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A Novel Cell-based Screening Assay for Cholesterol Biosynthetic Pathway Inhibition Using the RapidFire HTMS Platform

CYP51A1 is a heme-thiolate monoxygenase which participates in an obligatory step in the cholesterol biosynthetic pathway and catalyzes the formation of critical intermediates in humans. This enzyme is also a critical component of the ergosterol biosynthetic pathway in fungi. Inhibitors targeting this enzyme have been successfully marketed as antifungal agents for decades, while statins targeting HMG-CoA reductase (upstream of lanosterol in the cholesterol synthetic pathway) have dominated the cholesterol-lowering drug market. It is known that increased cholesterol synthesis is an important feature of actively proliferating cancer cells, and clinical trials have tried to assess the efficacy of statins as anti-cancer agents. However, conflicting evidence links statin intake to higher incidences of cancer-related death. Researchers exploring alternatives to statins for inhibiting the cholesterol synthetic pathway thus turned to CYP51A1 as a potential anticancer target, and antifungal CYP51A1 inhibitor drugs are being tested for anticancer efficacy.

As a full service drug discovery CRDO, Aragen was commissioned to enable one such project. The client was exploring the possibility of re-purposing an internal collection of antifungal CYP51A1 inhibitors for targeting cancers. The first step was to understand whether the compounds would be able to inhibit the cholesterol synthetic pathway in human cells, which required the quantitation of cholesterol or other intermediates within the cell.

The options for cholesterol detection included a simple kit-based enzyme assay or a cumbersome method using gas chromatography (GC). While the kit method did not have the necessary sensitivity, the GC method was time-consuming, lacked acceptable throughput, and was not cost-effective. To overcome these limitations, we devised a method using the RapidFire 365 HTMS system to develop the assay. RapidFire is a label-free technology which uses mass spectrometry for monitoring multiple analytes in complex sample mixtures. The short cycle time of 7 s per sample enables a high throughput analysis of required analytes and this platform is thus ideal for performing rapid screening of large compound libraries.

Method development was undertaken for cholesterol, as well as for the CYP51A1 substrates lanosterol and Dihydrolanosterol. All the three analytes were adequately detectable with clear separation in pure samples, as well as in sample mixtures. However, detection and quantitation of the same analytes in the context of a mammalian cell lysate presented several challenges. Analysis of lysates from untreated cells and those from cells treated with oxiconazole (a CYP51A1 inhibitor) showed that all analytes were detectable, but presented unique challenges (Fig 1A, 1B, and 1C). Our observations are given below:

Cholesterol

Though this sterol was detectable, the background signal was high, which masked the signal from the cholesterol synthetic pathway. Since we were using a label-free system, this result effectively ruled out cholesterol detection as a way forward for assay set up.

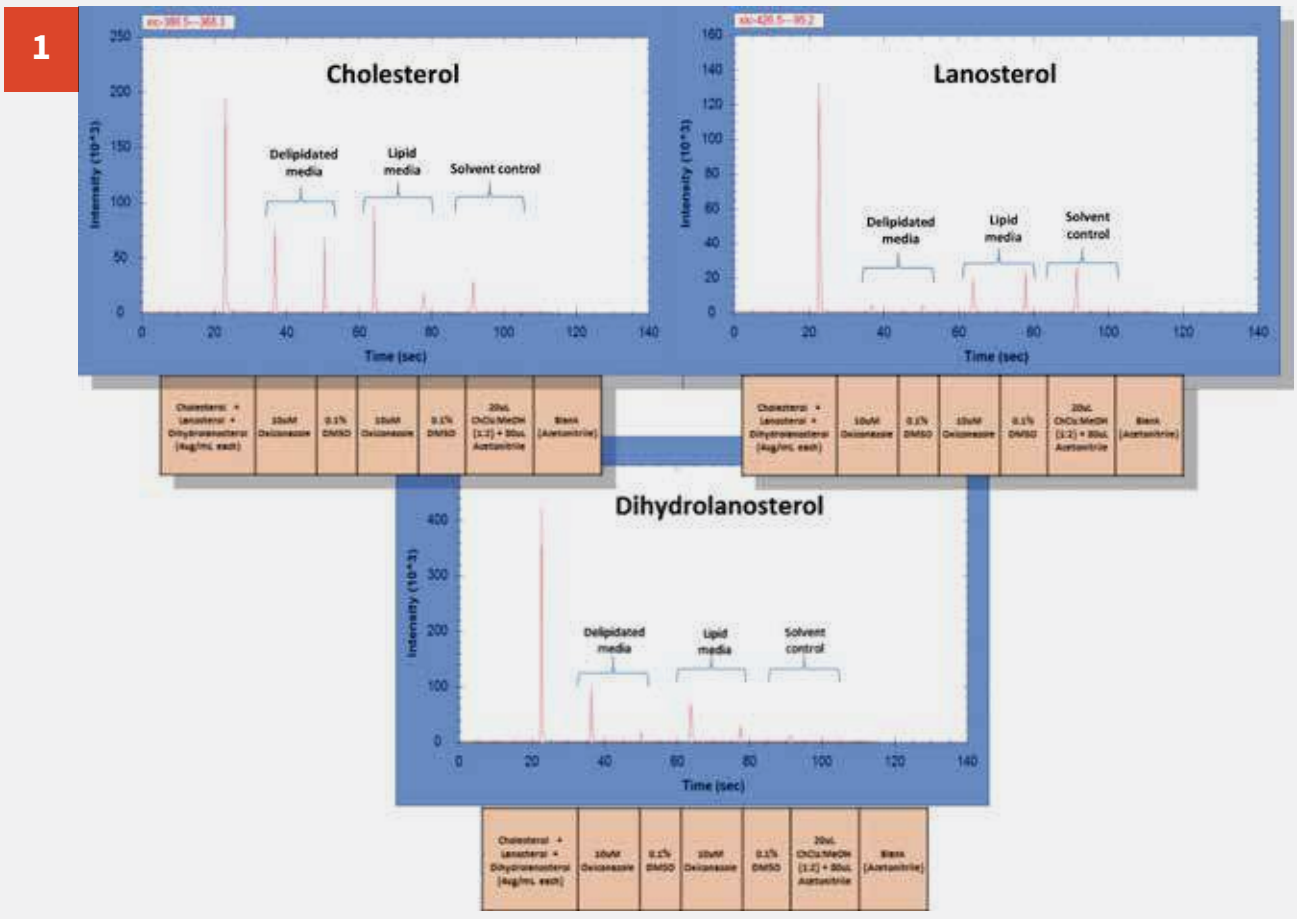
Lanosterol

This sterol is a direct substrate of CYP51A1, and as such an inhibitor of this enzyme should cause an accumulation of lanosterol and a higher lanosterol peak in the chromatogram. However, this was not borne out by the data (Fig 1B, delipidated media). Lanosterol was low with or without oxiconazole, a CYP51A1 inhibitor. A second enzyme (dehydrocholesterol reductase; DHCR24) converts lanosterol to 24, 25 Dihydrolanosterol, feeding into the Kandutsch-Russell pathway of cholesterol biosynthesis. This explained why we were not able to detect lanosterol accumulation in inhibitor-treated cell samples. It was clear that lanosterol was not the best analyte to monitor for CYP51A1 activity.

24,25 Dihydrolanosterol

This sterol is a direct substrate of CYP51A1, and a clear accumulation of this analyte was observed in cells treated with oxiconazole. There was little to no interference from other analytes or from background signals. We thus chose this compound as the most appropriate candidate to measure as a surrogate for CYP51A1 activity.

Fig 1. Detection of Cholesterol (A), Lanosterol (B), and Dihydrolanosterol (C) in pure mixtures and in HaCaT cells treated with 0.1% DMSO or 10 µM Oxiconazole.

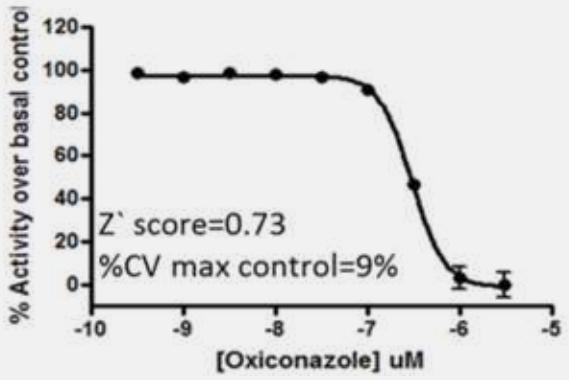


Our next task was to evaluate the effectiveness of this assay format for inhibitor screening by generating the IC^{50} value of a known inhibitor of CYP51A1 in mammalian cells. A dose response curve for oxiconazole was obtained using the optimized protocol. An IC^{50} value of 306 nM was obtained, which was in close agreement with the value generated at the client site by using the gas chromatography method (Fig 2).

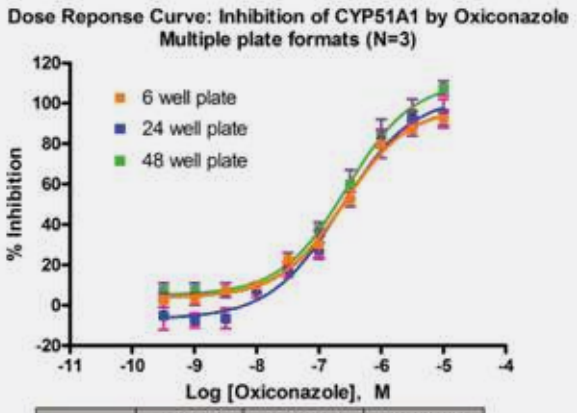
While this was a big step forward, the assay still required a 6-well format, and thus was not at the required throughput. Optimizations of various parameters such as cell density, growth and media conditions, and extraction procedure were performed to further miniaturize the assay. We were able to miniaturize the assay to the level of a 48-well plate with a high signal-to-background ratio of 20. The IC^{50} of oxiconazole was used as a measure of effectiveness of the 48-well format, and as Fig 3 shows, there was close agreement between IC^{50} values obtained in the 6-well, 24-well, and 48-well formats, as well as with the client-generated value using the gas chromatography method.

The CYP51A1 inhibitor screening assay using the RapidFire HTMS method was thus deemed to be optimized, and screening of client compounds was enabled with an acceptable throughput and in a cost-effective manner. This project then went on to yield high potency compounds for the client which allowed them to further their drug discovery plans.

2 Fig 2. Dose response curve of Oxiconazole inhibition of CYP51A1 in HaCaT cells performed in a 6-well plate format using the RapidFire HTMS method. The assay yielded an IC50 value of 306 nM.



3 Fig 3. Dose response curves of oxiconazole inhibition of CYP51A1 activity in HaCaT cells in 6-well, 24-well, and 48-well formats. The IC^{50} values obtained from all the three formats were in close agreement with each other.



	6-well	24-well	48-well
IC50, nM	267 ± 51	236 ± 67	292 ± 78
HillSlope	0.83 ± 0.1	0.76 ± 0.06	0.82 ± 0.11

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