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14. ABSTRACT We have developed an efficient assay capable of detecting assembly of the core ligase complex involved in nonhomologous end joining (NHEJ) of DNA double-strand breaks. The assay can also detect inhibition of complex formation upon linkage to a large protein such as estrogen receptor. We have shown that Cernunnos is an ideal target for disrupting NHEJ. Using recombinant proteins, we have found regions in Cernunnos that can be targeted for disrupting repair only in cells expressing estrogen receptor. Finally, we have performed experiments to demonstrate the feasibility of each individual step in the chemical synthesis of the adapter.					
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Project title: Targeting breast cancer with a steroid adapter to inhibit DNA repair

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Introduction

Targeted therapy offers the hope of curing cancer without side effects. We propose to synthesize a molecular adapter that will chemically link a DNA repair protein to the estrogen receptor (ER). The adapter will consist of hydroxytamoxifen attached to a small molecule warhead that binds a protein involved in the repair of DNA double-strand breaks. Binding of the adapter to the repair protein will not affect DNA repair. However, in cells expressing ER, the hydroxytamoxifen component of the adapter will bind ER and the warhead will bind the DNA repair protein to create a complex that disrupts assembly of the complex and/or misdirects the repair protein away from double-strand breaks to ER binding sites in the genome. The adapter will be administered together with agents that generate DNA double-strand breaks, such as ionizing radiation or doxyrubicin, which are already highly effective treatments for breast cancer. *The molecular adapter will make cells expressing ER hypersensitive to already effective treatments, while sparing tissues that do not express ER.*

Body

Task 1: Conduct feasibility studies for targeting XL

Subtask 1.1. Determine which NHEJ protein would be the best target for the molecular adapter.

Previously, we showed that Cernunnos stimulates the joining of blunt or mismatched ends in vitro (Tsai et al., 2007). Indeed, joining required Ku, XL, and Cernunnos, which catalyzed a mismatched end (MEnd) ligase activity. Potential targets for disruption the NHEJ pathway include XRCC4/Ligase IV (XL) and Cernunnos (C) (Fig. 1). The figure shows a molecular adapter linking Cernunnos to the estrogen receptor. In our strategy, the molecular adapter will interfere with NHEJ in the presence of ER protein by one of two mechanisms: direct interference with assembly of the MEnd ligase complex on DNA ends, or mis-localization of a key NHEJ protein to binding sites for the ER.

XL is a potential target because of its central role in NHEJ and its low abundance in cells. However, purification of XL requires growth in baculovirus infected insect cells, which is more cumbersome than growth in bacteria. We chose to change this specific aim from targeting XL to targeting Cernunnos for three reasons. First, the cellular abundance of Cernunnos is even lower than that of XL (Ahnesorg et al., 2006). Second, we can purify large quantities of Cernunnos protein in a bacterial expression vector.

Third, we discovered that the MEnd ligase complex contains multiple molecules of Cernunnos. An electrophoretic mobility shift assay (EMSA) showed the assembly of a very large complex on DNA ends, that grew progressively larger in a cooperative manner with increasing concentrations of Cernunnos (Fig. 2). We measured the amount of DNA in the complex by scanning for the radiolabeled DNA, and measured the amount of each protein in the complex by cutting the protein:DNA complex from the EMSA gel and resolving the proteins by quantitative SDS-PAGE. The complex contained multiple molecules of Cernunnos with an equal number of XL molecules (Fig. 3). Thus, Cernunnos is an excellent target for the molecular adapter because of its low abundance, ease of purification, and cooperative assembly in the MEnd ligase complex. This subtask was completed.

Subtask 1.2. Show that modification of XL to His-XL preserves its activity in NHEJ.

Previously, we purified modified XL (His-XL) containing 6 histidine residues at the C-terminus of Ligase IV. His-XL together with purified Ku and Cernunnos reproduced MEnd ligase activity. This demonstrated that we could modify the C-terminus of Ligase IV without affecting MEnd ligase activity. This subtask was completed.

Fig. 1. Strategy for disrupting NHEJ in cells expressing estrogen receptor.

The normal NHEJ pathway (left side of the diagram) requires Ku, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XL, and Cernunnos (C). DNA-PKcs facilitates synopsis of the DNA ends (DeFazio et al., 2002). Ku, XL and Cernunnos join even DNA with mismatched ends, which we term MEnd ligase activity (Tsai et al., 2007). Polymerase and nuclease process the DNA to permit ligation of both DNA strands.

As depicted in the legend (upper right corner), the molecular adapter consists of hydroxytamoxifen (black) covalently linked to a small molecule (red) that binds Cernunnos. The adapter preserves NHEJ in the absence of ER, but disrupts NHEJ in the presence of ER due either to mislocalization to ER binding sites or to interference with Cernunnos' interactions with other NHEJ proteins.

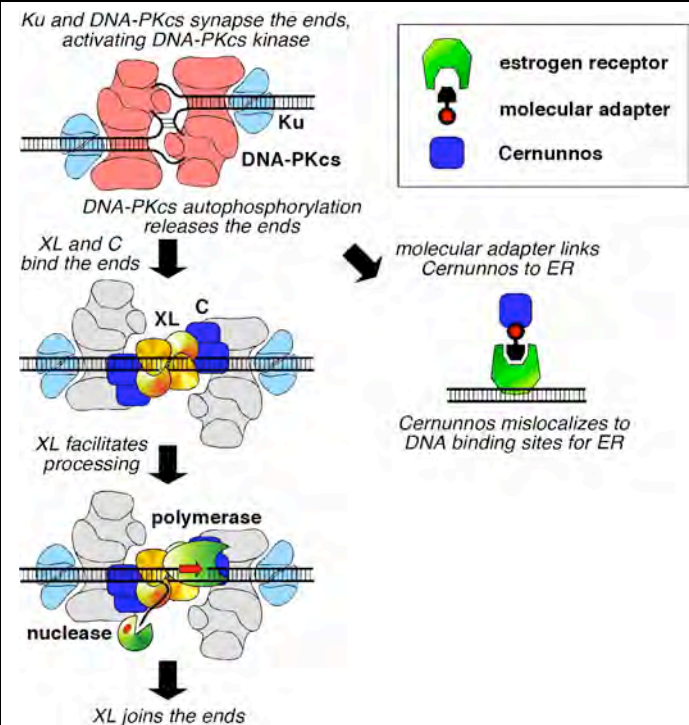


Fig. 2. EMSA for MEnd ligase complex on DNA

DNA labeled with ^{33}P was incubated with purified proteins: Ku, 2.4 nM; XL, 9.6 nM; and Cernunnos (C), 4.8 nM, 9.6 nM and 19.2 nM. Non-denaturing gel electrophoresis resolved complexes of DNA bound by Ku (lane 2), Ku and XL (lane 3) and Ku, XL, and Cernunnos (lanes 4-6). Free DNA migrated to a position near the bottom of the gel. Ku:XL:Cer denotes the position of the MEnd ligase complex. Increasing concentrations of Cernunnos generate progressively larger MEnd ligase complexes on DNA. We performed quantitative analysis of the proteins contained in the MEnd ligase complex by cutting the band out of the EMSA gel and resolving the proteins by SDS-PAGE in parallel with a titration of purified proteins. Scanning the gel for the ^{33}P -label quantified the DNA in the Ku:XL:Cer band. Analysis showed that the complex contains up to 6 Cernunnos and 6 XL molecules per DNA molecule.

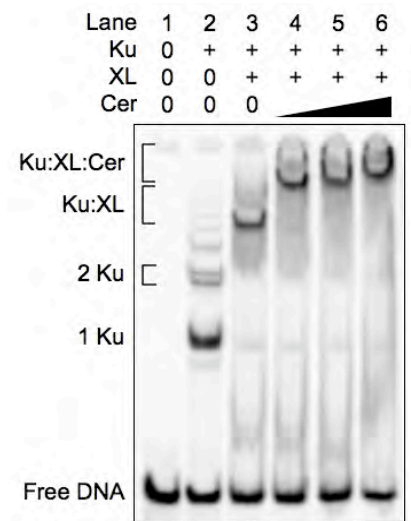
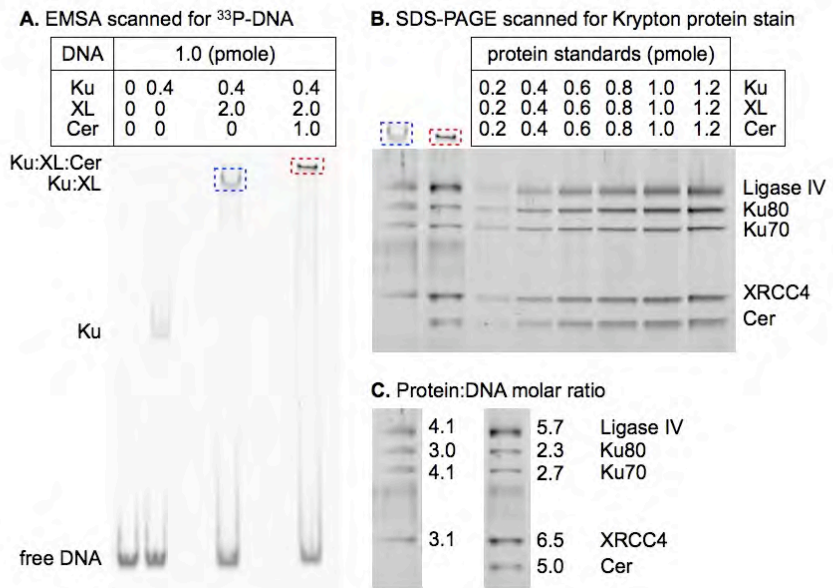


Fig. 3. MEnd ligase stoichiometry

We determined the stoichiometry of proteins contained in the MEnd ligase complex by cutting the band out of the EMSA gel (Panel A) and resolving the proteins by SDS-PAGE in parallel with a titration of purified proteins (Panel B). Scanning the gel for the ^{33}P -label quantified the DNA in the Ku:XL: Cer band. Analysis revealed a complex containing between 2 and 3 Ku molecules, and between 5 and 6 XL and Cernunnos molecules.

**Modified Subtask 1.3. Use ybbR-Cernunnos to test our cell free systems on a prototype adapter.**

To construct a prototype adapter, we are now using a ybbR tag, which is an 11 amino acid substrate for *B. subtilis* Sfp phosphopantetheinyl transferase. Sfp attaches CoA attached to small molecules to the ybbR substrate (Yin et al., 2005). We constructed recombinant Cernunnos with a ybbR tag at the N-terminus, C terminus and near the C-terminal end of a long non-conserved region in Cernunnos (Fig. 4).

We previously reported the expression and purification of biochemically active Cernunnos that had been modified with the ybbR tag at the N-terminus (construct I in Fig. 4). We used Sfp and biotin-CoA to label the ybbR tag with biotin, and purified the biotinylated protein on a streptavidin mutein (mutated streptavidin) matrix.

The N-terminal biotinylated Cernunnos molecule remained biochemically active. Attachment of streptavidin to biotinylated Cernunnos preserved MEnd ligase activity. Attachment of streptavidin (a 53 kDa tetramer) mimicked attachment of the estrogen receptor (a 134 kDa dimer) to Cernunnos. Thus, the N-terminus presents a potential target site for the molecular adapter, in which inhibition involves mislocalization of Cernunnos to ER binding sites.

We modified Cernunnos with the ybbR tag at the C-terminus (construct III in Fig. 4). However, this construct failed to support robust expression, and we did not study it further.

Next, we inserted the ybbR tag at an internal site of Cernunnos, the C-terminal end of the non-conserved region (construct II in Fig. 4). We are currently testing whether this construct supports robust expression. The insertion site for ybbR is close to the conserved basic region near the extreme C terminus, which we previously showed was required for DNA binding and NHEJ activity. We have now purified this recombinant Cernunnos molecule to near homogeneity and will test it for MEnd ligase activity once we have achieved homogeneity.

We will conjugate Cernunnos to a prototype adapter consisting of CoA linked to hydroxytamoxifen. After modifying the protocol of Trebley et al., we successfully attached hydroxytamoxifen to CoA (Trebley et al., 2006). We used tandem LC-mass spectroscopy to verify successful synthesis of the intermediates and the final product. Sfp will catalyze the attachment of the prototype adapter to ybbR-tagged Cernunnos. If the ybbR tag is located at the N-terminus, our results suggest that Cernunnos attached to hydroxytamoxifen should retain its ability to stimulate end joining by purified Ku and XL. Indeed, we will confirm that conjugated Cernunnos inhibits end joining only upon addition of ER α (Invitrogen). If inhibition increases with addition of circular DNA containing ER binding sites, we will conclude that our strategy can mislocalize the target protein away from DNA

ends. If addition of ER α molecules alone inhibits NHEJ, steric interference with the NHEJ machinery by ER can also be an effective mechanism.

Fig. 4. Cernunnos with ybbR tags

A. Cernunnos constructs. Cernunnos contains a non-conserved region from amino acid 233 through 277. We constructed three recombinant proteins: (I) a ybbR tag with a 2 amino acid linker is fused to the Cernunnos N-terminus; (II) a ybbR tag with linkers is inserted just after T277; (III) a ybbR tag is fused to the C-terminus. V5 epitope and His tag were inserted as indicated.

B. Cernunnos amino acid sequence. Conserved amino acids have colored backgrounds. Blue and red bars indicate α -helical and β -sheet structures. The green arrow marks the insertion site for the ybbR tag in Construct II.

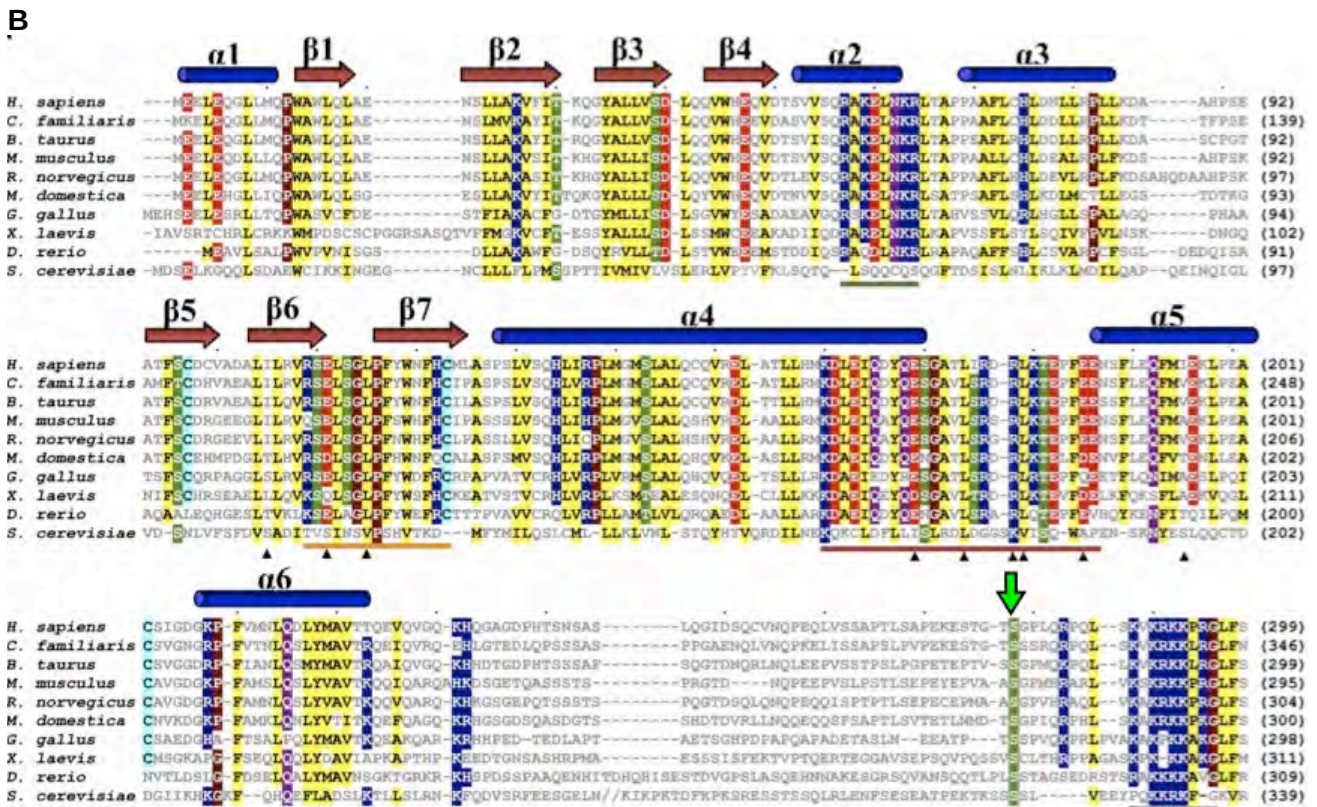
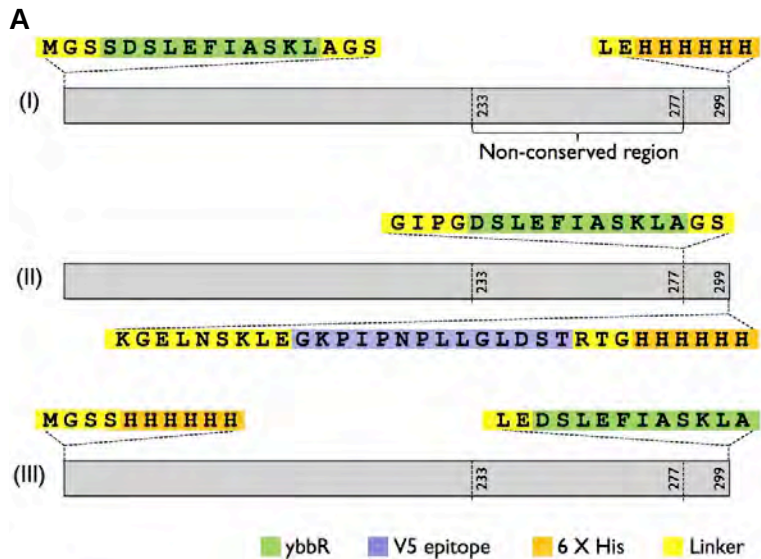


Fig. 5. Synthesis of the hydroxytamoxifen-CoA prototype molecule

The top line shows the starting compounds propiophenone, Compound 1, and 4,4'-hydroxybiphenyl, Compound 2 (both from VWR International). We synthesized the diphenol following the protocol of (Yu and Forman, 2003). We then modified the diphenol by addition of an azide group to yield Compound 3, following a modification of the protocol of (Trebley et al., 2006). The figure depicts Compound 3 in the Z (zusammen)-form, whereas the reaction utilizes either of the two hydroxyl groups to generate equal amounts of Z-form and E (entgegen)-form. Only the Z-form binds to ER, but this is not a problem, since Z and E-forms interconvert readily at room temperature.

The middle line shows conversion of CoA to an alkyne, Compound 4.

The bottom line shows tamoxifen, hydroxytamoxifen and the final product, hydroxytamoxifen-CoA, which was formed by joining the Compound 3 azide and the Compound 4 alkyne via the "click reaction", an azide-alkyne Huisgen cycloaddition (Kolb et al., 2001). Successful synthesis of each intermediate and the final product were verified by mass spectrometry (Fig. 6). Conversion of input CoA to the final product occurred with 35% efficiency, yielding 3.5 mg of hydroxytamoxifen-CoA.

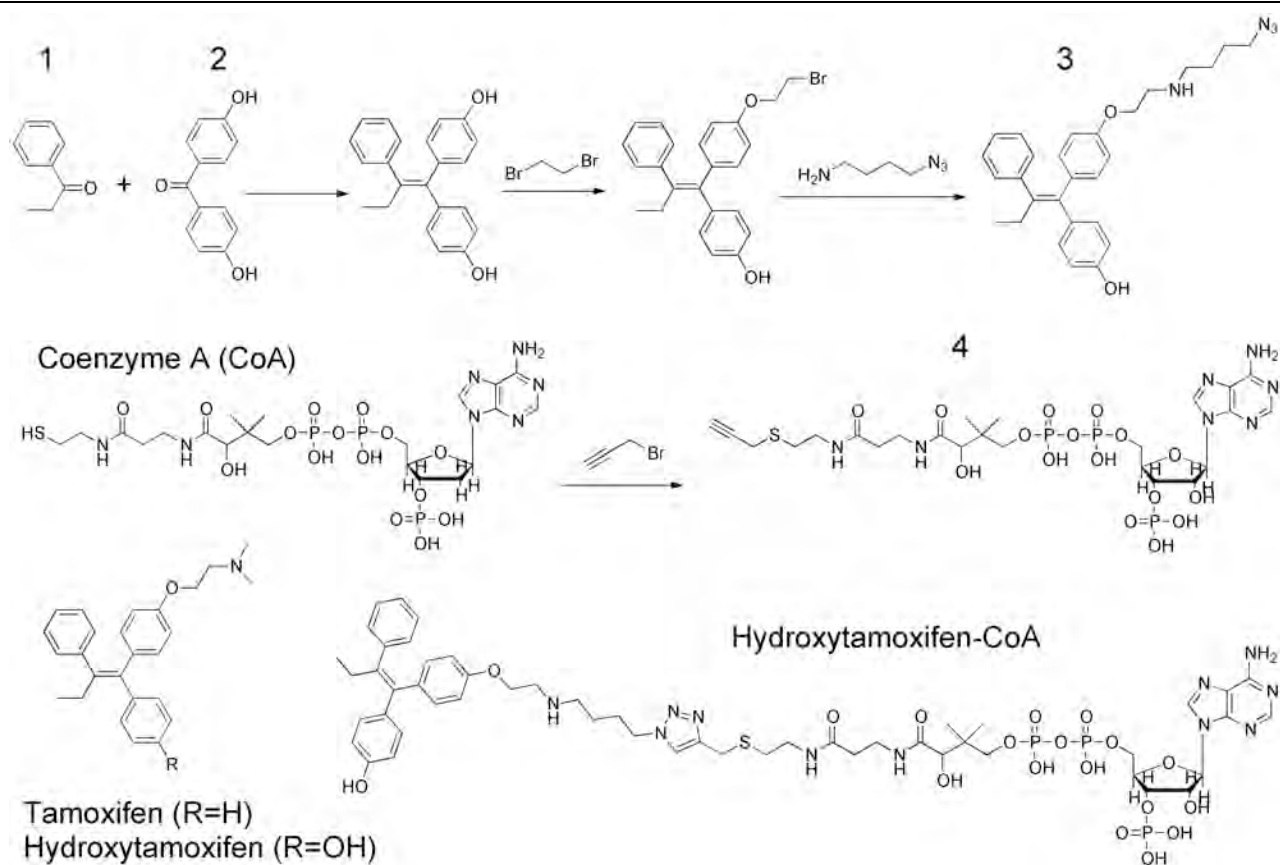
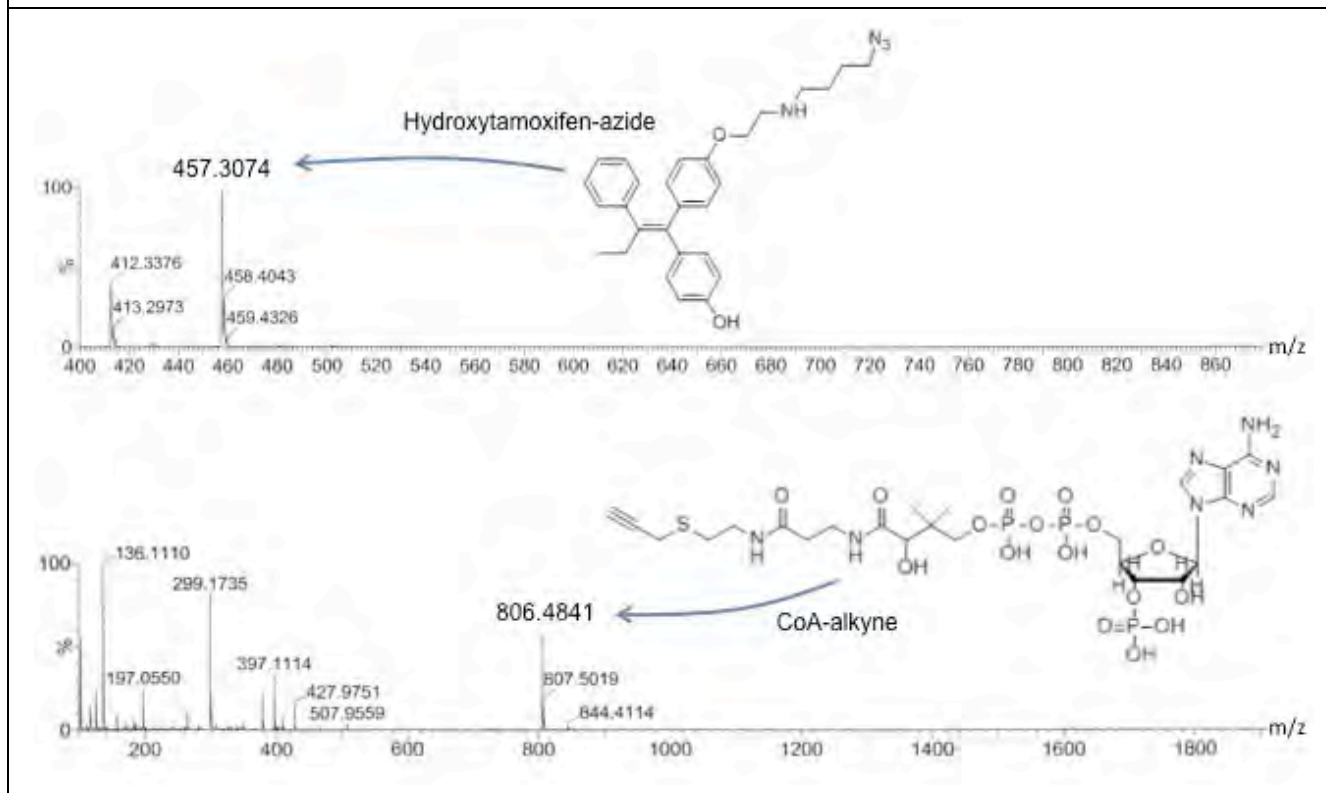


Fig. 6. Mass-spectroscopy confirms successful synthesis of alkyne and azide intermediates

The upper panel shows mass-spectroscopy analysis of the product of the series of reactions designed to synthesize compound 3 in Fig. 5, hydroxytamoxifen-azide. The bottom panel shows mass-spectroscopy analysis of the product of the reaction designed to synthesize compound 4 in Fig. 5, CoA-alkyne. The results demonstrate successful synthesis of both compounds.



Task 2: Select for small molecules that bind to Cernunnos or XL

Our collaborator, Pehr Harbury, has constructed a combinatorial tri-peptoid library on DNA with 2.2×10^{10} members. The library is constructed in 4 synthetic steps with final molecular weights averaging near 500 Daltons. This new scheme increases diversity of the scaffold, and allows other sources of building blocks. We will use the new library to select for small molecules that bind to Cernunnos or XL.

Task 3: Synthesize an adapter for the estrogen receptor that inhibits DNA repair.

Our progress in Task 1 allows us to test prototype adapters for the estrogen receptor utilizing hydroxytamoxifen, which has a very high binding affinity for the ER, and is the most potent form of tamoxifen. Furthermore, an adapter containing hydroxytamoxifen will have less potential for stimulating the growth of breast cancer cells upon binding to the ER.

Key Research Accomplishments

1. We showed that Cernunnos is an excellent target for disruption of NHEJ, because of its cooperative assembly into the MEnd ligase complex, and its ease of manipulation and purification.
2. We demonstrated that Cernunnos contains sites that can tolerate small modifications, but cannot withstand large modifications. This mimics the desired effect of the proposed molecular adapter.
3. We successfully tested each of the steps in the proposed synthesis of the molecular adapter by constructing a prototype adapter.
4. We have demonstrated the feasibility of using the ybbR tag to screen regions of Cernunnos that can serve as targets for disruption of NHEJ.

Reportable Outcomes

1. We are preparing a manuscript describing assembly of the MEnd ligase complex.
2. This grant has supported the employment of Dr. Chun Tsai as a research associate.

Conclusions

We have successfully conducted a series of experiments demonstrating that Cernunnos is a good target for the molecular adapter. We have also demonstrated the feasibility of each step in the proposed chemical synthesis of the molecular adapter. Further experiments with the prototype adapter must improve the yield of the click reaction, and develop a protocol for purifying the prototype adapter from other reaction products. Such experiments are feasible.

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Attachment 4: Revised Statement of Work

Justification for changes in the Statement of Work

1. Choice of Cernunnos as the target for the molecular adapter

Cernunnos provides an ideal target for several reasons. (A) Cernunnos shares with XL the desirable property of low levels in the cell, which would allow successful disruption of NHEJ even in breast cancers expressing modest levels of ER. (B) We have shown that multiple molecules of Cernunnos assemble cooperatively in the MEnd ligase complex at DNA ends. Thus, disruption of the complex would be more likely with sub-stoichiometric binding of the molecular adapter to the cellular pool of Cernunnos molecules. (C) Unlike XL, Cernunnos can be expressed and purified with high yields from *E. coli*.

2. Use of the ybbR tag to test a prototype adapter

The His tag on Cernunnos permits rapid purification, but the other NHEJ proteins in our in vitro assay system have also been purified with His tags. Addition of the ybbR tag provides a substrate for Sfp-catalyzed attachment of biotin. We have exploited the ybbR tag to attach biotin to different sites on the Cernunnos protein. We will synthesize a prototype adapter consisting of CoA-hydroxytamoxifen, and then use Sfp to attach the adapter to Cernunnos.

3. Substitution of hydroxytamoxifen for estradiol in the adapter

Hydroxytamoxifen provides several advantages over estradiol. (A) Hydroxytamoxifen provides a convenient substrate for chemical modification, as demonstrated by Trebley et al. Thus, hydroxytamoxifen will facilitate chemical synthesis of the prototype adapter as well as candidate small molecule adapters. (B) Hydroxytamoxifen has an extremely high affinity for ER. (C) In ER-expressing breast cancer cells, hydroxytamoxifen inhibits growth, while estradiol potentially stimulates growth.

4. Clarification of text in Subtask 3.3

5. Test of adapters in breast cancer cell lines expressing different levels of ER

We will test candidate adapters in several cell lines rather than a single cell line to ensure that they can work against a wide range of breast cancers. We will forgo the experiments with siRNA because it will be technically difficult to transduce the cells without affecting cell viability in the face of challenge with ionizing radiation and doxorubicin.

Task 1: Conduct feasibility studies for targeting Cernunnos

Subtask 1.1. Determine which NHEJ protein would be the best target for the molecular adapter.

Outcomes and deliverables: Cernunnos and XL emerged as ideal targets.

Timeline and milestones: completed.

Subtask 1.2. Show that modification of Cernunnos or XL with a His tag preserves activity in NHEJ.

Outcomes and deliverables: We purified recombinant XL (His-XL) that had been modified by insertion of 6 histidine residues at the C-terminus of Ligase IV. In the cell free system consisting of crude cell extracts, we showed that His-XL fully restored NHEJ to XL-depleted extracts. We also purified recombinant Cernunnos with His tag at the C-terminus. With purified proteins, we showed that these recombinant proteins efficiently supported joining of both cohesive ends and mismatched ends.

Timeline and milestones: completed.

Subtask 1.3. Use ybbR-Cernunnos to test our cell free systems for mislocalization of Cernunnos to ERE.

Outcomes and deliverables: We purified recombinant Cernunnos modified by the attachment of both a His tag (for rapid purification) and a ybbR tag (for conjugation to hydroxytamoxifen). To determine whether the prototype adapter, consisting of CoA-hydroxytamoxifen, can mislocalize recombinant Cernunnos, we will add estrogen receptor to the NHEJ proteins and determine whether circular DNA molecules containing estrogen receptor elements will inhibit end-joining activity. Cernunnos with an N-terminal ybbR tag will be used here because attachment of biotin and streptavidin preserved end-joining activity.

Timeline and milestones: partially completed.

Task 2: Select for small molecules that bind to Cernunnos

Subtask 2.1. Use DNA display to select for peptoids that bind to Cernunnos.

Outcomes and deliverables: We will use DNA display technology for in vitro evolution of peptoids that will bind to Cernunnos. We will perform the screen in collaboration with Dr. Pehr Harbury, who has won an NIH Director's Pioneer Award for further development of the technology.

Timeline and milestones: year 3.

Subtask 2.2. Screen for candidate peptoids that preserve NHEJ.

Outcomes and deliverables: After we identify a set of small molecules that bind to Cernunnos, we will perform a secondary selection for molecules that preserve NHEJ. Candidate peptoids will be added to extracts, and screened for preservation of NHEJ. This secondary screen can be done efficiently for up to 50 candidate peptoids.

Timeline and milestones: year 3.

Subtask 2.3. Screen for candidate small molecules that will enter intact cells.

Outcomes and deliverables: Using an assay developed by Yu et al. we will conjugate candidate peptoids to hydroxytamoxifen. Entry of the conjugate into an MCF-7 breast cancer cell line expressing estrogen receptor will trigger nuclear transport of a fusion protein that activates a reporter.

Timeline and milestones: year 3.

Task 3: Synthesize an adapter for the estrogen receptor that inhibits DNA repair.

Subtask 3.1. Synthesize a set of adapters from candidate peptoids.

Outcomes and deliverables: Each small peptoid warhead will be chemically linked to hydroxytamoxifen, adopting the chemistry used by Marquis et al. Successful synthesis will be confirmed by mass-spectroscopy and NMR spectroscopy.

Timeline and milestones: year 3.

Subtask 3.2. Determine which adapters can successfully link Cernunnos to ER.

Outcomes and deliverables: Using anti-Cernunnos and anti-ER antibodies, we will test for co-immunoprecipitation of Cernunnos and ER that is dependent on the presence of the molecular adapter.

Timeline and milestones: year 3.

Subtask 3.3. Determine which adapters inhibit NHEJ in cell extracts.

Outcomes and deliverables: To show that inhibition depends on the presence of ER, we will assess NHEJ in extracts from MCF-7 cells expressing ER. Our strategy seeks to inhibit NHEJ by attaching the ER to Cernunnos. This would cause either steric hindrance of interactions between Cernunnos and other NHEJ proteins, or mislocalization of Cernunnos to EREs in the genome and away from DNA ends. To test for steric hindrance, we will determine whether attachment of candidate adapters to Cernunnos inhibit NHEJ directly. To test for mislocalization, we will determine whether attachment of candidate adapters inhibit NHEJ in the presence of circular DNA. We will require that immunodepletion with anti-ER antibodies reverse the inhibition of NHEJ.

Timeline and milestones: year 3.

Subtask 3.4. Determine which adapters inhibit NHEJ in intact cells.

Outcomes and deliverables: We will add the molecular adapter to cultured breast cancer cell lines expressing different levels of ER. We will test the cells for increased sensitivity to ionizing radiation and to doxorubicin.

Timeline and milestones: year 3.