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Affinity enhancement of protein ligands by reversible covalent modification of neighboring lysine residues

Alberto Dal Corso,[#] Marco Catalano,[#] Anja Schmid, Jörg Scheuermann and Dario Neri*

Abstract: The discovery of protein ligands, capable of forming a reversible covalent bond with amino acid residues on protein target of interest, may represent a general strategy for the discovery of potent small molecule inhibitors. We analyzed the ability of different aromatic aldehydes to form imines by reaction with lysine using ^{1}H-NMR techniques. 2-Hydroxybenzaldehyde derivatives were found to efficiently form imines in the millimolar concentration range. These benzaldehyde derivatives could increase the binding affinity of protein ligands towards the cognate protein target. Affinity maturation was achieved not only by displaying ligand and aldehyde moieties on two complementary locked nucleic acid strands, but also by incorporating the binding fragments in a single small molecule ligand. The affinity gain was only observed when lysine residues were accessible in the immediate surroundings of the ligand binding site and could be abrogated by quenching with a molar excess of hydroxylamine.

Ligands capable of covalent binding to protein targets of interest are important for pharmaceutical applications and as chemical biology tools.[1,2] Aspirin, penicillin, omeprazole and clopidogrel are examples of successful covalent drugs. Moreover, the selective modification of cysteine residues close to the active site of kinases with suitable Michael acceptors has led to the development of covalent inhibitors for cancer therapy, such as ibrutinib (BTK inhibitor, Imbruvica™; AbbVie), afatinib (EGFR inhibitor, Gilotrif™; Boehringer) and osimertinib (EGFR inhibitor, Tagrisso™; AstraZeneca). Recently, researchers at AstraZeneca have reported the generation of highly potent inhibitors of induced myeloid leukemia cell differentiation protein (Mcl-1), featuring a formyl- or an acetyl-phenylboronic acid moiety in *ortho* position.[3] This additional group is capable of forming a *reversible* covalent bond with the ε-amino group of a specific lysine residue.[4,5] Imine formation is a well-known reaction of amines and several enzymes take advantage of Schiff base formation with a pyridoxal phosphate (PLP) cofactor.[6] The latter has been investigated as useful tool for different applications in chemical biology. For instance, Francis and coworkers had previously investigated the use of PLP and ortho-hydroxy-pyridinecarboxyaldehydes for site-specific modification of *N*-terminal amino acids.[7] Moreover, PLP derivatives have been recently designed to be integrated into the cell metabolic processing, enabling the discovery of new protein families, such as the full complement of PLP-dependent enzymes in *Staphylococcus aureus*.[8]

The use of functional groups, capable of forming a reversible covalent bond with lysine residues, could be useful for pharmaceutical applications, provided that the reactivity of those chemical moieties was not too high, leading to a blockade by human serum albumin in blood. In this Communication, we have systematically analyzed the equilibrium constants for the reaction of various benzaldehydes with primary amines. Using four different protein targets, which display a different density of lysine residues, we have investigated how the incorporation of suitable aromatic aldehydes into the ligand structure can lead to an affinity enhancement.

Figure 1A depicts how Schiff base formation for benzaldehyde derivatives may be in competition with the hydration of the aldehyde moiety and how these equilibria can be perturbed by neighboring functional groups. The two reactions can be monitored and quantified using ^{1}H-NMR methods [**Figure 1B**]. We observed that the least reactive benzaldehyde derivatives [e.g., benzaldehyde (**1**)] exhibited no detectable Schiff base formation at pH 7.4 in the presence of 20 mM N_α-Acetyl-lysine. By contrast, efficient imine formation could be detected for 2-hydroxybenzaldehydes [e.g., (**13**)], with an apparent dissociation constant K_D = 23 mM [**Figure 1C**]. The *ortho*-formyl-phenylboronic acid (**14**) moiety, which had been used for the generation of reversible covalent Mcl-1 inhibitors[3], exhibited a K_D value of 17 mM, while phthaldialdehyde (**16**) strongly reacted to form an irreversible 1-isoindolinone adduct.[9]

We have previously shown that the display of chemical moieties on complementary DNA strands may facilitate the discovery and characterization of synergistic binding fragments to protein targets.[10] For example, we and others have shown that the potency and selectivity of Michael acceptors towards cysteine-containing kinases may be increased by the incorporation of binding fragments on complementary nucleic acids strands, with a “dock and lock” mechanism.[11-12]

In this work, we initially assessed the contribution of benzaldehyde derivatives to protein binding for various ligands, using chemical derivatives of short (12-bp) locked nucleic acids (LNAs) [**Figure 2A**].[13] We tested our methodology on three protein targets: human serum albumin (HSA), bovine carbonic anhydrase II (CAII) and human interleukin-2 (IL2). For these targets, we and others had previously described small molecule ligands.[14-18] The binding sites of these ligands on the cognate proteins differed in terms of local density of lysine residues [**Figure 2B**].

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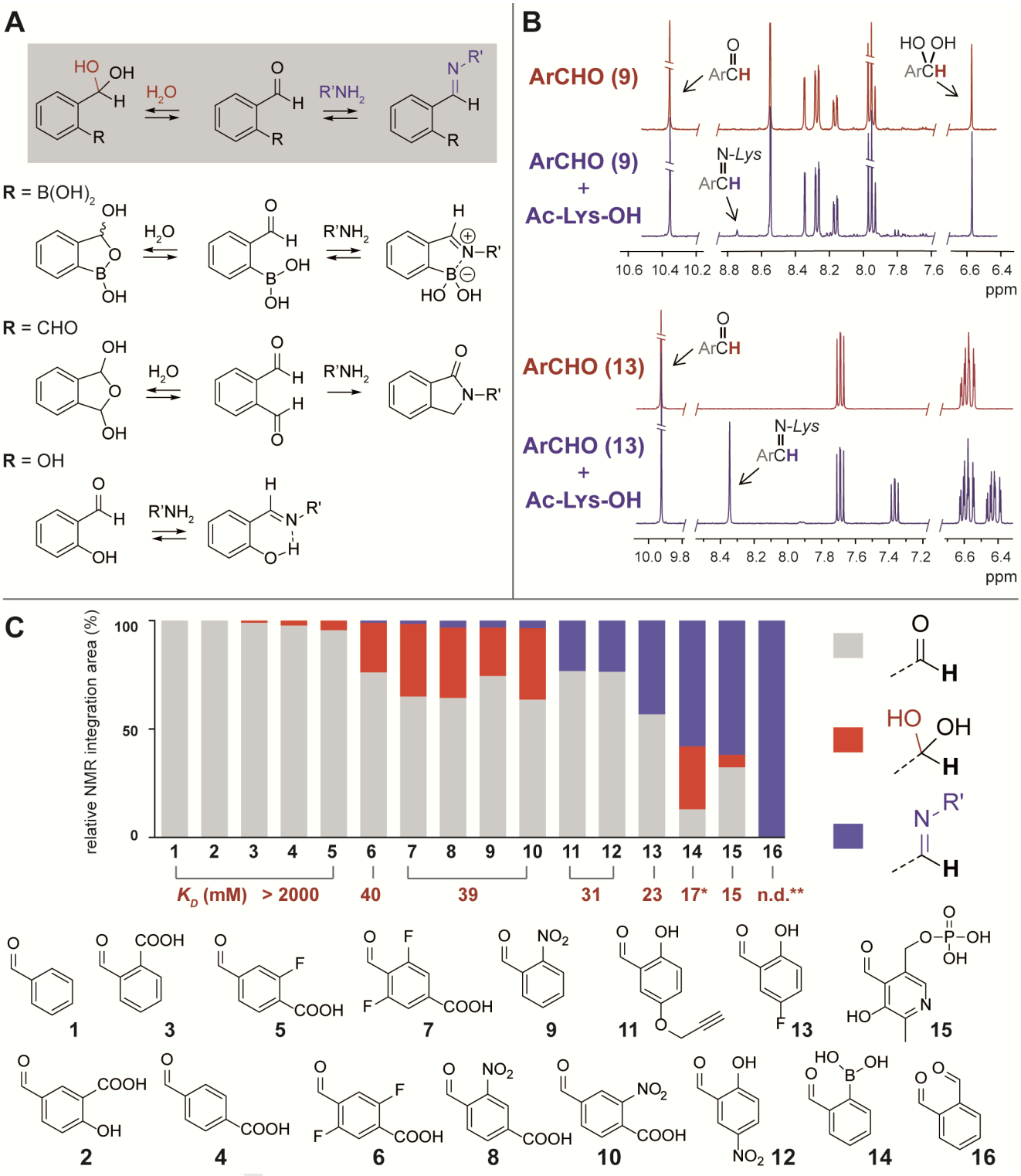

Figure 1. Reversible covalent modification of primary amines with benzaldehyde derivatives. **(A)** Aldehyde hydration and Schiff base (imine) formation as alternative reaction routes for benzaldehydes and other examples of imine formation assisted by functional groups in the *ortho* position; **(B)** ^{1}H-NMR spectra of the reaction products of benzaldehydes **9** and **13** (2 mM) with 20 mM of Nα-Acetyl-lysine (Ac-Lys-OH), performed at room temperature in phosphate buffer, pH = 7.4, dissolved in D_2O]. While for **9** the aldehyde hydration reaction is predominant, compound **13** yields a stable imine product. **(C)** The percentage of hydration product (red) and/or imine formation (blue) was quantified using ^{1}H-NMR and used to measure K_D constants. The chemical structures of the compounds and the corresponding numbers are indicated. *Data referred to the integration of broad NMR signals; ** Data referred to the 100% aldehyde conversion, according to the mechanism depicted in **Figure 1A**.

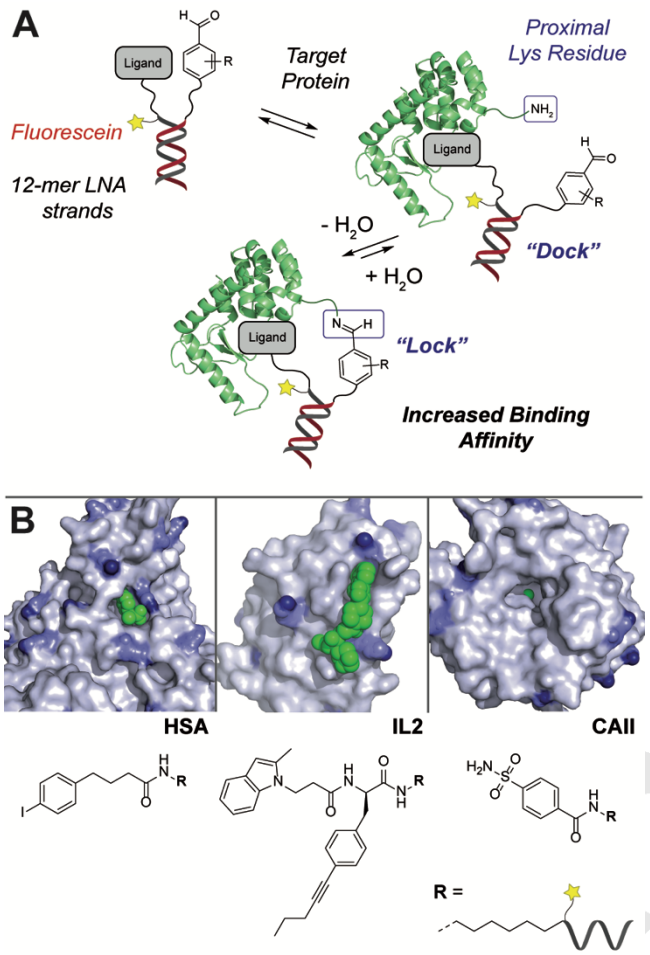

Figure 2. (A) Schematic representation of a "dock-and-lock" procedure, featuring bifunctional structures displayed at the extremity of a fluorescently-labeled nucleic acid. In a first step, a non-covalent ligand interacts with the cognate site on the target protein of interest. Subsequently, a benzaldehyde moiety may form a Schiff base with a neighboring lysine residue, thus stabilizing the complex. **(B)** Chemical structures of ligands to human serum albumin (HSA), carbonic anhydrase II (CAII) and interleukin-2 (IL2), whose binding characteristics had previously been described.[16-18] Space-fill representations of the three-dimensional structures of the target proteins, with lysine sidechains depicted in blue, reveals a high density of primary amines around the active sites of HSA and IL2, but not for CAII. Ligands of HSA and IL2 are depicted in green in the crystal structures, while only the zinc atom in the active site is depicted in green for CAII (Brookhaven Protein Data Bank accession numbers 2BXG, 1PW6 and 1V9E).

We synthesized LNA conjugates, starting from amino-tagged locked nucleic acid precursors, bearing a primary amino group with a C6 spacer. The LNA strand, which was modified at the 5' extremity, also carried a fluorescein moiety on the last base, facilitating affinity measurements in solution using fluorescence polarization.[19,20] In physiological buffer conditions, the incorporation of a 2-hydroxybenzaldehyde moiety (**11-LNA**) on the complementary LNA strand enhanced the affinity of 4-(4-iodophenyl)butanoic acid to HSA by 5-fold, compared to benzaldehyde (**1-LNA**) or phenyl (**0-LNA**) derivatives [**Figure 3**].

A similar affinity gain (4-fold) was observed for the IL2 ligand, but in this case also the benzaldehyde derivative **1-LNA** exhibited an improved dissociation constant compared to **0-LNA**. As expected, the binding potentiation of HSA and IL2 ligands could be quenched by the addition of a molar excess of hydroxylamine [**Figure 3** and **Supplementary Figure S1**].
By contrast, no affinity gain could be detected for the CAII ligands, as CAII lacks primary amines in close proximity to the active site [**Figures 2** and **3**].

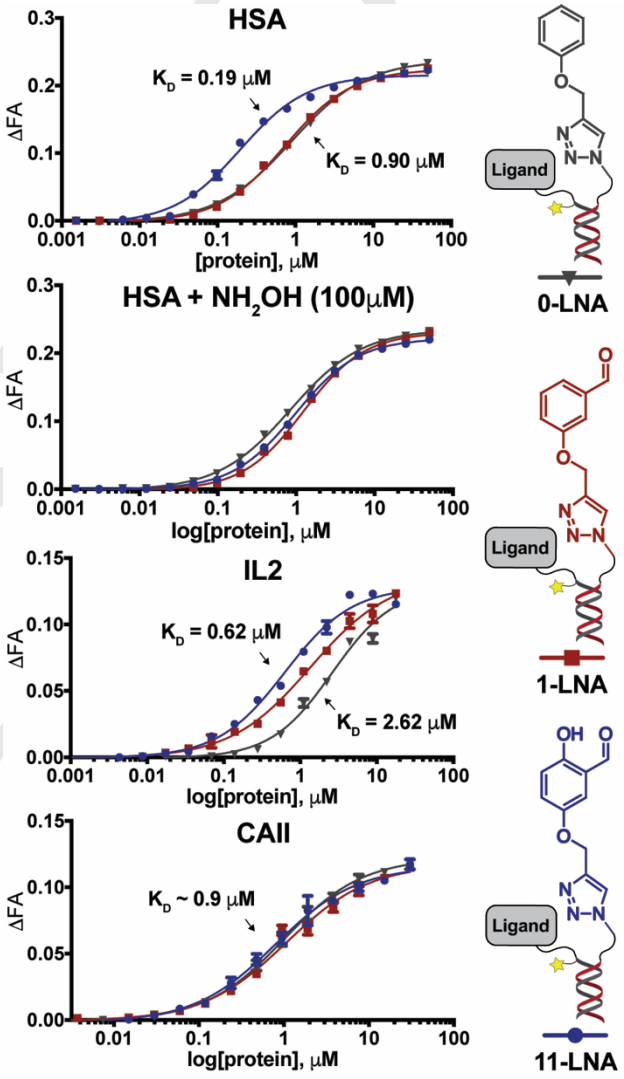

Figure 3. Affinity measurements of protein ligands using fluorescence polarization methodologies and LNA derivatives. An affinity gain for LNA derivatives featuring the reactive **11-LNA** moiety was observed for HSA and IL2, but not for CAII. The affinity gain could be abolished by incubation with a molar excess of hydroxylamine. FA = Fluorescence Anisotropy

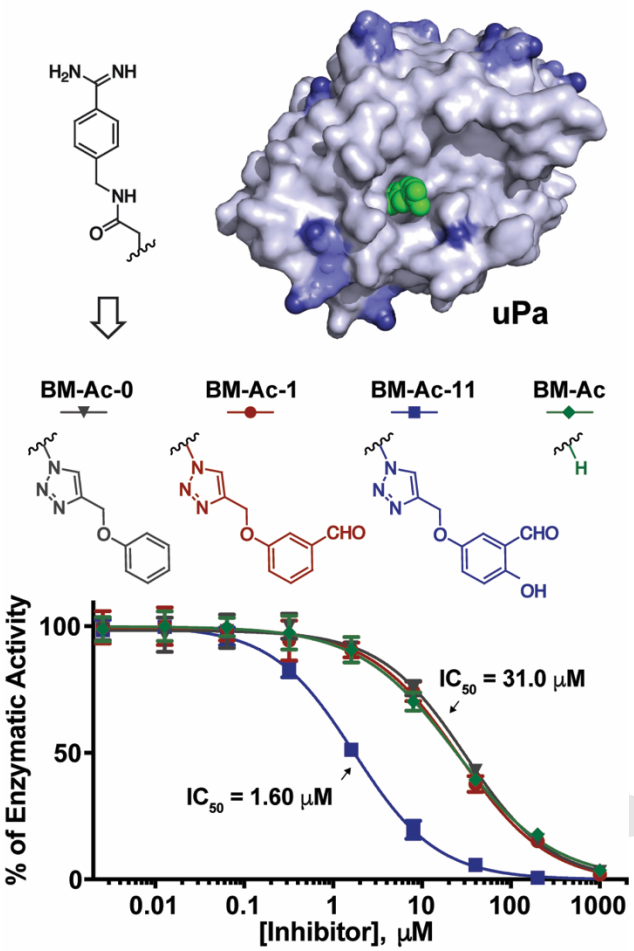

Figure 4. Enzymatic inhibition assay for benzamidine derivative against urokinase-type plasminogen activator (uPa). The space-fill representation of the three-dimensional structure of the protein (Brookhaven Protein Data Bank accession numbers 3MWI) reveals an ε-amino group of a lysine residue in close proximity of the active site (depicted in blue). In an inhibition assay in presence of Z-GGR-AMC as a fluorogenic substrate, four benzamidine derivatives were tested. Only the compound bearing the 2-hydroxybenzaldehyde moiety (**BM-Ac-11**) showed a 20-fold improvement of the inhibition capacity.

The generation of reversible covalent protein binders through the incorporation of 2-hydroxybenzaldehyde moieties is potentially applicable to various small molecule ligands. We had previously shown that the substitution of an LNA moiety with suitable chemical linkers can lead to an additional affinity gain.[21] With this aim, we studied the possibility to generate small benzamidine derivatives as urokinase-type plasminogen activator (uPa) inhibitors. uPa over-expression in breast cancer correlates with poor prognosis and benzamidine (a broad spectrum serine protease inhibitor) displays a rather modest inhibitory activity against uPa [180 μM K_D].[22]
The ε-amino group of the Lys143 of uPa is in close proximity to the benzamidine binding site [**Figure 4**]. We generated four benzamidine derivatives devoid of the LNA scaffold and studied their inhibitory activity towards uPA. The molecule containing the 2-hydroxybenzaldehyde moiety showed a 20-fold improved potency compared to *N*-(4-carbamimidoylbenzyl)acetamide (**BM-Ac**), as well as the **BM-Ac-0** and **BM-Ac-1** compounds, used as negative controls. [**Figure 4**].

The concept of using a bidentate binding mode in order to obtain a high-affinity protein recognition has previously been investigated both for small organic ligands and for recombinant antibodies.[23-24] The simultaneous engagement of two binding sites has been exploited in dynamic ligation screening, forming covalent bonds between synergistic fragments in the presence of a target protein of interest.[25] Large compound collections, displayed at the extremities of complementary DNA strands which can combinatorially self-assemble, can be screened using dual-pharmacophore encoded chemical library technology.[26] DNA-encoded libraries have previously been used to discover irreversible covalent inhibitors.[27] Similar procedures could be implemented for the discovery of reversible covalent binders, by incorporating 2-hydroxybenzaldehyde or similar moieties during library construction. Encoded self-assembling chemical (ESAC) libraries are particularly attractive for the discovery of covalent inhibitors, since DNA conjugates can be individually purified to homogeneity, thus preserving the integrity of reactive functional groups.[11,19] Moreover, DNA structures have been used to probe the magnitude of affinity gain by polydentate engagement with multivalent protein target.[28]

It will be interesting to investigate whether more reactive lysine modifiers (or modifiers for other amino acids) can be used for pharmaceutical applications. From a thermodynamic viewpoint, practical applications would be limited by the molar concentration of amino acid residues in serum and in tissues. However, since Schiff base formation is a relatively slow reaction, it may be conceivable that a “dock-and-lock” procedure may allow the selective *in vivo* modification of target proteins, if kinetic parameters are judiciously chosen. *N*-hydroxysuccinimido derivatives of aptamers have recently been used to promote a site-specific protein modification in cell cultures.[29] It is therefore possible that moieties more reactive than 2-hydroxybenzaldehyde may be compatible with pharmaceutical and chemical biology applications.

Notes

D.N. is a co-founder and shareholder of Philogen (www.philogen.com), a Swiss-Italian Biotech company that operates in the field of DNA-Encoded Chemical Libraries. J.S. is a board member of Philochem AG (www.philochem.ch).

Acknowledgements

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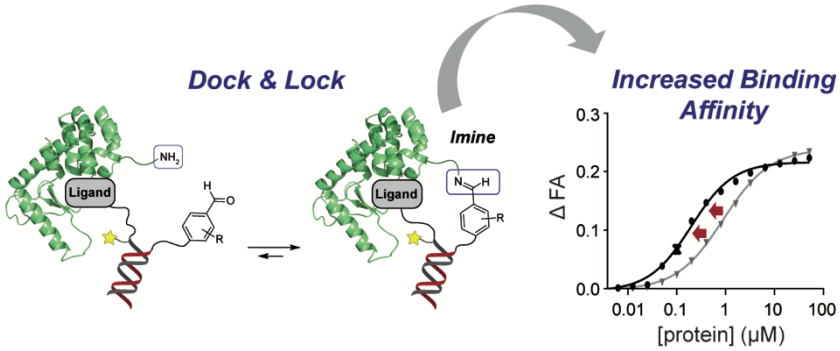

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The simultaneous display of protein ligands and of 2-hydroxybenzaldehyde derivatives on either two complementary LNA strands or small-organic molecule lead to an enhanced binding affinity by reversible covalent interaction with proximal lysine residues.