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Salicylates are interference compounds in TR-FRET assays

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ABSTRACT

Given the importance of high-throughput screening in drug discovery, the identification of compounds that interfere with assay readouts is crucial. The pursuit of false positives wastes time and money, while distracting development teams from more promising leads. In the context of TR-FRET assays, most interfering compounds are dyes or aggregators. In the course of our studies on the PD1–PDL2 interaction, we discovered that salicylic acids, an extremely common compound subclass in screening libraries, interfere with TR-FRET assays. While the precise mechanism of interference was not established, our data suggest that interaction of the salicylate with the cryptand-ligated europium FRET donor is responsible for the change in assay signal.

The existence of pan-assay interference compounds (PAINS) is a well-studied problem in the high-throughput screening field.[1,2] These may be loosely defined as molecules that appear as false positives in assays against multiple different targets, or otherwise present as 'frequent hitters' that give flat structure activity relationship (SAR) profiles upon attempted medicinal chemistry optimization.[2] In some cases a compound's recurrent appearance as an unoptimizable hit is understood to arise from nonspecific electrophilic alkylation of proteins,[1,3] promotion of protein aggregation,[4] or redox effects.[5,1] In other cases, the mechanism underpinning PAIN-like behavior for a particular class of molecules is unknown.[6]

For many PAINS, the problem stems not from nonspecific interactions with the proteins in the assay, but from interference with the assay readout itself. For example, colored or fluorescent compounds interfere with spectrophotometric detection, tertiary amines interfere with AlphaScreen techniques by quenching the singlet oxygen required for signal generation,[1] and thiol-reactive compounds interfere with screens that depend on sulfhydryl reporters.[7]

Whereas traditional high-throughput screens seek to identify molecules of modest molecular weight (typically <500 g/mol) that already have reasonable potency (K_d in the low micromolar range or better) and that can be optimized through structural adjustments, *fragment-based* lead discovery requires identifying smaller molecular weight molecules (typically <300 g/mol) that are much less potent on their own (K_d up to the low-millimolar range) but that can subsequently be used as the basis for lead compounds through fragment-growing or fragment-linking approaches.[8] Because fragment-based methods often require testing compounds at higher concentrations than is typical for other high-throughput screening campaigns, interferences can be especially problematic.

In this Letter, we report an unusual assay interference, wherein salicylate-containing molecules persistently appeared as false-positives in a TR-FRET assay (time-resolved fluorescence resonance energy transfer). We attribute this interference to unexpected binding of the salicylate to the chelated Eu^{3+} donor that is used in the assay, which in turn changes the photophysical properties of the donor.

We recently embarked upon a computationally driven screening program to identify lead compounds and fragments capable of allosterically modulating the interaction between programed death 1 (PD1) and its ligands PDL1 and PDL2. These proteins collectively exert a critical control over the immune system, in that binding of PD1 to either ligand induces T-cell anergy. The PD1–PDL interaction prevents T-cells from attacking healthy tissue, but the process often suffers from misregulation. Agents that promote or antagonize the PD1–PDL interaction have emerged as potential therapeutics in areas as diverse as diabetes,[9] autoimmune diseases,[10,11] and oncology.[12,13]

We used the PocketFinder program[14] to identify two putative pockets on the PD1 surface, and two more on the PDL1 surface. Each pocket was selected based on its potential ability to accommodate small organic molecules, as well as the likelihood that occupancy of the pocket might change the conformational preferences of the protein in such a way as to alter its affinity for its

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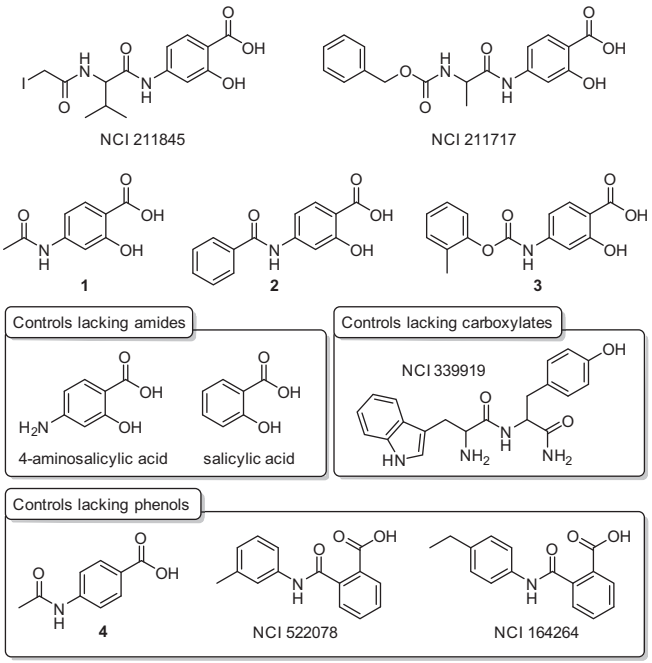

Figure 1. Small molecules discussed in this Letter.

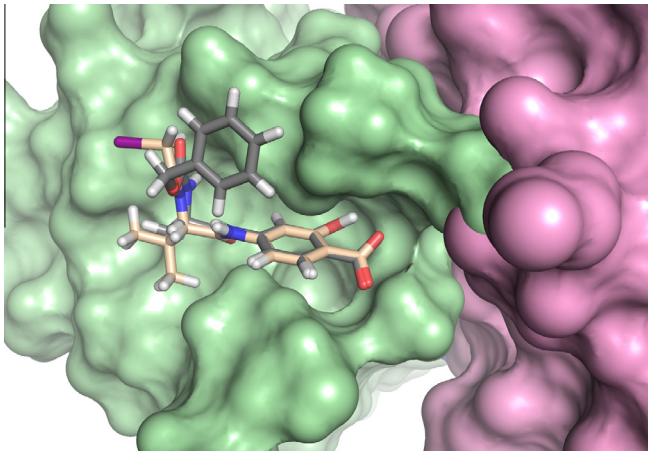

Figure 2. Docked complexes of NCI 211845 (beige) and NCI 211717 (gray) bound to an *N*-terminal PD1 pocket (green). The proximity of the PDL interface to this pocket (PDL1 shown in magenta), together with the proximity of several residues known to be important for PDL binding[22] suggested the possibility that small molecules bound to the pocket could allosterically modulate ligand binding. Visual inspection of the in silico modeled complex suggests that the salicylate headgroups of the two NCI molecules are particularly critical for binding.

binding partner. We then used ICM (Internal Coordinate Mechanics, Molsoft LLC)[15] to perform molecular modeling, molecular docking and in silico screening of prospective small-molecule modulators, based on energy calculations and global energy optimization[16] using the NCI database (>200,000 compounds) and ZINC 'Drugs Now' database (>10,000,000 compounds).[17]

From our ranked lists of putative binders, we chose >80 compounds to bring into the wet lab for in vitro testing. Among these were NCI 211845 and NCI 211717 (Fig. 1), which were predicted to bind to an N-terminal pocket on PD1 that lies near to the PDL binding domain (Fig. 2). Neither molecule is promising as a lead compound—NCI 211717 contains an undesirable lipophilic group (actually a CBZ protecting group) while NCI 211845 incorporates an electrophilic iodoacetamide function that could nonspecifically alkylate a protein. On the other hand, the shared 4-aminosalicylate

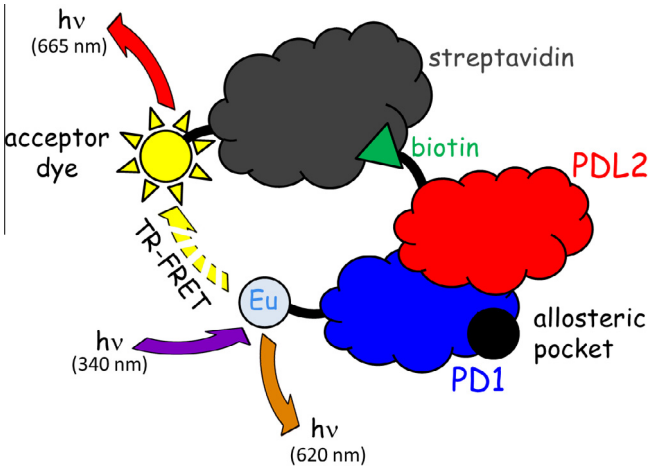

Figure 3. Cartoon illustration of the TR-FRET assay used in the current study. The signal indicative of complex formation is derived from the ratio of emission at 665 nm, to that at 620 nm. A 100 μs delay time was used to minimize interferences from test compounds that contained chromophores.

motif present in each structure was attractive as a possible fragment. Salicylates in general (and aminosalicylates in particular) are well established among the pantheon of known drug compounds (e.g., olsalazine, balsalazide and sulfasalazine are all prodrug forms of 5-aminosalicylate, which is administered directly as mesalazine), and are also well represented in screening libraries (e.g., the ZINC database has >2700 4-aminosalicylates and >3800 5-aminosalicylates available for purchase[18]). Moreover, simpler amide derivatives of 4-aminosalicylic acid would fit within the generally-accepted guidelines for fragment selection: they would have molecular weights well under 300 g/mol, they could certainly contain <18 heavy atoms, and their log*P* and hydrogen-bond donor values would be consistent with the so-called 'rule of 3'.[19] Although the number of hydrogen-bond acceptors might be considered slightly high, this criterion is now recognized to be a poor predictor of fragment viability.[20] We therefore chose to see these two predicted PD1 binders as being representative of a promising molecular fragment, laden with distinctly suboptimal amide sidechains. In keeping with this view, we elected to test the parent fragment **1** (*N*-acetyl-4-aminosalicylic acid) alongside the two NCI compounds. In addition, we tested salicylic acid and 4-aminosalicylic acid as controls, and compounds **2** and **3** as simple analogs.[21]

Evaluation of fragment binding is often done using biophysical methods. NMR is particularly common,[23,24] but SPR[25,26] and ITC[27] are also used. In this case however, we were less interested in evaluating small-molecule *binding* than in determining whether or not that binding event contributed to a change in PD1 function.

Our labs have previously had success using fluorescence-polarization assays (FP) to evaluate inhibitors of protein–protein interactions.[28,29] We considered employing similar techniques here, and indeed briefly evaluated the use of FP, thermal shift and ELISA assays to test the efficacy of our computationally-selected test compounds. However, we ultimately settled on the use of a commercial PD1–PDL2 assay based on TR-FRET as our primary screen,[30] to be supported by subsequent SPR analysis for promising compounds.

One of the principal reasons for our selection of a TR-FRET assay was that we expected it to be less prone to interferences than other fluorescence-based methods. Unlike FP or traditional FRET measurements, in which background scattering or test-compound fluorescence can contribute substantially to the signal readout, in TR-FRET assays the lifetime of the donor (usually Eu^{3+} or Tb^{3+}) is orders of magnitude longer than the lifetime of either scattered