Published in final edited form as:

ACS Chem Biol. 2015 May 15; 10(5): 1198–1208. doi:10.1021/cb500757u.

Peptide Library Approach to Uncover Phosphomimetic Inhibitors of the BRCA1 C-Terminal Domain

E. Railey White^{†,‡}, Luxin Sun^{⊥,‡}, Zhong Ma^{‡,‡}, Jason M. Beckta^{‡,||}, Brittany A. Danzig^{†,‡}, David E. Hacker^{†,‡}, Melissa Huie[‡], David C. Williams^{‡,§}, Ross A. Edwards[⊥], Kristoffer Valerie^{‡,||}, J. N. Mark Glover[⊥], and Matthew C. T. Hartman^{†,‡,*}

[†]Department of Chemistry, Virginia Commonwealth University (VCU), 1001 West Main Street, P.O. Box 842006, Richmond, Virginia 23284, United States

[‡]Massey Cancer Center, Virginia Commonwealth University, 401 College Street, Richmond, Virginia 23298, United States

[§]Department of Pathology, Virginia Commonwealth University, 401 College Street, Richmond, Virginia 23298, United States

^{||}Department of Radiation Oncology, Virginia Commonwealth University, 401 College Street, Richmond, Virginia 23298, United States

[⊥]Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Abstract

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*Corresponding Author: mchartman@vcu.edu.

#Author Contributions

L.S. and Z.M. contributed equally. E.R.W. and Z.M. optimized the incorporation of unnatural AAs and performed the *in vitro* selection. E.R.W. and B.D. synthesized the peptides and performed the FP experiments. D.H. analyzed the cyclic structure by MS and performed the radioactivity bead binding assay. M.B.H., E.R.W., B.D., and D.W. prepared the GST-BRCA1 and TR-BRCA1 proteins. E.R.W. performed the ITC experiments. L.S. performed the crystallization studies. L.S., R.E., and J.G. solved the crystal structure. L.S. prepared the BRCT mutants and performed the FP experiments with them. J.B. performed the foci assays. M.C.H. directed the *in vitro* selection, peptide synthesis, and binding analysis; J.G. guided the crystallization studies; and K.V. directed the foci assays. E.R.W., M.C.H., J.G., and K.V. analyzed the data and together wrote the manuscript.

Accession Codes

Coordinates and structure factors have been deposited in the Protein Data Bank under the accession code 4OFB.

The authors declare the following competing financial interest(s): Z. Ma is employed by Ra Pharmaceuticals, which is further developing the technology described in this paper. Ra Pharmaceuticals may benefit from the publication of this paper. M.C.T. Hartman holds shares in and receives consulting fees from Ra Pharmaceuticals.

Supporting Information

Supporting Tables S1–S8 and Supporting Figures S1–S17, details on the preparation of reagents, procedures for cloning and protein expression, peptide synthesis and purity analysis, determination of binding affinity, and cell culture growth conditions. This material is available free of charge via the Internet from <http://pubs.acs.org>.

Many intracellular protein–protein interactions are mediated by the phosphorylation of serine, and phosphoserine-containing peptides can inhibit these interactions. However, hydrolysis of the phosphate by phosphatases, and the poor cell permeability associated with phosphorylated peptides has limited their utility in cellular and *in vivo* contexts. Compounding the problem, strategies to replace phosphoserine in peptide inhibitors with easily accessible mimetics (such as Glu or Asp) routinely fail. Here, we present an *in vitro* selection strategy for replacement of phosphoserine. Using mRNA display, we created a 10 trillion member structurally diverse unnatural peptide library. From this library, we found a peptide that specifically binds to the C-terminal domain (BRCT)₂ of breast cancer associated protein 1 (BRCA1) with an affinity comparable to phosphorylated peptides. A crystal structure of the peptide bound reveals that the pSer-x-x-Phe motif normally found in BRCA1 (BRCT)₂ binding partners is replaced by a Glu-x-x-4-fluoroPhe and that the peptide picks up additional contacts on the protein surface not observed in cognate phosphopeptide binding. Expression of the peptide in human cells led to defects in DNA repair by homologous recombination, a process BRCA1 is known to coordinate. Overall, this work validates a new *in vitro* selection approach for the development of inhibitors of protein–protein interactions mediated by serine phosphorylation.

Many dynamic protein–protein interactions (PPI)s are controlled by phosphorylation. The phosphoproteome is primarily composed of phosphoserine, threonine, and tyrosine, with phosphoserine being by far the most abundant.¹ To mediate these interactions, nature has evolved a wide variety of structures that recognize phosphorylated proteins and peptides with high affinity and specificity.² Several phosphoprotein interactions are *bona fide* therapeutic targets, yet development of inhibitors for these interactions has been hindered by the poor pharmacokinetic properties of phosphorylated peptides. Phosphoserine-containing peptides are undesirable therapeutic agents for two major reasons: they are susceptible to dephosphorylation by phosphatases, and by virtue of their negatively charged phosphoserine, they are not typically cell permeable.

Proteins containing BRCA1 C-terminal domains (BRCT) are a class of phosphoprotein binding modules that offer intriguing possibilities for the development of medically useful inhibitors. BRCT domains are a common hallmark of nuclear proteins involved in DNA damage signaling. They often exist as tandem repeats that selectively bind to phosphorylated (phosphoserine or phosphothreonine) protein partners.^{3,4} Perhaps the best studied (BRCT)₂ domain protein is the breast and ovarian cancer-associated protein, BRCA1, which participates in a nuclear pathway that responds to DNA double strand breaks to ultimately drive the repair of these lesions by homologous recombination.^{5,6} The critical importance of the phosphopeptide binding activity for BRCA1 tumor suppressor function is underlined by

the fact that mutations that precisely target the phosphopeptide binding cleft and abrogate phosphopeptide binding have been found to be associated with increased breast cancer risks.⁷

The critical role of BRCA1 in DNA damage signaling is beginning to be exploited for breast cancer therapy. Mutations in BRCA1 that lead to defects in DNA damage signaling can sensitize cells to radiation and many DNA-targeting chemotherapies and likely are responsible for the increased sensitivity of BRCA1-deficient tumors to these agents.^{8,10} The finding that BRCA1 mutations impact homologous recombination repair and sensitize cells to the single strand break repair enzyme poly(ADP)ribose polymerase (PARP)⁹ has led to promising approaches to target BRCA-deficient cancers¹¹ although the development of resistance is a significant challenge.¹² In contrast, the majority of sporadic breast cancers are not thought to be driven by BRCA1 mutations. In these cases, chemical inhibition of BRCA1 could potentially offer a means to selectively sensitize breast and ovarian tissues to DNA-targeting therapies.

Peptide library screening revealed that the BRCA1 (BRCT)₂ selectively binds phosphopeptides containing a pSer-x-x-Phe motif^{3,13} and subsequent structural investigations revealed a phosphopeptide binding cleft spanning the two repeats.¹⁴ Typically, pSer-containing peptides containing this motif have K_D values in the 300 nM to 5 μ M range (for examples see refs 15 and 16). After some optimization of the native peptide excised from the protein BACH1, Natarajan and co-workers found that a tetrapeptide of the sequence Ac-pSer-Pro-Thr-Phe-OH had K_D of 190 nM,¹⁷ and they later found that attaching hydrophobic groups onto the N-terminus of this peptide further enhanced binding 7-fold.¹⁸ However, attempts to replace phosphoserine with phosphomimetic groups have led to a >2 orders of magnitude loss in binding affinity ($K_D > 50 \mu$ M).^{17,19} Very recently, Na and co-workers replaced pSer with a difluorophosphonate²⁰ to develop BRCA1 inhibitors with K_D values in the 250 nM to 1 μ M range.²¹

As an alternative route to inhibitor discovery, we reasoned that by using a peptide library of sufficient size and functional diversity, a peptide lacking a phosphate or phosphonate could be found that binds to the (BRCT)₂ domain of BRCA1. There are many methodologies that have been developed for creation of peptide libraries, but mRNA display^{22,23} provides advantages over other techniques because of the potential for incorporation of noncanonical amino acids as well as the ability to prepare cyclic peptide libraries with up to 10¹³ unique peptides,^{24–30} 3–6 orders of magnitude more diverse than is possible with on-bead synthesis or phage display. This increased diversity^{31,32} should improve our chances to find phosphomimetic peptides. Here, we show successful application of the mRNA display technology to discover the first nonphosphate or phosphonate-containing peptide that binds to a (BRCT)₂ domain with an affinity similar cognate pSer-containing peptides. This peptide is also able to block PPIs of the BRCA1 (BRCT)₂ domain in cell lysates and disrupt DNA repair by homologous recombination. Therefore, this work demonstrates a novel paradigm for discovery of phosphoprotein–protein interaction inhibitors.

RESULTS AND DISCUSSION

Creation of a Diverse Peptide Library Composed of Both Linear and Cyclic Peptides

In order to maximize our chance of finding a tight-binding peptide, we designed our library to contain both linear and cyclic sequences of a variety of ring sizes. The library was constructed to contain a 12 amino acid random region encoding the peptide sequence Met-Cys-X₁₂-linker-His₆, with the random, X, amino acids designated by the codon NN(G/T/C) (Supporting Information Table S2), which was chosen so that approximately 40% of the library would contain a second cysteine. For cyclization, we focused on the well-established and robust bis-bromomethylbenzene chemistry, which covalently cyclizes peptides with two cysteines.^{33–35} The random position of the second cysteine allows the BRCA1 protein to “choose” from a variety of ring sizes or linear peptides for an optimum fit. Based on our previous work describing amino acid analogues that can be incorporated by substitution in ribosomal translation,³⁶ a group of six unnatural amino acids (Figure 1, Supporting Information Chart S1) were optimized for use together with the other 14 natural amino acids to make up the building blocks for the peptide selection (Supporting Information Figures S1–S5 and Table S1). When the translation reactions were supplemented with all six unnatural AAs (along with the other 14 natural AAs), the resulting yield was 30% of the all-natural amino acid reaction.

In Vitro Selection Using BRCA1 (BRCT)₂ as Bait Leads to Phosphomimetic Peptides

The general scheme of the mRNA display selection process is shown in Figure 1. The mRNA peptide fusion library was prepared in the standard way.^{30,35} After translation in the presence of unnatural amino acids, a library of mRNAs covalently linked to the peptides they encoded was formed. The mRNA–peptide fusions were purified via Oligo-dT cellulose and cyclized on the resin, followed by reverse transcription and Ni-NTA purification. The translation was performed on a large (10 mL) scale for round 1 leading to the creation of 1.3×10^{13} mRNA–peptide fusions, each theoretically unique. The functional peptides were captured onto magnetic beads containing GST-BRCA1 (BRCT)₂ (Supporting Information Figure S6). After elution with glutathione, the surviving mRNA–peptide fusions were amplified and this process was iterated (Figure 1 and 2a). After the eighth and final round, cDNAs corresponding to the selected mRNA–peptide fusions were sequenced (Supporting Information Table S3).

As shown in Figure 2b, the peptides could be sorted into seven families, which we named 8.1–8.7 based on their relative abundance. The most notable motif is the recurring N-terminal Asp-4FPhe-x-4FPhe (4FPhe = 4-fluoro-L-phenylalanine) sequence that is found in 64 of the 85 sequences. Another 11 sequences (Families 8.5 and 8.6) have an N-terminal Asp-4FPhe as well as a C-terminal Asp/Glu-x-x-4FPhe motif. These Asp/Glu-x-x-4FPhe motifs are quite similar to the pSer-x-x-Phe motif shared by all known BRCA1 (BRCT)₂-domain binding proteins.³⁷ Previously oriented SPOT library screens have shown that peptides containing β -branched and aromatic AAs in the x-x positions show enhanced binding.¹³ This preference is also mirrored in many of our sequences. Finally, the lack of a second cysteine in nearly all of the sequences suggests that the BRCA1 (BRCT)₂ protein prefers binding to less constrained peptides.

Selected Peptides Compete with a Known Phosphoserine-Containing Peptide for Binding to BRCA1

We prepared linear peptides for all 8 families using solid phase peptide synthesis (SPPS) (Supporting Information Figure S7). During the synthesis, we incorporated all of the requisite unnatural AAs, except for the arginine analog canavanine (Cav), which was found only in sequences 8.1, 8.2, 8.6, and 8.7. Synthesis of a suitably Fmoc-protected Cav proved to be a challenge, and we showed using a bead capture assay of *in vitro* translated peptide 8.6 that the reversion to Arg did not affect binding affinity (Supporting Information Figure S8). In addition, we needed to determine the fate of peptides containing a single cysteine when reacted with our cyclization reagent, *m*-dibromoxylene, so we treated a peptide 8.3 derivative with *m*-dibromoxylene. Surprisingly, this peptide did cyclize, and as evidenced by MS-MS, made a small sulfonium-containing ring between the N-terminal Met and Cys (Supporting Information Figures S9 and S10, and Table S4).

Analysis of SPPS-synthesized peptide binding affinities was conducted with a fluorescence polarization binding inhibition assay in competition with a known BRCA1 (BRCT)₂ binding peptide derived from BACH1, FAM- β -A-pSPTF-NH₂.¹⁸ The peptides had IC₅₀s in the 10–100 μ M range, with peptide 8.1 and 8.6 serving as the most effective inhibitors (Table 1 and Supporting Information Figure S11). Cyclization with *m*-dibromoxylene did not enhance affinity (Supporting Information Table S5). Of all of the peptides tested, 8.6 had the lowest IC₅₀ value (10.5 μ M), and we analyzed the thermodynamic features of its binding with BRCA1 (BRCT)₂ using isothermal titration calorimetry (ITC). The K_D was determined to be 3.7 μ M, and its binding is driven by a large, negative ΔH Value (Figure 3). To validate our hypothesis that the Glu-x-x-4FPhe was mimicking the cognate pSer-x-x-Phe, we prepared a mutant peptide containing the Glu to pSer mutation (8.6E8pS). This peptide bound with a 10-fold lower K_D (Figure 3), bolstering this hypothesis. The enhanced interaction was driven by more favorable entropy.

Crystal Structure of BRCA1 (BRCT)₂ with Bound Peptide 8.6 Reveals Novel Contacts Not Found in pSer-Containing Peptides

In order to fully define the mechanism by which peptide 8.6 binds the BRCA1 (BRCT)₂, we determined the crystal structure of peptide 8.6 3–14 (which lacks the N-terminal Met-Cys and is more soluble) bound to BRCA1 (BRCT)₂ at 3.05 Å resolution (Figure 4, Supporting Information Figure S12 and Table S6). An alignment of this structure with the structure of the BRCA1 (BRCT)₂ bound to the BACH1/FancJ target peptide reveals that the Glu-x-x-4FPhe motif of peptide 8.6 tracks closely upon the pSer-x-x-Phe motif and the structures of the BRCA1 BRCT domains are essentially identical (RMSD 0.4 Å for all C α atoms) (Figure 4a). The 4FPhe residue at the +3 position of peptide 8.6 overlays closely upon the phenylalanine of the natural peptide (Figure 4b), while the phosphomimetic glutamate is positioned to provide two of the three hydrogen bond/salt bridge interactions within the phosphate binding pocket (Figure 4c). The two available oxygen atoms of the glutamate side chain hydrogen bond with the Ser1655 side chain hydroxyl and the Gly1656 main chain amide; however, this binding mode sacrifices the salt bridge to Lys1702. Instead, a long-range (~4.5 Å) electrostatic interaction between Lys1702 ϵ -amino group and the glutamate may partially stabilize binding (Figure 4c). The selected peptide also contains a Lys at +5,

which makes salt bridging interactions with Glu1836 and Asp1840, an interaction that is also observed in the complex formed with the natural BACH1/FancJ peptide (Figure 4e).

The substitution of the phosphoserine for a glutamate is expected to dramatically reduce peptide binding affinity for BRCA1,¹⁹ as evidenced by our Glu to pSer mutant (Figure 3). Our peptides compensate for the lack of pSer in the library by making a number of additional contacts to the (BRCT)₂. Residues N-terminal to the glutamate (residues 2 to 5) occupy a hydrophobic groove in the (BRCT)₂ domain between β 1 and α 1. In particular, the -2 4FPhe and the -4 Ile residues pack in this cleft, providing van der Waals contacts (Figure 4d). The peptide utilized in crystallization terminates at 5; however, peptides extended to 7 with a Met-Cys dipeptide potentially contain additional amino acids that could interact with this groove. In addition, residues at the +1, +2, and +4 positions also contact the (BRCT)₂ in ways that are predicted to enhance binding (Figure 4e). The selected Tyr at +1 stacks against Asn1774 and also packs at against the Phe at +3. The +1 position is either a proline or a tyrosine in all structures determined to date with pSer-x-x-Phe peptides. Arginine residues are selected at the +2 and +4 positions, which are oriented toward Glu1698, providing electrostatic contacts, which are not observed in any of the complexes determined with pSer-x-x-Phe peptides. A summary of the crystal structure contacts is shown in Figure 4f.

To further characterize the difference between peptide 8.6 and the native pSer peptide from BACH1 in interaction with the BRCA1 (BRCT)₂, we generated several BRCA1 (BRCT)₂ mutants and examined their affinity with both peptides using a fluorescence polarization assay (Figure 5a and Supporting Information Figure S13). As expected, mutations within the conserved pSer-x-x-Phe recognition groove (M1775R, S1655A, K1702M) completely abolished recognition of the pSer peptide. In contrast, with peptide 8.6, only M1775R completely abrogated binding. K1702 M only reduced binding ~6-fold and S1655A demonstrated binding that is indistinguishable from wild type. This suggests that the Phe recognition pocket at the interface between the two BRCT repeats is critical for peptide 8.6 binding and that the integrity of the pSer binding pocket, formed by Ser1655 and Lys1702, is less important. The finding that the K1702M mutation significantly impacts binding but the S1655A mutation does not, is consistent with the idea that the positive charge of Lys1702 acts to stabilize the negative charge of the glutamate. The fact that the S1655A mutation does not impact binding could be explained if the glutamate residue is able to rotate to hydrogen bond with the main chain NH of Gly1656 and Lys1702. The novel contacts involving Glu1698 and Asn1744 were also tested. The results reveal significant 11- and 26-fold reductions in binding affinity for E1698A and N1774A, respectively to peptide 8.6, but no significant change in affinity to the wild type pSer peptide.

We performed an alanine scan of the peptide to further understand how each residue contributes to the affinity of the peptide (Figure 5b and Supporting Information Figure S11). The N-terminal residues that we tested all affect competitive binding, including those that sit in the hydrophobic groove (Ile4 and 4FPhe6) and the N-terminal Met and Cys which were not present in the crystallized peptide. Based on the crystal structure, it was not surprising that the Glu-Tyr-Arg-4FPhe portion of the peptide contained the residues most essential for binding. Substituting either the Glu8 or the 4FPhe11 residues resulted in the largest reductions in competitive binding. The Arg10 alanine mutant also showed a large (16-fold)

reduction in affinity which validates the importance of the interaction with E1698 in the crystal structure. We were surprised to find that the Tyr to Ala mutant (Y9A) competed more effectively for binding than the wild-type 8.6, which suggests that either the interaction between the Tyr side chain and Asn1774 is unimportant or the loss in that interaction is compensated for by a reduction in the entropic cost of binding. Finally, the C-terminal Arg12 and Lys13, although they both form salt bridges in the crystal structure, were not required for strong competitive binding.

Peptide 8.6 Binds Selectively to BRCA1 (BRCT)₂ Compared to Other (BRCT)₂ Domains

With the unique features of the binding surface described, we then wondered if peptide 8.6 was selective for BRCA1 (BRCT)₂ vs other similar (BRCT)₂ domains. Using ITC, we tested the binding of 8.6 to both TopBP1 BRCT7/8 and the tandem MDC1 BRCT repeat. TopBP1 BRCT7/8 is structurally similar to the BRCA1 BRCT and binds a similar phosphoserine containing target peptide in BACH1/FancJ that contains hydrophobic residues at the +2 to +4 positions.³⁸ MDC1 is also very similar structurally to BRCA1 and binds a pSer-x-x-Tyr motif at the precise C-terminus of γ H2AX.³⁹ In spite of these similarities, ITC experiments demonstrated that peptide 8.6 did not detectably interact with either TopBP1 BRCT7/8 or MDC1 BRCT (Supporting Information Figure S14). An alignment of the BRCA1 BRCT complex with these two related BRCT domains suggests the basis for the inability of peptide 8.6 to bind these related proteins (Supporting Information Figure S14). While the overall folds and the phosphate binding pockets of the three proteins are similar, the additional surfaces in the BRCA1 BRCT that are critical for interactions with peptide 8.6 are not well conserved in either TopBP1 BRCT7/8 or the MDC1 BRCT domain.

Expression of 8.6 Nat in Human Cells Alters the DNA Damage Response

If peptide 8.6 serves as a BRCA1 inhibitor in cells, it should have a measurable effect on both the DNA damage response, and in particular, DNA repair through homologous recombination (HR) by blocking PPIs between BRCA1 and its binding partners. We decided to check this by overexpressing peptide 8.6 inside cells as a fusion to EGFP. We used ITC to verify that the substitution of the two 4FPhe with Phe (peptide 8.6 Nat) did not significantly abrogate binding (Supporting Information Figure S15). We appended 8.6 Nat onto the C-terminus of the EGFP protein, and using lentiviral infection, integrated this construct into T98 human glioblastoma cells using a doxycycline (DOX)-controlled promoter, creating the line T98-EGFP-8.6. A previous study has determined that EGFP overexpression leads to intracellular concentrations of 20 μ M,⁴⁰ well above the K_D of our ligand. We first verified that the parental T98 cells showed a typical DNA damage response by monitoring γ H2AX and Rad51 nuclear foci formation and resolution (Supporting Information Figure S16). Whereas γ H2AX foci are known to mark DNA double-strand breaks (DSBs) generated in response to radiation in general, Rad51 foci are specific markers of ongoing homologous recombination (HR). The disappearance of foci over time is a sign of active and functional DSB repair. Whereas both γ H2AX and Rad51 foci levels increased ~1.5-fold after radiation, only γ H2AX levels declined by 3 h (Supporting Information Figure S16). Rad51 foci continued to accumulate between 30 min and 3 h in line with the known slower kinetics of HR.⁴¹ Altogether, T98 cells show a typical response to radiation and have functional DSB repair.^{37,42}

With these baselines established, we tested T98-EGFP-8.6 cells. We first confirmed using fluorescence and Western blotting that T98 cells treated with doxycycline (DOX) expressed EGFP-8.6Nat (Supporting Information Figure S17) in a tightly controlled manner. We then analyzed both γ -H2AX and RAD51 foci in these cells. Cells treated with DOX to initiate induction of EGFP-8.6 Nat production showed a significantly (2.3-fold) higher background of γ -H2AX foci than untreated cells, suggesting that the induction of EGFP-8.6Nat increased DNA damage (Figure 6a). Upon irradiation, the level of γ -H2AX foci also increased in the presence of EGFP-8.6 Nat as compared to cells lacking DOX induction (6.8-fold vs 4.3-fold). Neither of these effects are observed in cells expressing EGFP alone, although the comparative data with these cells is somewhat obscured by a higher basal level of foci. A similar trend was observed when we observed Rad51 foci in these cells. Rad51 foci are increased in EGFP-8.6Nat cells after induction with DOX both in the presence and absence of IR. This enhancement is not observed in T98 cells expressing EGFP alone (Figure 6b). The Rad51 foci studies suggest that the inhibition of DNA repair is due to aberrant homologous recombination.

It is well established that Abraxas-RAP80, BACH1, and CtIP bind to the BRCA1 (BRCT)₂ domain and have critical functions in HR.⁶ We verified that synthesized peptide 8.6 Nat was able to block the binding of phospho-CtIP and BRCA1 in cell lysates (Figure 7), evidence that our peptide is altering DNA repair through interfering with BRCA1 function. Altogether, it is likely that the increase in foci after DOX treatment and interference with the removal of foci are the result of inducing dysfunctional DSB repair by the expression of EGFP-8.6Nat and concomitant blocking of BRCA1 (BRCT)₂ function.

There are now several examples of peptides containing sequences that replace phosphotyrosine with mimics or natural amino acids (mostly SH2 domain inhibitors).⁴³ However, most successful examples for replacement of phosphoserine focus on a single method involving difluorophosphonate replacement.^{20,21} More than a decade ago, Fu and co-workers used phage display to successfully uncover nonphosphorylated peptide inhibitors to a different class of phosphoserine-binding proteins, the 14-3-3 proteins.⁴⁴ In their work, the non-phosphorylated inhibitors bound to the amphipathic groove containing the phosphoserine binding site, yet the inhibitor interacted with a different, but overlapping, set of residues compared with a phosphorylated peptide binder.⁴⁴ In contrast, peptide 8.6 binds in a similar manner to the native pSer-x-x-Phe peptides, with the direct substitution of the glutamic acid for the phosphoserine and maintenance of some, but not all, interactions in the pSer pocket. The resulting peptide also makes additional contacts that are not found in any previous BRCA1-ligand structure. These additional contacts may explain the large, negative H values describing the binding of 8.6, which are significantly larger in magnitude (24.6 kcal mol⁻¹ vs 15 to 18 kcal mol⁻¹) than our pSer mutant and other pSer peptide binders.^{19,45} The unfavorable entropy term describing the binding of peptide 8.6 does not appear to be due to the additional contacts at the N-terminus, since the pSer mutant led to a favorable increase in entropy, even though it presumably maintains many of the same contacts. The additional contacts mediated by the inhibitor peptide are, however, likely responsible for the high degree of specificity of this inhibitor, which is unable to bind to the highly related MDC1 and TopBP1 (BRCT)₂ domains, which both bind related phosphopeptides.

In contrast to our previous selection for binders to thrombin,³⁵ our BRCA1 selection did not uncover unnatural peptide binders that were inactive upon natural AA reversion. The ability to find novel unnatural motifs is likely dependent on the number and types of unnatural AAs substituted as well as the binding preferences of the protein. For example, if we had used a more sterically demanding phenylalanine variant in our library that was unable to fit into the Phe binding pocket, it is likely that peptides containing a Glu-x-x-Phe analog motif (such as 8.6) would not have survived. But would we have found any alternatives? The fact that our library was handicapped because it lacked pSer, yet still contained effective binders, suggests that we would have found other, unique solutions to the binding problem given a different starting pool of amino acids. In fact, to the best of our knowledge peptide 8.6 is one of the only examples where a pSer in a *peptide* has been replaced with Glu or Asp with maintenance of binding affinity⁴⁶ (although this phenomenon has been reported in full-length proteins^{47–49}). We succeeded in substituting pSer with Glu while others have failed¹⁹ because our diverse library was able to compensate for the weaker interaction in the pSer pocket by optimizing adjacent interactions (Figure 4). Considering that carboxylates are much more commonly found in drugs⁵⁰ than phosphates or phosphonates due to their stability and improved permeability characteristics,^{51,52} our library approach holds promise for the development of future phosphomimetic peptides with carboxylate substitutions.

Although we used a peptide library that was theoretically 40% cyclized between two cysteines at random positions, all of the peptides found had the small N-terminal Cys-Met cycle. Analysis of the crystal structure complex strongly suggests why this is the case—peptide 8.6 binds in an extended conformation, similar to phosphoserine peptides. Cyclic peptides that encompass the Glu-x-x-Phe4FPhe motif would have been unable to adopt the extended conformation required for binding. This suggests that including a diversity of scaffolds, including linear peptides, in a library is likely to lead to the best chance of finding a high-affinity binder.

Our cell culture data demonstrated that T98 cells over-expressing peptide 8.6 Nat appended to EGFP are defective in DNA repair by HR relative to control cells expressing EGFP alone. Recently, cell studies have been conducted with a phosphorylated BRCA1 peptide inhibitor attached to a cell penetrating peptide⁵³ and with a difluorophosphonate-containing BRCA1 inhibitor.²¹ Addition of these molecules at high concentrations to cells led to several phenotypes associated with BRCA1 deficiency. Since BRCA1 deficiency leads to sensitivity to DNA damage, these inhibitors and ours are important leads for the development of chemo- and radio-sensitizers for tumors containing wild-type BRCA1, the vast majority of all cancers.

In summary, we have developed a new approach for the discovery of phosphomimetic peptides from extremely diverse libraries, and we have identified the first functional inhibitor of any BRCT domain that does not contain a phosphate or phosphonate. The crystal structure of our peptide shows how our peptide compensates for a suboptimal Glu for pSer mutation by making additional contacts to adjacent surfaces of the protein, many of which are not observed in any pSer BRCT peptide crystal structure. Future application of this technology to other BRCT domains as well as other phosphoserine-mediated PPIs will

lead to a collection of useful phosphomimetic peptide tools for unravelling the interwoven strategies mammalian cells use to repair damaged DNA.

METHODS

Unnatural Amino Acid Optimization

Optimizing the yield of the unnatural amino acids used for the selection was conducted with the PURE reconstituted translation system⁵⁴ and standard translation assays performed as previously described including templates MLEPQ-FLAG, MTINR-FLAG, MDYKM-H₆, and MHFSW-FLAG.⁵⁵ The sequence of the MVHM-H6 mRNA template is 5'-CUAAUACGACUCACUAUAGGGUUAACUUUAGUAAGGAGGACAGCUA-AAUGCACGUAAUGCAUCACCAUACACCACCAUAUGUAGUAG-3'. Final concentrations of amino acids used are shown in Supporting Information Table S1.

Preparation of mRNA–Peptide Fusions

The *in vitro* selection protocol has previously been outlined in detail³⁰ and is described here in brief. The sequence of the mRNA display library can be found in Supporting Information Table S2. The library mRNAs were photochemically cross-linked to a puromycin linker³⁰ after annealing, followed by exposure to UV irradiation, and were then used in translation to produce peptide-mRNA fusions. A 10 mL scale translation was used to generate peptides for the first round of selection. The mRNA peptide fusions were bound to Oligo(dT) resin via the poly-A sequence contained in the puromycin linker. Peptides were cyclized on the resin using *m*-dibromoxylene and eluted with water.³⁵ This was followed by reverse transcription of the mRNA, and Ni-NTA purification. The library was then dialyzed into selection buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 4 mM MgCl₂, 0.25% (v/v) Triton X-100) and used in the first round of selection. The yield of peptide fusions in the first round after all purification steps was 22 pmol, equivalent to 1.3×10^{13} peptides.

Selection for Binders to BRCA1 (BRCT)₂

Prior to the selection, glutathione beads containing GST and GST-(BRCT)₂ were prepared.

GST Beads—200 μ L of magnetic glutathione beads (Pierce) were washed three times with 1 mL GSH beads wash buffer (125 mM Tris-HCl pH 8, 150 mM NaCl). 1 mL of 10 μ M GST in GSH beads wash buffer was added to the beads and tumbled for 1 h at 4 °C. The supernatant was then discarded, and the beads were washed twice with 1 mL GSH beads wash buffer and once with 1 mL selection buffer.

GST-(BRCT)₂ Beads—A separate 400 μ L of bead suspension was washed three times with GSH beads wash buffer. 1 mL of 10 μ M GST-(BRCT)₂ was added and tumbled 1 h at 4 °C. GST-(BRCT)₂ bound beads were washed twice with GST beads wash buffer and once with selection buffer. The previously purified peptide-mRNA fusions were dissolved in 1200 μ L selection buffer.

Preclear—mRNA–peptide fusions were added to GST bound beads suspended in selection buffer and tumbled 1 h at 4 °C.

Selection (Round 1)—The supernatant containing the library was transferred to the GST-(BRCT)₂ bound beads. With an additional three washes, the total volume transferred to the GST-(BRCT)₂ beads was 1300 μ L. 13 μ L of 10 mg mL⁻¹ BSA was also added, and the tube was tumbled for 1 h at 4 °C. The beads were washed three times with 1 mL selection buffer. Peptide fusions were eluted along with the bound protein by the addition of 6 $\times$ 100 μ L freshly prepared GSH elution buffer (250 mM Tris-HCl pH 9, 500 mM NaCl, 100 mM reduced L-glutathione (Sigma), 1% (v/v) Triton X-100). Each addition of elution buffer was allowed to incubate for 5 min. Portions of selection input, flow through, washes, resuspended beads, and elution fractions were quantified by scintillation counting of ³⁵S-Met. Elution fractions were combined and dialyzed overnight at 4 °C into 0.1% (v/v) Triton X-100 prior to PCR amplification. The amplified cDNA was then transcribed and used for the subsequent round of selection.

Selection (Subsequent Rounds)—The scale of the second round of selection was based on a 1 mL translation, followed by rounds 3–8 that were based on 500 μ L translation reactions. Additionally, the preclear step was only performed in rounds 1 and 2.

Crystallization and Structure Solution

Human BRCA1-(BRCT)₂ (1646–1859) was expressed as an untagged recombinant protein in *E. coli* strain BL21(DE3) then purified as previously described.⁵⁶ The BRCA1 (BRCT)₂ was concentrated to 10 mg mL⁻¹ in storage buffer (400 mM NaCl, 10 mM Tris-HCl, pH 7.5) then incubated with peptide 8.6 3–14 at 1:3 protein to peptide ratio for 2 h at 4 °C. Crystals were grown using the sitting drop method by mixing 0.8 μ L of protein/peptide mixture with 1.0 μ L of crystallization buffer (0.1 M sodium cacodylate, 0.1 M magnesium acetate, 24% (v/v) PEG 6000, pH 6.5) and harvested after 24 h. Suitable crystals were cryo-protected by immersion in crystallization buffer supplemented with 20% (v/v) glycerol prior to freezing in liquid nitrogen. Data were collected at beamline 08B1-1 (Canadian Light Source) and the intensity data were processed with HKL2000.⁵⁷ Crystals belonged to the trigonal space group *P* 41 ($a = b = 37.8$ Å, $c = 176.0$ Å, $\alpha = \beta = \gamma = 90.0^\circ$) with a single protein/peptide complex in the asymmetric unit. The complex structure was solved with Phaser⁵⁸ using the BRCA1 (BRCT)₂ crystal structure (PDB ID code 1JNX⁵⁶) as a search model. The inhibitor peptide was then built in manually in Coot⁵⁹ based on the additional electron density around the conserved pSer-x-x-Phe motif recognition site. The BRCT domain from the higher resolution structure of the BRCA1 (BRCT)₂/ATRIP peptide complex (PDB ID code 4IGK⁶⁰) was superimposed on the current model and used as the basis for further refinement. Loop regions 1792–1807 and 1816–1819 were modeled based on their conformation in 1JNX. Due to a lack of density, residues Glu1817 and Asp1818 were left out of the final model. Statistics of data collection, processing, and refinement are provided in Supporting Information Table S7. Coordinates and structure factors have been deposited in the protein data bank under the accession code 4OFB.

DNA Damage Foci Formation Assay

T98-EGFP and T98-EGFP-8.6 cells were grown on Lab-Tek II (Thermo Scientific) glass chamber slides (using the aforementioned conditions). Doxycycline (1 μ g mL⁻¹, Sigma) was added 24 h after seeding. Seven days after the addition of doxycycline, cells were treated

with 5 Gy of radiation using an MDS Nordion Gammacell 40 research irradiator. After treatment, cells were fixed in 4% (v/v) paraformaldehyde, permeabilized for 10 min with 0.5% (v/v) Triton-X 100 in phosphate-buffered saline, and blocked with a 1× casein (Sigma) or 3% (v/v) goat serum (Gibco) mixture for 2 h at room temperature (RT). Cells were then exposed to a 1:500 primary antibody solution (either γ -H2AX (Millipore) or Rad-51 (Calbiochem)) overnight at 4 °C. The slides were then washed with phosphate-buffered saline and exposed to a 1:500 Alexa-Fluor 594 secondary antibody solution. Slides were washed again with phosphate-buffered saline, and nuclei were counterstained with VectaShield mounting media with DAPI. Images were acquired using a Zeiss LSM 710 confocal microscope and analyzed (including foci counting) with Perkin Elmer's Volocity software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by VCU's Massey Cancer Center (M.C.T.H.), the Concern Foundation (M.C.T.H.), the William T. and Kate F. Jeffress Fund (M.C.T.H.), Ra Pharmaceuticals (M.C.T.H.), the Canadian Institutes for Health Research (J.N.M.G.), and the National Institutes of Health (NIH) (R01CA166264 to M.C.T.H., R01NS064593 and R21CA156995 to K.V., F30CA171893 to J.M.B., and CA92584 to J.N.M.G.). We thank P. Grochulski and the staff at the Canadian Light Source CMCf beamline 08ID-1 for assistance with synchrotron data collection, J. Turner (VCU) for his help with fluorescence polarization experiments, C. Escalante and R. Jaiswal (VCU) for help with ITC experiments, and T. Cropp (VCU) for advice on the manuscript.

ABBREVIATIONS

BRCA1	Breast Cancer Associated Protein 1
BRCT	BRCA1 C-terminal domain
DOX	doxycycline
DSB	DNA double strand breaks
FP	fluorescence polarization
HR	homologous recombination
ITC	isothermal titration calorimetry
PARP	poly(ADP)ribose polymerase
PPI	protein-protein interaction
SPPS	solid-phase peptide synthesis

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Figure 1.

In vitro selection strategy to discover inhibitors of BRCA1 (BRCT)₂. The DNA library encodes a 12 amino acid random region with an N-terminal cysteine. During *in vitro* translation, the unnatural amino acids shown are incorporated into the peptide library along with 14 canonical amino acids. The single letter abbreviation denotes which amino acid is replaced by that analog (e.g., Fa replaces F). After mRNA–peptide fusion formation, peptides with a second cysteine are cyclized with dibromoxylene. Purified mRNA–peptide fusions undergo reverse transcription before being selected for binding to GST-(BRCT)₂ fusion immobilized on magnetic resin. Unbound peptides are washed away, and bound peptides are eluted, PCR amplified, and carried through another round of selection. Structural representation of BRCA1 (BRCT)₂ was adapted from PDB entry code 1T29 (ref 16) using PyMOL Molecular Graphics System (Schrodinger, LLC).

Figure 3.

Peptide 8.6 interaction with BRCA1 (BRCT)₂ domain characterized by isothermal titration calorimetry. Titrations were conducted with the indicated peptides into TR-(BRCT)₂ fusion protein in a buffer containing 300 mM NaCl, 2.7 mM KCl, and 10 mM phosphate, pH 7.4. All measurements were conducted at 25 °C with TR-(BRCT)₂ concentrations of 33 μM and 20 μM , and peptide concentrations of 426 μM and 214 μM for peptides 8.6 and 8.6 E8pS, respectively.

Figure 4.

Crystal structure of BRCA1 (BRCT)₂ bound to peptide 8.6. (a) Overall structure of the BRCA1 (BRCT)₂-peptide complex with the peptide in blue and the (BRCT)₂ domain in green. The structure is superimposed on the structure of the BRCA1 (BRCT)₂ bound to the BACH1 pSer-x-x-Phe containing peptide (PDB ID: 1T15; BACH1 peptide in pink). (b) Comparison of the recognition of the 8.6 peptide and BACH1 +3 residues by the BRCA1. Hydrogen bonds are highlighted (yellow). (c) Comparison of interactions in the BRCA1 (BRCT)₂ phosphate binding pocket between peptide 8.6 Glu and the BACH1 pSer. (d) Interactions between the N-terminal BRCT and residues 2 to 4 of peptide 8.6. Note that additional residues N-terminal to 5 could further interact with the hydrophobic surfaces provided by Tyr1665, Phe1662, and Leu1676 as indicated by the blue dashed line. (e) Interactions between the C-terminal BRCT and peptide +1 to +5 residues. (f) Summary of the crystal structure contacts of peptide 8.6 with the BRCA1 (BRCT)₂ domain. The Met and Cys residues at 7 and 6, respectively, are proposed interactions and are not observed in the crystal structure.

Figure 5.

Analysis of binding with protein and peptide mutants by fluorescence polarization. (a) Comparison of binding affinities of either the BACH1 peptide (FITC-SRSTpSTPFNK-NH₂) or peptide 8.6 (FAM- β -Ala-MCTIDFDEYRFRKT-NH₂) with a series of GST-BRCA1 (BRCT)₂ mutants. The K_D values of the peptide–protein interactions were determined by fluorescence polarization and were normalized against the K_D of the wild-type GST-BRCA1 (BRCT)₂ (Supporting Information Table S8). (b) Changes in competitive binding observed with alanine mutants of peptide 8.6. Each peptide was incubated at various concentrations with TR-BRCA1 (BRCT)₂ (2.5 μ M) and the peptide FAM- β -Ala-pSPTF-NH₂ (20 nM). The values reported were derived by dividing the IC_{50} of the mutant peptide (Supporting Information Table S6) with the IC_{50} of wild-type peptide 8.6 under identical conditions. ND = no binding or competitive binding detected. Fa refers to 4-fluorophenylalanine.

Figure 6.

Peptide 8.6 interferes with the DNA damage response. T98 cells transduced with either a TRIPZ-EGFP (T98-EGFP) or TRIPZ-EGFP-8.6 Nat (T98-EGFP-8.6) construct were treated with or without DOX for 7 days. DOX-treated T98-EGFP-8.6 cells had significantly ($p < 0.05$) higher (a) γ -H2AX and (b) Rad51 foci than untreated cells, an effect that was more pronounced after 5 Gy radiation. Cells were fixed 3 h after irradiation. Right panel: foci appearing in TRIPZ-EGFP-8.6 Nat cells as quantified in the bar diagram. Blue staining (DAPI) indicates nuclei, red staining indicates (a) γ -H2AX or (b) RAD51. Single cell inset shows digital magnification of representative 63 $\times$ field. Scale bar is equal to 33.5 μ m.

Figure 7.

Peptide 8.6 blocks BRCA1 (BRCT)₂ PPIs. Pull down-western showing the effect of increasing concentrations of the peptide 8.6 Nat on the capture of phospho-CtIP-myc from cell lysates with BRCA1 (BRCT)₂ beads. Only a portion of CtIP is phosphorylated; hence, the contrast in intensity between input and bound.

Table 1

Competitive Inhibition of pSer-x-x-Phe Fluorescent Peptide Binding by Library Peptides

name	sequence ^a	IC ₅₀ (μM) ^b
8.6	Ac-MCTID F DEYRFRKT-NH ₂	10 ± 1
8.1	Ac-MCND F IFRRST F RA-NH ₂	24 ± 2
8.5	Ac-MCYD F DTTNDHT F -NH ₂	26 ± 2
8.2	Ac-MCSD F IFSRRTY F -NH ₂	49 ± 5
8.4	Ac-MCHND F AFAKTSLY-NH ₂	56 ± 4
8.7	Ac-MCD F QFRKPSTTIY-NH ₂	56 ± 6
8.3	Ac-MCND F TFDKNLNHH-NH ₂	99 ± 8

^a Unnatural amino acids are designated by bold text.

^b IC₅₀ values are based on a competition FP assay with 20 nM of peptide FAM-β-A-pSPTF and 2.7 μM of thioredoxin-(BRCT)₂ fusion and variable concentrations of each peptide. IC₅₀ values were calculated by fitting with a four parameter logistic equation.