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RESEARCH ARTICLE

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Piptides: New, Easily Accessible Chemotypes For Interactions With Biomolecules

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Abstract: Small molecule probe development is pivotal in biomolecular science. Research described here was undertaken to develop a non-peptidic chemotype, *piptides*, that is amenable to convenient, iterative solid-phase syntheses, and useful in biomolecular probe discovery. Piptides can be made from readily accessible *pip acid* building blocks and have good proteolytic and pH stabilities. An illustrative application of piptides against a protein-protein interaction (PPI) target was explored. The *Exploring Key Orientations*, EKO, strategy was used to evaluate piptide candidates for this. A library of only 14 piptides contained five members that disrupted epidermal growth factor (EGF) and its receptor, EGFR, at low micromolar concentrations. These piptides also caused apoptotic cell death, and antagonized EGF-induced phosphorylation of intracellular tyrosine residues in EGFR.

Introduction

Small molecule probes that perturb biomacromolecular function are useful in medicinal science.[1] High throughput screening can be used to obtain such probes, but these strategies are not always fruitful, justifiable, or affordable.[2] Screening becomes more efficient if it features chemotypes that can be quickly, reliably, and inexpensively assembled into diverse libraries using combinatorial methods.[3] Such strategies are optimally useful if they can be performed by researchers possessing only basic synthetic skills. Further streamlining may be achieved using "privileged chemotypes" that have a bias to positively interact with the target biomacromolecules.[4]

Peptides are privileged chemotypes because they interact with biomolecules using native pharmacophores (Figure 1a). Cyclization[5] and *N*-methylation[6] strategies can make them even better probes by rendering them more rigid, proteolytically stable, and cell permeable,[6a, 7] but these modifications require more synthetic expertise.

Peptoids[8] (*N*-substituted oligoglycines, Figure 1b) with diverse *N*-substituents are easy to prepare, via solid-phase syntheses,[9] from commercially available amines. However, they tend to be *more* flexible than peptides, which must lose more entropy to interact with a target, hence rarely bind with dissociation constants <1μM.[10] Analogs with enhanced rigidity are known (*eg* cyclic[11] and ones with α-chiral centers[12]) but making them requires well-developed synthetic skills. Moreover, population of both *cis* and *trans* conformers of tertiary amide bonds, of which there are often many, complicate peptoid NMR spectra.

β-Peptides (Figure 1c) share some of the same disadvantages as peptoids, and they tend to be more flexible than peptides. An exception is systems comprised of α,β-amino acids (*eg* cyclic systems), but these cannot be readily functionalized with the full complement of side chains corresponding to genetically encoded amino acids.[13]

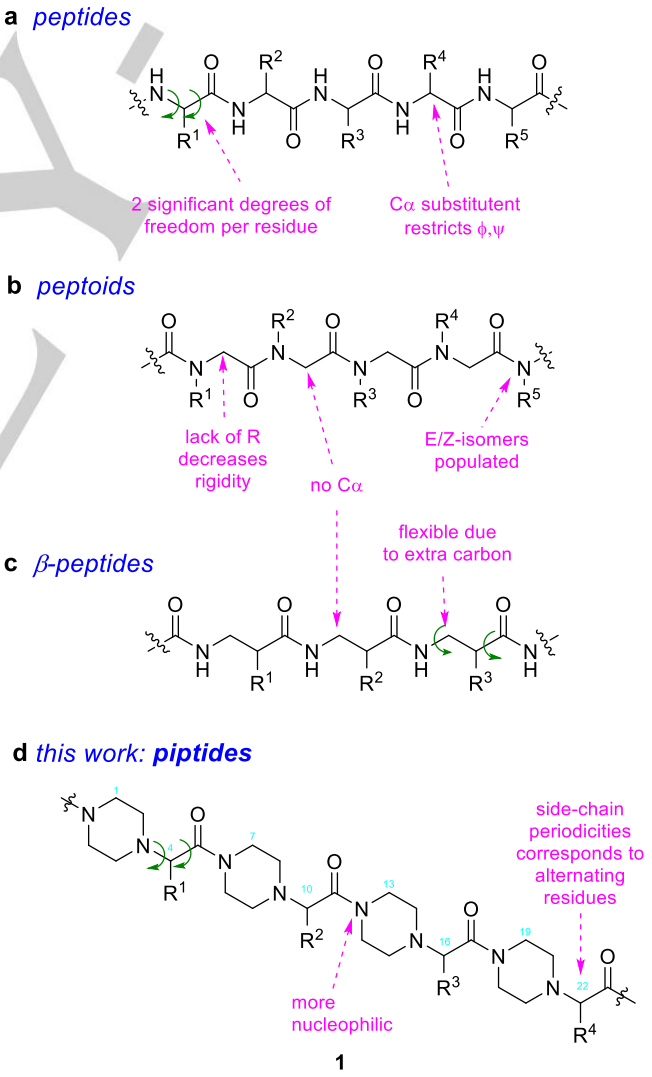


Figure 1. Comparison of **a** peptides, **b** peptoids, **c** β-peptides, and **d** piptides.

Recognizing the limitations outlined above, we set out to establish a new chemotype design with the following unique combination of attributes:

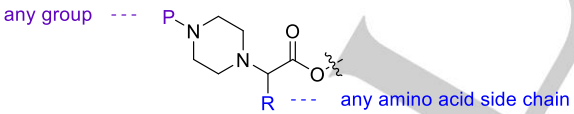
- ease of solid-phase syntheses;
- primary structure diversity that parallels peptides;
- more rigidity (less significant degrees of freedom) than corresponding peptides, peptoids, or side chain functionalized β-peptides;
- monomer repeats that spatially resemble alternate amino acids of the corresponding peptides (*ie* "side chain periodicities" of atomic spacings between side chains);
- incorporation of piperazine units, as in many pharmaceuticals;
- pH characteristics that vary around the physiological range;
- proteolytic and pH stability; and,
- structures that sample unexplored patent space.

It is non-trivial to devise chemotypes that meet all these criteria, but the one featured here does: piptides **1** (Figure 1d).

This contribution illustrates how researchers can design initial hit piptides to perturb protein-protein interactions (PPIs), with epidermal growth factor (EGF) and transforming growth factor-α (TGFα) interacting their receptor EGFR as an illustrative case. EGFR is a validated target for cancer therapeutics.[14] Modalities for regulating signals from EGFR are used in the clinic, but they all tend to feature biologicals (*eg* humanized monoclonal antibodies), or kinase inhibitors.[15] Attempts to block EGF•EGFR or TGFα•EGFR by targeting the PPI interfaces with small molecules have been largely unsuccessful, yet we were able to identify several preliminary hits from a library of only 14 piptide-based compounds.

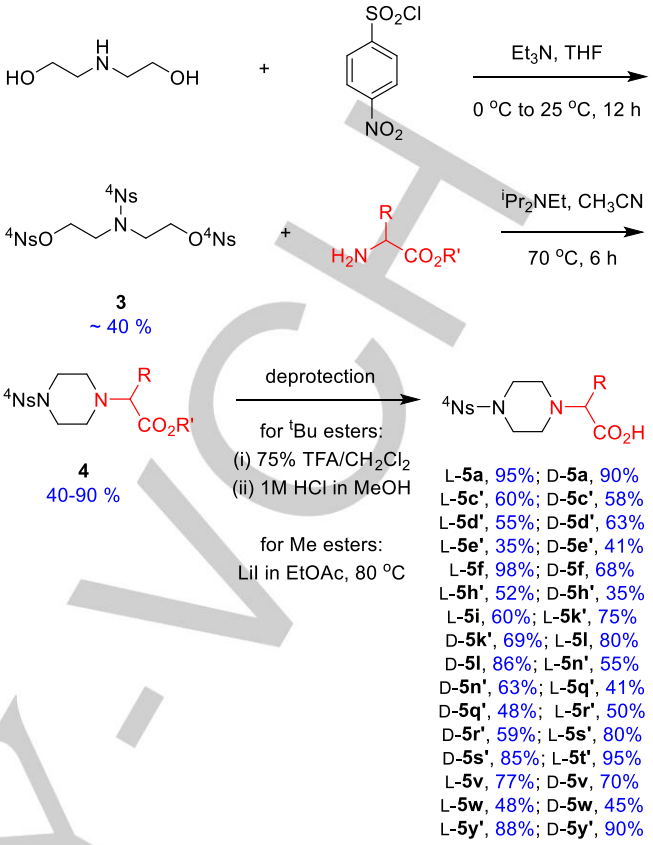
Results and Discussion

Piptides can be constructed from fragments **2,** *pip acids*. Prior to our work there were only two synthetic strategies to a small number of pip acids, and both of these rendered only *racemic* material.[16]



2
pip acid

Tri(4-nitrobenzene sulfonate) {tri-nosyl or tri-^{4}Ns} **3** had been reacted with primary arylamines under microwave conditions to give *N*-aryl,*N*-^{4}Ns-piperazines.[17] Compounds **3** could be converted into a variety of *N*-^{4}Ns-pip acid esters **4**, then selectively deprotected to their *C*-free *N*-protected forms **5** (and, under other conditions, to *C*-protected *N*-free analog **6**, *vide infra*; Scheme 1). These *N*-protected pip acids tend to be solids. They can be made from amino acid building blocks with protected-functionalized side chains, *ie* exactly the ones commonly used in the FMOC approach to peptides,[18] and appropriate for solid-phase syntheses on trifluoroacetic acid (TFA)-sensitive resins. This is important because incorporation of functionalized side chains into peptidomimetic chemotypes can be time-consuming and experimentally tedious.



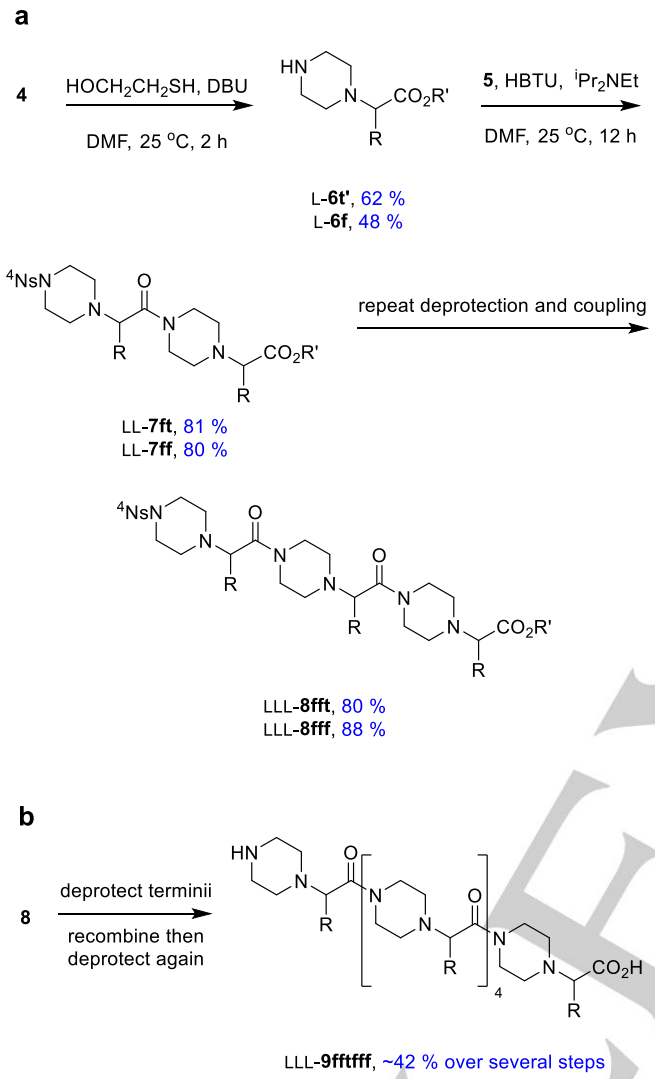
Scheme 1. Generalized syntheses of pip acids. Lower case one-letter codes are used to delineate amino acid side chains (R) and relate them to the closest amino acid; primed letters indicate protected side chains (*eg* **d'** for the –$CH_2CO_2{}^tBu$ of Asp and **k'** for the –$(CH_2)_4NHBoc$ of Lys).

Denosylation of Phe- and Thr(O^{t}Bu)-derived pip acids L-**4f** and L-**4t'**, respectively, gave the piperazines **6** used to initiate trial solution phase syntheses. Several denosylation methods were investigated, and the conditions shown in Scheme 2 were the best found.

(2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[19] was used to activate the *C*-free *N*-protected units **5** to give the *C,N*-diprotected dipiptides **7** then tripiptides **8** (Scheme 2a). Scheme 2b illustrates how piptides with repeating sequences can be made by dividing intermediates like **8**, selective deprotection of either terminus, then recombination.

Solid-phase piptide syntheses may be guided by the following concepts. The protection strategy is similar to common peptide syntheses because side chain-protected amino acids appropriate for FMOC-peptide syntheses[18a] are used to make the functionalized *N*-protected pip acid derivatives **5**. In that case, use of resins functionalized with TFA-sensitive handles allows successive couplings of side chain-protected pip acids with retention of their masking groups. Finally, cleavage of the target piptide with simultaneous side chain deprotection, could be achieved via treatment with scavenger cocktails containing TFA. Uncoupled, supported, piptide *N*-termini are better stained using the chloranil test[20] than via ninhydrin,[21] just as for peptides syntheses where Pro is the *N*-terminal residue. Thus, visual chloranil tests indicate situations in which two or more coupling cycles are necessary to drive the reactions to completion. Denosylation of supported piptides (*cf* for supported peptides[22]), and the coupling reactions used to form them, may

optionally be accelerated using microwave reactors (Scheme 3). Scheme 3 also illustrates how hybrids of piptides, and peptides can be produced easily by coupling α-amino acids to piptide *N*-termini, as in the synthesis of compounds **10** shown.

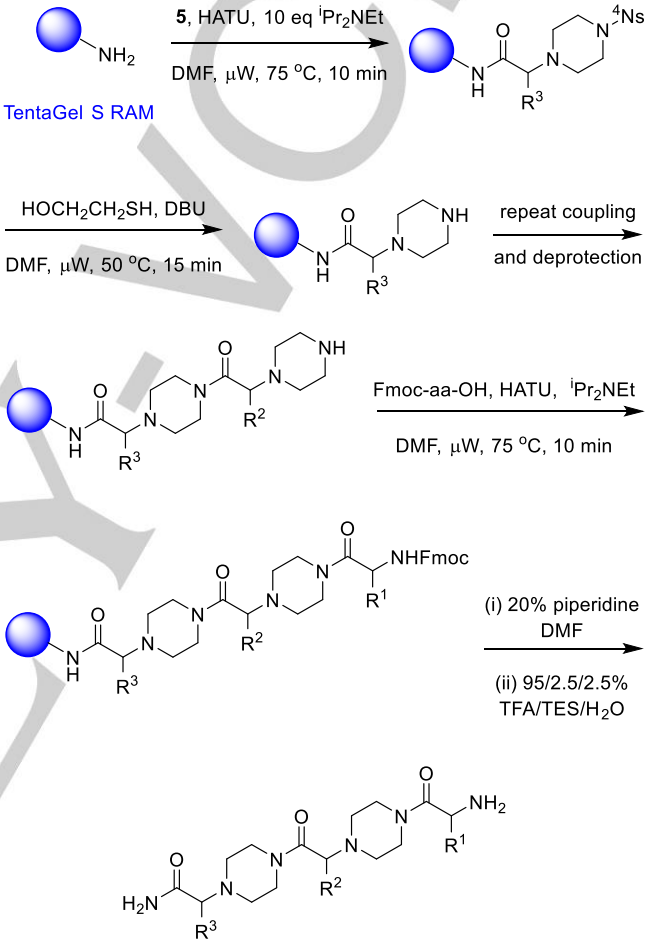


Scheme 2. **a** Linear, and **b** divergent-convergent, solution phase syntheses of piptides.

Isolated yields are indicated in Scheme 3, but these parameters are notoriously variable in solid-phase syntheses due to the small amounts of material cleaved from the support, losses in prep-HPLC purification, and because all yields based on support loading are hard to measure accurately. Analytical HPLC UV/ELSD analyses of crude materials from illustrative solid-phase syntheses are given in the SI; these data show that the purity of piptides cleaved from the resin is high. Moreover, solid-phase syntheses of piptides are not restricted to two or three repeat residues; Scheme S1 shows preparation of a 9-mer piptide.

Two experiments were performed to assess relative stabilities of piptides and peptides towards hydrolysis. To test enzymatic proteolysis, piptide LLL-**10fff** and the closely related peptide LLL-**11fff** (Figure S1a) were incubated at 37 °C in pH 7.4 PBS with Pronase® (a protease mixture used in proteomic studies).[23] The peptide degraded rapidly under these conditions ($t_{1/2}$ ~ 2 h), whereas the piptide showed no significant decomposition after 24 h (Figure S1b). To test for pH-mediated hydrolysis, the same piptide was maintained in aqueous media under acidic, basic, and neutral conditions; it showed essentially no decomposition at low pH but around 30 % decomposition was observed at pH 10 (Figure S1c).

Short piptide-containing sequences such as **10** would not be expected to fold into any preferred conformation in solution. Indeed, circular dichroism spectra collected for LLL-**10ysl** in methanol showed only very weak molar ellipticities, *ie* its CD spectrum was essentially flat (Figure S2).



Scheme 3. Illustrative solid-phase synthesis of piptides on TentaGel S-RAM resin (blue spheres).

Exploring Key Orientations on Secondary structures (EKOS)[24] was used to evaluate biases of preferred conformations of **10aaa**. In EKOS, conformations of the mimic are simulated, preferred ones (within 3 kcal/mol of the lowest energy conformation identified) are systematically overlaid on ideal secondary structures according to their three *C*α – *C*β vectors, and the fit of the superimpositions are evaluated in terms of the root mean square deviations (RMSDs) of the six *C*α and *C*β coordinates involved. We have shown,[24-25] superior secondary structure mimics overlay with RMSD values <0.5 Å. Figure S3 shows **10aaa** is an excellent mimic of strand-turn-strand, and parallel and antiparallel β-sheets. A Ramachandran plot (Figure S4) shows preferred conformers of **10aaa** are

concentrated in a narrow range of ϕ,ψ-bond angles, indicative of conformational rigidity.

Epidermal growth factor receptor (EGFR) is overexpressed at varying levels in most types of cancer cells.[14] High surface densities of EGFR favor dimerization, making cells overexpressing EGFR abnormally sensitive to stimulation by their complementary endogenous protein growth hormones (EGF and TGFα), hence promoting unconstrained cell growth.[26] For instance, secretion of TGFα is associated with various cancer types including breast, lung,[15a] kidney, melanoma, liver,[27] and glioblastomas.[26] In general, there seems to be a causal link between increased EGF or TGFα expression and tumor development.[28] Conversely, blockade of EGFR suppresses tumor cell growth *in vitro* and *in vivo.*

EGFR antagonists used for cancer chemotherapy can be divided into "biologicals" (typically antibodies, *eg* cetuximab, panitumumab, and trastuzumab) that perturb ligand•EGFR interactions,[15a-f] and kinase inhibitors (*eg* gefitinib, erlotinib, lapatinib) that suppress signaling from the receptors.[15e-h] However, small molecule inhibitors of the EGF kinase domain are not ideal probes for EGFR-mediated signaling because they tend to inhibit other kinases. Furthermore, in the clinic, EGFR kinase inhibitors (and even humanized mAbs for this target) are vulnerable to intrinsic and acquired resistance.[15b]

The non-ligated, "inactive" form of EGFR rests in an autoinhibited conformation. On introduction of TGFα or EGF, two of the same protein ligands bind two identical domains along the EGFR periphery causing conformational changes, but the EGFR•EGFR interface is maintained.[29] Mutagenesis of EGF and of TGFα have revealed key residues for interactions with EGFR. For instance, affinity between TGFα and EGFR seems to disproportionately rely on ^{42}Arg and ^{48}Leu of the ligand.[30] A molecular dynamics study[31] and X-ray crystallography (1MOX) has also revealed several residues of TGFα that are involved in hydrogen bonding and salt bridge formation with EGFR, including ^{44}Glu and ^{46}Ala.

In the current study, the EKO strategy[32] was used to evaluate piptide-based chemotypes for their potential to disrupt protein ligand•EGFR interactions. Briefly, EKO compares favored conformations of small molecules that present three amino acid sidechains, with PPI interface regions, based on degree of fit of side chain *Cα* and *Cβ* coordinates. Validation for the strategy has been reported in the context of the HIV-1 protease dimer,[32] the anti-thrombin dimer,[33] uPA•uPAR,[34] and PCSK9•LDLR.[35]

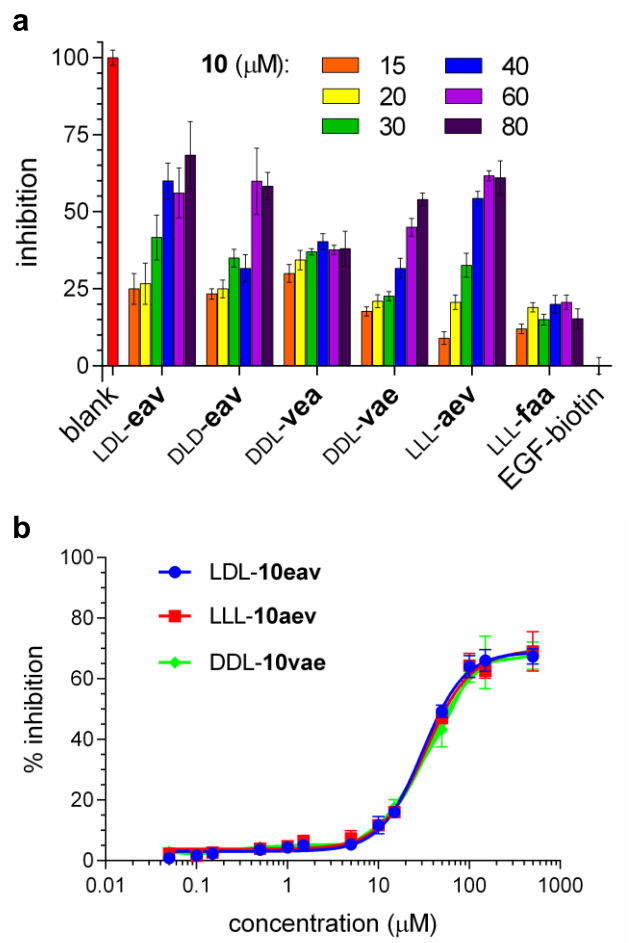


Figure 3. ELISA assays to **a** detect inhibition of binding 10 μM EGF-biotin to EGFR anchored to a microplate, and to **b** determine IC_{50} values.

Here, EKO analyses indicated preferred conformers of chemotype **10** overlaid on EGF or TGFα at the TGFα•EGFR interface (Figure 2). Figures 3b – g illustrates different stereoisomers of **10aaa** overlaid well on ^{39}Val, ^{44}Glu, and ^{46}Ala, in both possible orientations (*ie N*-to-*C*, and *C*-to-*N*). Thus, Figure 2b shows the *C*-terminus of DLD-**10aaa** superimposed on ^{39}Val, whereas DDD-**10aaa** best matched its *N*-terminus on ^{39}Val in Figure 2e. Observations such as these, where "*pseudo*-symmetrical" chemotypes, like **10,** overlay with either *N*-to-*C* polarity, are unsurprising because EKO considers *only* the side chain orientations and not the scaffold core.

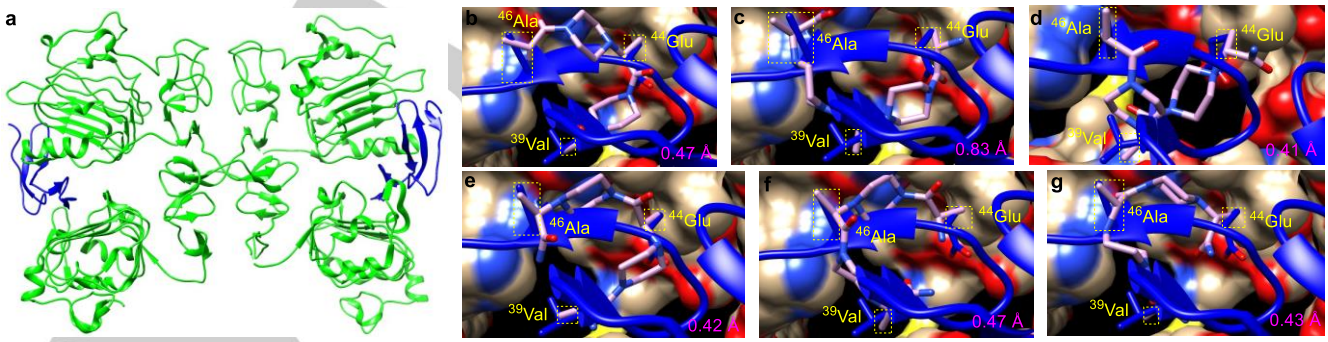


Figure 2. EKO analyses of piptide-based chemotypes on TGFα•EGFR. **a** TGFα•EGFR dimer interface (1MOX). **b-g** EKO implicated stereoisomers of **10aaa** overlaid on TGFα: ^{39}Val, ^{44}Glu, and ^{46}Ala. Note structure 1MOX indicates ^{44}Glu and ^{46}Ala are involved in *H*-bonding with EGFR. Figures in purple represent the root mean squared deviation of mimic and protein-ligand at the six Cα and Cβ coordinates involved.

ELISA primary assays revealed five of the 14 EKO-implicated chemotypes perturbed EGF•EGFR at micromolar concentrations with satisfactory dose-response profiles. Figure 3 calibrates their inhibition of EGF•EGFR relative to a blank with no protein ligand (equivalent to 100% "inhibition" because there is no EGF binding to EGFR) and to 10 μM EGF-biotin (under these conditions EGF-biotin saturates the available EGFR hence "inhibition" is set to 0%). That graphic also includes data for LLL-**10faa**, which is a "partial control" insofar as it has the same chemotype core, but some of the side chains and stereochemistries are *not* ones predicted to be appropriate from the EKO analyses. Compound LLL-**10faa** also has hydrophobic sidechains, which could promote non-specific binding, and, indeed, in the event, that partial control showed only relatively low inhibition with no dose-response (Figure 3a). Concentrations of the three best inhibitors from the primary ELISA were varied at closer intervals to obtain IC_{50} values (μM throughout; Figure 3b): LDL-**10eav**, 30.5 ± 1.2; LLL-**10aev**, 33.3 ± 1.7; DDL-**10vae**, 35.8 ± 2.2.

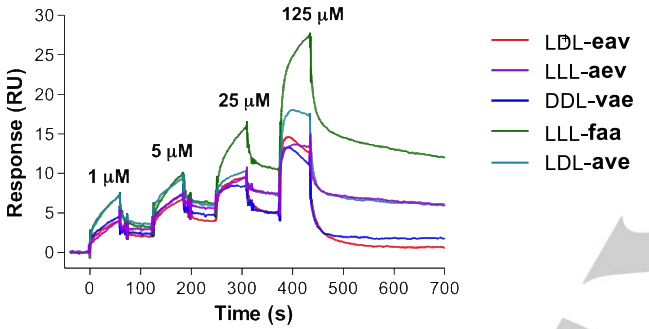


Figure 4. Direct binding of compounds **10** to EGFR immobilized on Biacore sensor chip surface (SPR) shown as a sensorgram.

In SPR experiments, binding of LDL-**10eav** and DDL-**10vae** to EGFR was shown to be reversible as indicated by complete dissociation to baseline level. However, LLL-**10aev** showed more significant secondary binding and aggregation on the receptor surface, as indicated by incomplete dissociation after extended washing. The K_d values calculated from SPR were LDL-**10eav**, 41.1 ± 13.2; LLL-**10aev**, 50.6 ± 13.8; DDL-**10vae**, 30.7 ± 10.2 μM (Figure 4). Competition experiments were performed to complement these direct binding assays. In these, the compounds were tested in competition with 30 nM TGFα or 27 nM EGF on immobilized EGFR. Ratios of K_d values of EGF or TGFα for EGFR with and without the featured compounds were obtained; values greater than 1 indicate diminished binding of the natural protein ligands to EGFR. In the event, the compound predicted to have the lowest K_d for EGFR in the direct binding SPR studies, DDL-**10vae**, had the most negative impact on the binding of TGFα to EGFR in the competitive assay, as expected (Figure S5c).

Cytotoxicity assays were performed on the five hit compounds featured in Figure 5a with the expectation that reduced viabilities would result if these bind EGFR. A549 human lung cancer cells were used in this study because they overexpress EGFR.[36] Figure 5a shows all the five of these featured compounds reduced viability more than the partial negative control LLL-**10faa**, but less than gefitinib (a FDA-approved EGFR kinase inhibitor).[37] Compounds were also tested using HEK293 (EGFR-negative) cells and showed little to no cytotoxicity (Figure S6).

Flow cytometry experiments indicated gefitinib and LDL-**10eav** caused cytotoxicity via early apoptosis (annexin V staining), more than necrosis (propidium iodide; Figure 5b and S7). Western blot assays were performed to determine if an illustrative piptide-based probe impedes EGF-induced pTyr at EGFR. Figure 5c illustrates that phosphorylation could be suppressed almost completely by LDL-**10eav** in competition with EGF.

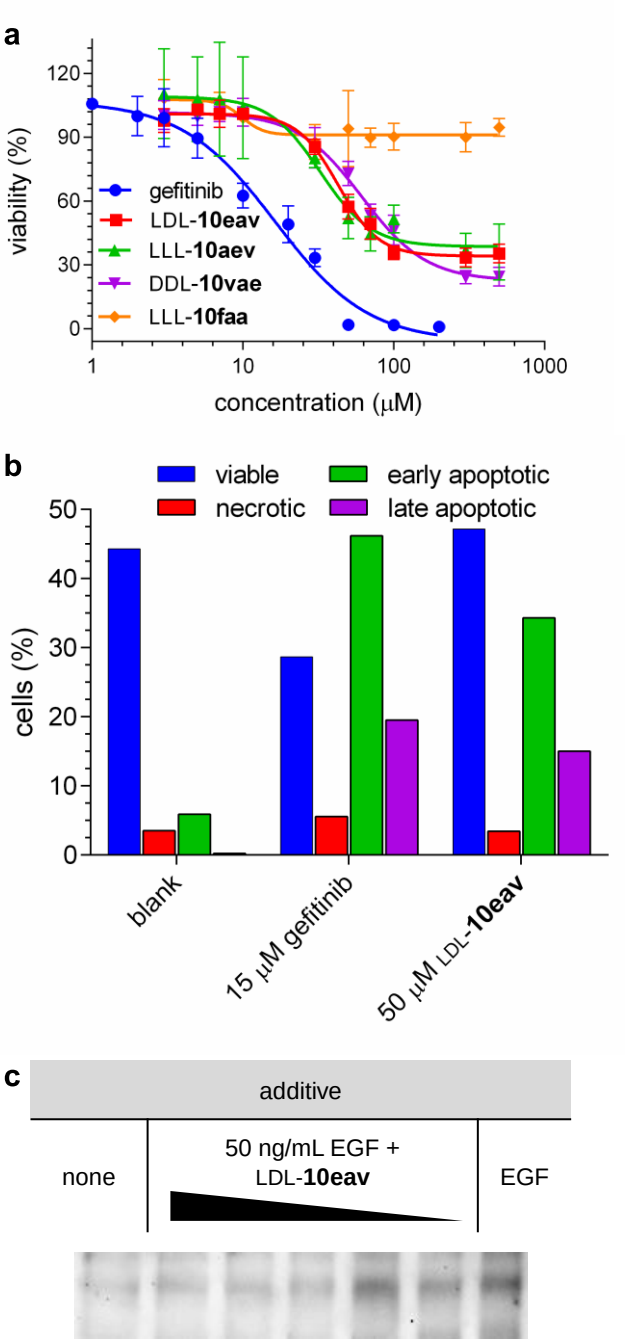


Figure 5. Cellular data for piptide-based chemotypes. **a** A549 cell viabilities as monitored using alamarBlue. **b** Flow cytometry data for A549 cells treated with either 15 μM gefitinib or 50 μM LDL-**10eav**. **c** Inhibition of EGFR pTyr for LDL-**10eav** at concentrations (μM) decreasing from 112.5, 75, 50, 25, 12.5 in competition with EGF (uniformly used at 50 ng/mL).

Conclusion

Piptides can be prepared expeditiously by researchers with only moderate synthesis skills; and they would become even more accessible if pip acids became commercially available. Our data on EGFR indicate piptide-based chemotypes can be starting point probes for PPI targets. Five viable hits emerged from a library of only 14 molecules selected by evaluation with EKO; their K_d values (30 – 90 μM) are modest, but detection of any measurable binding is a notable success in studying small molecules to perturb PPIs, particularly because solubilized EGFR is probably not in a native conformation when immobilized on a gold surface for SPR. Moreover, the best binders from these five hits were active in cellular assays (induce cytotoxicity via apoptosis and inhibit EGF-mediated phosphorylation of intracellular Tyr residues in EGFR).

This study does not feature intracellular targets, but researchers might be interested in applying the strategy to such PPIs. An attractive feature of piperazines is that they can be reversibly protonated, and this can facilitate passive diffusion through membranes. Piptides with appropriate side chains might conceivably be somewhat cell permeable, and Table S1 shows some calculated data to explore this point.

The closest parallel to piptides in the literature is oligooxopiperazines.[38] Oligooxopiperazines have been used for disrupting PPIs,[39] but are harder to access, and the range of side chains so far incorporated is less. Further, the two chemotypes are simply different, thus probably suitable for complementary applications.

Acknowledgements

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Keywords: cancer • EGF • peptidomimetics • protein–protein interactions • solid-phase synthesis

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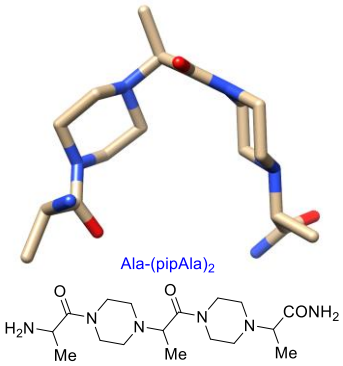

Piperazines + peptides = piptides! These new chemotypes are accessible from natural amino acids in either configuration, hence can conveniently display all the side chain pharmacophores found in peptides and proteins. They can be made in solution or on supports and are proteolytically-/pH-robust. Piptide-based disruption of EGF•EGFR was chosen as an initial challenge and Exploring Key Orientations (EKO) was used to evaluate candidates.