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Structure Activity Relationship Studies to Probe the Phosphoprotein Binding Site on the Carboxy Terminal Domains of the Breast Cancer Susceptibility Gene 1

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Abstract

The carboxy terminal BRCT domains of the breast cancer susceptibility gene 1 (BRCA1) bind to a plethora of phosphorylated proteins through a pSXXF consensus recognition motif. BRCT-protein binding regulates key cellular functions such as lipogenesis, cell-cycle checkpoint control and DNA damage response. Identification of the minimal binding sequence and defining the key interactions responsible for biological activity are critical steps in the peptidomimetic design process. Here, we report a systematic structure activity relationship study that maps the BRCT(BRCA1)-pSXXF binding interface. The study has led to the identification of peptides with nanomolar binding affinities comparable to the previously reported 13-mer peptides. The results also provide a clear description of the pSXXF-BRCT interface, which is essential for developing small molecule inhibitors *via* the peptidomimetic approach.

INTRODUCTION

Protein-protein interactions are the driving forces that underlie multiple signaling events and the corresponding cellular functions. Due to the transient nature of these interactions, classical biochemical techniques are limited in their ability to precisely and temporally perturb these systems. Chemical probes are emerging as a viable alternative to dissect complex networks of protein-protein interactions.¹ Recently, high-throughput screening (HTS) has been used to identify chemical probes for protein-protein interactions.^{2, 3} Current methodologies result in low hit rates and weak affinities suggest the need for alternate approaches must be developed. The rational design of peptidomimetics starting from a small peptide with low micromolar affinity can be used to overcome the shortcomings of HTS.^{4, 5}

The tumor suppressor protein BRCA1 maintains the integrity of the genome by mediating DNA damage response.⁶ The carboxy terminal domains of BRCA1 (BRCT) recognize and bind phosphorylated protein partners in response to DNA damage, such as Abraxas and BACH1.^{7, 8} The interaction of BRCT and phosphorylated proteins are mediated through a pSXXF consensus recognition motif. The BRCT(BRCA1)-pSXXF interaction is anchored

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SUPPORTING INFORMATION PARAGRAPH. A table with the thermodynamic parameters used for analysis and LC traces and mass spectra of the peptides are included.

through a two-point binding mode: a hydrophilic contact made by the phosphorylated serine (**pS**) residue and a hydrophobic contact made by the phenylalanine (**F**) residue.⁹⁻¹⁴

A set of reproducible fluorescence polarization (FP) assays were developed to dissect BRCT-phosphoproteins interaction and identify small molecules that will specifically bind to the pSXXF binding site on BRCT.^{15, 16} Tetrapeptides which bind BRCT with micromolar affinities were identified using FP assays.¹⁷ These tetrapeptides bind to the BRCT at the exact same site as the 13-mer peptides (ISRSTpSPTFNKQT) previously reported.^{18, 19} A combination of orthogonal techniques, FP and Isothermal Titration Calorimetry (ITC), was used in this study to determine the structure activity relationship (SAR) and the enthalpic and entropic contributions associated with the BRCT-tetrapeptide interactions were modeled using a thermodynamic optimization plot (TOP).²⁰ These studies resulted in the identification of peptide **18** (Ac-pSPTF-CO₂H) with nanomolar binding affinity for BRCT(BRCA1).

RESULTS AND DISCUSSION

IC₅₀ values obtained from the screen are summarized in **Table 1**. A SAR of BRCT-tetrapeptide was constructed and the structural basis is pictorially summarized in **Figure 1**.

Modification to the pS (P position) residue

Peptides **2-4** containing phosphoserine mimics were used to explore the phosphate-binding site. Previously, our laboratory has shown that substituting the phosphoserine mimic using glutamate (**E**) into the peptide sequence renders it inactive.¹⁷ Structural studies showed that the three oxygen atoms on the phosphate group are in close proximity to the side chains of Lys₁₇₀₂, Ser₁₆₅₅ and to the amide nitrogen of Gly₁₆₅₆ on the N-terminus BRCT domain.⁹⁻¹⁴ Consistent with this observation, peptide **3** containing a gamma-carboxy-glutamate (**γcE**) side chain, with four oxygen atoms and two –OH groups, resulted in a partial rescue of the inhibitory activity (compared to **2**), confirming that at least three heteroatoms are needed to make the hydrophilic contacts. The phosphothreonine (**pT**) peptide **4** was used to explore the size of the phosphate-binding site. Remarkably, this peptide was inactive. Analysis of the crystal structure reveals that rotation of the C_α-C_β bond on **pT** could minimize the steric interaction (**Figure 1A**, see arrow) between the methyl group on **pT** and the Thr₁₇₀₀ side chain on BRCT. This conformational change will force the phosphate group on **pT** to orient away from the hydrophilic contacts on BRCT, resulting in the complete loss of activity.

Modification to the P+1 residue

The role of the Pro residue in pSPTF binding to BRCT was investigated using the pSATF peptide **5**. This modification resulted in a ~ 3-fold loss of activity compared to the parent peptide **1**. This suggests that the Pro residue is critical in restricting the peptide to the bound conformation.

Modification to the P+2 residue

Five peptides **6-10** with systematic changes to the P+2 positions were used to explore the role of (a) –OH on the side chain and (b) steric interaction by the β-branching on the Thr (**T**) side chain. Interestingly, compared to the peptide **1**, both the Ala substituted peptide **6** and the Ser substituted peptide **8** resulted in ~7-fold loss of activity, while the Val substituted peptide **7** showed slightly improved activity. This result indicates that the steric interaction is more critical than the –OH functionality.

Peptides with the isopentane side chains, the Ile substituted peptide **9** and the Leu substituted peptide **10**, which are β and γ branched respectively, were screened in order to further

explore this steric interaction. Both peptides were less active compared to peptides **1** and **7** with the Thr/Val side chains. It is interesting to note that peptide **9** with the β -branching is more active than peptide **10** with γ -branching. Together, the SAR at the P+2 position clearly shows that β -branched peptides (**1**, **7** and **9**) are ~3- to 8-fold more active than unbranched (**6** and **8**) or γ -branched (**10**) peptides. This analysis and examination of the crystal structure reveal that the steric interaction with the side chain of Glu₁₆₉₈ on BRCT contributes to the BRCT-peptide interaction and that β -branching is optimal at this position (**Figure 1B**).

Modification to the P+3 residue

Peptides **11** and **12** were synthesized to incorporate unnatural amino acids with naphthyl side chains (¹Nap and ²Nap) to explore the size and shape of the hydrophobic patch. Both modifications resulted in partial loss of activity (Table 1), however, the ²Nap substitution is an order of magnitude better than the ¹Nap substitution. This change suggests that the Phe binding pocket is asymmetric and will better accommodate substitution at the *para* position on the phenyl ring compared to the *ortho* position. The Tyr containing peptide **13** was used to explore the effect of *para* substitution and possible hydrogen bonding with the amide carbonyl of Asn₁₇₇₄ on BRCT. This substitution, however, resulted in a 3-fold loss of activity and suggests that this pocket does not tolerate even small extensions to the phenyl ring.

Combinatorial effects

The pSATF peptide **5** and the pSPAF peptide **6** are ~ 3-fold and ~ 7.5-fold less active than **1**, respectively. Remarkably, the pSAAF peptide **14** with the combined substitution is ~ 21-fold less active than **1**. This result highlights that the interactions made by the middle residues are linked and contribute to the overall binding in a combinatorial manner.

Modification to the pSXXF backbone

Peptides **15** and **16** were synthesized to explore the contribution of the backbone to BRCT-peptide binding. It is known that proline dimers tend to adopt the polyproline type II (PPII) secondary structure due to minimization of the pseudo allylic (1,3) strain (see supplementary material, **Figure S1**).²¹ The PPII secondary structure is defined by the backbone dihedral angles $\phi_{\text{-CO-N-CA-CO-}} = -75^\circ$, $\psi_{\text{-N-CA-CO-N-}} = +145^\circ$. Peptides containing Pro-Pro motifs will adopt the PPII conformation and, therefore, will be conformationally constrained, resulting in rigid scaffolds.²² The pSPPF peptide **15**, wherein a Pro-Pro motif is introduced, was used to explore this effect. This modification resulted in > 50-fold loss of activity compared to the parent peptide **1**, suggesting that rigidifying the pSXXF peptide to the PPII conformation is not favorable.

Reversing the peptide sequence, combined with D amino acids, is a common approach used to stabilize peptides to cellular degradation.²³ The reversed-sequence peptide will have the amide bonds reversed, but the side chains will orient similar to the parent-L-peptide. To test the effect in our system, we generated peptide **16** which has the side chains positioned correctly to facilitate optimum contact with the protein (see supplementary material, **Figure S2**). Peptide **16**, however, was not active, indicating that the backbone is involved in some undefined interaction or the termini functionality is critical for binding. Analysis of the BRCT(BRCA1)-peptide crystal structure suggested the latter, which led us to generate peptides **17-19** with changes to the termini of the pSPTF peptide **1**.

Modification to the pSXXF termini

Peptide **17** without the acetyl protecting group on the α N of pSer and peptide **18** with an isosteric replacement of $-\text{CONH}_2$ with $-\text{COOH}$ at the P+3 Phe residue were synthesized to

probe the termini contribution to BRCT-peptide binding. Removal of the acetyl group in peptide **17** resulted in a ~2-fold loss of activity. Interestingly, the replacement of –CONH₂ with –COOH (peptide **18**) resulted in a ~5-fold increase in activity. A possible salt bridge with the Arg₁₆₉₉ on the BRCT(BRCA1) could be responsible for the increased activity.¹⁹ Since the peptide pSPVF **7** showed marginal improvement compared to peptide **1**, peptide **19** was generated with the carboxylic acid at the C-terminus of pSPVF. As hypothesized, peptide **19** also showed ~3-fold improvement in activity compared to peptide **1** suggesting the possible salt bridge proposed above as a potential contact. This observation can be exploited during inhibitor design and carboxylic acid mimics incorporated into the C-terminus of this peptide currently under investigation.

Isothermal titration calorimetry (ITC)

Direct binding experiments using ITC were conducted to determine the dissociation constants (K_d) and the thermodynamic parameters (ΔG , ΔH and ΔS) of a subset of peptides listed in **Table 1**. A correlation with an $R^2 = 0.9$ between the IC₅₀ values obtained from the competitive FP assay and the K_d values obtained from the binding studies using ITC (supplementary material **Figure S3**) was observed. A thermodynamic optimization plot (TOP) has been suggested as an analysis method for the optimization of drug candidates by dissecting the enthalpic and entropic contributions associated with each chemical modification.²⁰ A TOP was generated using the thermodynamic parameters (**Figure 2**) for a subset of peptides containing a single change compared to the parent peptide **1**. Each region of the TOP is associated with some change in the thermodynamic signature of the binding event. The thermodynamic components of binding of each region are as follows: (a) regions I and II, binding entropy is more favorable while the enthalpy is less favorable. In region I the entropic gain is greater than the enthalpic loss resulting in a net gain in binding affinity, (b) region III, loss in both enthalpy and entropy, (c) region IV, enthalpy is more favorable but not sufficient to overcome the associated loss in entropy, (d) region V, enthalpy gains are able to overcome the entropy loss and (e) region VI, both enthalpy and entropy are favorable.

The enthalpic gain of peptide **3** is not sufficient to overcome the entropic loss, resulting in a lower binding affinity compared to peptide **1**. The enthalpic loss associated with the modifications of peptides **8** and **17** is not offset by the entropic gain leading to slightly reduced binding affinity. Peptide **7** has a slightly higher binding affinity as the enthalpic gain is greater than the entropic loss. Finally, peptide **18** shows both an enthalpic and an entropic gain. This profile explains the increase in binding affinity of peptide **18** as compared to peptide **1**. From the TOP analysis, we conclude: (a) the β -branching with the Val_(P+2) substitution in peptide **7** results in an enthalpic gain derived from the hydrophobic contact with the Glu₁₆₉₈ side chain on BRCT. This gain is sufficient to offset the entropic loss due to the lack of a previously described intramolecular hydrogen bond in peptide **8**¹⁷ and (b) the carboxylic acid at the C-terminus in peptide **18** is a significant contributor to this interaction as both an enthalpic and entropic gain is observed with this substitution.

Thermodynamics of the BRCT(BRCA1)-pSPXF interactions

The origin of the differential BRCT(BRCA1) affinities of the pSPXF–CONH₂ and –CO₂H peptide pairs (**1** and **18**, **7** and **19**) (see supplementary material, **Table S1**) has been dissected using the thermodynamic signatures derived from ITC. The similar thermodynamic profile in all four tetrapeptides (**1**, **7**, **18** and **19**, **Figure 3**) suggests that the binding is anchored by pS and F contacts, consistent with reported studies of the longer peptides.^{17, 24}

Peptides **18** and **19** with the carboxylic acid terminus display higher binding affinity than the carboxamide peptides **1** and **7**. More importantly, the binding affinity of the tetrapeptide **18**

Ac-pSPTF-CO₂H ($K_d = 0.19 \pm 0.03 \mu\text{M}$) is now comparable to the decapeptide Ac-SRSTpSPTFNK-CONH₂ ($K_d = 0.44 \pm 0.06 \mu\text{M}$, our data)¹⁷ and the recently reported 13-mer peptide ISRSTpSPTFNKQT ($K_d = 0.41 \pm 0.02 \mu\text{M}$).²⁴ A crude extrapolation of this result suggests that Abraxas (mimicked by peptide **18**), a BRCA1 binding partner, with the pSPTF motif at the C-terminus, probably binds BRCT(BRCA1) with a higher affinity compared to BACH1 (mimicked by the 13-mer peptide), another BRCA1 binding partner, with the pSPTF motif in the middle of the protein.

Calorimetric titrations at different temperatures (298K - 310K, see supplementary material **Table S1** and **Figure S4** for the raw data and binding isotherms respectively) were performed to probe the effect of temperature on the binding energetics of Ac-pSPTF-CONH₂ and Ac-pSPTF-CO₂H. Linear Van't Hoff plots (eq. 1) for peptides **1** and **18** (supplementary material **Figure S5A**) yield H_{vH} values of $-14.24 \pm 1.26 \text{ kcal}$ and $-16.45 \pm 3.25 \text{ kcal}$, respectively. This analysis shows that the -CO₂H modification in peptide **18** results in a ~1-2 kcal enthalpy driven binding event.

$$\ln K_d = -\Delta H/RT + \Delta S/R \quad (\text{eq. 1})$$

$$\Delta C_p = d\Delta H/dT \quad (\text{eq. 2})$$

The change in heat capacity at constant pressure, ΔC_p , for the BRCT-tetrapeptide complex formation was determined using the standard thermodynamic relationship (eq. 2). The heat capacities for the tetrapeptides **1** and **18** were calculated from the slopes obtained by fitting the plot of ΔH as a function of temperature (supplementary material **Figure S5B** and **S5C**). The ΔC_p values (Ac-pSPTF-CONH₂ = $-303 \pm 27 \text{ cal mol}^{-1}\text{K}^{-1}$ and Ac-pSPTF-CO₂H = $-350 \pm 69 \text{ cal mol}^{-1}\text{K}^{-1}$) of both peptides are comparable, suggesting similar binding modes. These values are also comparable to the previously reported value for the 13-mer peptide (ISRSTpSPTFNKQT = $-340 \pm 30 \text{ cal mol}^{-1}\text{K}^{-1}$),²⁴ further establishing that the tetrapeptide is the minimal unit required for BRCT(BRCA1)-peptide binding.

CONCLUSION

A systematic SAR study was carried out in this study which to definition of the following: (a) at least three oxygen atoms are required to make the hydrophilic contact and the side chain of Thr₁₇₀₀ on BRCT(BRCA1) makes this binding site specific for pSer, (b) the conformationally constraining-residue at the P+1 position helps to correctly orient the hydrophilic and hydrophobic contacts on the protein,¹⁷ (c) β -branching at the P+2 position is optimal and (d) a salt bridge with Arg₁₆₉₉ at the P+3 position serves as an additional contact to enhance binding. This result is consistent with a structural study that reported similar findings.¹⁹ We have identified tetrapeptides **18** and **19** with nanomolar binding affinity for BRCT(BRCA1) and have shown that their thermodynamic signatures are identical to longer peptides. These peptides will serve as leads for optimization for future BRCT(BRCA1) inhibitors.

EXPERIMENTAL METHODS

Peptide synthesis

The peptides were synthesized using standard Fmoc-chemistry either in house or by the Tufts peptides core. The peptides were synthesized on Rink Amide NovaGelTM resin (0.25 mmol) (EMD, Gibbstown, NJ) using N- α -Fmoc-protected amino acids (EMD, Gibbstown, NJ) or unnatural N- α -Fmoc protected amino acids (3B Scientific Corporation, Libertyville,

II or Fischer Scientific, Pittsburgh, PA) and TBTU-HOBt coupling chemistry on a Focus XC synthesizer (Aapptec, Louisville, KY). Fmoc-acid (5eq) and TBTU/HOBt (4eq) (Chem-impex international, INC., Wood Dale, IL) were dissolved in 2-3 mL of NMP. DIEA (Sigma, St. Louis, MO) (15 eq) was added to mixture and incubated for 5 min. This mixture was then added to Fmoc-deprotected peptide resin and allowed to couple for 1 h. Each coupling step was monitored using the Kaiser test (Sigma, St. Louis, MO). To avoid derivatives with deletion, after the coupling step the N-terminal extremities were capped with a 5% acetic anhydride (Sigma, St. Louis, MO), 5% DIEA, 5% HOBt, and 85% NMP. After each coupling and deprotection step, the resin was thoroughly washed with DMF, MeOH and DCM. At the end of the synthesis, the N-terminus of the desired peptide was acetylated as described above. The peptides were then cleaved from the resin using trifluoroacetic acid (TFA) (Sigma, St. Louis, MO)/TIS (Sigma, St. Louis, MO)/water (95:2.5:2.5) over a 3 h period. The crude peptides were precipitated in cold ether and air-dried overnight. Purification was performed on a preparative Agilent LC system (Agilent Technologies, Santa Clara, CA) using an Agilent C18 reverse-phase column Zobrax 300SB-C18 (21.2×150 mm, 5 micron). Buffer A was water with 0.05% TFA and buffer B was acetonitrile with 0.05% TFA. Gradient was buffer B from 5 to 40% in 20 min then 40 to 100% in 5 min at 20 ml/min flow rate. The peptide fractions were lyophilized on a sharp freeze -110 (Aapptec, Louisville, KY). The purity of the peptides were determined by HPLC analysis with a Agilent C18 reverse phase column (4.6×50 mm, 3.5 micron) with similar buffers but a gradient from 5 to 50 B in 20 min and a gradient from 50 to 100 B in 5 min with a 1 ml/min flow rate. Electrospray mass spectrometry was carried out on an Agilent HPLC-MS system. Amino acid analyses were performed on a model Hitachi L-8800 amino acid analyzer.

Fluorescence Polarization assay

The assays were carried out in 384-well low volume Corning plates. The polarization and fluorescence were measured on a Spectramax M5 (Molecular Devices) plate reader. The peptides were titrated into a mixture of protein (1000 nM) and fluorescently labeled tetrapeptide (100 nM). The data was processed using SigmaPlot 11.0., and the IC₅₀ values were determined by non-linear least square fitting.

Isothermal Titration Calorimetry

A Microcal VP-ITC instrument was used to measure heat changes arising from the protein-peptide interaction. All experiments were carried out at 25°C (except the temperature dependent studies). The protein was dialyzed three times for 2 h each and the buffer after the third dialysis was used to fill the reference cell and to dissolve the peptides (50mM KH₂PO₄ pH = 7.2). All solutions were degassed with stirring for 10 min using the Thermovac and filtered through a 2 m filter before loading into syringe or cell. The peptides (100 – 400 μM) were loaded in the syringe and the cell was charged with the protein (10 – 20 μM). The first injection of 3 μL of peptide into protein was followed by 29 injections of 10 μL / injection, every 3 minutes. The solution was constantly stirred at 307 rpm. Dilution curves were obtained by titrating the peptide into buffer under similar conditions. This curve was subtracted from the sample curve to get the corrected heats obtained for the reaction. The data obtained was integrated using Origin 7 software supplied with the Microcal instrument.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BRCA1	BRCA1 Cancer susceptibility gene 1
BRCT	BRCA1 Carboxy Terminal
HTS	High Throughput Screening
BACH1	BRCA1 Associated Carboxy Helicase 1
FP	Fluorescence Polarization
ITC	Isothermal Titration Calorimetry
SAR	Structure Activity Relationship
TOP	Thermodynamic Optimization Plot
PPII	Poly-L-Proline type II

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Figure 1.

A and B are two orientations of the pSPTF-BRCT(BRCA1) binding interface. The tetrapeptide is shown as green sticks while the protein is shown in blue. The potential hydrophilic contacts are shown by broken dotted lines and the hydrophobic contacts are shown either as surface or by dots. The red double headed arrow shows the contact made by the β-carbon on pSer with the Thr₁₇₀₀ side chain on BRCT. (PDB ID: 1T29)

Figure 2.

Thermodynamic optimization plot (TOP): The optimization line was generated using two experimental data points ($-T\Delta S$, ΔH) and (0, ΔG) for peptide **1**. Horizontal and vertical lines were drawn through the ($-T\Delta S$, ΔH) data point of peptide **1** to establish the six regions labeled I to VI. Peptides that fall in regions I, V and VI result in the gain of binding affinity while those that fall in regions II, III and IV result in loss of binding affinity.

Figure 3.
 (A) Graphical representation of the thermodynamic parameters of the pSPXF peptides. (B) Representative binding isotherm of peptide **18**.

Table 1

SAR of tetrapeptides

#	Peptide	IC ₅₀ (μM)
1	Ac-pSPTF-CONH ₂	4.6 ± 0.9
2	Ac-EPTF-CONH ₂	> 250
3	Ac-γcEPTF-CONH ₂	52.8 ± 1.6
4	Ac-pTPTF-CONH ₂	>250
5	Ac-pSATF-CONH ₂	15.0 ± 1.7
6	Ac-pSPAF-CONH ₂	35.0 ± 7.9
7	Ac-pSPVF-CONH ₂	3.2 ± 0.8
8	Ac-pSPSF-CONH ₂	30.1 ± 7.2
9	Ac-pSPIF-CONH ₂	7.1 ± 1.4
10	Ac-pSPLF-CONH ₂	18.4 ± 1.8
11	Ac-pSPT ² Nap-CONH ₂	17.7 ± 2.3
12	Ac-pSPT ¹ Nap-CONH ₂	171.7 ± 10.2
13	Ac-pSPTY-CONH ₂	14.9 ± 2.8
14	Ac-pSAAF-CONH ₂	98.4 ± 23.1
15	Ac-pSPPF-CONH ₂	> 250
16	Ac-FTApS-CONH ₂ (All D Amino Acids)	>250
17	H-pSPTF-CONH ₂	10.8 ± 1.4
18	Ac-pSPTF-COOH	1.0 ± 0.2
19	Ac-pSPVF-COOH	1.6 ± 0.3