Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 347–352

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

MONOCARBOXYLIC-BASED PHOSPHOTYROSYL MIMETICS IN THE DESIGN OF GRB2 SH2 DOMAIN INHIBITORS

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Received 16 September 1998; accepted 14 December 1998

Abstract: Three monocarboxylic-containing analogues, O-carboxymethyltyrosine (cmT, **5**), 4-(carboxymethyl)phenylalanine (cmF, **6**), and 4-(carboxydifluoromethyl)phenylalanine (F_2cmF, **7**) were utilized as phosphotyrosyl (pTyr) replacements in a high affinity ß-bend mimicking platform, where they exhibited IC_{50} values of 2.5 μM, 65 μM and 28 μM, respectively, in a Grb2 SH2 domain Biacore binding assay. When a terminal Nα-oxalyl axillary was utilized to enhance ligand interactions with a critical SH2 domain Arg67 residue (αA-helix), binding potencies increased from 4- to 10-fold, resulting in submicromolar affinity for cmF (IC_{50} = 0.6 μM) and low micromolar affinity for F_2cmF (IC_{50} = 2 μM). Cell lysate binding studies also showed inhibition of cognate Grb2 binding to the $p185^{erbB-2}$ phosphoprotein in the same rank order of potency as observed in the Biacore assay. These results indicate the potential value of cmF and F_2cmF residues as pTyr mimetics for the study of Grb2 SH2 domains and suggest new strategies for improvements in inhibitor design.

Src homology 2 (SH2) domains are protein motifs that recognize and bind with high affinity to phosphotyrosyl (pTyr, **1**)-containing proteins. These modules serve critical roles in protein-tyrosine kinase (PTK) pathways by facilitating protein-protein oligomerization in response to specific signalling events.[1] The growth factor receptor-bound 2 (Grb2) SH2 domain is a key link in mitogenic Ras pathways, functioning as a bridging element between cell surface growth factor receptors and the Ras protein. Grb2-mediated activation of the Ras pathway is highly relevant to a number of diseases, including breast cancer, where members of the epidermal growth factor receptor (EGFR) PTK family such as erbB-2 (HER-2/neu) are frequently over-expressed.[2] Progress in the development of novel high affinity Grb2 SH2 domain ligands[3-5] is typified by **11**,[6] whose design is based on binding of a larger pTyr-containing peptide to the Grb2 SH2 domain in ß-bend fashion.[7] While these inhibitors take advantage of high affinity interactions with SH2 domains outside the pTyr-binding pocket, finding suitable phosphatase-resistant replacements for the pTyr residue itself has continued to be a challenge.[8] In the area of non phosphorus-containing phosphate alternatives, analogues (**9**) of 4,5-dideoxyshikimate-3-phosphate (**8**) first demonstrated the utility of the malonate group as a phosphate mimetic.[9] Based on this work, the dicarboxylic-containing O-malonyl-L-tyrosine (OMT, **4**)[10] was prepared as a non phosphorus-containing pTyr mimetic. Although OMT residues are moderately potent against a variety of SH2 domains,[11] they share with parent pTyr the disadvantage of two negative charges. As reported herein, efforts are therefore underway to identify monocarboxylic-based pTyr mimetics for use in Grb2 SH2 domain antagonists.

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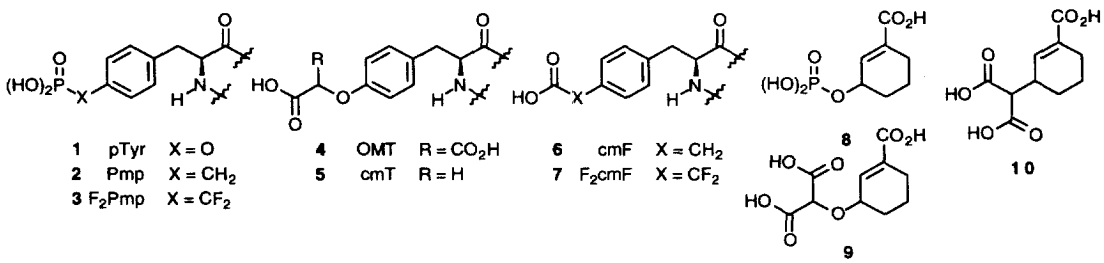
PII: S0960-894X(98)00740-9



Materials and Methods

Inhibitors. Phosphotyrosyl mimetics 4-(O-carboxymethyl)-L-tyrosine (cmT, **5**),[12] 4-(carboxymethyl)phenylalanine (cmF, **6**) and 4-carboxydifluoromethyl-L-phenylalanine (F_2cmF, **7**)[13] were prepared with orthogonal protection suitable for Fmoc-based chemistry, and incorporated into final ß-bend mimicking structures bearing terminal N^{α}-acetyl groups (**12**, **13**, and **14**, respectively) and oxalyl groups (**15**, **16** and **17**, respectively) in manners similar to that described.[14]

SH2 domain using Surface Plasmon Resonance (SPR). SPR was used to measure IC_{50} values for inhibition of Grb2 SH2 domain binding. IC_{50} values were determined by mixing inhibitor with recombinant Grb2 SH2 domains and measuring the amount of binding at equilibrium to an immobilized SHC phosphopeptide in a manner similar to that previously reported.[15]

Inhibition of Grb2 SH2 domain binding in cell lysates. Cell lysates were prepared from serum-treated erbB-2 overexpressing breast cancer cells (MDA-MB-453) using 1% TritonX-100 in PBS containing 0.2 mM $NaVO_4$. Lysates were incubated with 20 µM concentrations of inhibitory compounds for 30 minutes. Grb2 and associated Grb2-binding proteins were immunoprecipitated from each lysate (500 µg) with anti-Grb2 antibodies and collected using protein A sepharose using methods previously described.[16] Immunoprecipitated proteins were separated by SDS PAGE on 4-20 % gradient gels (Novagen). pTyr containing proteins were detected by Western blotting using antiphosphotyrosine antibodies (Upstate Biochemicals Inc.). Previous experiments have shown that a major tyrosine phosphorylated protein in these cells is the $p185^{erbB\text{-}2}$, which is overexpressed as a consequence of gene amplification.[17]

Results and Discussion

Monocarboxylic pTyr mimetics in Grb2 SH2 domain ligands. The first monocarboxylic analogue examined, cmT (**5**), was derived by simple deletion of one carboxyl from OMT (**4**).[12] A distinguishing feature of **5** is the presence of an ether oxygen linking the carboxymethyl group to the phenyl ring. Substitution of cmT for pTyr in the previously disclosed high affinity platform **11**[6] gave **12**, which exhibited a Grb2 SH2 domain affinity (IC_{50} = 65 µM, Figure 1) approximately 1000-fold less than that of the pTyr-containing parent **11** (IC_{50} = 0.07 µM[14] and 0.047 µM[6]). Molecular modelling confirmed that the bridging ether oxygen of the cmT residue (**5**), which joins the carboxymethyl group to the phenyl ring, orients the carboxylic group in the SH2 domain pTyr binding pocket in a suboptimal fashion (Figure 2).

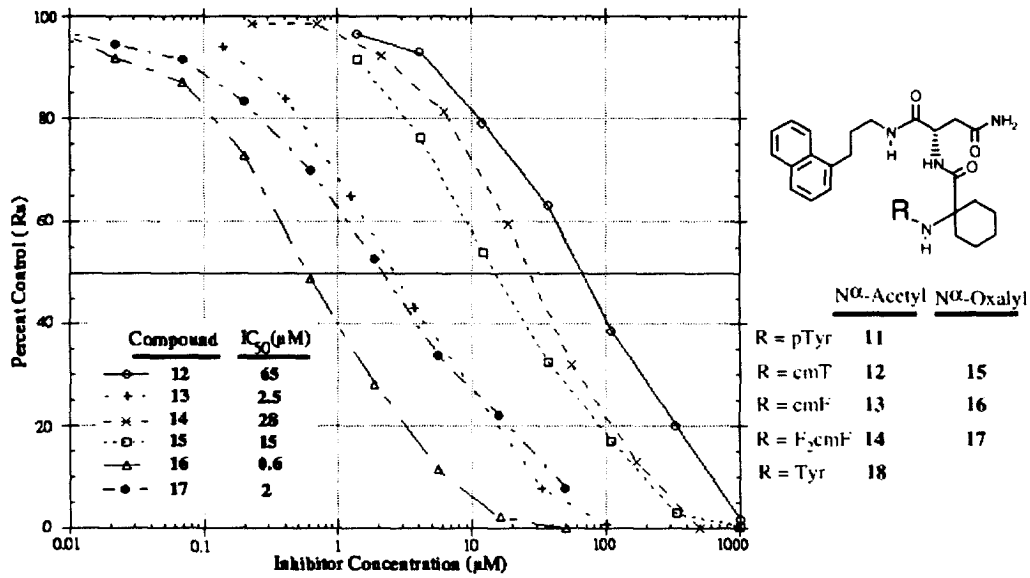


Figure 1. Biacore binding curves determined as explained in the Materials and Methods section. Under similar conditions pTyr-containing **11** and Tyr-containing **18** gave IC_{50} values of 0.07 μM and >>100 μM.[14]

The cmF residue as a pTyr mimetic. Sikorski had earlier found in the 4,5-dideoxyshikimate-3-phosphate series, that removal of the bridging ether oxygen (going from compound **9** to compound **10**) significantly reduced inhibitory potency.[9] In our study, removal of the ether oxygen from cmT (**5**) gave cmF (**6**). Previously (+/-)-cmF has been used as a pTyr mimetic in a src SH2 domain-directed peptide, where it exhibited an IC_{50} value of 940 μM.[18] More recently, a similar peptide containing L-cmF has been shown to bind to the $p56^{lck}$ SH2 domain with K_d of 42 μM.[19] In both of these reports an approximate 400-fold loss of potency was observed for the L-cmF residue relative to the pTyr-containing parent. Consistent with recent X-ray results of a cmF residue bound to the $p56^{lck}$ SH2 domain,[19] our molecular modelling studies indicated that alignment of the cmF residue in the Grb2 SH2 domain could achieve good spatial overlap of its carboxylic oxygens with two of the pTyr phosphate oxygens (Figure 2). In accord with these predictions, compound **13** showed an approximate 25-fold increase in binding affinity (IC_{50} = 2.5 μM) relative to ether-containing **12**.

The F_2cmF residue as a pTyr mimetic. X-ray studies of several ligated SH2 domains including src,[20] $p56^{lck}$ [21] and Grb2,[7] have shown the importance of the pTyr phosphate ester oxygen. Loss of interactions with this oxygen has been postulated to partially contribute to the reduced SH2 domain affinity of Pmp residues (**2**), where it has been replaced by a methylene unit.[22,23] As with Pmp, in place of the phosphate ether oxygen, cmF residues employ a methylene unit which could reduce the binding affinity of **13**. In the phosphonate series of pTyr mimetics, fluorines were added to the Pmp bridging methylene (giving F_2Pmp, **3**) to partially restore these lost interactions.[22] An analogous modification of cmF analogue **6** results in the new pTyr mimetic, F_2cmF (**7**).[13] In this case however, contrary to binding enhancement as was previously observed when fluorines were added to Pmp to yield F_2Pmp,[22] introduction of fluorines into the cmF residue resulted in a 10-fold reduction in potency (**14**; IC_{50} = 28 μM) (Figure 1).[24]

The N^{α}-Oxalyl moiety as an auxiliary which enhances Grb2 SH2 domain binding. Thermodynamic,[25] NMR[26] and X-ray[7] analysis Grb2 SH2 domains ligated to pTyr-containing ligands have highlighted key binding interactions between the positively charged Arg67 (αA-helix) and Arg86 (ßC-strand) residues and the two negatively charged phosphate oxygens. One limitation of mono-anionic carboxylic-based pTyr mimetics used in the current study is their inability to faithfully enter into interactions with both of these Arg residues. The tyrosyl α-nitrogen does provide a site from which additional critical interactions with Arg67 as was recently shown n a series of dicarboxylic-based pTyr mimetics where appending an oxalyl moiety to the tyrosyl α-nitrogen resulted in beneficial interaction with the positively charged Arg67 residue.[14] Molecular modelling studies indicated that similar enhanced binding interactions are possible if an N^{α}-oxalyl auxiliary were introduced into the cmF-containing **13** to yield the new inhibitor N^{α}-oxalyl-cmF **16** (Figure 2). Accordingly, a series of N^{α}-oxalyl-containing variants were prepared (**15**, **16**, and **17**) of the three monocarboxylic-based inhibitors **12**, **13** and **14**, respectively. As shown in Figure 1, the N^{α}-oxalyl group uniformly enhanced binding potencies of inhibitors from 4- to 10-fold: **15** (IC_{50} = 15 μM), **16** (IC_{50} = 0.6 μM) and **17** (IC_{50} = 2 μM).

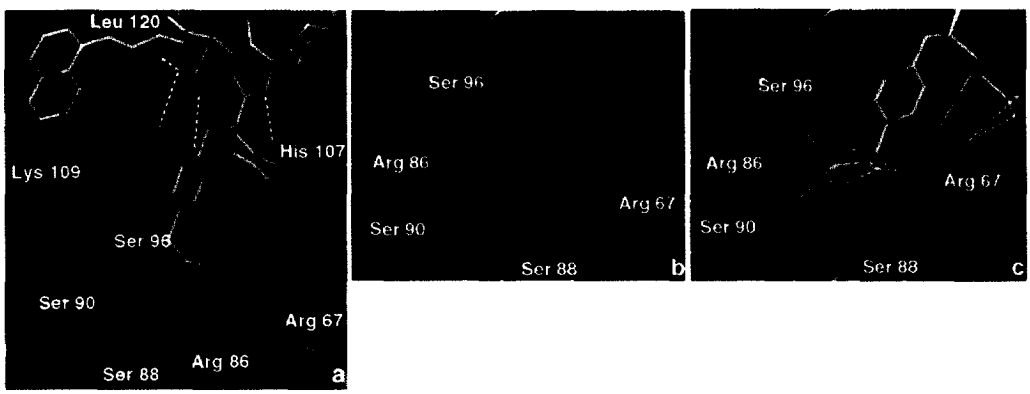


Figure 2. Comparison of the modeling results and the crystal structure of K-P-F-pY-V-N-V-NH_2 bound to Grb2 SH2; the protein backbone atoms are superimposed; heavy atoms of each ligand and the interacting side chains for the respective ligand are depicted; dash-dot line represents weak hydrogen bonds; (a) **12** (red), **13** (magenta) and **16** (yellow); (b) heptapeptide (blue, only Cα and carbonyl group of Phe are shown), **12** (red) and **13** (magenta), hydrogen-bonds between **13** and the protein are omitted; (c) **13** (magenta) and **16** (yellow).

Cell-lysate studies. Biacore data shown in Figure 1 reflect binding of isolated Grb2 SH2 domain fusion protein to a reference phopsho-SHC(Y317) peptide. While such studies are useful indicators of molecular interactions, they are limited since in physiological contexts Grb2 SH2 domains exist as subunits of larger Grb2 proteins that bind to pTyr-containing proteins. Therefore, in order to examine the ability of synthetic analogues to inhibit the interaction of native Grb2 with cognate $p185^{erbB\text{-}2}$ in cells, cell lysate were conducted. MDA-MB-453 cells were used, which are derived from a human breast cancer where there is amplification of *erbB*-2 gene. When Grb2 is immunoprecipitated from these cells, the $p185^{erbB\text{-}2}$ can be detected as a co-precipitating protein. This precipitation requires the Grb2 SH2 domain interaction with pTyr residues on $p185^{erbB\text{-}2}$. As shown in Figure 3 at 20 μM concentration, control (**18**), N^{α}-acetyl-cmT (**12**), N^{α}-acetyl-F_2cmF (**14**) and N^{α}-oxalyl-cmT (**15**)

showed strong p^{185} bands, indicating little inhibition of Grb2 binding to the $p185^{erbB\text{-}2}$. Much weaker p^{185} bands were observed for cells treated with N^{α}-acetyl-cmF (**13**), N^{α}-oxalyl-cmF (**16**) and N^{α}-oxalyl-F_2cmF (**17**), indicating good binding inhibition. The relative order of potencies for the cell lysate study, which reflects inhibition of cognate Grb2 binding to $p185^{erbB\text{-}2}$, is consistent with that observed in the Biacore assays (Figure 1).

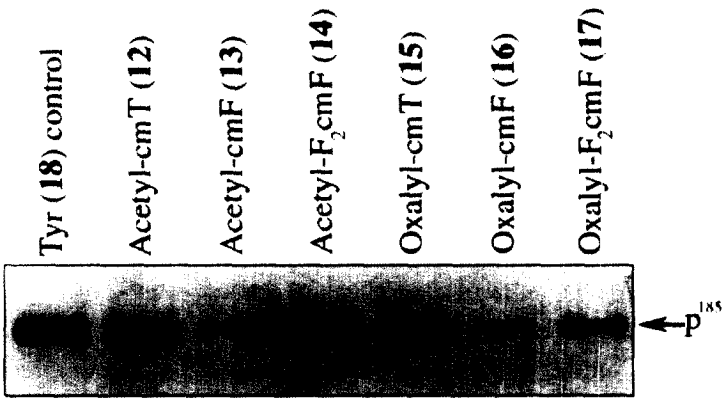


Figure 3. Antiphosphotyrosine blot of cell lysates following incubation of MDA-MB-453 cells with 20 μM of indicated compounds as described in the Materials and Methods section. The p^{185} band represents $p^{185erb\text{-}2}$ which has been co-precipitated using anti-Grb2 antibodies.

Conclusions

In this study three monocarboxylic pTyr mimetics, **5**, **6**, and **7**, have been examined in Grb2 SH2 domain binding systems. When incorporated into a high affinity ß-bend mimicking platform (**13**), cmF exhibited low micromolar affinity in an isolated Grb2 SH2 domain binding assay. The much poorer binding of the cmT residue, in spite of the presence of a key ether oxygen found in the parent pTyr residue, can be attributed to the non optimal positioning of the carboxyl group. A critical part of the current study was to examine the effect of adding fluorines to the cmF carboxymethylene.The failure of F_2cmF to bind with higher affinity than cmF, is unexpected and may suggest that binding interactions at the benzylic methylene position are not critical. A further goal of this work was to examine the utility of the N^{α}-oxalyl auxilary in conjunction with monocarboxy pTyr mimetics. The N^{α}-oxalyl residue uniformly increased binding potencies from 4- to 10-fold and resulted in submicromolar affinity for cmF-containing **16** and low micromolar affinity for F_2cmF-containing **17**. These are among the most potent non-phosphorus containing SH2 domain inhibitors yet reported. Cell lysate binding studies showed that these analogues also exhibited the same rank order of potency when measured against cognate Grb2 and $p185^{erbB\text{-}2}$. These results indicate the potential value of cmF and F_2cmF residues as pTyr mimetics for the study of Grb2 SH2 domains.

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