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Abstract (Maximum 200 Words)
Linkage analysis suggests that BRCA1 mutations leading to premature termination of the protein will confer high cancer risk. Up to this date, to remove both breasts and the ovaries is the only effective preventive measure for women carrying such mutations. 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: a) Screen aminoglycosides in yeast to determine if they could suppress a nonsense mutation in BRCA1; b) Determine the ability of selected antibiotics in human cells to suppress a nonsense mutation in BRCA1. Using human cells we show that gentamicin and paromomycin suppress BRCA1 nonsense mutations and may provide a preventive strategy for individuals carrying these mutations. Given the dearth of alternatives for these individuals and the exciting results obtained in our studies the use of antibiotics merits further investigation.

Subject Terms
BRCA1, antibiotics, breast cancer, ovarian cancer, prevention

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INTRODUCTION

Inherited mutations in BRCA1 account for the majority of cancer cases in families with hereditary breast and ovarian cancer. Current evidence indicates that BRCA1 is involved in several cellular processes including transcriptional activation, cell cycle regulation, DNA damage repair and maintenance of genomic stability.1,2 Deleterious alterations in BRCA1 may result in disruption of any or all of these processes and lead to cancer. Missense, nonsense and frameshift mutations that disrupt the function of BRCA1 confer cancer predisposition to individuals carrying the mutation. Most of these mutations are highly penetrant and confer 56-85% lifetime risk for breast cancer, which is significantly higher than the 11% lifetime risk in the general population. Linkage analysis suggests that all mutations leading to premature termination of the protein documented so far will confer high cancer risk.

For women carrying predisposing mutations, one particularly dramatic recourse is to preventively remove both breasts and the ovaries. Up to this date, this drastic surgical procedure is the only effective preventive measure within reach for these individuals. Theoretically, restoring BRCA1 function in these patients as a preventive measure might result in a significant decrease in breast and ovarian cancer risk. One strategy to restore the function of a mutant gene is through gene therapy, where an exogenous wild-type copy of the gene is inserted and expressed in the target tissue. However, this technology is still in its early stages and many technical problems remain. In this application we proposed to test an alternative strategy to restore BRCA1 function by using antibiotics. The underlying rationale is that the restoration of BRCA1 function with antibiotics may form the basis for a preventive treatment regimen to prevent breast cancer in families carrying nonsense mutations in BRCA1.

It has been already shown that, in eukaryotic cells, a class of common antibiotics (aminoglycosides) interacts with ribosomal RNA and relaxes codon recognition allowing normal tRNAs to insert an amino acid at a codon specifying a stop.3 As a result, the translational machinery will read through a nonsense mutation and produce a full-length protein that can potentially restore the protein’s original function. The clear advantages to this treatment are that antibiotics have limited and well characterized side effects and relatively low cost. This approach has been used by research groups studying different genetic diseases such as muscular dystrophy and cystic fibrosis with exciting results in cell lines and in vivo.3 This also includes a Phase I clinical trial 4. Here, we propose to apply and extend these findings into the area of breast and ovarian cancer prevention.

BODY

During the past year we focused our efforts in the following specific 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 a nonsense mutation in BRCA1.

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.
Yeast System

The yeast experiments were performed with *Saccharomyces cerevisiae* strain EGY48 [MATa, 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 b-galactosidase under the control of 8 LexA operators. 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. 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.

Growth assay. 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 (OD600 ~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 OD600 of 0.0002. Parallel cultures were set and incubated at 30°C and growth was assessed by measurement at OD600 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 analysis. 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 (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.

**The 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.
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, bekamycin, 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.

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".
- Grant application (in preparation) to extend the project proposed in the present application.

CONCLUSIONS

We have completed the experiments proposed for this project. 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.

Our work on the effects of aminoglycosides on BRCA1 mutations is now being extended. We are currently developing a system to test the antibiotics we have identified in cells derived from patients carrying naturally occurring BRCA1 nonsense mutations. We intend to analyze the effect of the antibiotics on the full-length endogenous protein.
REFERENCES


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.