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Technology Note

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A novel fluorescence competition assay for retinoic acid binding proteins

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ABSTRACT: Vitamin A derived retinoid compounds have multiple, powerful roles in the cellular growth and development cycle, and as a result have attracted significant attention from both academic and pharmaceutical research in developing and characterizing synthetic retinoid analogues. Simplifying the hit development workflow for retinoid signaling will improve options available for tackling related pathologies, including tumor growth and neurodegeneration. Here we present a novel assay that employs an intrinsically fluorescent synthetic retinoid, DC271, which allows direct measurement of the binding of non-labeled compounds to relevant proteins. The method allows for straightforward initial measurement of binding using existing compound libraries, and is followed by calculation of binding constants using a dilution series of plausible hits. The ease of use, high throughput format, and measurement of both qualitative and quantitative binding, offer a new direction for retinoid-related pharmacological development.

Retinoids are a class of molecules derived from Vitamin A which play a wide and active role within the growth and development cycle of mammalian cells.[1] This control over cell differentiation, proliferation and apoptosis is mediated by the interaction of all-*trans* retinoic acid (ATRA, Figure 1) with the retinoic acid receptor (RAR) proteins, which form DNA binding heterodimers with the related retinoid X receptors (RXR).[1-2] The recruitment of these retinoid-bound heterodimers to retinoic acid response elements (RARE), based around a DR5 hexameric core motif -(A/G)G(G/T)TCA, triggers expression of proteins and subsequent phenotype change. [1, 3-4]

Retinoids also form an important class of therapeutic agents used in chemotherapy, as well as in alleviating severe skin conditions.[5] Additionally, there is growing evidence to suggest that ATRA is capable of reducing levels of amyloid-β built up in neurological tissues which may offer a route to the treatment of Alzheimer's disease, and other conditions thought to be related to amyloid-β.[6] As a family, retinoids exhibit powerful control over the workings of the human body, and offer great promise for the modification of these systems as drug candidates.[7-8]

Cellular retinoic acid binding proteins, CRABP-I and CRABP-II, are small, intracellular shuttling proteins associated with retinoic acid trafficking in the cytosol.[9] The two exhibit significant differences in expression levels and localization, with CRABP-I being expressed in most adult cells, and CRABP-II being found mainly in the skin and early development tissues.[10]

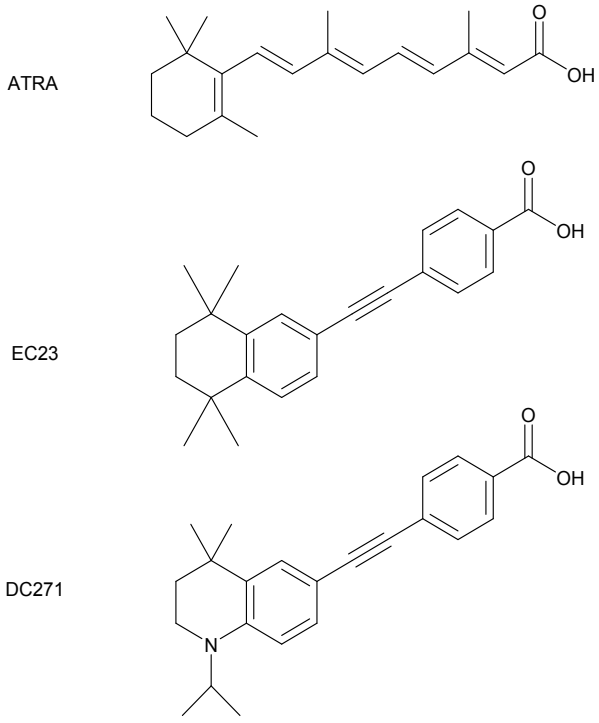

Figure 1: Natural CRABP-II ligand all-*trans* retinoic acid (ATRA), synthetic derivative EC23 and fluorescent retinoid DC271.

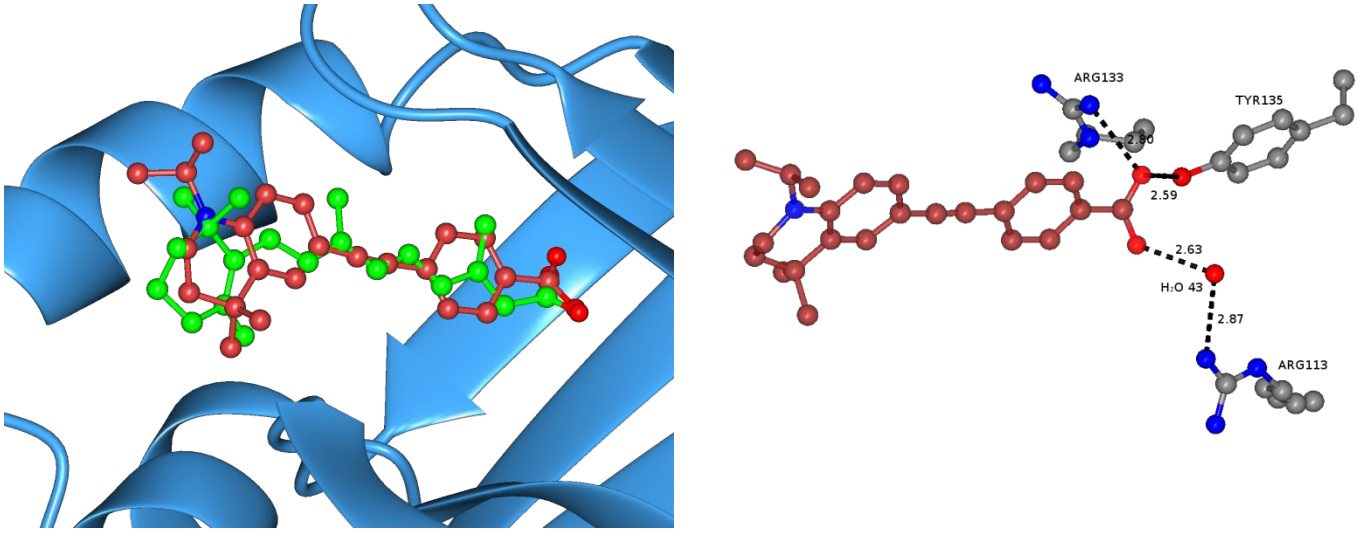

Figure 2: Left – Structural alignment of new CRABPII crystal structure 6HKR (DC271 bound – red) with reference structure 2FR3 (ATRA bound - green). Close agreement of the carboxylic acid head group can be seen. Right – Enhanced view of the binding triad residues of 6HKR including dashed hydrogen bonds, lengths marked in □.

CRABP-II alone is capable of nuclear localization, and is demonstrably responsible for increasing the transcriptional output of the retinoic acid signaling pathway. Accordingly, it is understood that it is responsible for trafficking ATRA to the nucleus, and to the DNA binding transcription factors, after its arrival in the cytoplasm.[11] Due to this deep involvement in the retinoid signaling pathway CRABP-II can be considered a protein of significant biological importance.

ATRA, specifically its conjugated polyene tail, is prone to isomerization and degradation under 300-400 nm light. The resulting mixture of ATRA, 9-*cis* and 13-*trans* isomers is far less effective as a biological agent and in turn impacts the reliability and consistency of quantitative work undertaken using the compound.[12] To circumvent this instability synthetic retinoids have been developed that mimic the shape of ATRA - particularly the elongation and terminal carboxylate function - but that do not share the compounds predilection to isomerisation.[13-15] One important example is EC23, a synthetic analogue of ATRA that is entirely light stable and carries out the same signaling role in stem cell differentiation assays, but with improved potency.[13, 16]

DC271 is a further development of EC23, based on a donor acceptor diphenylacetylene structure, which exhibits solvatochromic fluorescence when excited at wavelengths suitable for microscopy and as a fluorescent probe (absorbing at 340-410 nm).[17-18] The compound's emission is highly dependent on the local environment – intense and blue-shifted in nonpolar solvents, weak and red-shifted in polar solvents. In an aqueous environment in particular, the hydrophobic structure of DC271 causes severe quenching of the emission, ostensibly due to aggregation. This characteristic behavior can be used to monitor incorporation of DC271 into the hydrophobic binding pocket of a retinoid binding protein (such as CRABP-II) where the insulated and hydrophobic environment significantly increases the quantum yield analogous to a change into a non-polar solvent.[19,20]

The unique fluorescence characteristics of DC271 offers a novel and convenient method for direct measurement of binding to retinoid-binding proteins, something previously only achieved with complex and costly radio-labelling techniques or multi-step binding assays. Demonstrated herein is the measurement of the binding of small molecules to CRABP-II, in competition with DC271, as part of a high-throughput capable assay. The action of CRABP-II as a carrier protein makes this an ideal *proof-of-principle* experiment for screening retinoid related molecules for potential pharmaceutical effect, a technique equally applicable to retinoic acid receptor (RAR) proteins with vital downstream effects on phenotype.

To demonstrate the binding of synthetic retinoid DC271 to CRABP-II, and confirm the molecular basis of ligand binding, crystallization trials in an informed range of conditions were carried out resulting in a 1.5 Å resolution structure which was refined to R/R_{free} 0.14/0.17 (Figure 2 – PDB:6HKR). The fitting of DC271 into the ligand binding site was carried out using the unbiased density map, before refinements to side chains and solvent molecules were carried out. The final binding pose of DC271 was found to be highly congruent with that of endogenous ligand ATRA, where the carboxylate head group interacts with the same Arg112 Arg133 Tyr135 binding triad (Figure 2 - Right). This structure demonstrates that DC271 is capable of binding to CRABPII, and that it occupies the retinoid binding site of the protein. This, coupled with its inherent solvatochromic fluorescence, makes it an excellent candidate for use in a displacement assay.

A fluorescence displacement assay was carried out using a series of endogenous retinoids as well as structurally related fragments of DC271 (Figure 3 - A). This qualitative study was carried out at multiple concentrations, and gives an indication of binding as a function of ligand displacement (relative reduction in fluorescent signal compared to the control) of DC271 from the binding site. Black, non-binding surface 96 well plates were loaded with an 100 nm concentration of CRABPII and DC271, as well as an aliquot of the tested ligand or a relevant control. Each

plate was read using a Biotek Synergy H4 plate reader, in fluorescence intensity mode, exciting at 355 nm and reading emission at 460 nm.

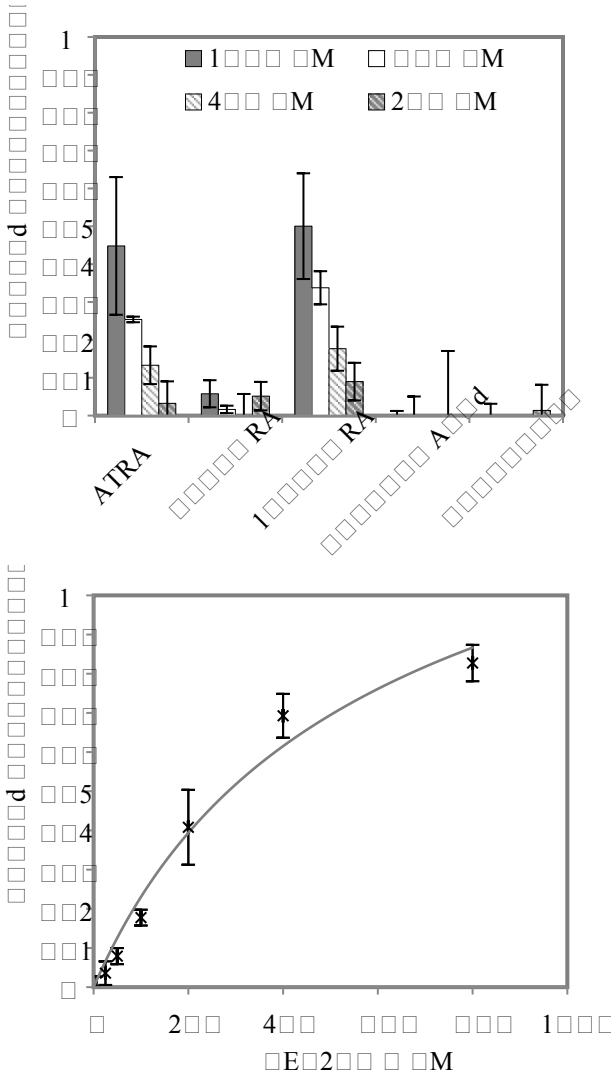

Figure 3: A - Qualitative displacement of DC271 from the binding site of CRABP-II by select retinoids and retinoid related structural components (n=3, α=0.05). B - Quantitative measurement of displacement of DC271 by EC23 synthetic retinoid (n=3, α=0.05). Curve fit using DynaFit[21] in a competitive mode.

These wavelengths were deemed the most suitable by examination of the absorption/emission spectra, and demonstrate the useful near-visible fluorescence of the compound.[19] A Z' value of > 0.60 was routinely achieved and the assay shows several compounds with binding affinity including ATRA and 13-*cis* retinoic acid. 9-*cis* retinoic acid, with its bent shape a poor fit for the CRABP-II binding site, demonstrated comparatively ineffective displacement of DC271, with synthesis fragments benzoic acid and quinoline also having no effect. These qualitative results conform to the known binding affinity of the respective compounds with CRABPII[22], demonstrating that the assay is truly indicative of active site binding.

The reduction in fluorescence observed when competing ligands displace DC271 from the CRABP-II binding site can also be applied to create a quantitative binding curve from a serial dilution. The result of this experiment is shown in Figure 3 as a competitive binding screen using a varying concentration of synthetic retinoid EC23. Subsequent fitting of the curve using DynaFit[21] gives an estimated binding constant (K_d) of 160 nM, consistent with the *in vivo* activity of EC23 and within the same nanomolar range previously reported for ATRA binding to both CRABPII and CRABPII.[16, 23-24]

This quantitative method is becoming increasingly relevant and offers more insight into a compound's binding efficiency than a single concentration library screen alone.[25] Analogous to a full competition titration, but using less material and equipment time, the rapid evaluation of potential compounds for binding efficiency will prove invaluable for hit-to-lead progression, as well as in the development of isoform specific binding compounds.

As a more powerful tool, it can be used most effectively once an initial screen has been carried out, or in complement to *in-silico* screening where a study can be focused on a smaller number of higher likelihood candidates. Alternatively, an entire library of compounds could be subjected to a qualitative screen, generating a rich dataset and reducing the opportunity for false negatives/positives that can occur at single concentrations. Given the wide range of compounds in the fluorescent DC series[18], adaptation to use a weaker binding compound would allow for investigation of weakly binding compounds and fragments which may otherwise prove hard to study.

The method demonstrated here comprises a simple 96 well assay, which can be employed in medium to high throughput screening, and offers a quick indicator of binding affinity to CRABP-II, the cytosolic carrier protein that is crucial to retinoid signaling activity at a cellular level. With further effort to optimize libraries and generate dilution series, a quantitative experiment can be carried out to determine binding constants, generating vital data for both hit-to-lead development programs and in the development of compounds with isoform specificity.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures and additional crystallography data (PDF)

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ABBREVIATIONS

ATRA – All-*trans* retinoic acid
CRABPII – Cellular retinoic acid binding protein II
RAR – retinoic acid receptor

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