies with MDA-MB-175VII, SK-BR-3, and HeLa, cells were seeded at 3,500/well in 96 well plates. For cell viability studies with iPSC-CM, cells were seeded into 12-well plates at 290,000 viable cells/well based on the supplier’s protocol. For apoptosis studies, NRCM were seeded at 50,000 cells/well in a 96 well plate. 24h later, medium was aspirated and replaced by new medium with and without NRG or NN in the presence of 1µM DOXO and incubated for an additional 24h. Caspase 3/7 activation was detected using the SensoLyte® Homogeneous AFC Caspase 3/7 Assay Kit (Anaspec).
Statistical Analysis – Data in Figure 1D were analyzed by a Wilcoxon rank-sum test.

Data in Figures 3-6 and in Table 1 were initially analyzed by a Kruskal-Wallis test with Dunn's multiple comparison post-tests to determine statistical significance and $P$ values were then determined using a Wilcoxon rank-sum test with an appropriate Bonferroni correction. For Table 1 and Figure 6, only endpoint data were analyzed and thus no conclusions about intermediate data points were drawn.
Supplemental Figure I: Histological analysis of animals from chronic doxorubicin (DOXO) toxicity \textit{in vivo} study. Chronic DOXO-induced cardiomyopathy was induced with weekly serial 4mg/kg intraperitoneal (ip) injections of DOXO as shown in Figure 7. Beginning on the day of the initial DOXO injection for each 4 week series, mice were injected daily for 7 days with the vehicle solution, 0.2% BSA in PBS (DOXO), neuregulin-1β (NRG), or bivalent NRG (NN) at 50\(\mu\)g/kg ip per day. Mice that were not injected with DOXO (Vehicle) were used as controls. Representative hematoxylin and eosin (H&E) stained slides revealed pockets of tissue that appeared necrotic (arrows), and these were most prevalent in the DOXO group. Representative Masson’s trichrome (Trichrome) stained slides revealed tissue that appeared fibrotic (arrows) in all DOXO-treated animals, with the greatest prevalence seen in the DOXO group. Scale bar = 10\(\mu\)m.
Supplemental Figure II: Bivalent neuregulin-1β (NN) induces ErbB receptor phosphorylation \textit{in vivo}. 8-12 week old female C57Bl/6 mice were intraperitoneally injected with 2.5\(\mu\)g/animal of neuregulin-1β (NRG) or NN and hearts were excised and homogenized at the indicated times post injection. Immunoblot analysis of ErbB receptor phosphorylation was carried out by standard methods and expression levels were quantified by densitometry using ImageJ software and normalized to GAPDH expression (n=3).
SUPPLEMENTAL REFERENCES