Institution: Swansea University		
Unit of Assessment: UoA3		
Title of case study: Shining a light on azole selectivity: use of a novel CYP protein expression and purification technology in antifungal development		
Period when the underpinning research was undertaken: 2004-2018		
Details of staff conducting the underpinning research from the submitting unit:		
Name(s):	**Role(s) (e.g., job title):**	**Period(s) employed by submitting HEI:**
Prof SL Kelly	Research Lead	2004-present
Prof DE Kelly	Research Lead	2004-present
Dr JE Parker	Senior Researcher	2005-present
Dr CL Price	Senior Researcher	2011-present
Dr A Warrilow	Senior Researcher	2004-present
Period when the claimed impact occurred: 2014-2020		
Is this case study continued from a case study submitted in 2014? No		

1. Summary of the impact

Against a background of growing antibiotic resistance, environmental sustainability considerations and tighter registration regulations to combat safety concerns, novel antifungal discovery and development has necessarily had to adapt and adopt screening strategies to identify appropriate candidates. Exploiting the differences between fungal and host Cytochrome P450 (CYPs) enzymes, researchers at Swansea university have developed a suite of CYPs, used to understand selectivity for the fungal CYP51 enzyme over the human homolog to assist in the design of the next generation of azole antifungals for agriculture as well as the clinic. This research has been integral to the discovery and development of BASF's blockbuster broad spectrum fungicide Mefentrifluconazole (Revysol®), currently being used to successfully increase global crop yields for forty crops in over sixty countries worldwide., The technology has also supported the extension of the exclusive use period for Prothioconazole and had clinical impacts in supporting the development of a new antifungal, Oteseconazole, for recurrent vulvovaginal candidiasis (RVVC), a debilitating condition that affects an estimated 138 million women annually.

2. Underpinning research

Azole antifungals, also known as DMI (14-alpha demethylase inhibitors), are widely used in both agriculture (to control plant diseases and preserve yield and quality of crops) and in the clinic (as therapy for the treatment of invasive fungal infections and for anti-estrogen therapy for breast cancer). Azoles work by inhibiting the Cytochrome P450 (CYP) dependent enzyme lanosterol 14-α-demethylase (CYP51), which converts lanosterol to ergosterol, the main sterol in the fungal cell membrane. Depletion of ergosterol damages the cell membrane resulting in cell death[1]. However, azole inhibition of P450 cytochromes is not always specific to fungal CYP51 and inhibition of human CYPs that contribute to drug metabolism can cause drug-drug interactions, and interactions with human CYPs involved in primary metabolic pathways (e.g., cholesterol biosynthesis) need to be considered. The Swansea P450 research group are focussed on determining the differences that exist between fungal and host cells which may be exploited to combat fungal infection without harming the host.

Previous experimental studies on the specificities of azoles with respect to human and fungal enzymes had relied upon the expression of recombinant proteins, assaying their activities in reconstituted systems and determining the 50% inhibitory concentrations (IC_{50}) of drugs. Functional CYP51 was extremely difficult and time consuming to express, purify, and reconstitute with a highly lipophilic substrate and reductase partner. In 2008 the group produced a strain of *Saccharomyces cerevisiae* containing human CYP51 (huCYP51) targeted to replace the native *S. cerevisiae* CYP51 (ScCYP51) at the chromosomal locus and under the control of the yeast CYP51 promoter and used it to test the specificity of azoles **(R1).** Importantly, the method they described allowed for rapid, high-throughput and reproducible direct side-by-side comparisons of the specificities for new drugs. Their unique suite of azole binding tools was expanded in 2011 to include successful expression of *Mycosphaerella graminicola* CYP51

(MgCYP51) in *E.coli* **(R2),** enabling spectrophotometric analysis using the purified 62-kDa MgCYP51 protein and the first description of an observed selectivity for the market leading Prothioconazole to MgCYP51 from classical azole fungicides. In 2013, this observation was demonstrated to be the result of a chemical breakdown of the product to fungicidal Prothioconazole-desthio **(R3).** In 2015, the group went further and characterised the *M.graminocola* cytochrome P450 reductase (MgCPR)/MgCYP51 redox supporting the conclusion that prothioconazole is a profungicide **(R4).** These represented important developments in fungicide design, producing a functional method to evaluate the effects of agricultural azole fungicides and established the group as international experts within the field, attracting industrial interest as evidenced by 24 linked patents **(R2)** held by Agrosciences industry leaders including *BASF SE and BAYER CROPSCIENCE.*

Previous comparisons of activity had not included agricultural azoles and pure CYP51 forms but had relied instead on microsomal fractions. In 2013, the group expressed *Candida albicans* CYP51 (CaCYP51) and the full-length form and a solubilized form of *Homo sapiens* CYP51 (HsCYP51) in *Escherichia coli*, purifying all three to homogeneity. They performed detailed azole binding studies using UV-visible spectroscopy under oxidative conditions and using therapeutic azole antifungal drugs and agricultural azole. In addition, 50% inhibitory concentration (IC_{50}) determinations for each azole were made using a CYP51 reconstitution assay system. The potential inhibitory action against CaCYP51 and the undesired side effect of inhibiting HsCYP51 were assessed, and the relative selectivity of each compound for the fungal enzyme was determined. The group subsequently described the worrying lack of selectivity of some agricultural fungicides that could impact human steroid production **(R5).**

In 2011, the US company Viamet Pharmaceuticals commissioned the Swansea P450 group to establish the modes of action for their lead antifungal compounds. The group used their purified recombinant CaCYP51 and HsCYP51 to measure the binding and inhibition properties of the candidate molecule VT-1161, and compare them against established treatments (clotrimazole, fluconazole, itraconazole, and voriconazole). These studies demonstrated potent inhibition of *Candida albicans* CYP51 and culture growth without inhibition of human CYP51, indicating a >2,000-fold selectivity, and supporting the potential utility of their candidate molecules in the treatment of Candida infections **(R6).**

[1]https://www.drugs.com/drug-class/azole-antifungals.html

3. References to the research

All outputs represented below are published in peer reviewed journals. All have been supported by either EU Framework 6 or BBSRC/DEFRA funding. R2 is referenced in 24 patents in Europe, US and World IP organizations. R1and R5 were submitted to REF2014, R6 has been submitted to REF2021.

R1. Parker JE, Merkamm M, Manning NJ, Pompon D, **Kelly SL, Kelly DE**. **(2008)** Differential azole antifungal efficacies contrasted using a *Saccharomyces cerevisiae* strain humanized for sterol 14 alpha-demethylase at the homologous locus. Antimicrob Agents Chemother. 52, 3597-3603. DOI:10.1128/AAC.00517-08.

R2. Parker JE, Warrilow AG, Cools HJ, Martel CM, Nes WD, Fraaije BA, Lucas JA, **Kelly DE, Kelly SL. (2011)** Mechanism of binding of prothioconazole to *Mycosphaerella graminicola* CYP51 differs from that of other azole antifungals. Appl Environ Microbiol. 77(4):1460-5. DOI: 10.1128/AEM.01332-10.

R3. Parker JE, Warrilow AG, Cools HJ, Fraaije BA, Lucas JA, Rigdova K, Griffiths WJ, **Kelly DE, Kelly SL. (2013)** Prothioconazole and prothioconazole-desthio activities against *Candida albicans* sterol 14-α-demethylase. Appl Environ Microbiol. 79(5):1639-45. DOI: 10.1128/AEM.03246-12.

R4. Price CL, Warrilow AGS, Parker JE, Mullins JGL, Nes WD, **Kelly DE, Kelly SL**. **(2015)** Novel substrate specificity and temperature sensitive activity of *Mycosphaerella graminicola* CYP51 (MgCYP51) supported by the native NADPH cytochrome P450 reductase (CPR). Appl Environ Microb. 81:3379-3386 DOI: 10.1128/AEM.03965-14.

R5. Warrilow AG, Parker JE, Kelly DE, Kelly SL. (2013) Azole affinity of sterol 14 alpha-demethylase (CYP51) enzymes from *Candida albicans* and *Homo sapiens*. Antimicrob Agents Chemother. 57(3), 1352-1360. DOI: 10.1128/AAC.02067-12.

R6. Warrilow AG, Hull CM, **Parker JE**, Garvey EP, Hoekstra WJ, Moore WR, Schotzinger RJ, **Kelly DE, Kelly SL**. **(2014)** The clinical candidate VT-1161 is a highly potent inhibitor of *Candida albicans* CYP51 but fails to bind the human enzyme. Antimicrob Agents Chemother. 58(12):7121-7. DOI: 10.1128/AAC.03707-14.

Supporting Grants:

G1. STEROLTALK: Functional genomics of complex regulatory networks from yeast to human: crosstalk of sterol homeostasis and drug metabolism; EU Framework 6; 2005-2008; S&D Kelly PIs: EUR2,000,000.

G2. EURESFUN (EUropeanReistanceFUNgal): EU Framework 6; 2005-2008; S Kelly PI; EUR2.755,000.

G3. Antifungal resistance via CYP51 mutation. BBSRC Govt partner award with DEFRA; S&D Kelly; 2008-2011; GBP650,000.

4. Details of the impact

The breakthrough technology developed by researchers at Swansea University's P450 group **(R1-R6)** has enabled selectivity testing for azole development by both AgroSciences and Pharmaceutical industries, leading to the discovery and development of new antifungals for both the field and the clinic.

Impact on agrichemical product development

Mefentrifluconazole (Revysol®)

The P450 Group's major advance in CYP expression and purification technology allowed BASF to establish a high-throughput *in-vitro* screening system enabling them to test synthesised compounds on target fungal enzymes. BASF used the system to steer new product profiles from more than 4000 triazole molecules **(C1).** This system aimed to identify candidates with both high fungicidal activity and minimal likelihood of adverse side effects thought to arise from aromatase inhibition. This high-throughput program identified Revysol® as a result, the UK now has the first new azole to be registered as a fungicide for 15 years, with wide-reaching impacts (see Table 1). The impact the research had on product selection and development was acknowledged by the BASF principal scientist **(C2):** *"Your expertise, technology and purified proteins turned out to be breakthrough technology. They have a high impact on this program and are a major contribution to success."*. The success of the selection strategy has been illustrated for Revysol by the absence of off-target toxicity that would indicate an endocrine disrupting potential **(C3).**

The global fungicides market was valued at **USD13,601,000,000** in 2019[1], highlighting the importance of crop-protection but there is increasing pressure on the AgroSciences industry to maintain a pipeline of novel candidates. Emerging global challenges such as antibiotic resistance, sustainability and tighter regulations have led industry leaders to incorporate selectivity assays early in the development process. Revysol® represents the first and only isopropanol azole of its kind in the market, providing fast-acting and long-lasting disease control for a broad range of crops and disease combinations.

Table 1 The impacts of Revysol®, an exemplar of the advantages of using selectivity assays early in the development process.

Impact type	Benefit
Regulatory	Revysol® was selected and designed to meet the highest level of regulatory standards, see Fig. 1 for global reach of registration as of October 2020 **(C4).**
Health	Revysol® reduces the risk of human endocrine disruption, as attested by EU registration **(C5).**

Figure 1 Global registration status for Revysol® as of October 2020 ***(C4)***

Impact on other fungicides and antifungal compounds.

Prothioconazole (Proline®)

In 2014 Bayer submitted a petition to the United States Environmental Protection Agency (EPA) for a three-year extension of the exclusive use period for their product Prothioconazole **(C6).** Prothioconazole (Proline®) is a triazolinthione fungicide for the control of disease in winter and spring wheat, Durum wheat, winter rye, winter and spring barley, winter and spring oats and for disease control in winter oilseed rape. In their petition the research output, **R4,** was central to their claim that *"substrate binding studies have indicated that Prothioconazole has a different binding site on the CYP51 enzyme compared with other DMI which may explain why it has retained performance where others are weak or ineffective due to a shift in resistance. As a result, Prothioconazole helps reduce further resistant strain selection to the weakening DMI class by offering a more efficacious rotational treatment within the full spray program.*" The petition was granted by the EPA on April 15, 2015 **(C7).**

Oteseconazole (VT-1161)

Currently in Phase 3 clinical studies (see Fig. 2), the clinical candidate VT-1161 is an orally available inhibitor of fungal CYP51 that has shown high potency and selectivity. The molecule VT-1161 was shown to be highly selective for fungal CYP51 **(R6)** and is therefore likely to avoid the side effects that limit the use of current antifungals, including fluconazole and terbinafine, which are often prescribed for acute episodes of vulvovaginal candidiasis and onychomycosis, respectively. **(C8).**

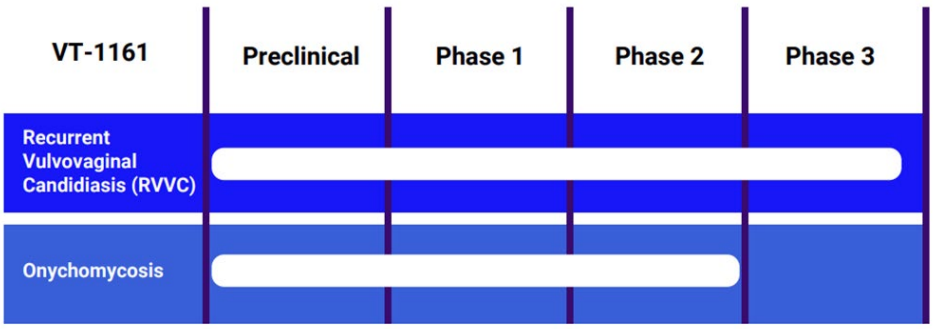

Figure 2 Clinical Trial Status of VT-1161 as of December 2020 (Mycovia Pharmaceuticals: RVVC: NCT02267382; NCT03562156; NCT03561701. Onychomycosis NCT02267356),

5. Sources to corroborate the impact

C1. CCPB proteins used in screening for Revysol®
https://www.youtube.com/watch?v=ZA2_P6VVJSU

C2. Testimonial from Principal Scientist, BASF SE, Dec 2020.

C3. Tesh *et al*. Innovative selection approach for a new antifungal agent mefentrifluconazole (Revysol®) and the impact upon its toxicity profile. Regul Toxicol Pharmacol. 2019 Aug;106:152-168. doi: 10.1016/j.yrtph.2019.04.009.

C4. Data taken from BASF website (https://agriculture.basf.com/global/en/innovations-for-agriculture/innovation-at-a-glance.html).

C5. EU grants approval for BASF's mefentr.ifluconazole, 04/03/19.

C6. Prothioconazole petition 3year extension, submitted by Bayer CropScience Jan 2014. Page 10.

C7. Review of Justification for Extension of Exclusive Use Period for Prothioconazole, EPA, May 2015.

C8. Mycovia Pharmaceuticals Pipeline for VT-1161, https://www.mycovia.com/pipeline