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Macrocyclization in the Design of a Conformationally Constrained Grb2 SH2 Domain Inhibitor

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Abstract—Grubbs' olefin metathesis reaction was utilized to prepare a macrocyclic variant of a linear Grb2 SH2 domain antagonist in an attempt to induce a β-bend conformation known to be required for high affinity binding. In extracellular Grb2 SH2 domain binding assays, the macrocyclic analogue exhibited an approximate 100-fold enhancement in binding potency relative to its linear counterpart. The macrocycle was not as effective in whole cell binding assays as would be expected based on its extracellular binding potency.

Src homology 2 (SH2) domains are phosphotyrosyl (pTyr) motif-binding proteins, which serve as important mediators of tyrosine kinase (PTK)-dependent signal transduction. Among SH2 domains, the Grb2 family participates in ErbB-2 (HER-2/neu) signalling which has been implicated in the etiology of certain breast cancers. Because disruption of SH2 domain complexes potentially represents a new therapeutic approach, development of Grb2 SH2 domain inhibitors has become an important area of research.[1,2] Binding of natural pTyr-containing ligands to Grb2 SH2 domains takes place in type-1 β-bend fashion.[3] Previous reports have disclosed high affinity β-bend mimicking tripeptide platforms such as **1**,[4] which contain both pTyr and critical pY + 2 Asn residues. We envisioned that modification of **1** through incorporation within macrocyclic structures exemplified by **2** could potentially increase affinity by introducing conformational constraints approximating those needed for binding. To examine this concept, analogue **3** was designed as a synthetically simplified variant lacking functionality at the tyrosyl α-position and containing an enzymatically stable phosphonate-based phosphate mimetic[5] (Fig. 1).

In order to approximate in macrocycle **3**, the Grb2 SH2 domain-binding β-bend conformation of platform **1**, important design considerations included selection of appropriate length and absolute configurations of points of attachment, of the ring-closing segment. While molecular modeling studies clearly indicated the necessity of (*S*,*R*) configurations respectively, at the naphthylpropyl and pTyr mimetic ring junctions, optimal chain length was more ambiguous. Although a two-carbon spacing appeared to orient the naphthyl ring too proximal to the tyrosyl ring and out of alignment with the hydrophobic protein binding region, a three-carbon chain length seemed to be suitable on both of these considerations (Fig. 2).

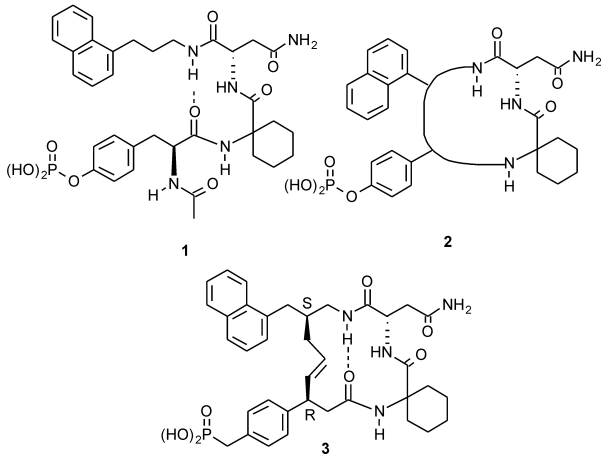

Figure 1. Structures of linear and macrocyclic ligands.

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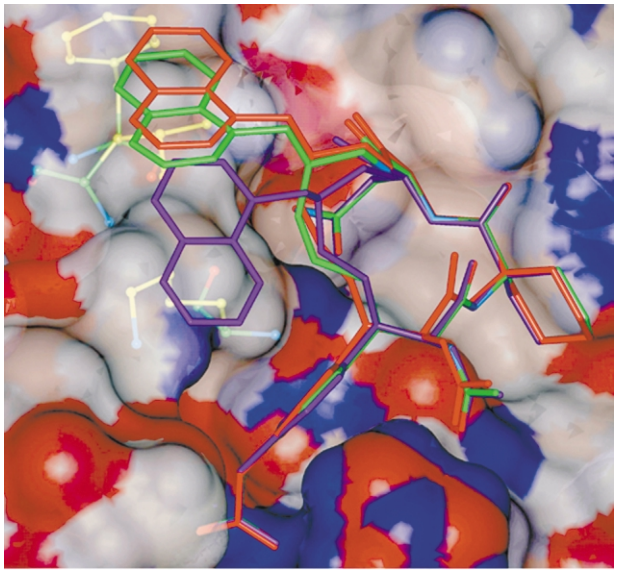
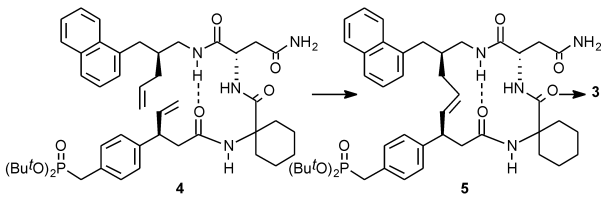


Figure 2. Molecular modeling simulations of Grb2 SH2 domain-bound macrocyclic variants of N$^{\alpha}$-acetyl-containing **1** showing effects of varying the ring-closing segment. Purple: two-carbon spacer; green: three-carbon spacer without double bond; red: three-carbon spacer with double bond.

The proven ability of Grubbs' olefin metathesis reaction to generate large macrocyclic peptides,[6] led us to adapt this approach toward ring closure. Accordingly, protected open-chain **4** was subjected to ruthenium-catalyzed metathesis, with resulting tertiary butyl protected **5** being deprotected (trifluoroacetic acid) and purified by preparative HPLC to yield final product **3** (Scheme 1).[7,8] One synthetic consequence of the olefin metathesis reaction was the introduction of a double bond at the site of juncture. Molecular modeling studies indicated that the presence or absence of this unsaturation would not significantly affect either the placement of the naphthyl ring or the overall orientation of the macrocycle (Fig. 2).

Effect of Deleting N$^{\alpha}$-Functionalization

Structure–activity studies have shown that important interactions of small molecule/peptide ligands with SH2 domains occur within the pTyr-binding pocket.[9] For the Grb2 SH2 domain, key binding interactions exist between negatively charged oxygens on the pTyr phosphate moiety and positively charged Arg αA2 and Arg βB5 guanidino groups.[2] Additional important ligand interactions with the Arg αA2 residue also originate from functionality appended to the tyrosyl α-amino group. Changes in these latter interactions induced by



Scheme 1. Synthetic approach toward macrocycle **3**.

variation of N$^{\alpha}$-derivatization can significantly affect binding potencies of Grb2 SH2 domain ligands.[2] Exemplary of this, we have previously disclosed differences in Grb2 SH2 domain binding potencies of open-chain N$^{\alpha}$-acetyl-containing **6** versus N$^{\alpha}$-oxalyl-containing **7**, wherein molecular modeling studies attributed enhanced potency of **7** to interactions of its N$^{\alpha}$-oxalyl group with the Arg αA2 residue.[10] In the current study, where the primary objective was induction of global conformational constraint, deletion of N$^{\alpha}$-functionality seemed an acceptable means of facilitating synthetic objectives, in spite of the fact that this would result in a loss of critical binding interactions. Des-amino analogue **8** was therefore prepared as an open-chain reference with which to compare the final macrocycle (**3**).

To examine the Grb2 SH2 domain-binding affinity of synthetic ligands, an ELISA-based assay was employed as described.[11] Similar to our previous observations,[10] N$^{\alpha}$-functionalization had a pronounced effect on binding potency. In the current experiments N$^{\alpha}$-acetyl-containing **6** ($IC_{50} = 0.055\,\mu M$) was less potent than the N$^{\alpha}$-oxalyl-containing congener **7** ($IC_{50} = 0.02\,\mu M$). Furthermore, important binding interactions were lost by removal of N$^{\alpha}$-functionalization, as des-amino analogue **8** ($IC_{50} = 2\,\mu M$) exhibited an approximate 100-fold loss of potency relative its N$^{\alpha}$-oxalyl counterpart **7** (Fig. 3).

Effect of Macrocyclization in Extracellular Binding Assays

The primary intent of the current study was to examine macrocyclization as an approach toward enhancing Grb2 SH2 domain binding potency. Preliminary molecular modeling experiments indicated that target macrocycle **3** should bind to the Grb2 SH2 domain in a fashion similar to its open-chain homologues. Binding enhancement was envisioned to arise from macrocycle-induced restrictions in solution conformations of unbound **3** relative to open-chain flexible parent **8**, such that a larger population would exhibit conformations appropriate for binding. Resulting reductions in entropy penalties incurred on binding of **3** were anticipated to be reflected in increased binding affinity. In extracellular ELISA binding studies, macrocycle **3**

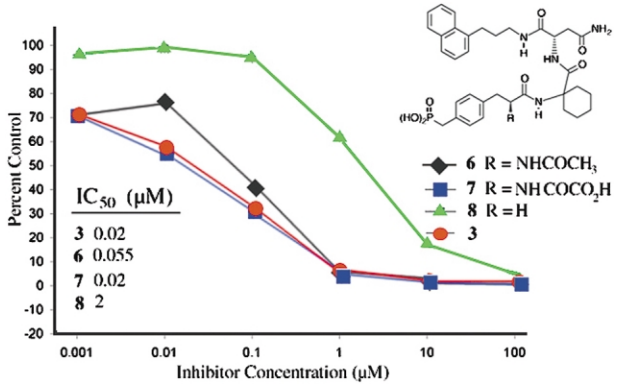


Figure 3. ELISA Grb2 SH2 domain binding assay of indicated compounds. Note: results from compound **6** are from a separate experiment.

exhibited a binding affinity ($IC_{50} = 0.02\,\mu M$), which was approximately 100-fold more potent than open-chain reference compound **8** (Fig. 3). Therefore, macrocyclization effectively restored binding affinity originally lost in the open-chain analogue **8** by removal of N^{α}-oxalyl functionality.

Grb2 SH2 Domain Binding Studies in Whole Cell Assays

The above ELISA results reflect the ability of synthetic ligands to inhibit binding of small phosphopeptides to isolated Grb2 SH2 domain fusion protein. Of greater interest however, is a measure of inhibitory potency in whole cell assays, where full length Grb2 interacts with native protein and where considerations of membrane transport come into play. Accordingly, ErbB-2 overexpressing human breast cancer cells (MDA-MB-453), were treated with inhibitors (25 μM) for 3 h and intracellular binding of Grb2 binding determined from cell lysates as previously described.[10,11] As shown (Fig. 4), relative intracellular binding potencies of N^{α}-acetyl (**6**) and N^{α}-oxalyl (**7**) containing analogues are consistent with binding affinities determined in cell-free assays using ELISA analysis (Fig. 3). Surprisingly however, macrocycle **3** exhibited significantly reduced potency relative to either **6** or **7**, showing much poorer intracellular efficacy than would be expected based on extracellular binding affinity against fusion protein (Fig. 3).

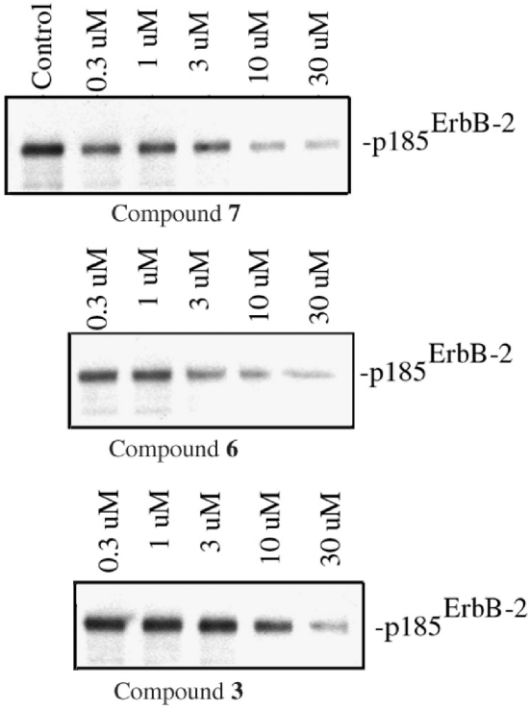


Figure 4. Inhibition of Grb2 SH2 domain binding in whole cells. Cells were treated with inhibitor at the indicated concentrations prior to stimulation with growth factor. Cells were then washed, lysed, and immunoprecipitated with anti-Grb2 antibody, then pTyr Western blots against ErbB-2 protein were run.

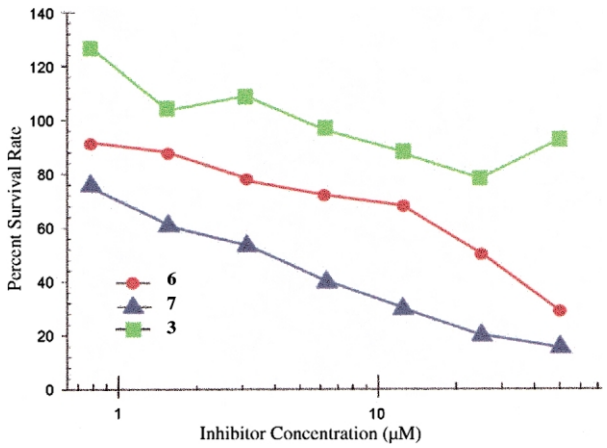


Figure 5. Effect of Grb2 inhibitors on growth of MDA-MB-453 cells.

Cell Growth Inhibition

Finally, it was of interest to examine the ability of macrocycle **3** to inhibit the growth of human breast cancer cells, which are mitogenically driven through the ErbB-2 pathways. For these studies, MDA-MB-453 cells were plated into 24-well plates and treated with Grb2 inhibitors at appropriate concentrations for 8–10 days, then cell number was determined by direct counting. As shown (Fig. 5), both N^{α}-acetyl (**6**) and N^{α}-oxalyl (**7**) containing inhibitors were able to inhibit cell growth in dose dependent fashions consistent with results of both extracellular ELISA assays and whole cell immunoprecipitate assays. Surprisingly, macrocycle **3** showed much reduced potency relative to either **6** or **7**, similar to intracellular binding results (Fig. 4).

Reported herein is the first example of a macrocyclic Grb2 SH2 domain inhibitor designed to examine the effects of global conformational constraint. Using a structurally simplified tripeptide platform in which the terminal pTyr-mimicking residue lacked N^{α}- functionality, a model macrocycle was prepared based on molecular modeling design considerations, which sought to induce a β-bend conformation similar to that needed for Grb2 SH2 domain binding. Consistent with expectations, macrocyclization resulted in a large enhancement of binding potency, as measured in an extracellular ELISA assay. Surprisingly however, intracellular potency of the macrocycle as measured in terms of both Grb2 SH2 domain binding and ErbB-2 dependent cell growth inhibition, were significantly less than expected based on extracellular binding results. The reasons for these discrepancies are unclear and further work is in progress to explore the potential utility of macrocyclization in the design of inhibitors directed against Grb2 and other families of SH2 domains.

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References and Notes

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