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14. ABSTRACT
Breast cancer is a major disease in the Western world affecting a large percentage of adult women. An effort to find potential chemical agents for the treatment of this disease has been made in many research laboratories. Our studies have focused on a protein called heregulin (HRG), which is a factor involved in the growth of the tumor mass as well as in the progression of the breast carcinomas to a more aggressive type of breast cancer. HRG has been shown to induce tumor growth in animal models. Similar to a growing number of growth factors, HRG was also found in the nuclear compartment of breast cancer cells after the cells were treated with HRG, or in cells in which the HRG protein has been expressed. An unexpected facet of this protein has been discovered: when cancer cells are treated with HRG, they become more favorably sensitive to some chemotherapeutic drugs. Interestingly, we found that a specific region of HRG sensitizes breast cancer cells to conventional chemotherapy, and moreover that it does not promote the growth of breast cancer cells. We hypothesize that HRG and specific regions of HRG are involved in distinct cellular processes and can be used to develop a universal chemosensitizer. The proposed study will address the possible mechanism responsible for the sensitization function of the HRG protein and its derived mutants on chemotherapeutic drugs. The proposed study will advance our understanding of breast cancer progression and will add new targets for improvements in chemotherapy. The functions of HRG that will be investigated are of great importance to breast cancer treatment, and have not previously been addressed. This study will confirm the novel idea that this protein does not act only as a growth factor as previously asserted, but also, and significantly, as a major player involved in other processes. The experiments that we propose will advance our understanding, and open new avenues for successful chemotherapy of breast cancer.

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ABSTRACT

BACKGROUND: Heregulin (HRG) expressing cells are markedly more sensitive to doxorubicin (DOXO). Cells genetically engineered to overexpress HRG are aggressive and have the ability to bypass the normal estrogen requirements for growth. These HRG expressing cells are antiestrogen independent *in vitro* and *in vivo*. It has been shown that HRG internalizes to the nucleus, its localization from the nucleus to a diffused cytoplasmic localization modulated by a deletion of the putative nuclear localization signal. Moreover, a deletion of the transmembrane domain and the NLS confers a marked increased sensitivity to DOXO, a commonly used drug for the treatment of hormone-independent breast carcinomas. The increased sensitization achieved by this mutant HRG protein has been attributed to its entrapment in the cells, making it incapable of being secreted to the media and thereby activating the HER-2 signaling pathway. We identified a minimal sequence derived from the HRG protein that is capable of sensitizing breast cancer cells to DOXO without any perceptible adverse effect. Furthermore, we have recently generated small cDNA's sequences derived from the HRG full length sequence and transfected breast cancer cell lines to conclude that these sequences, which do not contain the EGF-like domain of HRG, do not promote cell growth but induce the sensitization of breast cancer cells to most chemotherapeutic drugs used for the treatment of breast cancer including DOXO, Taxol and cisplatin. The GFP-HRG-mutant-protein was localized to the perinuclear region of breast cancer cells after transfection. Further experimentation is critical for the full understanding of the mechanism of response to conventional agents and to evaluate biological compound as chemosensitizer.

RATIONALE/PURPOSE: The identification of a region within the HRG sequence capable of sensitizing breast cancer cells to a wide variety of chemotherapeutic agents without inducing growth prompted us to recognize this molecule as a potential “universal chemosensitizer” to be use clinically in combination with many of the chemotherapeutic clinically used today for the treatment of breast cancer. We propose to develop a “biomolecule” derived from these HRG sequences, conjugate it to a protein delivery reagent and test it in breast cancer cells. The purpose of developing such compound is to use it in chemotherapeutic regimens in order to minimize the dosage and systemic toxicity of chemotherapeutic drugs, thus improving their efficacy in breast cancer treatment. We are entering an era in which basic research and clinical medicine are being united by molecularly directed therapeutic decisions. These experiments will allow the effective and use of tools while also suggest the optimum way in which to combine new biological agents with older established drugs.

OBJECTIVES: The objective of this concept proposal is to prove the theory of generating a chemosensitizer for breast cancer cells. To develop a biomolecule based on the HRG-mutant-protein as a “universal chemosensitizer” commonly used chemotherapeutic drugs based therapies for the treatment of breast cancer patients, and to determine its ability to sensitize breast cancer cells *in vitro* and *in vivo*.

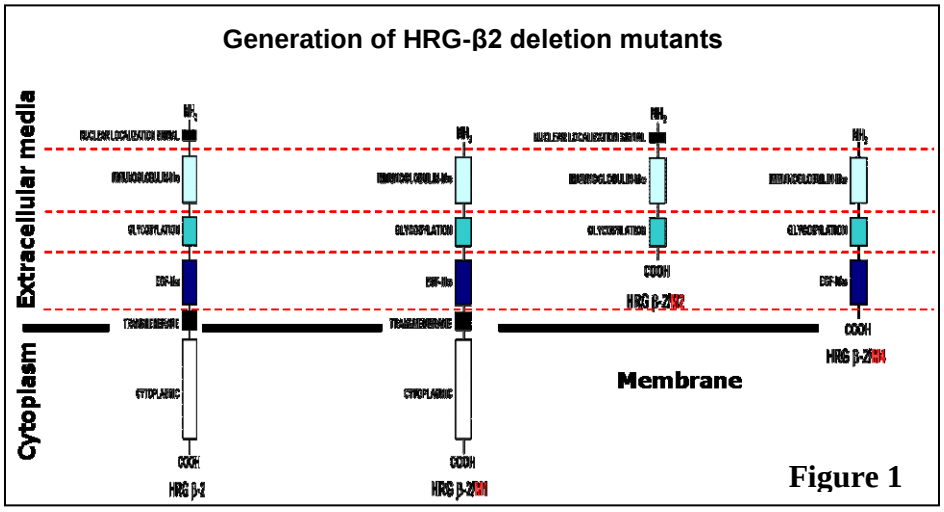
METHODS: The putative “universal chemosensitizer” derived the HRG-mutant-protein will be generated in large quantities as recombinant protein using conventional baculovirus technology, already used in our laboratory. The protein will then delivered into cells in culture using the protein delivery system “*Chariot”* using the BioPorter protein delivery system. This delivery system has a unique lipid formulation that allows direct translocation of proteins into living cells. We will perform anchorage-dependent and -independent growth assays in the presence of the “putative chemosensitizer” and chemotherapeutic drugs, including DOXO, Taxol, cisplatin (CDDP), VP-16, 5FU and others. IC50’s will be determined for the drugs in the presence and absence of increasing concentrations of the “chemosensitizer”. After *in vitro* assays are concluded, we will extend our studies to the *in vi*vo models generated in our laboratory (1, 2). For the in vivo studies we will take two approaches, initially we will use the breast cancer cells developed to expressed the chemosensitizer’s cDNA (already generated in our laboratory) and treat the mice with chemotherapeutic drugs in a dose and time dependent manner. Tumor growth, regression and sensitization will be some endpoints.

BENEFIT: The benefit of this study will be the generation of “universal chemosensitizer” specific for breast cancer. The use of this compound in combination with chemotherapeutic drugs should result in the increased efficacy of a diverse type of chemotherapeutic regimens by lowering their systemic cytotoxicity and preventing,

or delaying the acquisition of drug-resistance. At a minimum, we will prove that that this HRG-mutant sequence sensitizes three chemotherapeutic drugs used today for the treatment of breast cancer.

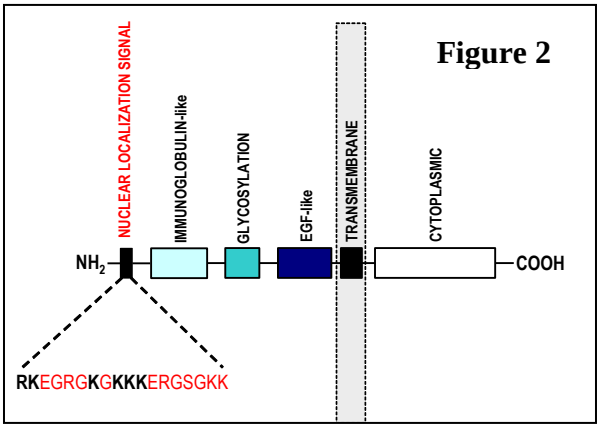
RESULTS:

1: Generation of HRG deletion mutants and recombinant HRG mutant proteins: We have generated four deletion mutants of HRG, as illustrated in Figure 1. Two strategies were followed: 1) we generated deletion mutants which were cloned into the pRC-CMV expression vector, then transfected each one into MCF-7 cells and assessed their biological effects, and 2) we generated recombinant proteins of each mutant and assessed their biological activity using the pMALcx$_2$ system (Fig 1). We also cloned the mutants into pGFP-N1-3 expression vectors for visualization of the cellular localization, since in this system HRG is expressed as a fusion protein with a green fluorescence protein (GFP).



HRG: Wild type (WT), HRG-M4: This mutant is lacking the C-terminal and N-terminal domains of HRG. NH2 terminal deletion will prevent this mutant from exerting intracrine action. Deletion of the C-terminal region, which is variable among different HRG subtypes, might affect binding preferences of HRG to different subtypes of its receptors, and thus modulate its physiological effects as mediated through erbB receptors.

2. HRG exhibits nuclear-associated functions. It well-characterized autocrine action involving transactivation of the erbB-2 orphan receptor, we and others previously demonstrated that HRG can also localize in the nuclei of actively dividing cells transfected with the HRG cDNA or in cells treated with ^{125}I-HRG protein. These findings strongly suggest that HRG may be a novel member of the nuclear matrix proteins and/or a DNA-binding protein. Moreover, considering that HRG on its own actively modulates breast cancer chemosensitivity in a erbB-2-independent manner, it is reasonable to suggest that its nuclear localization may be a key molecular feature determining the ability of HRG to regulate breast cancer cell sensitivity to DNA-damaging agents through the regulation of a nuclear component differentially responding to these agents. We recently envisioned that the nuclear localization of HRG may be a key molecular feature determining its ability to enhance DOXO-induced cytotoxicity. We have concluded that HRG must have a NLS at the NH$_2$-terminal. We have identified a novel NLS in the extracellular domain of the HRG-protein between the fourth and the sixteenth amino acids, which does not fully resemble any of the known nuclear localization sequences, but has close homology to the NLS that is found in the p53 protein (Figure 2).



To confirm its functionality, we deleted the first 33 amino acids of the HRG sequence, containing the putative NLS. Then, the ΔNLS-HRG construct was cloned into a mammalian expression vector as fusion protein with the green fluorescence protein (GFP) to allow its visualization. The HRG-negative, mammary epithelial breast cancer cell line MCF-7, was transiently transfected with the ΔNLS-HRG-GFP or full–length HRG-GFP fusion expression plasmids, and the localization of the fusion proteins was analyzed using indirect immunofluorescence and confocal microscopy. Remarkably, the deletion of the putative NLS domain of HRG completely prevented its transport to the nucleus. Moreover, a striking peri-nuclear accumulation of the HRG protein lacking the NLS sequence was clearly seen in most cases (Figure 3a).

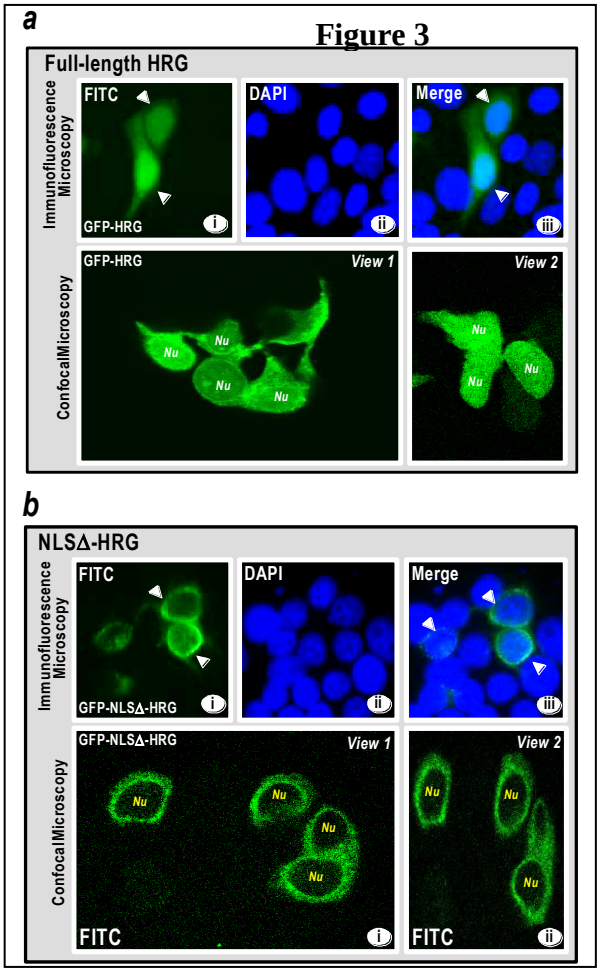


Figure 3

This cellular localization of ΔNLS-HRG was markedly different from the evidently nuclear localization of the full-length HRG protein (Figure 3b). These results confirm that HRG contains a functional NLS, which is essential for the translocation of the growth factor to the nucleus in breast cancer cells. We have previously shown that breast cancer cells engineered to overexpress HRG exhibit an enhanced susceptibility to the lethal effects of the topoisomerase II inhibitor DOXO. To determine whether the impairment of the NLS-dependent nuclear localization of HRG negatively regulated the ability of the full-length HRG protein to sensitize breast cancer cells to DOXO-induced cytotoxicity, MCF-7 cells were retro-virally transduced with the full-length HRG cDNA (MCF-7/HRG cells), with the structural deletion mutant of HRG (MCF-7/ΔNLS-HRG), or with the empty vector (MCF-7/pBabe). Stable cell lines were characterized.

3. PM2 Blocks Erbb-2 Tyrosine Phosphorylation: After the identification of the minimum PM sequence that would still block the activation of the erbB-signaling pathway induced by HRG. In this way we conducted phosphorylation assays and treated cells with HRG in the presence or absence of increasing concentrations of PM2 (A). We used PM1 (B) as a control. As seen in Figure 4, inhibition of erbB-2 phosphorylation was determined by inducing transactivation of the erbB receptor by HRG and the blockage using PM2 inhibitor: (A-top panel) while it does not block phosphorylation of EGFR (A- bottom panel).

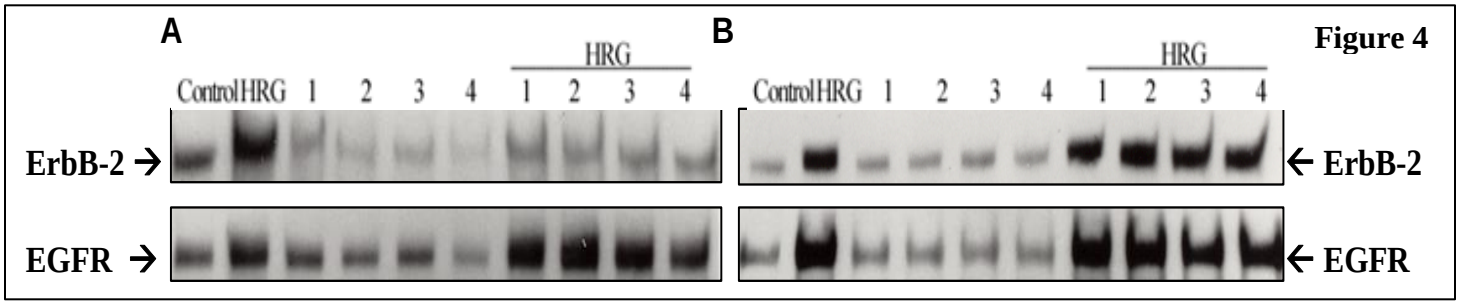
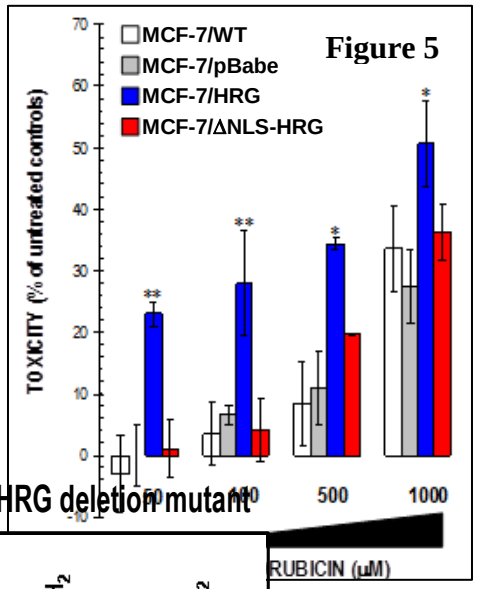


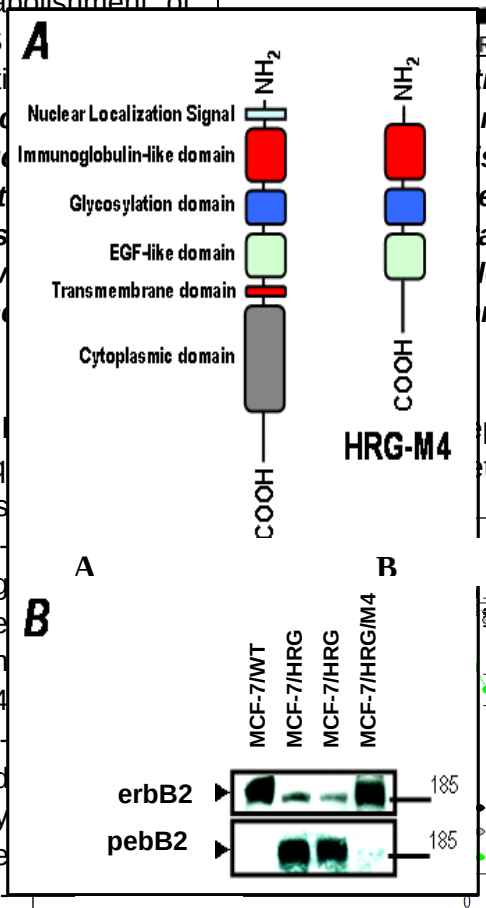
Figure 4

From these studies we concluded that PM2 is a potential candidate to block erbB2 signaling and perhaps develop it as a therapeutic targeted therapy. **We further determined that PM2 blocks dimerization of erbB2 with other erbB- receptors and that PM2 blocks cell growth induced by heregulin. In addition, we showed that PM2 blocks tumor formation of erbB2 overexpressing breast cancer cells when inoculated in athymic nude mice.**

4. HRG induces the sensitivity of breast cancer cells to DOXO requires a nuclear localization: Exponentially growing cells were incubated with increasing concentrations of DOXO for 60 minutes. After drug incubation and rinse, cells were incubated in drug-free media for 1 hour repair period. Then, a single-cell suspension was prepared by a 5-min exposure to 0.1% trypsin in PBS, ~500-1,000 cells/200 µl/well were re-plated in 96-well plates, and cell viability was determined after 7 days using a MTS assay. As expected, MCF-7 cells stably overexpressing the full-length HRG protein became more sensitive to DOXO, when compared to the parental or the empty vector-transduced MCF-7 cells. This sensitization effect was particularly significant when very low concentrations of DOXO (50 and 100 nM) were used. Interestingly, the cells transduced with the ΔNLS-HRG mutant did not show any increase in DOXO sensitivity. Indeed, the exclusion of HRG from the nucleus returned breast cancer cell's sensitivity to DOXO to the levels observed in wild-type MCF-7 and matched control MCF-7/pBabe cells (Figure 5). Since overexpression and/or erbB-2 activation correlates with the sensitivity profile of cancer cells to DOXO, we finally investigated whether the abolishment of DOXO hypersensitivity mediated by deletion of the NLS related to changes in the levels of erbB-2 phosphorylati ***trongly suggest that the nuclear function of HRG in breast cancer c regulator of its chemosensitizing function irrespective of HRG-indu s clear by now that a growing number of growth factors are ident e physiological implications and consequence of this localization is able to say the least. Nevertheless, the fact that many of the grow leus during the mitogenesis process indicates that a nuclear fun roteins in that compartment.***



Scheme 1A-B: HRG deletion mutant



6: ΔNLS-HRG deletion mutant (HRG-M4) sensitizes l pendently from erbB2 activation: We tested whether HRG-M4 was seq ted to act on an autocrine fashion. To do so, we performed experiments using HRG-M4 recombinant-protein and tested the auto-activation of erbB2 receptor. HRG and Maltose binding protein (MBP) were used as positive and negative controls, MDA-MB-453 cells were treated with conditioned media (CM) derived from the different M4 clones, to assess the ability, if any, of the secreted HRG-M4 protein to induce erbB2 tyrosine phosphorylation, and to assess whether the HRG-M4 secrete a biologically active HRG mutant, MCF-7/V (CM), c) we analyzed the MCF-7/HR/GM4 cells for their ability to auto-phosphorylation the erbB receptors (data not shown). To define whether an autocrine action of HRG/M4 was taking place, cell lysates derived from MCF-7 parental cells, (MCF-7-WT, MCF-7 cells transfected with the full-length HRG cDNA (MCF-7/HRG), and MCF-7 cells transfected with ΔNLS-HRG deletion mutant cDNA (MCF-7/HRG/M4) were collected in after 24 hours of serum free conditions (Figure 6A) ***Our results demonstrated that a decrease in erbB2 expression with an increase in perbB2 phosphorylation. Determining that while HRG downregulates the expression of erbB2 it induces its transactivation.*** MCF-7 cells stably transfected with the HRG-M4 construct were assessed for the sensitivity to Doxo and cell viability using the MTS assay Figure 6B. As a comparison we have added an additional figure in which we are comparing side by

side the sensitivity achieved via the introduction of either the HRG full length CDNA and the HRG mutant into MCF-7 cells. ***Although the wild type HRG does induce DOXO sensitivity of breast cancer cells, it is t is important to emphasize that the HRG mutant induces a much higher degree of sensitization. This was achieved merely by the deletion of the NLS and the COOH domains. Other HRG mutants generated in the laboratory did not induce DOXO sensitivity. We have also demonstrated a similar increase in DOXO sensitivity***.

7. A deletion mutant of HRG lacking N-terminus sequence and the cytoplasmic domain of the HRG protein (MCF-7/HRG/M4) enhances CDDP effectiveness. *HER-2* activation in HRG-promoted CDDP resistance: It was not clear, however, which functions can be attributed to the nuclear HRG and which functions, if any, can be independent of HER receptor activation. Therefore, we finally investigated whether secretion of HRG and therefore activation of *HER-2/neu* receptor are *necessary* and/or *sufficient* events in the HRG-promoted cellular resistance to CDDP. To determine whether a HGR-dependent transactivation of *HER-2* was required for HRG-promoted resistance to CDDP, we constructed a structural mutant of HRG that lacks N-terminus sequences (a putative nuclear localization signal –NLS- that will be discussed later in the preliminary data section) and the cytoplasmic domain of HRG (HRG/M4). HRG/M4 protein, although stably expressed in MCF-7 cells, is sequestered into a cellular compartment and is not secreted into the culture media, in other words, cannot act in an autocrine manner, Accordingly, Western blotting analysis of MCF-7/HRG-M4 cells demonstrated neither down-regulation of erbB2 receptor (66) nor increase in p erbB2 when compared with the matched control cells (Figure 7). ***CDDP effectiveness was enhanced up to 2.5-fold in MCF-7/HRG-M4 cells. These results demonstrate that deletion of the cytoplasmic domain, in addition to the putative NLS sequence consisting of the first 33 amino acids of the full-length HRG protein, abolishes the capability of HRG to promote CDDP resistance in breast cancer cells.*** Figure 7a: *Top panel:* Herceptin exposure significantly down-regulate HRG-promoted transactivation of erbB2 receptor in *erbB2*-negative MCF-7/HRG cells after exposure to 20 µg/ml Herceptin for 24, 48, and 72 h. *Bottom panel*: Analysis of the nature of interaction between Herceptin and CDDP in MCF-7/HRG cells. For each pair of columns, the height of the columns of the *left* represents the sum of the effect of each agent alone and, therefore, the expected cell toxicity if their effect is additive when used in combination. The total height of the columns on the *right* indicates the observed cell toxicity when the agents are used in combination. The difference between the heights of the paired columns reflects the magnitude of synergism on cell toxicity ($^{*}p< 0.05$; ** $p< 0.005$, Student's *t* test). Figure 7b: MTT assays on MCF-/PBabe and MCF-7/M4 cells in the presence and absence of increasing concentrations of CDDP.

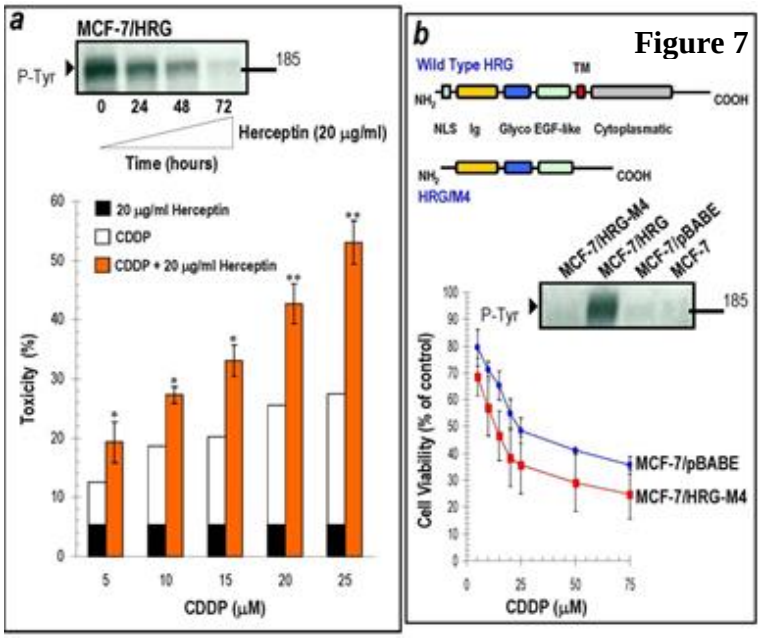


Figure 7

Overall, these data derived from MCF-7/HRG, MCF-7/HRG-M4 cells strongly suggest that activation of the erbB-2 signaling is sufficient to induce a prominent degree of resistance to CDDP in breast cancer cells. Moreover, transactivation of erbB-2 through the –COOH region of the HRG structure is necessary for trastuzumab-induced sensitization to CDDP-induced cytotoxicity and apoptosis. Nevertheless, it should be noted that a largely unknown function of the nuclear HRG is necessary to promote the highest level of cell protection against CDDP-induced cell death.

8. Alanine Modifications of PM2 to PM2-congers and Activity as measured by blockage of erbB-2 phosphorylation. Each of the amino acids of the core PM2 will be replaced with alanine as shown in Table 1. After each replacement, peptide mimetics were generated and tested for activity. As seen, changing the four first amino acids does not change the great activity to inhibit erbB-2 phosphorylation.

Table 1

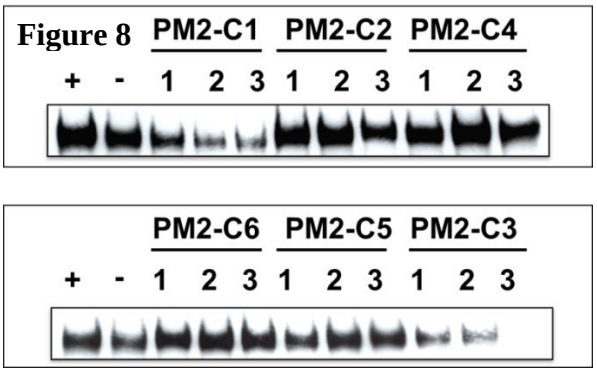
PM2-CONGER	ACTIVITY
LGLRSLRE	++++
LGLRSLRA	-
LGLRSLAE	-
LGLRSARE	-
LGLRALRE	+/-
LGLASLRE	++
LGARSLRE	++++
LALRSLRE	++++
AGLRSLRE	++++

Table 2

PM2-CONGERS	ACTIVITY
LGLRSLRE	++++
LGLRSLRE-(NH2)	-
Ac-LGLRSLRE	++++
Ac-LGLRSLRE-(NH2)	-

With that in mind we decided to develop more stable peptide mimetic congers and design modifications at the N-terminal, C-terminal and both. As seen in Table 2, the modifications at the C-terminal retain the activity of the PM2-congers. It remains to determine what other modifications could be achieve to attain a much stable PM-Conger that will still block activation of the erbB pathway and induce sensitization to chemotherapeutic drugs.

9. Generation of PM2-C-modification: To reach the best PM2 conger and the best chemosensitizer we generated and tested many peptide-mimetic compounds and screen them initially in a kinase assay. Second, we tested the leading PM-congers to block phosphorylation of the erbB signaling pathway. An example of six different PM2-C (Congers) tested (Figure 8). ***Our results show that while PM2-C2 and C4 fail to inhibit erbB-2 phosphorylation, PM2-C1 and C3 significantly inhibit the erbB2 phosphorylation.***



10. PM2 induces breast cancer cell sensitivity Cisplatin (CDDP) or Doxorubicin (DOXO): CDDP cytotoxicity in breast cancer cells was determined following a 5-days treatment with graded concentrations of CDDP (5 → 75μM) or DOXO (50-1000 μM). The metabolic status of CDDP-treated cells was first evaluated using a MTT-based cell viability assay and constructing dose-response curves. CDDP concentrations required to produce the median effect (D_m, analogous to the IC_{50} value) were calculated using the Chou & Talalay approach. CDDP sensitivity was significantly induced (Table 3) as well as the sensitivity to DOXO (Table 4).

Table 3	Dm	% Sensitivity to CDDP
PM1-C1	55	12
PM1-C3	57	11
PM2-C2	68	19
PM2-C4	70	21
PM2-C1	**10**	**87**
PM2-C3	**8**	**91**
PM2-C5	**9**	**93**

Table 4	Dm	% Sensitivity to DOXO
PM1-C1	75	12
PM1-C3	89	11
PM2-C2	72	19
PM2-C4	80	21
PM2-C1	**3**	**98**
PM2-C3	**2**	**94**
PM2-C5	**4**	**97**

A median effect plot of CDDP in cells where f_a = the fraction of cells, f_u = the fraction of cells unaffected, and D = drug dose. $\mathbf{D_m}$ is the dose required to produce the median-effect (a dose required for 50% decrease of cell viability; analogous to the IC_{50} value) and it was obtained from the of the *X*-intercept of the median-effect plot *X* = log (D) *versus Y* = log [f_a/f_u] or $D_m = 10^{-(Y\text{-intercept})/m}$, were f_a is the fraction affected by dose D (*e.g.*, 0.9 if cell viability is decreased by 90%) and f_u is the unaffected fraction (thus, $f_u = 1-f_a$). **m** is the Hill coefficient. ***r*** is the

linear regression correlation coefficients (**r**-values) of the median-effect plots reflect that the dose-effect relationships for CDDP conform to the principle of mass action (in general, **r**-values $>$ 0.9 confirm the validity of this methodology).

In addition, we performed assays, Taxol, VP-16 and 5FU. We determined the IC50's for each one of the drugs in the presence and absence of increasing concentrations of the best PM2-Cogerns. The overall conclusion was that PM2-C3 had the chemosensitization effect using three different chemotherapeutic drugs: CDDP, DOXO and Taxol. A more stable PM2-C3 would need to be sensitized to continue with *in vivo* experiments.

During the year of our studies we accomplished the generation of a universal chemosensitizer specific for breast cancers resistant tee mayor chemotherapeutic agents. The identification of this compound results in the possible increased efficacy of a diverse type of chemotherapeutic regimens by lowering their systemic cytotoxicity and preventing, or delaying the acquisition of drug-resistance. At a minimum, we will prove that that this HRG-mutant sequence sensitizes three chemotherapeutic drugs used today for the treatment of breast cancer.