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<b>13. ABSTRACT (Maximum 200 Words)</b> BRCA2 mutations leading to premature termination of the protein confer high cancer risk. Currently very few clinical options are possible for women carrying predisposing mutations. So far, the only one to provide a significant reduction in risk is prophylactic mastectomy and oophorectomy. Restoring BRCA1 function in these patients might result in a significant decrease in cancer risk. We proposed to restore BRCA1 function using antibiotics. In eukaryotic cells, aminoglycosides interact with ribosomal RNA and relaxes codon recognition allowing normal tRNAs to insert an amino acid at a codon specifying a stop. As a result, the ribosome will read through the mutation and produce a full-length protein that can potentially restore the protein's original function. We focused on the following aims: Screen aminoglycosides in yeast to determine if they could suppress a nonsense mutation in BRCA1; Determine the ability of selected antibiotics in human cells to suppress different types of mutations and on the wild type BRCA1. We show that gentamicin suppresses BRCA1 nonsense mutations is able to restore some of the wildtype protein levels and may provide a preventive strategy for individuals carrying these mutations. Given the dearth of alternatives for these individuals and the results obtained in our studies the use of antibiotics merits further investigation.				
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## **FINAL REPORT**

### **INTRODUCTION**

Germline mutations in *BRCA1* account for the majority of cancer cases in families with hereditary breast and ovarian cancer syndrome. *BRCA1* is part of the DNA damage response but its molecular function has remained elusive. It has been suggested that *BRCA1* may be involved in several cellular processes including transcriptional activation, cell cycle regulation, DNA damage repair and maintenance of genomic stability<sup>1,2</sup> but it is still unclear which of these functions are required for tumor suppression. Deleterious alterations in *BRCA1* may result in disruption of any or all of these processes and lead to cancer. Mutations that disrupt the function of *BRCA1* confer cancer predisposition and most of these mutations are highly penetrant. These deleterious mutations confer 56-85% lifetime risk for breast cancer, significantly higher than the 11% lifetime risk in the general population. Results from segregation analysis and allelic association studies indicate that all mutations leading to premature termination of the protein will confer high cancer risk.

Currently very few clinical options are possible for women carrying predisposing mutations. So far, the only one to provide a significant reduction in risk is prophylactic mastectomy and oophorectomy. Our working hypothesis is that restoration *BRCA1* function in these patients as a preventive measure might result in a significant decrease in cancer risk. Gene therapy is a possible strategy to restore the function of a mutant gene, where an ectopic wild-type copy of the gene is inserted and may be expressed in the target tissue. However, a major hurdle to be transposed is an effective and specific delivery system. In this application we proposed to test an alternative strategy to restore *BRCA1* function by the use of common antibiotics. The rationale is that the restoration of *BRCA1* function with antibiotics may form the basis for a treatment regimen to prevent breast cancer in families carrying nonsense mutations in *BRCA1*.

In eukaryotic cells, a class of common antibiotics (aminoglycosides) interacts with ribosomal RNA and relaxes codon recognition. This interaction allows the insertion of an amino acid at a codon specifying termination<sup>3</sup>. This will result in a read through by the translation machinery and will produce a full-length protein that can potentially restore the original function. The advantages of this strategy are that antibiotics have limited and well characterized side effects and relatively low cost. This approach has been recently explored by research groups studying different genetic diseases such as muscular dystrophy and cystic fibrosis with exciting results in cell lines and *in vivo*<sup>3</sup>. This also includes Phase I clinical trials for muscular dystrophy<sup>4</sup> and cystic fibrosis ([http://www.cff.org/research/protein\\_assist\\_and\\_chloride\\_channel\\_therapies\\_gentamicin\\_treatment.cfm](http://www.cff.org/research/protein_assist_and_chloride_channel_therapies_gentamicin_treatment.cfm)). Here, we propose to apply and extend these findings into the area of breast and ovarian cancer prevention.

### **BODY**

During the period of this grant (including the no-cost extension we focused our efforts in the following specific aims (some of these have been described in our previous report and are also described here in this final report):

- Screen aminoglycosides in yeast to determine if they could suppress a nonsense mutation in *BRCA1*.

- Determine the ability of selected antibiotics in human cells to suppress a nonsense mutation in BRCA1.
- Determine the effects of antibiotics on different types of mutations (missense, frameshift and nonsense mutations) and on the wild type BRCA1.
- Use of human cells from BRCA1 patients to test the effect of antibiotics.

The basis for our assays is the ability of wild type BRCA1, but not cancer-associated nonsense, frameshift and missense mutations, to activate transcription when fused to a heterologous DNA binding domain. If treatment with antibiotics suppresses the mutations the resulting protein will display transcription activation activity. We have also investigated by western blot analysis the effect of antibiotics on protein levels.

### *Yeast System*

The yeast experiments were performed with *Saccharomyces cerevisiae* strain EGY48 [*MAT $\alpha$* , *ura3*, *trp1*, *his3*, 6 *lexA* operator-*LEU2*] and the following vectors. Expression vector plex9 was used to express fusion proteins of BRCA1 and LexA DNA binding domain. The yeast reporter vector pSH18.34 expresses  $\beta$ -galactosidase under the control of 8 LexA operators<sup>5</sup>. Both vectors carry selectable markers to allow selection of yeast transformants in SD minimal medium lacking tryptophan (plex9) and uracil (pSH18.34). A BRCA1 wild-type fragment (aa 1392-1863) was used as the backbone for the insertion of mutations. Fragments of BRCA1 containing cancer-associated mutations M1775R or Y1769X were amplified by PCR. The mutant PCR fragments were digested by the restriction enzymes *SacI* and *BamHI* and ligated into plex9: BRCA1 aa 1392-1863 previously digested by the same enzymes.

To test a nonsense mutation we generated a variant, Y1769X (mutation at codon 1769 that creates a stop codon). This mutant has a dramatically reduced transcription activity when fused to a heterologous DBD<sup>6</sup>. The protein produced by this variant can be clearly distinguished from the wild-type construct in western-blot analysis since it lacks the last 95 amino acids at the C-terminus of the protein<sup>6</sup>.

EGY48 contain an integrated reporter and its activation leads to growth in the absence of leucine. Our experiments tested whether aminoglycosides could induce full-length translation of the Y1769X BRCA1 construct allowing it to induce activation of the reporter (growth in selective medium). Cells were transformed with the LexA DBD fusion constructs and plated in solid medium lacking tryptophan. At least three independent colonies for each construct were inoculated into liquid medium lacking tryptophan and grown to saturation ( $OD_{600} \sim 1.5$ ). Saturated cultures were used to inoculate quantitative (liquid) or qualitative (solid) fresh medium lacking tryptophan or medium lacking tryptophan and leucine. For cultures in solid medium, paper discs containing antibiotics were laid on the agar. For liquid cultures, yeast cells were inoculated to an initial  $OD_{600}$  of 0.0002. Parallel cultures were set and incubated at 30°C and growth was assessed by measurement at  $OD_{600}$  after 38 hr in non-selective and selective (lacking leucine) medium containing no antibiotics or containing gentamicin, paromomycin or streptomycin (used as a control in the literature) at 100, 200, 500 and 1000mg/ml. Cells were allowed to grow at 30°C with shaking for 48h. We observed growth of transformants carrying the Y1769X mutation around a disc containing paromomycin indicating its ability to suppress BRCA1 nonsense mutations (Figure 1a). However, we observed no growth in any culture

containing the Y1769X mutant even at higher doses of antibiotic (results not shown). Thus, the antibiotics tested showed no indication of translation read through and activation of the reporter in the yeast liquid growth assay.

Protein extracts were obtained from each culture condition and western blots were performed to analyze the sizes of the proteins being produced after antibiotic treatment. An interesting result emerged from the western blots analysis. We detected two bands reactive with an antibody against the LexA epitope of the fusion protein in cells transformed with the Y1769X variant and treated with 500mg/ml of Gentamicin (Figure 1b,c). Taken together, these results suggest that although the amount of full-length protein produced was not enough to activate the reporter in the liquid assays, there was a clear effect of gentamicin on the suppression of the Y1769X mutation. Given the difficulty to estimate the antibiotic concentration and stability in the yeast solid and liquid medium we decided to develop a mammalian system to address the effects of antibiotics.

### *Mammalian System*

After the promising result with gentamicin in yeast, we decided to test aminoglycosides in the mammalian system. For these assays, GAL4 DBD: BRCA1 (aa 1396-1863) fusion constructs were subcloned in CDNA3. BRCA1 containing mutations A1708E or Y1769X were also generated. by the same approach we used previously in the yeast assays. The constructs containing the wild type sequence and the mutations were then used to assess the ability of aminoglycosides to induce translational read through. The restoration of the BRCA1 transcription function by the antibiotic was assed by both quantitative and qualitative approaches.

Qualitative analysis was based on cotransfections of 293T cells with a vector containing the wild-type BRCA1 construct or BRCA1 constructs carrying the mutations and a reporter plasmid containing a Green Fluorescent Protein (GFP) under the control of 5 GAL4-binding sites. In the qualitative assay we tested medium containing no antibiotics or containing gentamicin, paromomycin, bekanamycin, lividomycin or streptomycin at concentrations ranging from 100 to 700 mg/ml in 100 mg/ml increments. The cells were incubated at 37°C and observed for fluorescence after 48h of treatment.

Approximately 60% of cells transfected with the wild-type BRCA1 construct showed fluorescence in both treated and untreated groups, consistent with the transcriptional activation observed with wild-type BRCA1 in other assays<sup>1,7</sup> (Figure 2). Conversely, cells transfected with a construct containing mutation A1708E did not show any detectable fluorescence in either treated or untreated groups, consistent with the fact that missense mutations are not affected by antibiotics (Figure 2). Interestingly, while untreated cells transfected with the nonsense mutation Y1769X displayed no fluorescence, both Gentamicin and Paromomycin were able to induce translational read through (~10% of cells were green), with concentrations of 300mg/ml and 400mg/ml being the most effective. Higher concentrations were slightly toxic to cells. Bekanamycin, Lividomycin and Streptomycin on the other hand had no effect.

Quantitative analysis was based on cotransfections of 293T cells with a vector containing the BRCA1 constructs (described above) and a reporter plasmid containing firefly luciferase under the control of 5 GAL4-binding sites. We used pRL-TK, which contains a *Renilla* luciferase gene under a constitutive TK basal promoter as an internal control. In the quantitative assay we tested medium containing no antibiotics or containing gentamicin or paromomycin at

concentrations of 300 mg/ml and 400 mg/ml. The cells were incubated at 37°C and harvested after 24h of treatment.

As expected, the wild-type BRCA1 construct had high and similar luminescence counts in treated and untreated groups. However, different from the previous experiment, no activity was detected in treated or untreated cells carrying either the A1708E or the Y1769X variants (results not shown). *Taken together, these results suggest that a small percentage of cells display efficient read through (qualitative assay) but that the population of cells, as a whole, displays undetectable read through (quantitative assay).* In order to reduce the variability due to cotransfection of the constructs we also developed a series of stable cell lines containing the integrated reporters (luciferase and GFP) but could not detect any activity, probably due to a very low level of the reporter protein produced from a single integrated copy.

We obtained lymphoblastoid cells from patients carrying a nonsense germline mutation in BRCA1 (GM13708). Preliminary experiments failed to show any detectable difference in the protein levels in this cell line using gentamicin. We are currently investigating other antibiotics as well as other methods that might be more sensitive to detect small changes in protein levels.

We have also investigated whether there was any effect of the antibiotic treatment on the levels of the wild-type protein as well as proteins with frameshift and missense mutations. To analyze that we treated cells with antibiotics and measured protein levels by western blot. We found a dramatic and reproducible increase (over 10-fold) in levels of the wild-type protein in presence of gentamicin. To determine whether this effect was specific to the BRCA1 protein we transfected cells with a construct of the bacterial DNA Gyrase B domain in the same vector backbone as the BRCA1 protein. We also observed a comparable increase in levels suggesting that the effect could be due to non-specific effects of the antibiotic on the CMV promoter. We are currently investigating this possibility.

Because it is common practice to culture cells with penicillin and streptomycin we investigated whether the continuous treatment could modify the cellular response to the antibiotic treatment. This could have implications for the chronic clinical use antibiotics as a prevention scheme. We compared cells that were cultured for at least three passages without penicillin and streptomycin with cells that were cultured in the presence of these drugs. These cells were then treated with gentamicin and streptomycin in the dose mentioned above used for the suppression experiments. We found that cells that had been cultured in the presence of antibiotics displayed a small reduction in the response to gentamycin (results not shown), suggesting that cells develop some resistance.

### **KEY RESEARCH ACCOMPLISHMENTS**

- Developed and tested a qualitative (growth in solid medium) and quantitative (growth in liquid medium) yeast system to address the ability of antibiotics to suppress *BRCA1* nonsense mutations.
- Developed and tested a quantitative (using a luciferase reporter) and qualitative (using a GFP reporter) mammalian system to address the ability of antibiotics to suppress *BRCA1* nonsense mutations.
- Tested gentamicin, paromomycin, bekanamycin, lividomycin and streptomycin in a range of concentrations.
- Showed that gentamicin and paromomycin can suppress *BRCA1* nonsense mutations in human cells providing a basis for future in vivo experiments.

- Demonstrated that continuous use of antibiotics can lead to a small resistance to effect of gentamicin.
- Obtained preliminary data showing that antibiotics might have non-specific effects on transcription by the cytomegalovirus promoter.

### **REPORTABLE OUTCOMES**

- Plasmid pLexA:BRCA1 (aa 1396-1863).
- Plasmid pLexA:BRCA1 M1775R (aa 1396-1863).
- Plasmid pLexA:BRCA1 A1708E (aa 1396-1863).
- Plasmid pLexA:BRCA1 Y1769X (aa 1396-1769).
- Plasmid pCDNA3 GAL4DBD:BRCA1 (aa 1396-1863).
- Plasmid pCDNA3 GAL4DBD:BRCA1 M1775R (aa 1396-1863).
- Plasmid pCDNA3 GAL4DBD:BRCA1 A1708E (aa 1396-1863).
- Plasmid pCDNA3 GAL4DBD:BRCA1 Y1769X (aa 1396-1769).
- Stable 293T cell lines: containing integrated firefly luciferase reporter under the control of 5 GAL4 binding site (stably transfected with pG5E1bLuc).
- Stable 293T cell lines: containing integrated green fluorescent protein reporter under the control of 5 GAL4 binding site (stably transfected with pG5E1bGFP).
- Abstract to the 2002 Era of Hope Meeting entitled: "Restoring BRCA1 function with antibiotics" (a copy of the poster is attached).
- Grant application (in preparation) to extend the project proposed in the present application.

### **CONCLUSIONS**

We have completed the experiments proposed for this project and have also, in the no-cost extension period extended our observations. We verified that although the yeast system is more amenable to a high throughput approach our experiments demonstrate that they may not be the most reliable system to use. Importantly, using the mammalian assay we show that aminoglycosides, gentamicin and paromomycin in particular, induce translation read through in BRCA1 nonsense mutations and may provide an alternative preventive strategy for breast and ovarian cancer in individuals carrying nonsense mutations in *BRCA1*. We believe that given the dearth of alternatives for these individuals and the exciting results obtained in our studies that the use of antibiotics merits further investigation.

Recently I have accepted an offer to become an Associate Professor in the Program of Cancer Control at the Moffitt Cancer Center, in Tampa, Florida. The Moffitt Cancer Center has a well-established drug discovery program. I have already started to explore the possibility to collaborate with scientist that could use the yeast platform and rational drug design in order to screen for derivatives of antibiotics or other small compounds that may restore wild type function to mutant BRCA1.

More generally, the laboratory is now five years old and during this time we were able to secure private, state and federal funding (including a NIH RO1) and to publish several articles on the biology of BRCA1. In conclusion, funding from the DoD grant was crucial for my establishment as an independent breast cancer researcher.

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## LEGENDS

**Figure 1.** Suppression of a BRCA1 nonsense mutation by antibiotics in yeast. **a.** Growth assay in solid selective medium. Filter paper containing antibiotics are laid on the agar. A dilute cell suspension is plated on the solid selective medium and incubated for several days. Note that yeast carrying a nonsense BRCA1 mutation Y1769X is able to grow in selective medium around a filter paper containing paromomycin but not water, gentamicin or streptomycin. **b.** Western blot analysis of BRCA1 proteins produced in yeast in cells treated with different concentrations (200, 500 and 1000 mg/ml) of streptomycin (S; used as control) or gentamicin (G; 100 and 200 mg/ml). The protein normally produced from a Y1769X cDNA (shown in panel c) is 49 kDa. When treated with gentamicin, cells suppress the nonsense mutation and produce a protein of 65 kDa (panel c).

**Figure 2.** Suppression of a BRCA1 nonsense mutation by antibiotics in human cells. Cells cotransfected with a GFP reporter under the control of GAL4 binding sites. Wild type BRCA1 (top panel) is unaffected by antibiotic treatment. Gentamicin is able to restore transcription activation to a GAL4 DBD:BRCA1 cDNA carrying a Y1769X mutation (middle panel). Bottom panel shows that antibiotic treatment does not suppress a missense mutation.

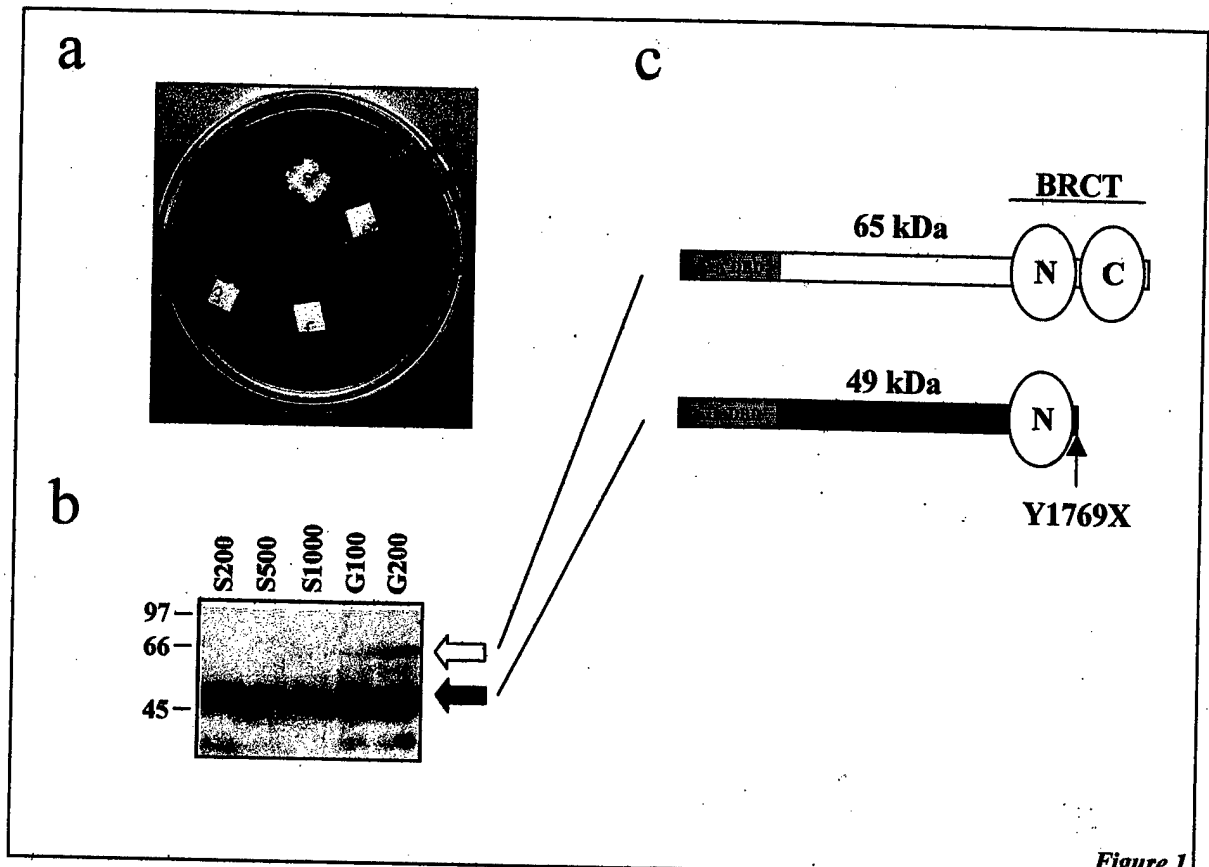


Figure 1

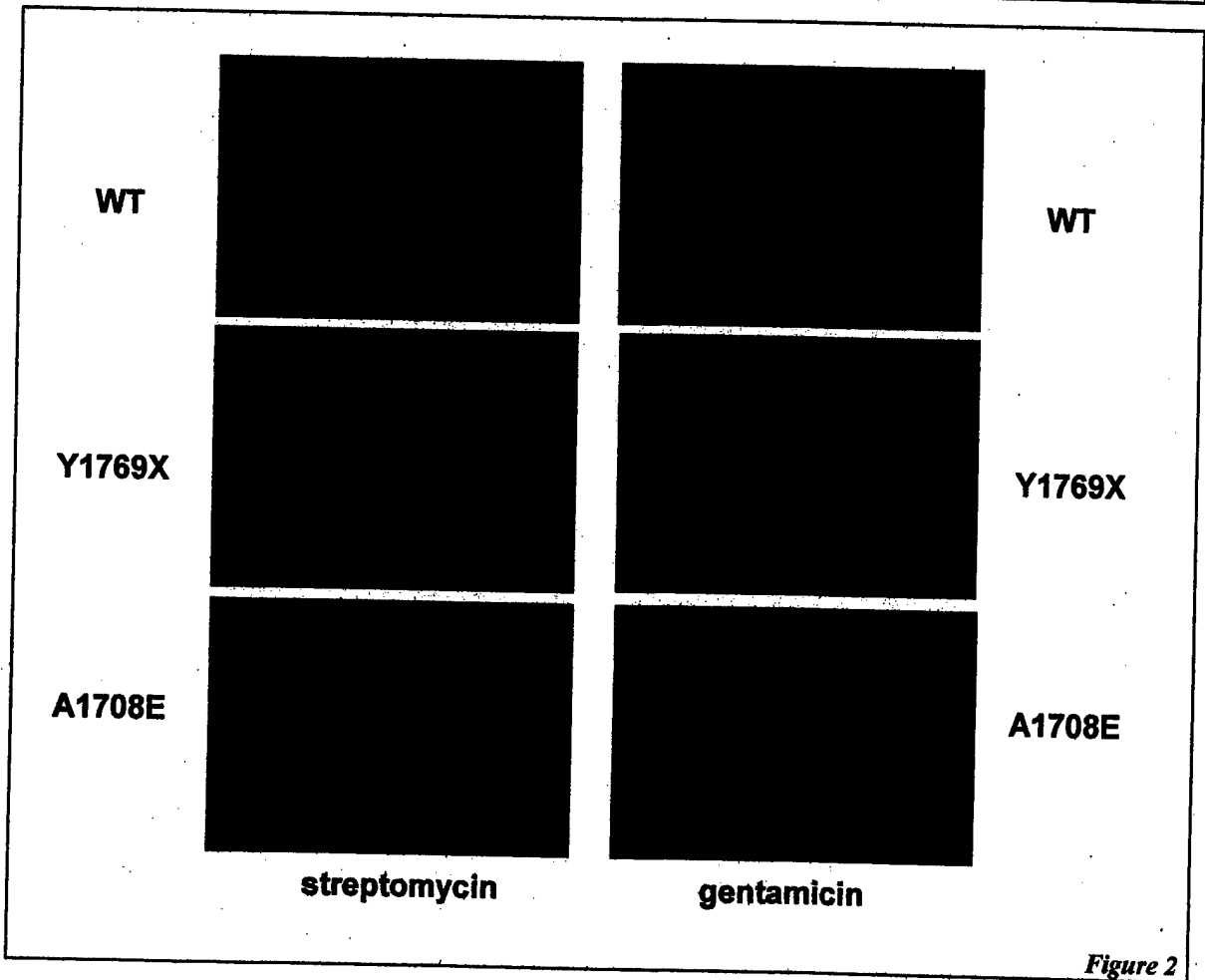


Figure 2

