Research Article

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Characterization of the mechanism of action of the fungicide fenpicoxamid and its metabolite UK-2A

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Abstract

BACKGROUND: Fenpicoxamid is a new fungicide for control of *Zymoseptoria tritici*, and is a derivative of the natural product UK-2A. Its mode of action and target site interactions have been investigated.

RESULTS: UK-2A strongly inhibited cytochrome *c* reductase, whereas fenpicoxamid was much less active, consistent with UK-2A being the fungicidally active species generated from fenpicoxamid by metabolism. Both compounds caused rapid loss of mitochondrial membrane potential in *Z. tritici* spores. In *Saccharomyces cerevisiae*, amino acid substitutions N31K, G37C and L198F at the Qi quinone binding site of cytochrome *b* reduced sensitivity to fenpicoxamid, UK-2A and antimycin A. Activity of fenpicoxamid was not reduced by the G143A exchange responsible for strobilurin resistance. A docking pose for UK-2A at the Qi site overlaid that of antimycin A. Activity towards *Botrytis cinerea* was potentiated by salicylhydroxamic acid, showing an ability of alternative respiration to mitigate activity. Fungitoxicity assays against *Z. tritici* field isolates showed no cross-resistance to strobilurin, azole or benzimidazole fungicides.

CONCLUSION: Fenpicoxamid is a Qi inhibitor fungicide that provides a new mode of action for *Z. tritici* control. Mutational and modeling studies suggest that the active species UK-2A binds at the Qi site in a similar, but not identical, fashion to antimycin A.

Keywords: fenpicoxamid; Inatreq™ active; UK-2A; cytochrome *b*; resistance; binding site; modeling

1 INTRODUCTION

Fenpicoxamid is a new fungicide with high potency towards a broad range of Ascomycete plant pathogens.[1] It is currently under commercial development for control of wheat leaf blotch caused by *Zymoseptoria tritici* and banana black Sigatoka caused by *Mycosphaerella fijiensis*. Fenpicoxamid is a derivative of UK-2A[2] (Fig. 1), a natural product that is structurally related to antimycin A and acts on the cytochrome bc_1 complex in the mitochondrial electron transport chain.[3–5] Conversion of fenpicoxamid to UK-2A by removal of its isopropylcarboxymethylether group occurs readily in *Z. tritici* and wheat cells.[1]

The cytochrome bc_1 complex has been a successful target for agricultural fungicides. It contains two quinone binding sites, known as the Qo and Qi sites. As the target site of the strobilurin fungicides (FRAC Code 11), the Qo site has been well exploited for fungicidal use.[6] UK-2A and antimycin A, as well as various other natural and synthetic compounds with fungicidal activity, act at the Qi site.[4,5,7–16] However, to date the only Qi site inhibitors that have been commercialized are the Oomycete-specific fungicides cyazofamid and amisulbrom.

This report summarizes studies undertaken to characterize the mechanism of action of fenpicoxamid and UK-2A at the target site and cellular level, and the risk for resistance development. Fenpicoxamid and UK-2A are compared with the strobilurins and antimycin A in terms of target site selectivity, their effects on mitochondrial membrane potential, and the impact of the alternative oxidase (AOX) on fungitoxicity. The potential for cross-resistance to other commercial fungicides is also discussed. To explore the potential for target site-based resistance and binding at the Qi site, resistant mutants of *Saccharomyces cerevisiae* were generated. A mechanism for binding of UK-2A to cytochrome *b* is proposed based on molecular docking and analysis of mutation effects.

2 METHODS

2.1 Preparation of mitochondrial membranes from *Z. tritici* and assay of cytochrome *c* reductase inhibition

Zymoseptoria tritici, strain ATCC 26518, was grown on potato dextrose agar (PDA) at 18 °C under black lights for 3-5 days. Flasks (2 L) containing 1 L of potato dextrose broth were inoculated with 7×10^5 spores mL^{-1} and incubated at 24 °C on a gyrotary shaker at 140 rpm for 48 h. Fungal mats were collected by vacuum filtration through cheesecloth over a 41-µm Spectra Mesh® nylon filter (Spectrum Laboratories, Rancho Dominguez, CA, USA) and

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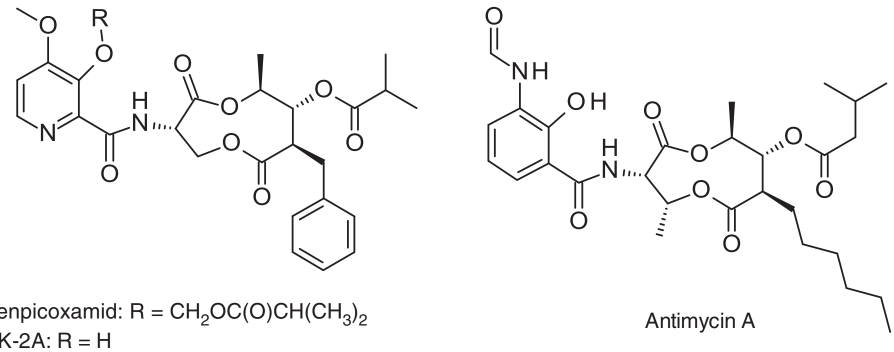

Fenpicoxamid: R = $CH_2OC(O)CH(CH_3)_2$
UK-2A: R = H

Antimycin A

Figure 1. Structures of fenpicoxamid, UK-2A and antimycin A.

resuspended in isolation buffer containing 0.44 M sucrose, 2 mM Na_2EDTA and 0.2% (w/v) bovine serum albumin in 50 mM Tris–HCl buffer, pH 7.3. After disruption in an ice-cooled Bead-Beater (BioSpec Products, Bartlesville, OK, USA) using 0.5-mm-diameter glass beads for 5 × 15 s, with 45-s cooling periods between pulses, the homogenate was centrifuged at 1910 *g* for 10 min at 4 °C. The supernatant was centrifuged at 100 000 *g* for 60 min at 4 °C and the resulting pellet was resuspended in isolation buffer by brief homogenization using a 55 mL Potter-Elvehjem tissue homogenizer (Kimble Chase Life Science, Vineland, NJ, USA), then frozen drop-wise in liquid nitrogen and stored at -80 °C. The protein concentration was 7.1 mg mL^{-1} as determined using the Bio-Rad DC™ protein assay (Bio-Rad, Hercules, CA, USA).

Cytochrome *c* reductase activity was assayed in a reaction buffer, modified from Hill *et al.*,[17] containing 0.2 mM Na_2EDTA, 250 mM sucrose, 0.01% Tween 20, 1 mM sodium azide, 2.5 mM freshly prepared potassium cyanide and 30 μM equine cytochrome *c* in 50 mM potassium phosphate buffer, pH 7.0. Mitochondrial membranes were diluted to 107 μg mL^{-1} in reaction buffer and the assay was initiated by addition of 40 μM decylubiquinol, prepared according to Fisher *et al.*[18] The initial rate of reduction of cytochrome *c* was determined by measuring the increase in absorbance at 550 minus 539 nm. Inhibitors were dissolved in dimethylsulfoxide (DMSO) and tested using 5-fold dilutions series with two replicates at each concentration.

2.2 Evaluation of effects on mitochondrial membrane potential in *Z. tritici* spores

Effects on mitochondrial membrane potential were detected using the MitoProbe™ JC-1 assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and a Guava EasyCyte Plus flow cytometer system (EMD Millipore, Billerica, MA, USA) equipped with a 488-nm laser excitation source.

Test compounds were dissolved in DMSO and 10-fold serial dilutions were prepared. Aliquots (1 μL) of each dilution were transferred to wells of flat-bottomed 96-well microtiter plates, with three replicate wells for each treatment and DMSO alone in control wells. Wells then received 100 μL of YMP broth (20 g of glucose, 3 g of K_2HPO_4, 3 g of KH_2PO_4 and 0.67% Difco™ yeast nitrogen base without amino acids per liter, from Difco Laboratories Inc., Detroit, MI, USA) containing 0.02% Tween 20. Microtiter plate wells were inoculated with 100 μL of *Z. tritici* spore suspension at 5×10^5 spores mL^{-1} in YMP broth and incubated at 23 °C for 2 h, then 2 μL of a 200 μM JC-1 stock solution in DMSO was added, mixed and incubated at 37 °C for 30 min. The plate was then moved to room temperature for 10 min in the dark before analysis. Loss of mitochondrial membrane potential was quantified based on the reduction in the percentage of cells with polarized mitochondria as determined by cytofluorometric analysis of 2000 cells per well from plots of yellow versus green fluorescence.

2.3 Cytochrome *b* mutants of *Saccharomyces cerevisiae*

A wild-type diploid strain of *S. cerevisiae* containing an intron-free version of the cytochrome *b* gene (*cyt b*) was used as the parental strain. The parent strain and two mutant strains, which were generated by site-directed mutagenesis and mitochondrial transformation[17,18] and contained the *cyt b* G143A and K228 M substitutions, were generously provided by Dr. Brigitte Meunier (CNRS Institute for Integrative Biology of the Cell, Gif-sur-Yvette, France).

Mutants resistant to fenpicoxamid (Inatreq™ active from Dow AgroSciences, Indianapolis, IN, USA) and antimycin A were generated using a manganese chloride mutagenesis procedure.[19] The wild-type strain described above was grown overnight in YPD broth (10 g of yeast extract, 20 g of Bacto peptone (Becton, Dickinson and Co., Sparks, MD, USA) and 20 g of dextrose per liter) at 30 °C with shaking at 200 rpm. Manganese chloride (1 mL of a filter-sterilized 100 mM solution) was added to 9 mL of cell suspension at 5×10^7 cells mL^{-1} in YPD broth and incubated for 5 h at 30 °C with shaking at 200 rpm. Cells (100 μL aliquots) were plated on YPG agar (10 g of yeast extract, 20 g of Bacto peptone, 30 ml of glycerol and 20 g of agar per liter) containing either 10 μg mL^{-1} fenpicoxamid or 0.05 μg mL^{-1} antimycin A. First, surviving colonies were streaked out on the same fungicide-containing medium for one or more cycles, and then single colonies were transferred to fungicide-free medium.

2.4 Sequencing the cytochrome *b* gene

The *cyt b* gene was sequenced for the parental *S. cerevisiae* strain, ten mutants which were selected for their ability to grow on plates containing fenpicoxamid and two mutants which were selected on plates containing antimycin A. For DNA preparation, the strains were grown overnight as 15-mL cultures in YPD broth. Cells were pelleted and resuspended in 400 μL of sterile distilled water. Total DNA was extracted from 200 μL of the resuspended cells using the ZR Fungal/Bacterial DNA Miniprep kit from Zymo Research (Irvine, CA, USA; Cat. #D6005). DNA was quantified using a NanoDrop 2000c instrument from Thermo Fisher Scientific and the concentration was adjusted to 10 ng μL^{-1}.

For PCR amplifications, a pair of primers was designed based on the *S. cerevisiae cyt b* gene sequence available in the National Center for Biotechnology Information with accession ID NM_001184362. The pair of primers amplified a 1155-bp fragment of the entire 1158 bp of the *cyt b* gene. The forward primer CYTBSCF1 was 5′-ATGGCATTTAGAAAATCAAATGTGTA-3′ and the reverse primer CYTBSCR1 was 5′-TTTATTAACTCTACCGATATAG-3′. Four 50-μL PCR solutions were pooled and purified using the QIAquick® PCR Purification Kit from Qiagen (Gaithersburg, MD, USA). Forward and reverse sequences were obtained using the primers described above. Also, a second pair of oligonucleotides was designed to generate additional forward and reverse sequences to increase reliability of the sequence. These oligonucletides were spanned by the primers described above. The forward primer CYTBSCF2 was 5′-GAGATGTGCATAATGGTTAT-3′ and the reverse primer CYTBSCR2 was 5′-GTATTACCTCTTACTACACT-3′. All PCR products were externally sequenced by Eurofins MWG Operon (Hunstville, AL, USA). All sequence alignments were carried out using ALIGNX from Vector NTI provided by Invitrogen Corporation, Carlsbad, CA, USA.

2.5 Fungitoxicity assay of *S. cerevisiae* mutants

Compounds were dissolved in DMSO and 3-fold dilutions in DMSO were prepared by serial dilution. Aliquots (1 μL) of each dilution were transferred to wells of flat-bottomed 96-well microtiter plates, with three replicate wells for each treatment, and DMSO alone in control wells. Wells then received 100 μL of YPG broth. Cells taken from overnight cultures grown on YPD medium at 30 °C were suspended in YPG broth at a cell density of 2×10^5 cells mL^{-1}. Wells were inoculated with 100 μL of cell suspension and the plates were incubated at 30 °C for 72 h. Growth was assessed using a NEPHELOstar Galaxy plate reader (BMG Labtech, Cary, NC, USA) and 50% effective concentration (EC_{50}) values for growth inhibition were determined using the CALCUSYN program (Biosoft, Cambridge, UK).

2.6 Molecular docking of ligands at the *S. cerevisiae* Qi site

The crystal structure of the *S. cerevisiae* cytochrome bc_1 complex[20] (PDB: 1EZV) was used as the protein target. The structure was first parameterized and then refined in the Protein Preparation Wizard module (Schrodinger Inc., Cambridge, MA) using the OPLS2005 force field. After the protein system had been well prepared, UK-2A, its protonated form, fenpicoxamid and antimycin A were docked into the Qi site using the Glide SP docking method from Schrodinger Inc. No constraints were used in the docking procedure.

2.7 Germination and growth inhibition assays with *Botrytis cinerea*

A grape isolate of *B. cinerea* (strain B131) was grown at 18 °C on PDA under black light in 9-cm-diameter Petri dishes for 10 – 14 days. To measure inhibition of growth from spore inoculum, fungicides were dissolved in DMSO and 3-fold dilutions were prepared by serial dilution. Aliquots (1 μL) of each dilution were transferred to wells of flat-bottomed 96-well microtiter plates, with three replicate wells for each treatment and DMSO alone in control wells. Wells then received 100 μL of Difco™ Sabouraud dextrose broth (SDB), with and without salicylhydroxamic acid (SHAM) at 500 μg mL^{-1}, followed by 100 μL of an aqueous spore suspension at 1.5×10^5 spores mL^{-1}. After incubation at 23 °C for 72 h, growth was assessed using a NEPHELOstar Galaxy plate reader. To measure inhibition of spore germination, plates were prepared as above, but incubated for only 6 h. Germination-associated spore adhesion was then assessed using sulforhodamine B staining.[21]

Table 1. *Zymoseptoria tritici* strains used for analysis of cross-resistance to commercial fungicides

		Resistance phenotype[a]		
Strain	Origin	Strobilurin	Azole	Benzimidazole
ATCC 26518	Minnesota	S	S	S
NZL12	New Zealand	S	S	S
LARS 15	Long Ashton, UK	S	R[b]	S
FRA-3	France	S	R[c]	R[d]
DEU2	Germany	R[e]	R[f]	R[d]
FRA-6	France	R[e]	R[g]	R[d]
GBR22	Marcham, UK	R[e]	R[h]	R[d]

[a] S, sensitive; R, resistant.
[b] Contains L50S, I381V and Y459D substitutions in sterol 14α-demethylase.
[c] Contains L50S, A379G, I381V, ΔY459/G460 and N531 K substitutions in sterol 14α-demethylase.
[d] Contains the E198A substitution in β-tubulin.
[e] Contains the G143A substitution in cytochrome *b*.
[f] Contains L50S, S188 N, A379G, I381V, ΔY459/G460 and N531 K substitutions in sterol 14α-demethylase.
[g] Contains L50S, I381V and Y459S substitutions in sterol 14α-demethylase.
[h] Contains L50S, I381V and Y459D substitutions in sterol 14α-demethylase.

2.8 Assay for cross-resistance to commercial fungicides

Zymoseptoria tritici strains representing different fungicide resistance phenotypes (Table 1) were kindly provided by the Fraaije laboratory, Rothamsted, Harpenden, UK. Compounds were dissolved in DMSO and 2-fold dilutions in DMSO prepared by serial dilution. Aliquots (1 μL) of each dilution were transferred to wells of flat-bottomed 96-well microtiter plates, with three replicate wells for each treatment and DMSO alone in control wells. Wells received 100 μL of YMP broth, and were then inoculated with 100 μL of spore suspension in YMP broth at 1×10^5 spores mL^{-1}. After incubation at 23 °C for 72 h, growth was assessed using a NEPHELOstar Galaxy plate reader.

3 RESULTS AND DISCUSSION

3.1 Inhibition of cytochrome *c* reductase from *Z. tritici*

UK-2A strongly inhibited cytochrome *c* reductase from *Z. tritici* (Table 2). In comparison with strobilurin fungicides, the inhibitory potency of UK-2A exceeded that of azoxystrobin and approached that of pyraclostrobin. The potency of UK-2A was equivalent to that of antimycin A, whereas in respiratory studies using bovine mitochondrial preparations with succinate as the substrate antimycin A was about 3-fold more potent than UK-2A.[4] *Cyt b* is highly conserved between organisms, [22] and the activity of UK-2A and antimycin A against both fungal and bovine enzymes can be explained by the high amino acid similarity at the Qi site (Fig. 2). Fenpicoxamid was 93-fold less potent than UK-2A in the cytochrome *c* reductase assay, consistent with UK-2A being the active species. We have detected slight conversion of fenpicoxamid to UK-2A by mitochondrial preparations, so the observed cytochrome *c* reductase inhibition by fenpicoxamid probably reflects production of small amounts of UK-2A rather than inhibition by fenpicoxamid itself.

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                 1                                          44
                                                     m      m
                          **  **     **  *      ** **   *  **
S. cerevisiae    M--A----FRKSNVYLSLVNSYIIDSPQPSSINYWWNMGSLLGLCLVIQI
Z. tritici       M--R----IWKSHPLFSLVNGYLIDSPQPSNLSYLWNFGSLLGFCLVIQI
M. fijiensis     ----------------------IID-PQPSNISYLWNFGSLLGFCLVIQI
B. taurus        MT-----NIRKSHPLMKIVNNAFIDLPAPSNISSWWNFGSLLGICLILQI

                 186                                             234
                              m
                      *  **  *  *     *           *    **   *
S. cerevisiae    VPFIIAAMVIMHLMALHIHG-SSNPLGITGNLDRIPMHSYFIFKDLVTVF
Z. tritici       LPFVLAALVLMHLIALHDTAGSGNPLGVSGNYDRLPFAPYFIFKDLITIF
M. fijiensis     FPFVLAALALMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFIFKDLITIF
B. taurus        LPFIIMAIAMVHLLFLHETG-SNNPTGISSDVDKIPFHPYYTIKDILGAL
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Figure 2. Comparison between species of cytochrome *b* sequence in the regions containing amino acids that form the Qi site, and locations of amino acid substitutions that confer resistance to fenpicoxamid and UK-2A. Amino acids located within 4.5 Å of antimycin A in the crystal structure of *Bos taurus* cytochrome bc_1 complex with bound antimycin A[28] are indicated with an asterisk. Sites at which amino acid substitutions confer resistance to UK-2A and fenpicoxamid in *S. cerevisiae* are indicated by "**m**". Amino acids conserved between *S. cerevisiae*, *Z. tritici* and *M. fijiensis* are indicated by shading. Amino acids conserved between all species are in bold. GenBank accession numbers for the sequences used were NM_001184362 (*S. cerevisiae*), AY247413 (*Z. tritici*), AF343070 (*M. fijiensis*) and NC_006853 (*B. taurus*).

Table 2. Inhibition of cytochrome *c* reductase from *Z. tritici* by UK-2A, fenpicoxamid and other complex III inhibitors

Compound	IC_{50}
UK-2A	6.71 ± 1.33
Fenpicoxamid	622.5 ± 339.8
Antimycin A	7.14 ± 1.29
Azoxystrobin	156.1 ± 44.5
Pyraclostrobin	3.48 ± 0.32

Values represent mean 50% inhibitory concentration (IC_{50}) (nM) ± standard deviation from four separate experiments.

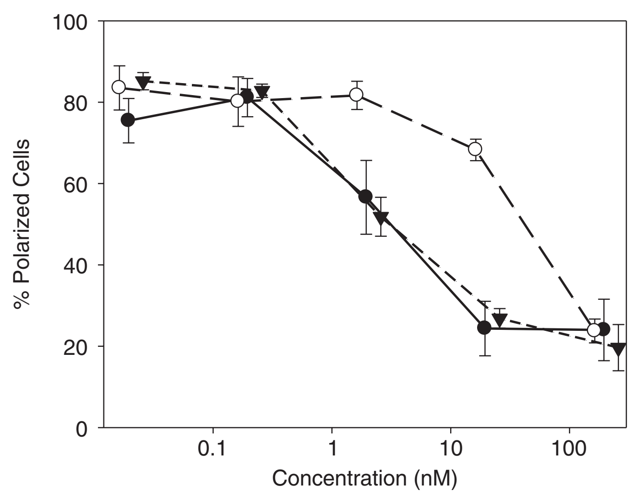

Figure 3. Effects of UK-2A (●), fenpicoxamid (○) and pyraclostrobin(▼) on mitochondrial depolarization in *Z. tritici* spores after treatment for 2 h. Values are mean ± SD of three replicates.

3.2 Effects of fenpicoxamid and UK-2A on mitochondrial membrane potential in *Z. tritici*

Electron transport is responsible for generation of the mitochondrial membrane potential across the inner mitochondrial membrane that drives ATP synthesis. The fluorescent dye JC-1 has been utilized for monitoring mitochondrial membrane potential in cells,[23] and was used in combination with flow cytometric analysis to characterize early effects of UK-2A and fenpicoxamid at the whole-cell level in *Z. tritici*.

At the cellular level, UK-2A, fenpicoxamid and pyraclostrobin caused a rapid loss of mitochondrial membrane potential in spores within 2 h of exposure (Fig. 3). Dose–response analyses of depolarization showed that UK-2A and pyraclostrobin have similar potencies at the cellular level, consistent with their comparable potencies against cytochrome *c* reductase. Fenpicoxamid was estimated to be 13-fold less potent than UK-2A in causing mitochondrial depolarization after the 2-h treatment, consistent with a gradual conversion to UK-2A by *Z. tritici* spores.

3.3 Isolation of resistant mutants in *S. cerevisiae* and analysis of cross-resistance

Saccharomyces cerevisiae was chosen as a model system to explore target site resistance to fenpicoxamid and to gain insight into the binding mechanism of UK-2A through analysis of mutation effects. The choice of *S. cerevisiae* was based on the availability of structural information on cytochrome bc_1,[20] as well as the ease of isolating *cyt b* mutants and extensive literature concerning the effects of mutations on sensitivity to inhibitors.[10–13,17,18,24–26]

Selection of mutants on medium containing fenpicoxamid yielded two types of single amino acid substitution in cytochrome *b*: L198F and G37C, with each substitution found independently in five mutants. Selection on medium containing antimycin A yielded the same L198F substitution as well as an N31K substitution. These mutants and strains containing the K228 M and G143A exchanges were used to analyze cross-resistance relationships between UK-2A, fenpicoxamid and other complex III inhibitors (Table 3).

Substitutions N31K, G37C and L198F conferred a high level of resistance to fenpicoxamid, UK-2A and antimycin A. Amino acid exchanges involving N31, G37 and L198 have been associated previously with resistance to antimycin A,[22,24–27] and these residues are located at the antimycin A binding site in published crystal structures of the bovine and chicken complexes.[28,29] Substitutions at L198 and G37 in *S. cerevisiae* have also been associated with resistance to the Qi site ligands ilicicolin H and funiculosin, two natural products which contain a pyridone ring system,[10,11] and the

Table 3. Effects of cytochrome *b* amino acid substitions in *S. cerevisiae* on sensitivity to fenpicoxamid, UK-2A and other complex III inhibitors

Compound		Parent strain	K228 M (Qi site)	L198F (Qi site)	N3IK (Qi site)	G37C (Qi site)	G143A (Qo site)
Fenpicoxamid	EC_{50}[a]	0.25 ± 0.01	0.10 ± 0.02	7.74 ± 0.88	7.33 ± 1.15	10.02 ± 1.09	0.18 ± 0.01
	RF[b]		0.4	31.0	29.3	40.1	0.7
UK-2A	EC_{50}	0.070 ± 0.014	0.015 ± 0.003	6.98 ± 1.37	6.83 ± 1.24	4.01 ± 0.09	0.048 ± 0.007
	RF		0.2	99.7	97.6	57.3	0.7
Antimycin A	EC_{50}	0.00083 ± 0.00021	0.020 ± 0.007	0.011 ± 0.001	0.044 ± 0.007	0.015 ± 0.003	0.00066 ± 0.00002
	RF		24.1	13.3	53.0	18.1	0.8
Azoxystrobin	EC_{50}	0.0096 ± 0.0011	0.0021 ± 0.0007	0.012 ± 0.001	0.0028 ± 0.0006	0.0058 ± 0.0008	3.92 ± 0.79
	RF		0.2	1.3	0.3	0.6	408.3
1[c]	EC_{50}	0.34 ± 0.13	0.12 ± 0.02	9.92 ± 2.03	8.17 ± 2.26	25.85 ± 3.52	0.14 ± 0.03
	RF		0.4	29.2	24.0	76.0	0.4
2[c]	EC_{50}	0.089 ± 0.016	8.14 ± 0.41	4.92 ± 1.2	12.11 ± 0.56	16.38 ± 2.26	0.045 ± 0.003
	RF		91.5	55.3	136.1	184.0	0.5
3[c]	EC_{50}	9.05 ± 0.48	8.31 ± 1.15	>25	>25	>25	5.80 ± 0.97
	RF		0.9	>2.8	>2.8	>2.8	0.6

[a] Values represent the mean EC_{50} (mg L^{-1}) ± standard deviation for three replicates.
[b] Resistance factor (EC_{50} value for mutant strain/EC_{50} value for parent strain).
[c] Compound structures are provided in Fig. 5.

N31K exchange conferred resistance to the respiratory inhibitor diuron.[26] The ability of substitutions involving N31, G37 and L198 to confer resistance to UK-2A clearly shows that UK-2A binds to the Qi site. These data also support previous evidence for binding of UK-2A to the Qi site of bovine cytochrome bc_1 based on spectral changes.[4] One Qi site substitution, K228M, conferred high resistance to antimycin A, but did not cause resistance to UK-2A. This suggests that interactions of UK-2A and antimycin A at the Qi site are not identical, as discussed in more detail below. Consistent with the action of fenpicoxamid and UK-2A at the Qi site, sensitivity was not reduced by the Qo site G143A exchange, which conferred a high level of resistance to azoxystrobin.

The Qi site is also a promising target for discovery of drugs to treat protozoal infections such as malaria and toxoplasmosis.[12–16] Various pyridones,[15] quinolones such as 1-hydroxy-2-dodecyl-4(1*H*)quinolone (HDQ)[12] and endochin-like quinolones (ELQs),[13,14] as well as the macrocylic inhibitors ML238 and BRD6323[16] act at the Qi site in protozoans, and Qi site amino acid substitutions have been shown to confer resistance to these compounds in *S. cerevisiae*,[12,13] *Plasmodium falciparum*[16] and *Toxoplasma gondii*.[14] While changes involving the particular amino acids associated with resistance to UK-2A and fenpicoxamid in our study have not been linked to resistance to these antiprotozoal agents, some amino acid substitutions reduced sensitivity to antimycin A; T222P, G33V and K228M, which conferred resistance to ELQ-316 in *T. gondii*,[14] BRD6323 in *P. falciparum*[16] and HDQ in *S. cerevisiae*, [12] respectively.

The Qi inhibitor Oomycete fungicides cyazofamid and amisulbrom were launched commercially in 2001 and 2008, respectively. To date, reports of development of resistance to these compounds have been rare.[30] However, in recent resistance monitoring studies in France for grape downy mildew, isolates with specific resistance to these Qi inhibitors were detected (Ecophytopic website; http://viticulture.ecophytopic.fr/sites/default/files/actualites_doc/Note_technique_commune_Vigne_2017_Vdef.pdf). Information on possible mutations in *cyt b* for these isolates is not yet available.

For multiple Qi inhibitors and organisms, there is clear evidence that target site mutations can confer resistance and our results show that this is also true for fenpicoxamid. In relation to possible development of resistance to fenpicoxamid in the field, it is noteworthy that the amino acid subsitutions that conferred resistance in *S. cerevisiae* involve residues (N31, G37 and L198) that are conserved in *Z. tritici* and *M. fijiensis*, the causal agents of the principal diseases targeted by fenpicoxamid (Fig. 2). In *S. cerevisiae*, the L198F exchange did not impair cytochrome *b* reductase activity or respiratory growth,[10] while the impact of substitutions involving G37 appears to depend on the particular amino acid change. G37D, which conferred resistance to ilicicolin H, impaired respiratory growth and reduced cytochrome *b* reductase activity, whereas G37S, which conferred resistance to antimycin A, had little effect on enzyme activity and growth.[10] G37V is known to cause resistance to antimycin A in *S. cerevisiae*[24] and a *Z. tritici* laboratory mutant, selected for resistance to antimycin A, was reported to have the same amino acid change.[27]

For chemistries with various modes of action, it is commonly observed that many target site mutations associated with fungicide resistance in the laboratory are not detected during resistance monitoring studies in the field. Fitness penalties associated with a mutation, the life cycle of the particular pathogen, fungicide use practices, and environmental factors may influence resistance development. As fenpicoxamid will be the first Qi inhibitor fungicide to be used commercially, other than for Oomycete disease control, the emergence in the field of particular mutations conferring resistance in *Z. tritici* or *M. fijiensis* is difficult to predict, justifying further research especially in those target pathogens.

3.4 Molecular docking of UK-2A at the Qi site and comparison with antimycin A

Antimycin A was docked to the published crystal structure for cytochrome bc_1 from *S. cerevisiae*. We found a very similar binding pose and key interactions to those described for the bovine and chicken antimycin – cytochrome bc_1 complexes (Fig. 4). The 3-formylamino salicylamide head binds deeply in the Qi site and forms a hydrogen bond network with nearby residues. The carboxyl group of D229 binds to the NH of the formylamino group

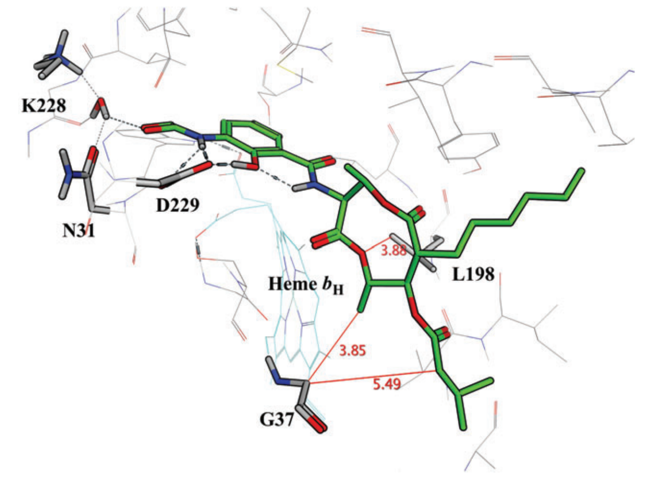

Figure 4. Docking pose of antimycin A bound to the Qi site of *S. cerevisiae* cytochrome bc_1. Red lines indicate distances (Å) between antimycin A and amino acids at which substitutions conferring resistance in *S. cerevisiae* were found.

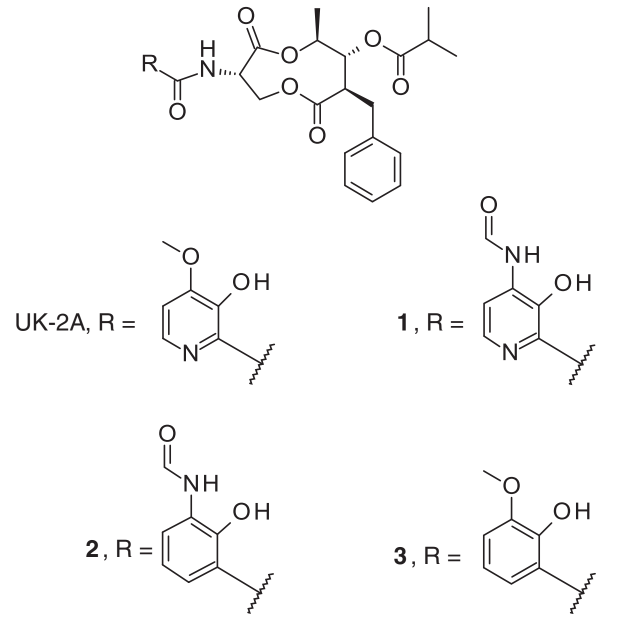

Figure 5. UK-2A analogs with different head groups.

and the OH of the salicylamide head of antimycin A. Another hydrogen bond exists between the carbonyl of the formylamino group and a water molecule, which interacts by hydrogen bonding with K228 and N31. Also, there is an intra-molecular hydrogen bond between the NH of the amide linker and the ring hydroxyl group. Besides multiple hydrogen bonds, hydrophobic interactions also contribute significantly to the binding of antimycin A, mainly resulting from the van der Waals contacts between the nine-member bislactone ring and the hydrophobic side chains of surrounding residues.

UK-2A differs structurally from antimycin A in several areas (Fig. 1). Instead of a 3-formylamino salicylamide head, UK-2A has a 3-hydroxy-4-methoxy-picolinamide head, while on the bislactone ring, UK-2A has 2-methyl propanoate instead of 3-methyl butanoate, and a benzyl instead of a hexyl side chain.

Initial modeling studies suggested that the docking pose for UK-2A could be similar to that of antimycin A, with D229 binding to the hydroxyl group of the picolinamide head. However, mutant sensitivity data for other analogs (Table 3) suggest that the binding conformation for the head group of UK-2A is different from that of antimycin A. Compound **1** (Fig. 5), which differs from UK-2A only by replacement of the 4-methoxy group on the picolinamide with a formylamino substituent, retained strong activity and its activity was not reduced by the K228 M substitution, as would be expected if the picolinamide bound in the same way as the salicylamide of antimycin A. In contrast, the activity of compound **2**, which lacks the pyridyl N and has the same salicylamide head as antimycin A, was greatly reduced by the K228 M exchange, suggesting a similar binding mode to antimycin A. These results suggest that the pyridyl N of compound **1** prevents the interaction of its formylamino group with K228. Furthermore, in UK-2A the importance of the pyridyl N is reinforced by greatly reduced activity of compound **3,** in which pyridine is replaced by a phenyl ring.

Assuming a pivotal role for the pyridyl N of UK-2A in binding, another possible docking pose for UK-2A was proposed (Fig. 6). The new binding mode revealed the overall structural overlap between UK-2A and antimycin A in the Qi binding pocket; however, the pyridine head of UK-2A is flipped by 180 degrees. In this model, the N atom in the pyridine ring is protonated, introducing a positive charge. As a result, the protonated N atom can form a strong salt bridge interaction with the carboxyl group of D229. Statistically, a salt bridge interaction contributes ~2 kcal mol^{-1} to the binding strength and is much stronger than a hydrogen bond (~1 kcal mol^{-1}) between D229 and the OH of the picolinic acid head of UK-2A would be if UK-2A bound similarly to antimycin A. A salt bridge could provide the driving force needed to flip the pyridine ring by 180 degrees. As a result, this docking pose has a more favorable docking score (GlideScore = -10.30) than the pose in which OH interacts with D229 (GlideScore = -9.88). As in the case of antimycin A,[28] our proposed docking pose for UK-2A predicts an intra-molecular hydrogen bond between the ring OH group and the NH of its amide bond which should stabilize the bound conformation. D229 plays a key role in binding Qi site inhibitors, including antimycin A,[28] pyridones such as GSK932121,[15] ascochlorin[31] and 2-nonyl-4-hydroxyquinoline N-oxide (NQNO).[32] However, the interaction between D229 and these inhibitors involves hydrogen bonding with an O atom, in contrast to its proposed involvement in a salt bridge interaction with the pyridyl N atom of UK-2A.

Possible mechanisms underlying effects of mutations that reduce sensitivity to UK-2A and antimycin A can be inferred from our model. The K228 M substitution, which conferred resistance to antimycin, would weaken binding by breaking the hydrogen bond network between K228 and the formylamino group in antimycin A. The different binding mode of UK-2A, and lack of interaction with K228 in the model, can explain why the K228 M exchange did not reduce activity of UK-2A or analog **1,** in which methoxy is replaced by a formylamino group.

The N31K exchange, which reduced activity of both UK-2A and antimycin A, would probably affect the nearby D229 residue as a result of electrostatic Lys-Asp attraction. This would be expected to affect UK-2A binding by disrupting the proposed salt bridge between D229 and the pyridyl N of UK-2A. In the case of antimycin A, the same Lys-Asp attraction could disrupt hydrogen bonds between D229 and the head group.

In the binding models of UK-2A and antimycin A, the bislactone ring of both ligands occupies the same location in the Qi binding site. G37 is close to the exocyclic methyl groups and the ester tails on the bislactone rings of UK-2A and antimycin A, while L198 is

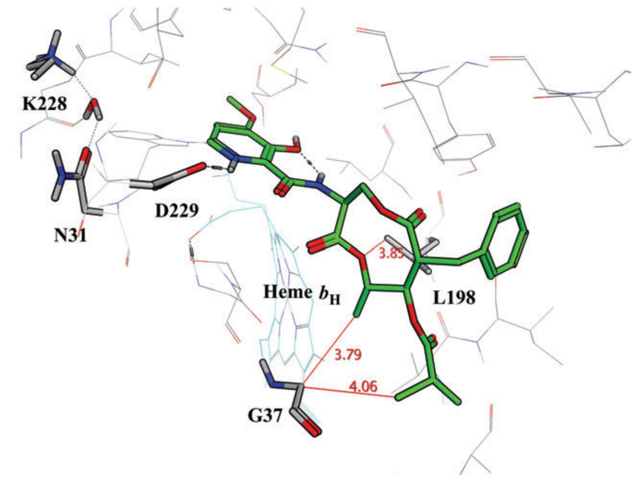

Figure 6. Docking pose of UK-2A bound to the Qi site of *S. cerevisiae* cytochrome bc_1. Red lines indicate distances (Å) between UK-2A and amino acids at which substitutions conferring resistance in *S. cerevisiae* were found.

near the bislactone ring of both ligands. As the G37C and L198F substitutions result in larger residues, reduced sensitivity to UK-2A and antimycin A that results from these mutations is probably caused by displacement of the nine-membered rings from their optimal binding position through steric interference.

Based on its low inhibitory potency towards cytochrome *c* reductase (Table 2), fenpicoxamid would be expected to bind much more weakly than UK-2A, or not at all. Consistent with this prediction, attempts to dock fenpicoxamid in the Qi site showed that the large isopropylcarboxymethylether group prevents it from adopting the same pose as UK-2A. The fact that amino acid substitutions at the Qi site had a similar effect on sensitivity of *S. cerevisiae* to fenpicoxamid and UK-2A can be explained by the ability of *S. cerevisiae* to convert fenpicoxamid to UK-2A as the fungicidally active species.

3.5 Influence of the alternative oxidase on activity of UK-2A and fenpicoxamid

Inhibition of complex III can be circumvented by a pathway involving the alternative oxidase (AOX), which either can be induced in response to inhibition of complex III or may be constitutive.[33,34] This has been well documented for strobilurins,[33,35–39] ametoctradin,[27] and the Qi site inhibitors antimycin A[34] and cyazofamid.[7] Although the AOX provides a bypass around complex III, energy production is less efficient as AOX does not contribute to proton pumping. AOX expression differs between fungal pathogens, and in a given pathogen AOX expression can change depending on growth stage.[33,37–39] As a new fungicide acting on complex III, it is important to establish whether fenpicoxamid is affected by the AOX.

Salicylhydroxamic acid (SHAM) is a well-known inhibitor of AOX, and is a useful tool for characterizing AOX function through its ability to synergize the *in vitro* activity of complex III inhibitors.[7,33,35,37,39] To establish whether AOX affects fenpicoxamid activity, the effects of SHAM on the activities of fenpicoxamid, UK-2A, antimycin A and pyraclostrobin were compared. *Botrytis cinerea* was chosen for these experiments as the role of AOX in overcoming inhibitory effects of strobilurins has been well studied in this organism.[37,39] Strobilurins strongly inhibit germination of *B. cinerea* spores, but only delay rather than prevent germination in the absence of an AOX inhibitor.[39] After spores have germinated, subsequent mycelial growth is relatively insensitive as a consequence of constitutive expression of AOX.[37]

After incubation for 6 h, fenpicoxamid and UK-2A strongly inhibited spore germination. UK-2A showed comparable potency to pyraclostrobin, whereas fenpicoxamid was less active than UK-2A (Table 4). At this early time-point, both Qo and Qi site inhibitors were highly active in the absence of SHAM, and inclusion of SHAM produced only a modest increase in activity. However, after prolonged incubation (72 h) the compounds maintained strong growth inhibition in the presence of SHAM, but not in its absence. These results demonstrate that fungitoxicity of fenpicoxamid and UK-2A is affected by activity of the AOX similarly to other complex III inhibitors. High expression of AOX may explain why fenpicoxamid has shown only moderate activity against *B. cinerea* in greenhouse testing despite its potent inhibition of spore germination *in vitro* (Table 4).

Whereas *in vitro* studies have shown that the AOX can overcome fungitoxicity of complex III inhibitors, effects on field performance are poorly understood. In the case of ametoctradin, field isolates of *Plasmopara viticola* that overexpressed AOX were much less sensitive *in vitro* but appeared to have reduced fitness.[27] However, *P. viticola* strains with reduced sensitivity to ametoctradin, cyazofamid and amisulbrom attributable to AOX have recently been reported in France with resistance progressing, suggesting an ability of AOX-overexpressing strains to compete under field conditions (Ecophytopic website; http://viticulture.ecophytopic.fr/sites/default/files/actualites_doc/Note_technique_commune_Vigne_2017_Vdef.pdf).

In *Z. tritici*, certain field isolates have been described which appeared less sensitive to strobilurins as a consequence of the AOX.[40] However, this mechanism has had no apparent effect on strobilurin field efficacy, possibly because the alternative pathway is less efficient in energy production and renders these isolates less fit. The primary mechanism responsible for loss of disease control by strobilurins in cases of field resistance involves target site mutation, particularly the G143A substitution in cytochrome *b*.[6] Because cytochrome *b* is encoded by a mitochondrial gene, with many copies per cell, a single point mutation event would not confer a high degree of resistance without further selection of resistant mitochondria. It has been speculated that AOX may be involved in development of strobilurin resistance by enabling continued slow growth of *Z. tritici* in the presence of a Quinone outside Inhibitor (QoI) fungicide *in planta*, allowing resistance mutations in mtDNA to predominate.[40] However, evidence for this role of AOX under field conditions is lacking.

3.6 *In vitro* evaluation of cross-resistance between fenpicoxamid, UK-2A and commercial fungicides against *Z. tritici*

The ability to control pathogens that are resistant to existing commercial fungicides is critical for success of a new fungicide. As fenpicoxamid is the first Qi site inhibitor to be developed outside the Oomycete fungicide market, cross-resistance to commercial fungicides based on target site mutations would not be anticipated, but nevertheless must be investigated. A representative set of *Z. tritici* field isolates exhibiting sensitivity or target site-based resistance to strobilurin, azole or benzimidazole fungicides (Table 1) was tested for sensitivity to fenpicoxamid and UK-2A. Field isolates with resistance to benzimidazoles (carbendazim) or reduced sensitivity to azoles (epoxiconazole) did not show cross-resistance to fenpicoxamid and UK-2A (Table 5). Also, strobilurin-resistant

Table 4. Effect of SHAM on inhibition of *B. cinerea* by Qi and Qo site inhibitors

	Inhibition of germination at 6 h[a]		Inhibition of growth at 72 h[a]	
Compound	Minus SHAM	Plus SHAM	Minus SHAM	Plus SHAM
Fenpicoxamid	0.047 ± 0.0064	0.028 ± 0.00019	>2	0.047 ± 0.015
UK-2A	0.0035 ± 0.00040	0.0016 ± 0.000074	>0.2	0.0051 ± 0.0036
Antimycin	0.029 ± 0.0028	0.012 ± 0.000090	>0.2	0.0091 ± 0.0030
Pyraclostrobin	0.0047 ± 0.00064	0.0028 ± 0.000017	>0.2	0.0097 ± 0.0025

[a] Values represent the mean EC_{50} (mg L^{-1}) ± standard deviation for three replicates.

Table 5. *In vitro* sensitivity of fungicide-resistant *Z. tritici* field isolates to fenpicoxamid and UK-2A[a]

Strain	Fenpicoxamid	UK-2A	Azoxystrobin	Epoxiconazole	Carbendazim
ATCC 26518	0.0073 ± 0.00064	0.00055 ± 0.00013	0.0021 ± 0.000021	0.0022 ± 0.00089	0.028 ± 0.0092
RF[b]			0.98	0.96	1.0
NZL-12	0.012 ± 0.0013	0.00068 ± 0.000085	0.0022 ± 0.00027	0.0024 ± 0.00058	0.028 ± 0.0045
RF			1.0	1.0	1.0
LARS 15	0.022 ± 0.0015	0.0014 ± 0.00016	0.0061 ± 0.00020	0.048 ± 0.010	0.032 ± 0.0045
RF	2.3	2.3	2.8	21	1.1
FRA-3	0.011 ± 0.00048	0.0019 ± 0.00010	0.0064 ± 0.00094	0.072 ± 0.018	>5
RF	1.1	3.1	3.0	31.3	>179
DEU-2	0.0033 ± 0.00039	0.00081 ± 0.00008	2.04 ± 0.051	0.049 ± 0.025	>5
RF	0.34	1.3	949	21.3	>179
FRA-6	0.0018 ± 0.00026	0.00047 ± 0.00011	1.48 ± 0.017	0.071 ± 0.00080	>5
RF	0.19	0.76	688	30.9	>179
GBR-22	0.0031 ± 0.0011	0.00019 ± 0.000066	1.54 ± 0.24	0.03 ± 0.018	>5
RF	0.32	0.31	716	13	>179
Average wild-type sensitivity[c]			0.0022	0.0023	0.028

[a] Values represent the mean EC_{50} (mg L^{-1}) ± standard deviation for three replicates.
[b] RF = EC_{50} value/mean of EC_{50} values for strains ATCC 26518 and NZL-12.
[c] Mean of EC_{50} values for strains ATCC 26518 and NZL-12.

isolates containing the G143A exchange in cytochrome *b* were fully sensitive to fenpicoxamid and UK-2A, consistent with results described earlier for the analogous substitution in *S. cerevisiae* (Table 3).

The strobilurin fungicides provide an interesting benchmark in considering the potential for development of resistance to fenpicoxamid as they have also been used to control *Z. tritici* and *M. fijiensis*, act on the same mitochondrial-encoded cytochrome *b* protein, and appear to be affected similary by the alternative oxidase pathway. The first strobilurins, kresoxim-methyl and azoxystrobin, were introduced commercially in 1996 and 1997, respectively.[6] Resistance was reported in *M. fijiensis* in 2000[41] and in *M. graminicola* in 2002,[42,43] and in both organisms was caused by the G143A substitution. After their detection, the frequency of these isolates increased rapidly such that the ability of strobilurins to control these pathogens has been severely compromised. Whether a comparable mutation conferring a high level of resistance to fenpicoxamid can arise in the field population is unknown.

Inhibitors of succinate dehydrogenase (SDHIs) represent a major new class of fungicides for control of *Z. tritici*. Field resistance to SDHIs has occurred in a number of diseases and is starting to emerge in *Z. tritici*.[44] SDHI-resistant field isolates of *Z. tritici* have not yet been tested for sensitivity to fenpicoxamid and UK-2A; however, SDHI-resistant laboratory mutants of *Z. tritici* do not show reduced sensitivity to fenpicoxamid (Fraaije B, unpublished). Cross-resistance between fenpicoxamid and SDHIs caused by target site mutations would not be anticipated based on their different modes of action.

In recent years, monitoring studies have identified *Z. tritici* isolates in which a reduced sensitivity to fungicides with unrelated modes of action results from overexpression of efflux pumps, especially the major facilitator superfamily drug transporter MgMfs1.[45–47] These multi-drug-resistant (MDR) strains were less sensitive to the three classes of unisite fungicides currently used to control *Z. tritici* [sterol 14α-demethylation inhibitors (DMIs), QoIs and SDHIs], but sensitivity to the multi-site fungicide chlorothalonil was not reduced.[46] *In vitro* sensitivity testing has shown that MDR strains also exhibit reduced sensitivity to fenpicoxamid (Walker A-S, unpublished). Levels of resistance conferred by overexpression of efflux pumps are generally low to moderate[45–47] and the impact of this resistance mechanism alone on performance of fenpicoxamid in the field would probably be limited. Nevertheless, as exemplified by resistance to DMIs, in combination with other resistance mechanisms multi-drug resistance may contribute to high levels of resistance.[46]

As a novel unisite fungicide acting at the Qi site, fenpicoxamid provides a much needed new mode of action for control of *Z. tritici*, and implementation of an appropriate resistance management and monitoring strategy for fenpicoxamid is important to maintain its effectiveness. While target site mutations probably present the

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greatest resistance risk for fenpicoxamid, induction of the AOX and efflux pump overexpression might also contribute to resistance development; we hope that future studies will further advance our understanding of these mechanisms and provide guidance for resistance management.

4 CONCLUSION

The fungicidal activity of fenpicoxamid involves action of its hydrolytic breakdown product UK-2A at the Qi site of complex III in the mitochondrial electron transport chain. At the cellular level, fenpicoxamid and UK-2A resemble other complex III inhibitors in their rapid effects on mitochondrial membrane potential and the influence of AOX on activity. Analysis of mutations conferring resistance in *S. cerevisiae* and molecular docking studies show that UK-2A binds at the same site as antimycin A. Fenpicoxamid offers a new fungicidal mode of action for control of *Z. tritici* and other pathogens and is not cross-resistant to other fungicides.

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