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for Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Hamid Band, M.D.

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, NE 68198-6810

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14. ABSTRACT The endocytic trafficking pathway is the site of action for receptor-targeted drug-delivery strategies, including Antibody-Drug-Conjugates (ADCs) and nanoparticle drug-delivery systems. Effective drug-release requires trafficking of the endocytosed receptor-bound cargo into the lysosomes for efficient disintegration. However, cancer-cell specific alterations that lead to receptor recycling, instead of lysosomal-degradation, can dampen the efficiency of drug delivery. Such changes include receptor overexpression, increased association with the molecular chaperone, chaperones such as HSP90 or alterations in regulators of recycling versus lysosomal pathways (Rab GTPases, c-Src, deubiquitinases). While substantial effort has gone into designing receptor-targeted drug delivery systems, the consequence of factors leading to altered recycling versus lysosomal trafficking on the efficiency of drug delivery have not been considered. The receptor tyrosine kinases ErbB2 and EGFR, which are often overexpressed in breast cancer, are examples of cell surface receptors used for evaluating nanoparticle-based targeted drug delivery systems. However, several studies have established that the ErbB2 receptor is either endocytosis-impaired or undergoes rapid recycling, suggesting that the strategies to enhance receptor internalization and lysosomal routing could further enhance the efficacy of cytotoxic drug being delivered. While, the molecular chaperone HSP90 is critical for maintaining oncogenic ErbB2-activity, it also is thought to be responsible for the altered trafficking of the receptor. The objective of this synergistic DOD-IDEA grant proposal was to evaluate our innovative hypothesis that HSP90 inhibitors can facilitate ErbB2-targeted delivery of chemotherapeutic payloads. As hypothesized, studies reported here demonstrate that the HSP90 inhibitor 17-AAG indeed facilitates the uptake and delivery of a model chemotherapeutic agent Doxorubicin specifically into ErbB2-overexpressing breast cancer cells. These novel findings should pave the way for endocytic mechanism-based enhancement of receptor-targeted drug delivery.					
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Introduction:

Breast cancer continues to remain the leading type of cancer among women and second leading cause of cancer-related deaths in the U.S according to the statistics released by the American Cancer Society. In 2011, there were more than 2.6 million breast cancer survivors and over 230,480 new cases of invasive breast cancer were expected to be diagnosed (<http://www.breastcancer.org>). Nearly a third of breast cancer patients are diagnosed to be positive for the Human Epidermal Growth Factor Receptor 2 (Her2; also known as ErbB2 or Neu) and therefore represent a major therapeutic target. The humanized anti-ErbB2 monoclonal antibody, Trastuzumab (Trast a ercept n enentech an ranc sc C s n) is an essential part of treatment for ErbB2-overexpressing breast cancers. Trastuzumab is currently administered with other chemotherapeutics, like microtubule stabilizing agents (Docetaxel, Paclitaxel), DNA binding drugs (Doxorubicin, Epirubicin, Cisplatin) or alkylating agents (Cyclophosphamide). However, clinical data indicate a less than satisfactory response rate in patients and importantly, most patients that do respond initially eventually develop resistance. In addition to the tumor cells acquiring resistance, the patients also have to endure the effects of the chemotherapeutics on the normal tissue. Anti-ErbB2 antibody-conjugated polymeric nanoparticles with a capacity to load multiple drugs at high concentrations represent a promising alternative to circumvent these problems. However, success of ErbB2-targeted drug-delivery into the cytosol using nanotechnology platforms critically depends on the efficiency of internalization. Optimal targeting must therefore take into account the biology of endocytic trafficking of ErbB2 receptor (1-3). Specifically, the low rate of endocytosis is attributed to constitutive association of ErbB2 with Heat Shock Protein 90 (HSP90) (2, 3). This Synergistic IDEA project seeks to develop a novel strategy to effectively target ErbB2-overexpressing breast cancer with anti-ErbB2 antibody-coated nanogels carrying potent chemotherapeutics in combination with HSP90 inhibitors to enhance the endocytosis of ErbB2 receptor-bound nanogel cargo. A successful outcome of our studies will provide a new therapeutic approach against a particularly difficult form of breast cancer and may provide a template for therapeutic targeting of other forms of breast cancer and other cancers. Success in preclinical models would also provide strong rationale for clinical translation of this technology with the ultimate goals of selective delivery of imaging and therapeutic modalities to tumors.

Grant Hypothesis/Objective and Specific Aims: In this grant, we had hypothesized that the efficacy of treatment of ErbB2-overexpressing cancers using targeted delivery of cytotoxic payload encapsulated in nanogels based on copolymer micelles can be vastly improved by simultaneously targeting ErbB2-HSP90 complex using HSP90 inhibitors and optimizing ErbB2 endocytosis. The specific aims for this proposal were:

- 1) Develop novel nanogels based on copolymer micelles with ionic cross-linked cores carrying anticancer chemotherapeutics (single drugs and combinations) and decorated with an anti-ErbB2 antibody for targeting to ErbB2-overexpressing breast cancer cells;*
- 2) Optimize the intracellular delivery of targeted nanogels carrying cytotoxic drugs to ErbB2-overexpressing breast cancer cells based on HSP90 inhibitor-facilitated internalization of ErbB2;*
- 3) Demonstrate enhanced efficacy of 17-AAG to deliver cytotoxic cargo encapsulated in targeted nanogels using in vivo xenograft mouse models.*

Statement of Work Proposed prior to award administration in Jan 2011.

According to the proposed Statement Of Work (SOW) prior to the initiation of the award in Jan 2011, the following goals were projected towards achieving the above listed objectives:

Aim 1 (PI - Bronich): Develop novel nanogels based on copolymer micelles with ionic cross-linked cores carrying anticancer chemotherapeutics (single drugs and combinations) and decorated with anti-ErbB2 antibodies for targeting to ErbB2-overexpressing breast cancer cells. (Year 1: Months 1-12)

Task 1 (PI - Bronich). Preparation and characterization of Dox-loaded nanogels from Poly(ethylene oxide)-*b*-poly(methacrylic acid) block copolymers (PEO-*b*-PMA) of structures will be used as building blocks for nanofabrication of nanogels. (Year 1: Months 1-6 various).

Task 2 (PI - Bronich). *In vivo* drug release studies (Year 1: Months 6-9; Number of mice = 40)

Task 3 (PI - Bronich). Preparation and characterization of Anti-ErbB2 antibody (Trastuzumab)-conjugated nanogels loaded with Dox (Year 1: Months 6-12).

Aim 2 (PI – Band): Optimize the intracellular delivery of targeted nanogels carrying cytotoxic drugs to ErbB2-overexpressing breast cancer cells based on HSP90 inhibitor-facilitated internalization of ErbB2. (Year 1: Months 8-12; Year 2: Months 1-4)

Task 1 (PI – Band). Compare the ErbB2-specific uptake of Trastuzumab-conjugated nanogels carrying cytotoxic drugs specifically into ErbB2-overexpressing cells, in the presence or absence of HSP90 inhibitors. (Year 2: Months 1-5)

Task 2 (PI – Band). To establish that co-treatment with 17-AAG leads to enhanced cytotoxicity by drug-loaded nanogels specifically in ErbB2-overexpressing cells. (Year 2: Months 3-6).

Aim 3 (PI – Band): Demonstrate enhanced efficacy of 17-AAG to deliver cytotoxic cargo encapsulated in targeted nanogels using *in vivo* xenograft mouse models. (Year 2: Months 6-12)

Task 1 (PI – Band). Establishment of tumor xenograft in immune-compromised mice (NOD-SCID) for experiments to be done in Tasks 2 & 3 described below. (Year 2: Months 6-8; Number of mice = 180)

Task 2 (PIs – Band & Bronich). Characterization of the ability of Trastuzumab-conjugated nanogels to selectively home in on ErbB2-overexpressing tumors using *in vivo* imaging. (Year 2: Months: 7-8; Number of mice = 10 out of the 180 animals with xenografts established in task 1 will be used for task 2)

Task 3 (PIs – Band & Bronich). Test the delivery of nanogels encapsulating cytotoxic drugs with and without 17-AAG co-treatment (Year 2: Months 8-12; Number of mice = 170 mice of the 180 mice with the tumor xenograft established in task 1 will be used in task 3)

Body of the Report:

Summary: In the first year report, we described the: (i) synthesis and characterization of nanogels with cross-linked ionic cores from poly(methacrylic acid) chains and a hydrophilic shell from poly(ethylene oxide) (PEO) chains; (ii) optimization of the structure and composition of drug-loaded nanogels; (iii) development of procedures for conjugation of anti-ErbB2 antibody (Trastuzumab) to nanogels; (iv) initial studies demonstrating the principle that HSP90-inhibition facilitates the uptake of ErbB2-targeted nanogels (without any drugs loaded).

Building up on the our studies from year 1, the second year report describes our *in vitro* results demonstrating enhanced cytotoxicity of Trastuzumab-coated nanogels carrying Doxorubicin specifically in ErbB2-overexpressing breast cancer cells. As maintaining the functionality of trastuzumab on drug-loaded nanogels required additional optimization, the schedule for our *in vivo* studies listed in Aim 3 was delayed, but these studies are currently ongoing and we anticipate completing these by the end of March 2013. In preparation for future expansion of our collaborative work, we have continued to expand on the goals of original aim 1 towards the development of novel polypeptide-based NGs with a greater capacity for encapsulating hydrophobic drugs like 17-AAG and Paclitaxel along with ionic chemotherapeutics such as Cisplatin. These studies are projected to serve as solid preliminary data for future grant applications. To allow completion of our proposed studies and to follow up findings from ongoing *in vivo* analyses and additional drug encapsulation studies, we have sought and received permission from DOD to continue the project on a non-cost extension basis until January 31, 2014.

Second Year Progress:

1) Demonstration that HSP90-inhibition facilitates trafficking of Trastuzumab-nanogels into lysosomal compartments (Aim 2; Task 1) – In the first year progress report we had described studies demonstrating that HSP90 inhibition indeed enhances the uptake of Trastuzumab-conjugated nanogels into

punctate intracellular vesicular structures (see Fig. 14 & 15 of first year progress report). However, we had not yet confirmed whether the nanogel cargo trafficked into the lysosomal compartment (the desired sub-cellular organelle that will facilitate the disintegration of the nanogel structure and release the encapsulated drugs). Confocal Immunofluorescence analysis done on SKBr-3 cells, as described in Fig. 16 (the figure numbering follows year 1 report in order to reflect continuity), confirmed that the nanogels were found in LAMP-1 (a lysosomal marker)-positive compartments.

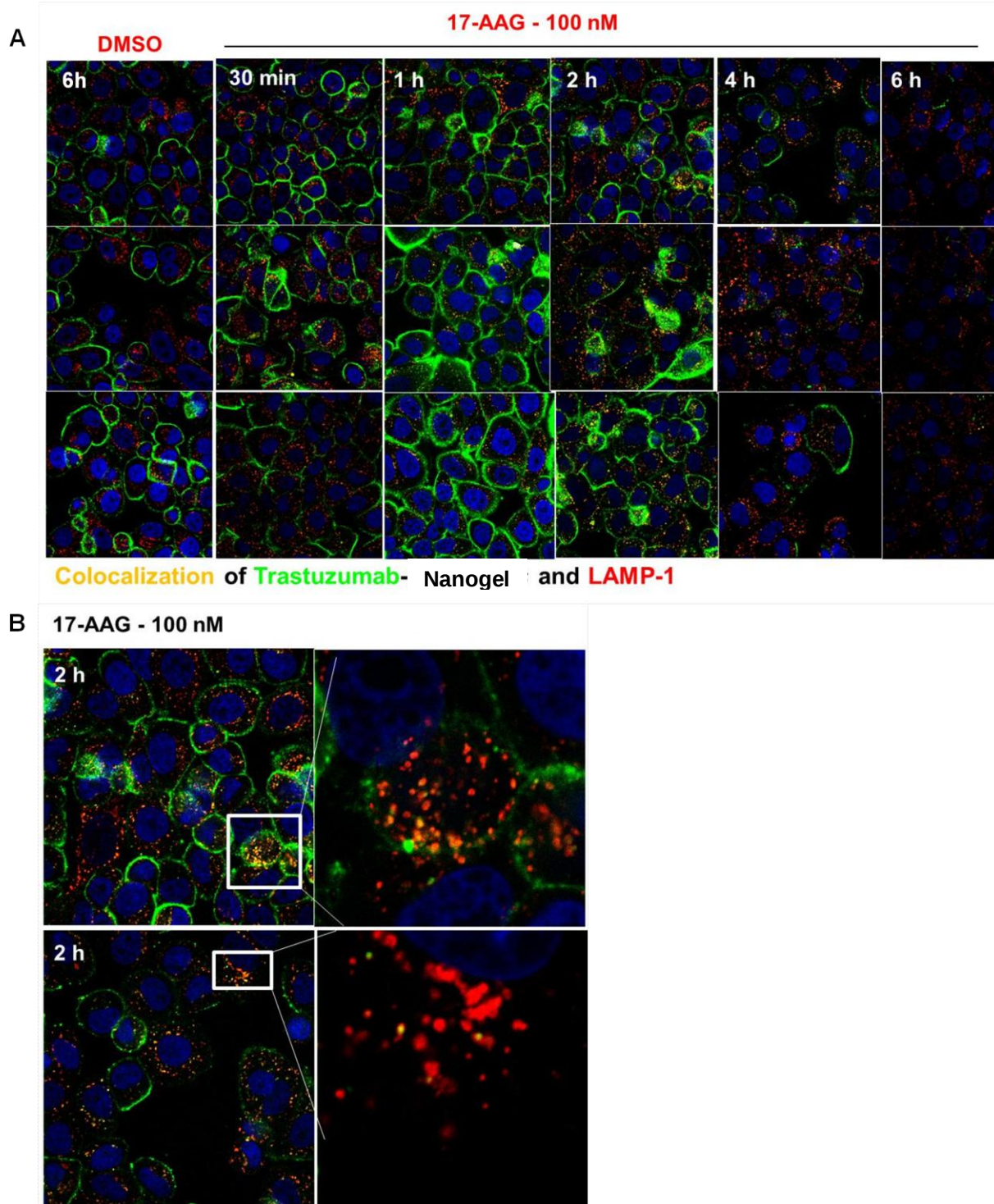
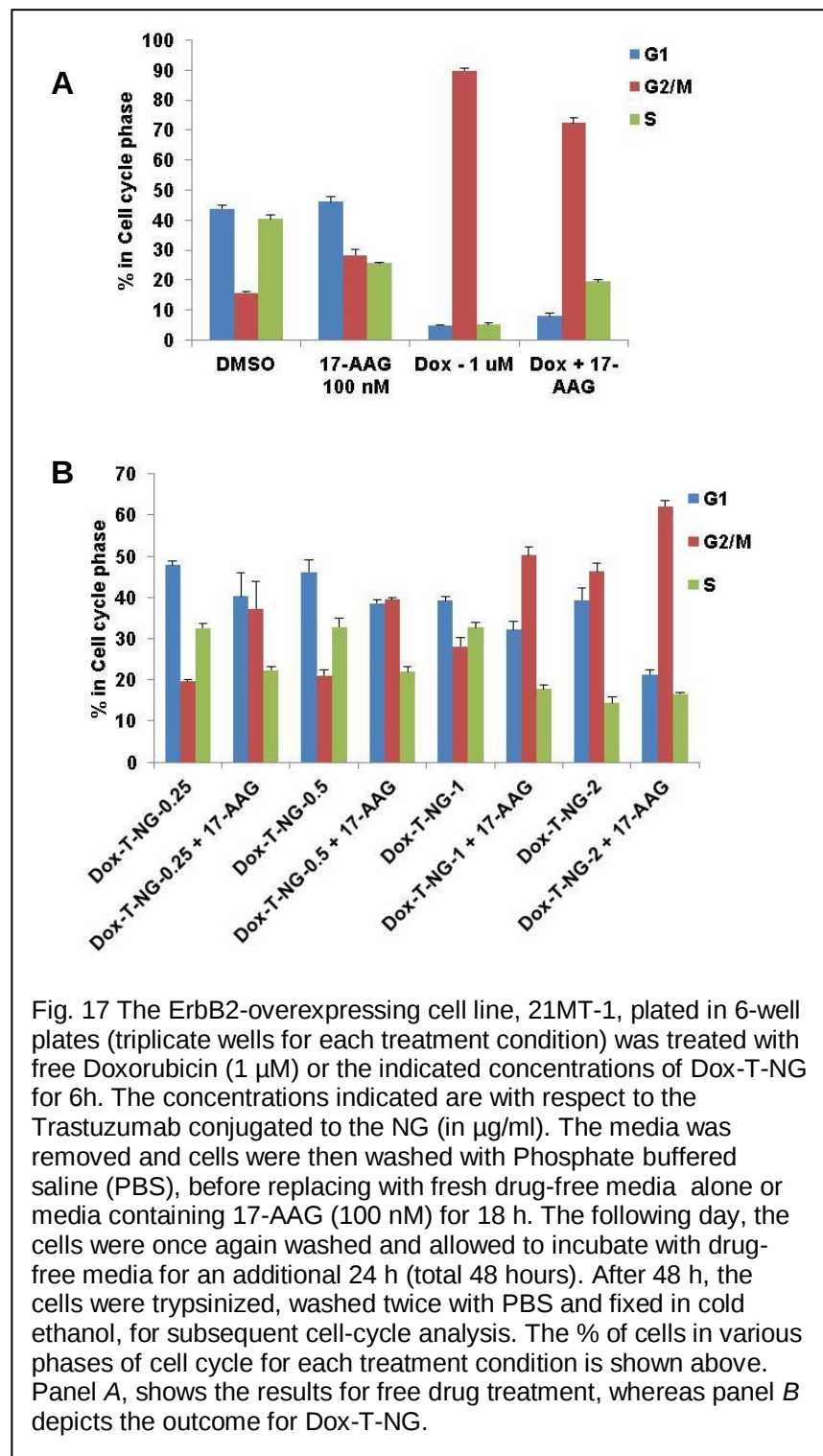


Fig. 16 A, Trastuzumab nanogels (without drugs) was bound to ErbB2-overexpressing SKBr3 cells, plated on glass coverslips, for 1h. 17-AAG (100 nM) was added for the indicated times, after which the slides were washed and fixed in 4% paraformaldehyde. Nanogels were visualized by staining the Fc portion of Trastuzumab using FITC-conjugated anti-Human antibody (green), whereas the lysosomal marker LAMP-1 was stained using mouse anti-human LAMP-1 mAb, followed by Alexa-594-conjugated anti-mouse secondary. Shown here are three independent fields analyzed by confocal immunofluorescence microscopy. B, a zoomed in image of the boxed regions from two independent fields demonstrating co-localization (yellow) of nanogels (green) with LAMP-1 (red).

2) Demonstration that HSP90-inhibition enhances the cytostatic effect of Doxorubicin delivered via Trastuzumab-nanogels specifically in ErbB2-overexpressing breast cancer cells (Aim 2; Task 2) -

Having verified our central hypothesis that HSP90-inhibition facilitates the routing of Trastuzumab-nanogels (without drugs) into the lysosomes, we evaluated the ability of 17-AAG to enhance the delivery of Doxorubicin (a model chemotherapeutic) encapsulated within the nanogels. The ErbB2-overexpressing cell line, 21MT-1 was used for these experiments. Doxorubicin-loaded Trastuzumab-conjugated nanogels (Dox-T-NG; at varying concentrations) were incubated with 21MT-1 cells for 6 hours, following which cells were either left alone (control) or treated with 100 nM 17-AAG for 18 hours to trigger internalization and lysosomal routing of the targeted nanogel cargo. Similar experiments on the ErbB2-low cell line MCF-7 were done in order to confirm ErbB2-specific delivery. Biological effects were assessed by evaluating Doxorubicin-induced G2/M arrest. The results are shown in Fig. 17-19. As expected, there was clear increase in the % of ErbB2-overexpressing 21MT-1 cells that underwent G2/M arrest upon 17-AAG-treatment as compared to cells that were not treated with 17-AAG, at varying doses of Dox-T-NG tested (see Fig. 17). On the other hand, no appreciable difference of 17-AAG was noted in similar experiments conducted on the ErbB2-low MCF-7 cells (Fig. 18) especially at lower concentrations of Dox-T-NG.



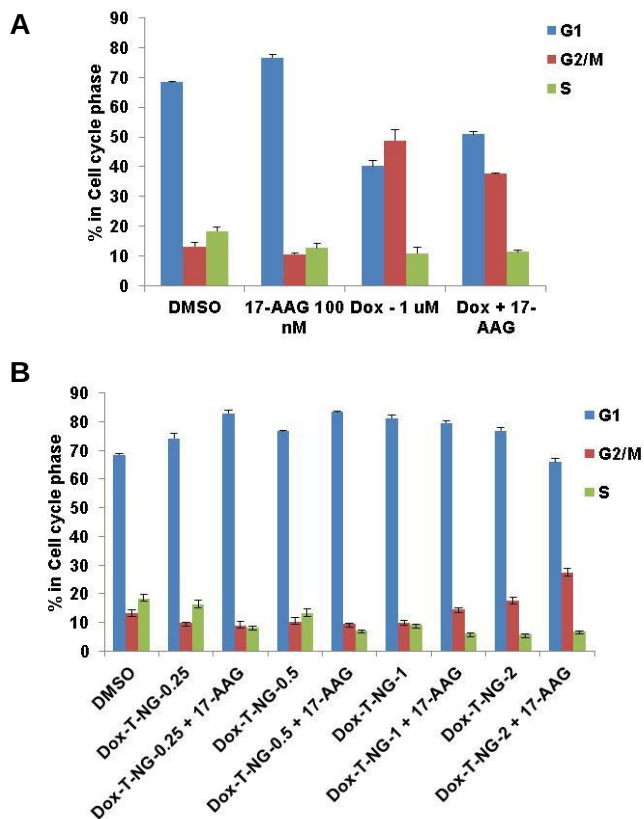


Fig. 18 The ErbB2-low cell line, MCF-7, plated in 6-well plates (triplicate wells for each treatment condition) was treated with free Doxorubicin (1 μ M) or the indicated concentrations of Dox-T-NG for 6h. The media was removed and cells were then washed with Phosphate buffered saline (PBS), before replacing with fresh drug-free media alone or media containing 17-AAG (100 nM) for 18 h. The following day, the cells were once again washed and allowed to incubate with drug-free media for an additional 24 h (total 48 hours). After 48 h, the cells were trypsinized, washed twice with PBS and fixed in cold ethanol, for subsequent cell-cycle analysis. The % of cells in various phases of cell cycle for each treatment condition is shown above. Panel A, shows the results for free drug treatment, whereas panel B depicts the outcome for Dox-T-NG.

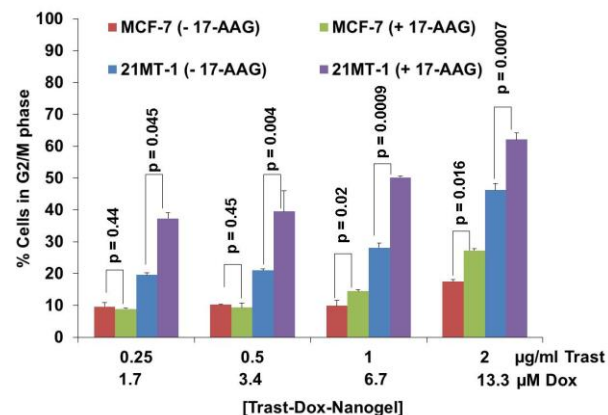
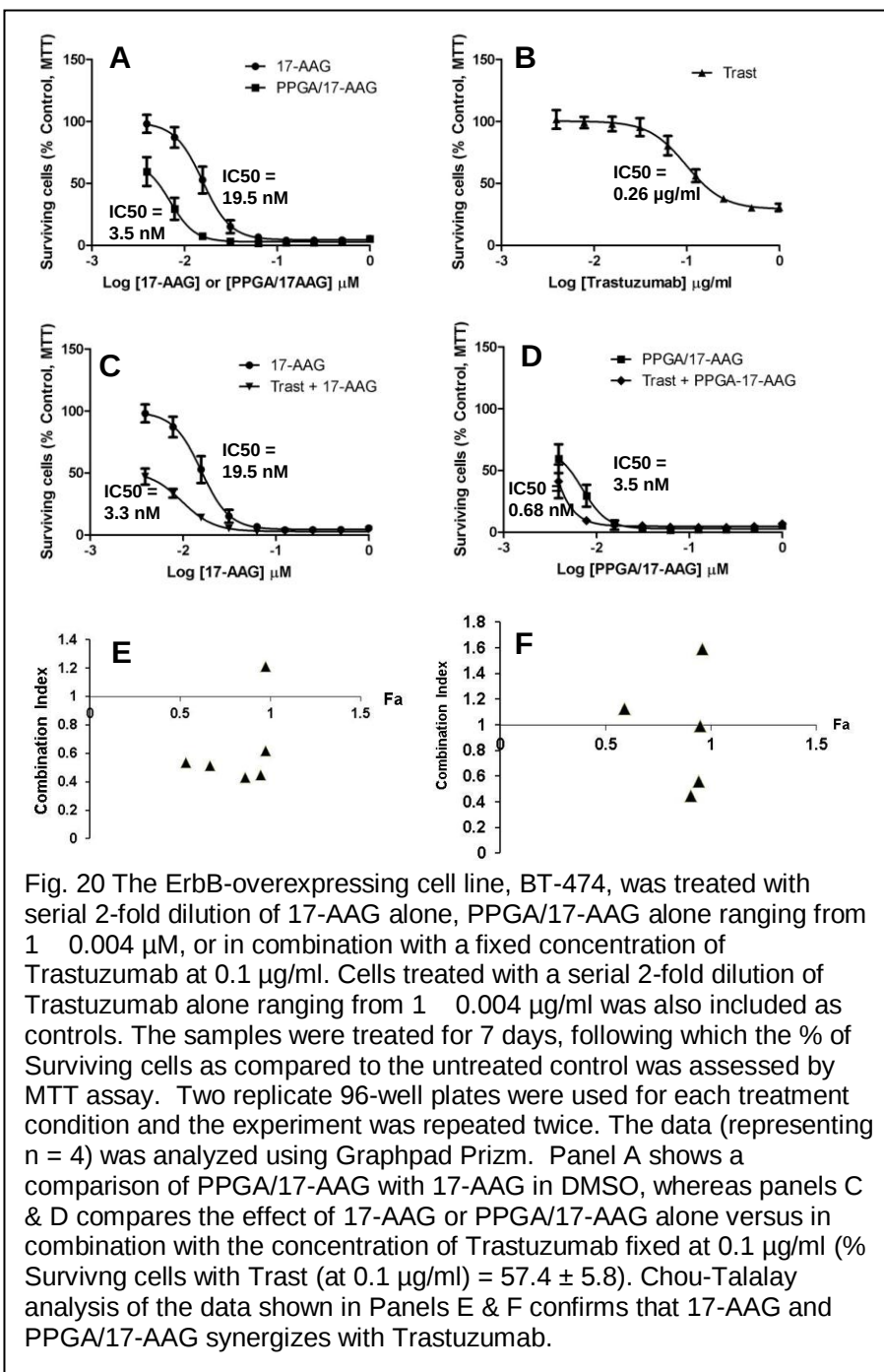


Fig. 19 Shown here is the comparison of the effect of Dox-T-NG, with/without 17-AAG treatment on 21MT-1 and MCF-7 cell line (data from Fig. 17 & 18).

3) Demonstrate enhanced efficacy of 17-AAG to deliver cytotoxic cargo encapsulated in targeted nanogels using in vivo xenograft mouse models (Aim 3) – Towards the completion of this aim, we have injected immune compromised mice with the ErbB2-overexpressing breast cancer cell line BT474 (6.7×10^7 cells/mice). As of 2-06-2013, about 30% of the mice have developed palpable tumors. The following treatment groups are planned: 1. Vehicle; 2. 17-AAG (2.5 mg/kg); 3. Non-targeted NG (no drugs); 4. Non-targeted Dox-NG; 5. Non-targeted Dox-NG + 17-AAG; 6. Trastuzumab-NG (no drug); 7. Trastuzumab-DoxNG; 8. Trastuzumab-DoxNG + 17-AAG. To complete the requisite groups, we are waiting for more mice to develop tumors, to initiate the treatment with sufficient number of mice per group. In the meanwhile we have begun the preparation and characterization of targeted and non-targeted nanomaterial regimens at a scale necessary for in vivo injections. The in vivo studies are expected to be completed by the end of March 2013.

1) Evaluate the synergistic cytotoxicity of the combination of Trastuzumab plus 17-AAG in PEG-b-PGA nanogel against ErbB2-overexpressing breast cancer cells – We have previously demonstrated that



HSP90-inhibitors such as 17-AAG enhances the ability of Trastuzumab to induce lysosomal-downregulation of ErbB2 and induce synergistic cytotoxicity in ErbB2-overexpressing breast cancer models (4). Indeed, phase I and II clinical studies have validated that the combination of HSP90-inhibitors with Trastuzumab is significantly more effective (5). Unfortunately, issues related to toxicities due to the currently used formulation of 17-AAG in Cremophor-based solvents seems to be one of the major factors limiting the progress of 17-AAG beyond phase II clinical trials (6). Novel formulations of 17-AAG are vigorously being pursued. Given the potential of our PPGA-17-AAG formulation, we assessed the ability of PPGA-17-AAG to enhance Trastuzumab-induced ErbB2 degradation. As we have previously demonstrated with 17-AAG (4), low concentrations of PPGA-17-AAG was found to synergize pharmacologically with Trastuzumab in ErbB2-overexpressing breast cancer cell line models. PPGA-17AAG seemed to be much more potent than 17-AAG in DMSO (see Fig 20A) even when used as a single drug (see Fig. 20A). Trastuzumab alone showed the expected dose-response against BT-474 cells (Fig. 20B). Notably, similar to 17-AAG/DMSO, PPGA-17-AAG was also synergistic with Trastuzumab (Fig 20C-F).

Key Research Accomplishments:

Year 1:

1. Representative panel of nanogels was synthesized using diblock copolymers of different structure and compositions.
2. The chemical structure of the block copolymer is a key parameter determining the formation of templates for the nanofabrication of nanogels.
3. The physicochemical characteristics of the nanogels (dimensions, swelling behavior) can be tuned by changing the cross-linking density of the cores of the nanogels or by using cross-linkers with different chemical structures. The incorporation of more hydrophobic cross-linkers into the core of the nanogels resulted in the formation of nanogels with smaller sizes and with higher density of cross-links.
4. Doxorubicin can be loaded into cross-linked cores of the nanogels with high loading capacity (up to 55 w/w%).
5. The nanogels containing drug combinations (doxorubicin and 17-AAG) were prepared and characterized.
6. Procedures for conjugation of nanogels with Trastuzumab antibodies in aqueous media were developed.
7. Biodistribution studies on non-targeted Doxorubicin-nanogels demonstrated that the nanogels showed an efficient systemic delivery of the drug to human tumor xenografts

Year 2:

1. Demonstration of 17AAG mediated HSP90 inhibition as a method to promote lysosomal targeting of trastuzumab-conjugated nanogels
2. Demonstration of functional trastuzumab-conjugated nanogels with highly concentrated anticancer drug cargo
3. Demonstration of biological activity of trastuzumab-conjugated and anticancer drug-loaded nanogels selectively against ErbB2-overexpressing cell lines in vitro.
4. Demonstration of synergism between 17AAG and anticancer drug-loaded nanogels against ErbB2-overexpressing cell lines in vitro.

Reportable Outcomes:

Overall Summary of Achievements:

- HSP90 inhibitor 17AAG as a means to promote nanogel targeting into lysosomes.
- Successful engineering of biologically functional nanogels carrying functional anti-ErbB2 antibody and anticancer drug payload.
- Generation of bulk lots of functional nanogels with anti-ErbB2 antibody and anticancer drug payloads suitable for preclinical testing in mice further in vivo studies currently underway.

- Conclusive new findings as a basis for new proposals for development of the nanogel platform for further preclinical and clinical studies.

Invited Presentations and Talks:

Year 1:

1. In vivo delivery of anti-HER2/neu antibody using nanogels for breast cancer treatment. S. M. Raja, J. Kim, S. Desale, N. V. Nukolova, H. S. Oberoi, H. Williams, H. Luan, V. Band, A. V. Kabanov, T. K. Bronich, H. Band. *American Association of Cancer Research*, 102nd Annual meeting, Chicago, IL, Mar 31-April 4, 2012.
2. Nanogels as a platform for drug delivery in cancer. S. M. Raja, J. Kim, S. Desale, N. V. Nukolova, H. S. Oberoi, H. Williams, H. Luan, V. Band, A. V. Kabanov, T. K. Bronich, H. Band. *American Association of Cancer Research*, 102nd Annual meeting, Chicago, IL, Mar 31-April 4, 2012.

Year 2:

1. Mechanism-based enhancement of ErbB2-targeted delivery of chemotherapeutics encapsulated in Trastuzumab-conjugated polymeric nanocarriers. **Srikumar M. Raja**, Jong Oh Kim, Swapnil S. Desale, Natasha V. Nukolova, Hardeep S. Oberoi, Stetson H. Williams, Haitao Luan, Vimla Band, Alexander V. Kabanov, Tatiana K. Bronich, Hamid Band. *American Association of Cancer Research*, 102nd Annual meeting, Chicago, IL, Mar 31-April 4, 2012.
2. Ionic nanogels for drug delivery in cancer. Tatiana K. Bronich; *Journal of Controlled Release*; Atlantic City, New Jersey; Dec 6 2012.
3. Dysregulated Endocytosis in Cancer. Tatiana K. Bronich; NIH NCI Workshop January 10-11, 2013

Manuscripts under preparation:

1. Desale SS., Luan H., Williams, S.H., Feng, D., Band, V., Band, H. *, Bronich, T.K. * and **Raja, S.M.*** (2013) Targeting the HSP90 chaperone enhances ErbB2-targeted drug-delivery via Trastuzumab-conjugated nanogels encapsulating chemotherapeutics by re-routing the receptor-bound cargo from recycling to lysosomal pathway.
2. Kim J.O., Nukolova, N., Oberoi, H., Williams, S.H., Luan, H., Band, V., Band, H.* and Bronich T.K.* **Raja, S.M.*** (2013). Novel ErbB2-targeted nanogels co-encapsulating 17-AAG and Doxorubicin for ErbB2-breast cancers.
3. **Raja, S.M.***, Bronich, T.K., Band, V. and Band, H.* (2013) ErbB-receptor endocytic pathway and targeted nanoparticulate drug-delivery systems in Breast Cancer treatment. (Review article)

Grants submitted:

1. Translating novel insights from EGFR-biology into nanomedicine therapies for Triple Negative Breast Cancers; NIH R21 (PI Raja, S.M.)

Conclusions:

In conclusion, the proposed hypothesis that facilitating ErbB2 traffic towards lysosomes will enhance the efficiency of nanogel-based drug delivery in ErbB2-overexpressing breast cancer has been tested and validated using in vitro studies, and in vivo animal studies are underway. The novel concept established here together with technical achievements to prepare scalable batches of nanogels with anti-ErbB2 antibody as a targeting moiety and high concentrations of anticancer drugs encapsulated in the core have opened a clear translational avenue to move the nanogels developed here towards clinical use in breast cancer treatment.

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6. Arteaga CL. Why is this effective HSP90 inhibitor not being developed in HER2+ breast cancer? *Clin Cancer Res* 2011;17(15):4919-21.