International Center
for Public Health

Update on resistance mechanisms: Highly active triazole antifungal agents

David S. Perlin, PhD
Executive Director and Professor
Public Health Research Institute / New Jersey Medical School

PHRI

28 January, 2012

UMDNJ
University of Medicine & Dentistry of New Jersey

COI: Opinion Leader panels and grant support from Merck, Pfizer, Astellas, Celgene and bioMerieux.

In the beginning……

Antimicrob Agents Chemother. 1997 Jun;41(6):1364-8.

Itraconazole resistance in *Aspergillus fumigatus*.

Denning DW, Venkateswarlu K, Oakley KL, Anderson MJ, Manning NJ, Stevens DA, Warnock DW, Kelly SL.

Abstract

……**In vitro resistance in *Aspergillus fumigatus* to itraconazole correlated with in vivo outcome has not been previously described.** For three isolates (AF72, AF90, and AF91) of *A. fumigatus* from two patients with invasive aspergillosis itraconazole MICs were elevated. A neutropenic murine model was used to establish the validity of the MICs. …….**The MICs for the three resistant isolates were >16 microg/ml. In vitro resistance was confirmed in vivo for all three isolates.** Molecular typing showed the isolates from the two patients to be genetically distinct……..

Drug Susceptibility, ECVs, Break Points and Resistance

Wild-type MIC distributions of three triazoles for six *Aspergillus* spp. from five laboratories, using CLSI M38-A2 microdilution method

Species	Antifungal agent	Total	No. of isolates With MIC (µg/ml):											
			≤0.01	0.03	0.06	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16	32
A. fumigatus	Itraconazole	2,554		22	114	461	752	963	211	31	(8)	(14)	(15)	
	Posaconazole	1,647	105	422	430	351	230	96	12	(7)	1			
	Voriconazole	2,778		1	16	123	1,193	1,091	291	39	17	7	(1)	
A. flavus	Itraconazole	536		7	30	151	121	194	31	2	(1)		(1)	
	Posaconazole	321		32	132	84	56	13	2	2			(1)	
	Voriconazole	590			1	15	115	290	158	10	(1)		(1)	
A. terreus	Itraconazole	369		24	48	72	104	73	48					
	Posaconazole	330		19	52	91	126	41		1				
	Voriconazole	462		2		24	99	217	106	10			1	3
A. niger	Itraconazole	427		2	7	13	42	119	178	66	(7)	(31)	(3)	
	Posaconazole	325		15	36	77	61	126	4	6				
	Voriconazole	479		3	5	19	59	174	169	47	3			(1)

Espinel-Ingroff et al. 2010 JCM

MIC distribution and ECVs for three triazoles and six *Aspergillus* spp. from five laboratories as determined by the CLSI M38-A2 microdilution method at 48 h

Species	antifungal	(no. isolates)	MIC (ug/ml)	mode (ug/ml)	ECV (ug/ml)	ECV (%)
A. fumigatus						
	Itraconazole	(2,554)	≤0.03-2	(0.5)	1	98.8
	Posaconazole	(1,647)	≤0.015-4	(0.06)	0.5	99.2
	Voriconazole	(2,778)	0.03-≥4	(0.25)	1	97.7
A. flavus						
	Itraconazole	(536)	0.03-2	(0.5)	1	99.6
	Posaconazole	(321)	≤0.03-2	(0.06)	0.25	94.7
	Voriconazole	(590)	0.06-4	(0.5)	1	98.1
A. terreus						
	Itraconazole	(369)	0.03-1	(0.25)	1	100
	Posaconazole	(330)	≤0.03-2	(0.25)	0.5	99.7
	Voriconazole	(462)	0.03->4	(0.5)	1	99.1
A. niger						
	Itraconazole	(427)	0.03-2	(1)	2	100
	Posaconazole	(325)	≤0.03-2	(0.5)	0.5	96.9
	Voriconazole	(479)	≤0.03-4	(0.5)	2	99.4

Espinel-Ingroff et al. 2010 JCM

CLSI Clinical Breakpoints and ECVs

- Clinical breakpoints are not available for mold testing by the CLSI methodology versus any antifungal agent.
- In the absence of clinical breakpoints, ECVs provide guidance to characterize the susceptibility of Aspergillus isolates to itraconazole, posaconazole, and voriconazole

EUCAST: Proposed interpretative breakpoints* for *A. fumigatus* and highly active azoles.

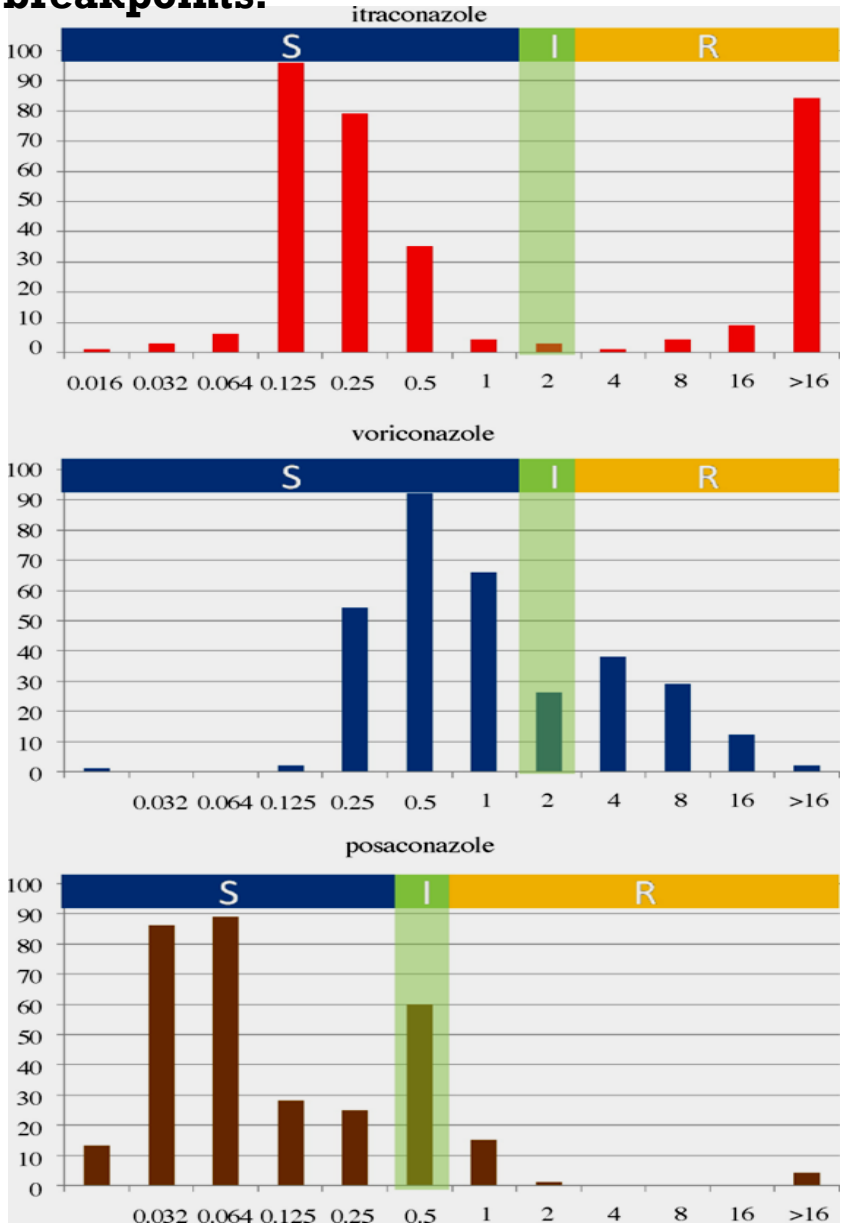
(MIC, mg/L)

Drug	Susceptible	Intermediate	Resistant
Itraconazole	<2	2	>2
Voriconazole	<2	2	>2
Posaconazole	<0.5	0.5	>0.5

*Breakpoints based on MIC distributions, pharmacokinetic and pharmaco-dynamic (PK/PD) parameters, animal data, and clinical experience have been proposed for the EUCAST reference method for *A. fumigatus* and the three triazoles.

Verweij et al. DRU *12 (2009) 141*

Relationship between MIC distribution of 325 consecutive MIC determinations of clinical *A. fumigatus* isolates from the Nijmegen fungus culture collection and the proposed breakpoints.



Verweij et al. DRU *12 (2009)*

Multiple-Triazole–Resistant *Aspergillus fumigatus*

Table 1. Characteristics of Nine Patients from Whom *A. fumigatus* Resistant to Multiple Triazoles Was Cultured.

Sex	Yr of Age	Underlying Disease	Date of Isolation	Site of Isolation	Disease Classification*	Previous Azole Exposure	Treatment	Outcome
Male†	15	X-linked chronic granulomatous disease	April 4, 2002	Sputum	Breakthrough invasive pulmonary aspergillosis, proven	Prophylaxis with itraconazole (for 6 yr)	Voriconazole (high-dose)	Survived
Male	73	None	Dec. 3, 2003	Ear swab	Invasive aspergillosis of mastoid cavity, proven	None	Surgery and topical therapy	Survived
Male	16	Hyper-IgE syndrome	Nov. 19, 2004	Bronchoalveolar-lavage fluid	Breakthrough invasive pulmonary aspergillosis, proven	Treatment with voriconazole (for 2 yr)	Surgery and posaconazole	Survived
Female	76	Pulmonary fibrosis	June 26, 2005	Sputum	Invasive pulmonary aspergillosis, possible	None	Voriconazole	Survived
Male	31	Chronic granulomatous disease	Nov. 1, 2005	Lung aspirate	Breakthrough invasive pulmonary aspergillosis, probable	Prophylaxis with itraconazole (for >10 yr)	Caspofungin and posaconazole	Survived
Female	68	Acute myeloid leukemia	Feb. 14, 2006	Bronchoalveolar-lavage fluid	Disseminated invasive aspergillosis, probable	None	Voriconazole	Died
Female	62	Chronic obstructive pulmonary disease	April 5, 2006	Bronchoalveolar-lavage fluid	Invasive pulmonary aspergillosis, possible	None	Voriconazole, amphotericin B, and posaconazole	Survived
Male	19	Chronic granulomatous disease	April 15, 2006	Bone	Breakthrough aspergillus osteomyelitis, proven	Prophylaxis with itraconazole (for >2 yr)	Voriconazole, caspofungin, and posaconazole	Survived
Male	45	Acute myeloid leukemia and allogeneic hematopoietic stem-cell transplantation	May 11, 2006	Nose swab	Breakthrough aspergillus sinusitis, proven	Prophylaxis with itraconazole (for 4 wk)	Posaconazole	Died

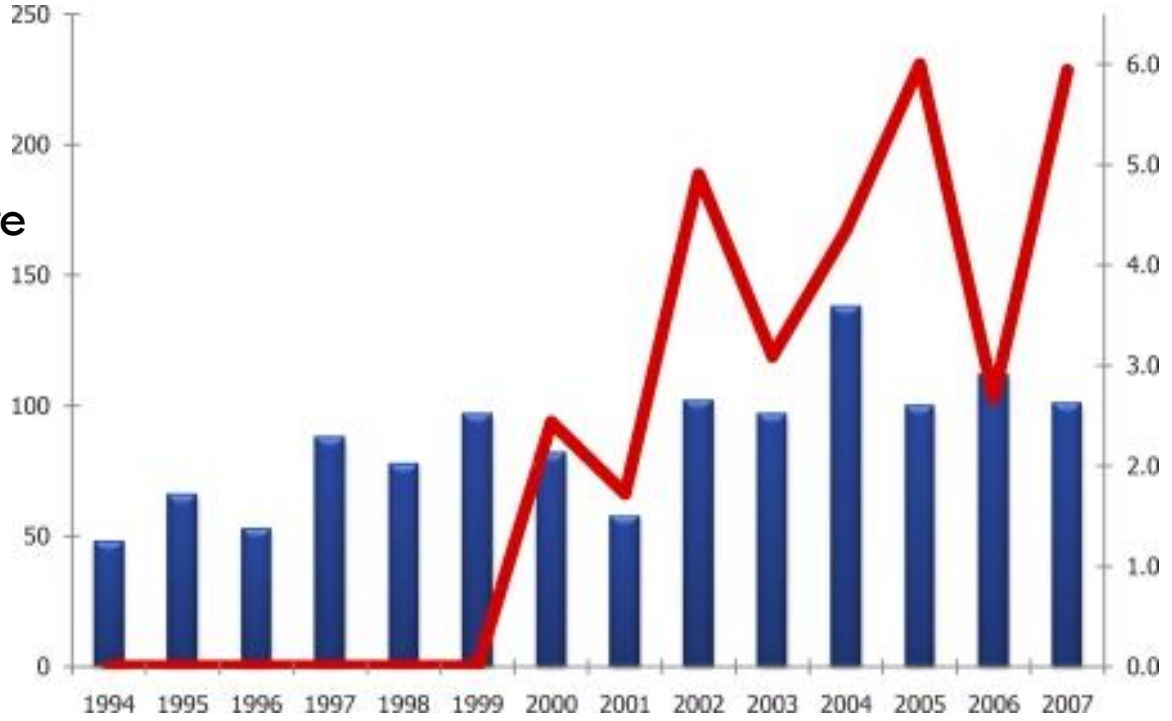
* Diseases were classified according to consensus criteria defined by the European Organisation for Research and Treatment of Cancer and the National Institute of Allergy and Infectious Diseases Mycoses Study Group.
† Information about this patient is from Warris et al.[3]

Factors: Extended duration prophylaxis with itraconazole or therapy with voriconazole; Inherent resistance dominated by a single mechanism Leu98/TR

Verweij et al. 2007 NEJM

Epidemiology of ITZ Resistance in the *A. fumigatus* Isolates from Nijmegen Medical Center

Patients with a positive *A. fumigatus* culture



percentage of those patients with an ITZ+ isolate

Snelders et al. PLoS Med. (2008).

Emerg Infect Dis. 2011 Oct;17(10):1846-54.

Clinical implications of azole resistance in Aspergillus fumigatus, The Netherlands, 2007-2009.

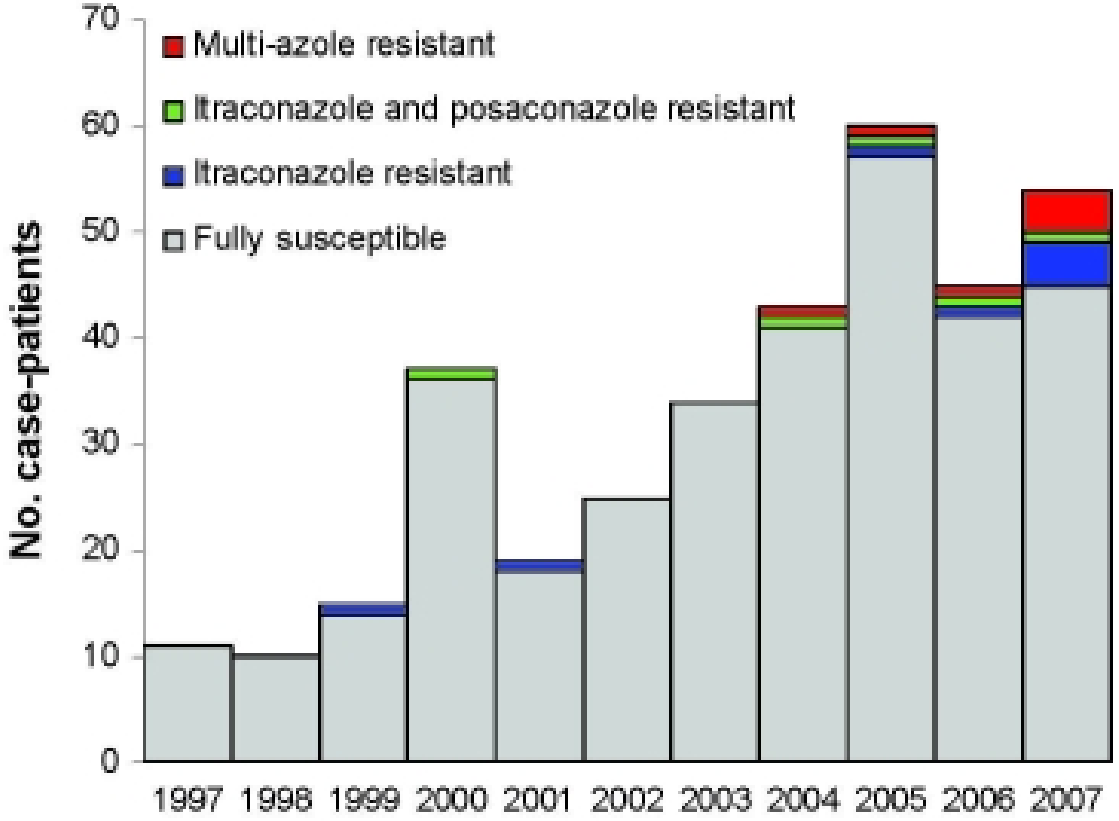
van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, Kuijper EJ, Van Tiel FH, Melchers WJ, Verweij PE.

From June 2007 through January 2009, all clinical Aspergillus spp. isolates were screened for itraconazole resistance. **In total, 2,062 isolates from 1,385 patients were screened; the prevalence of itraconazole resistance in *A. fumigatus* in our patient cohort was 5.3%** (range 0.8%-9.5%).

Patients with a hematologic or oncologic disease were more likely to harbor an azole-resistant isolate than were other patient groups ($p<0.05$). **Most patients (64.0%) from whom a resistant isolate was identified were azole naive,** and the case-fatality rate of patients with azole-resistant invasive aspergillosis was 88.0%.

Multiazole resistance in *A. fumigatus* is widespread in the Netherlands and is associated with a high death rate for patients with invasive aspergillosis.

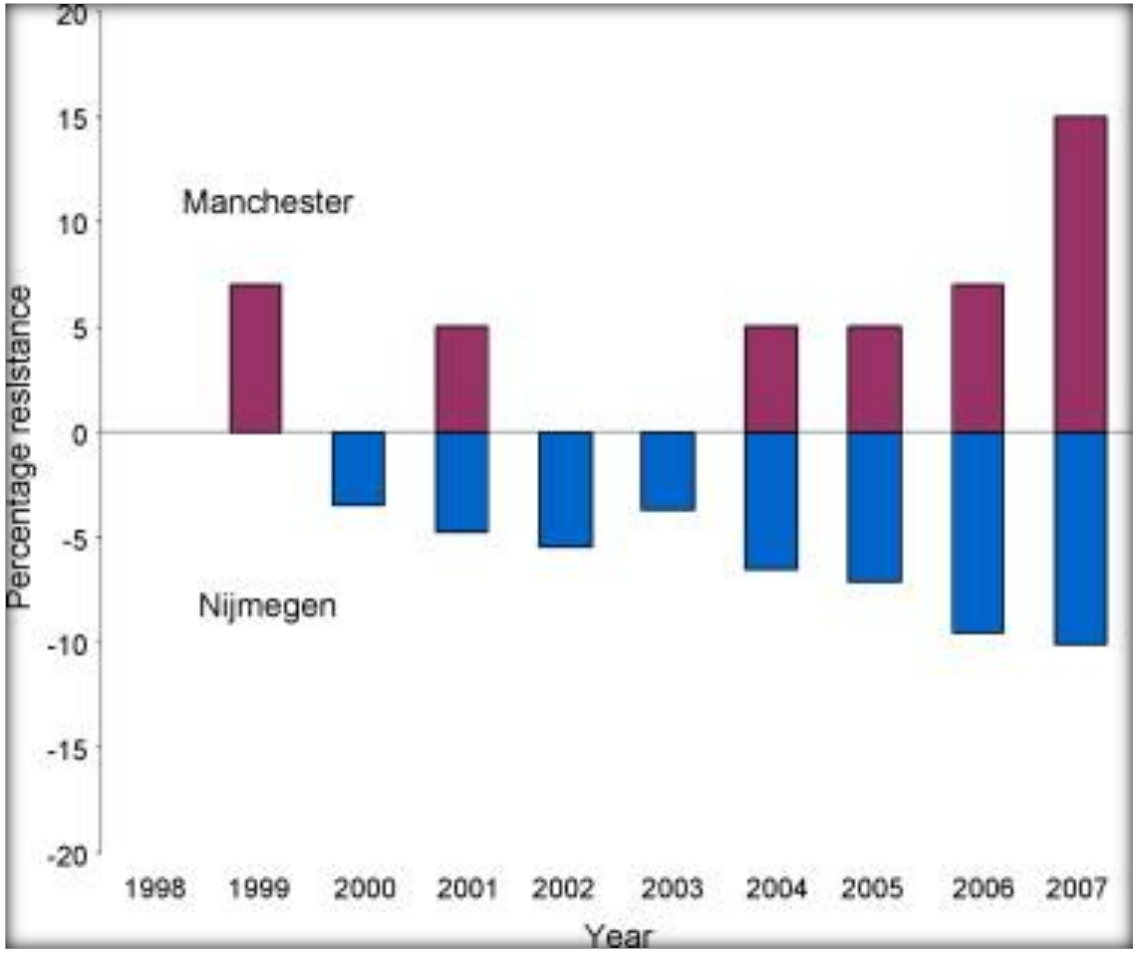
Azole resistance in clinical *A. fumigatus* isolates received in Regional Mycology Lab Manchester, UK, 1997–2007.



Multiazole resistant isolates are on the rise.

Howard et al. EID (2009)

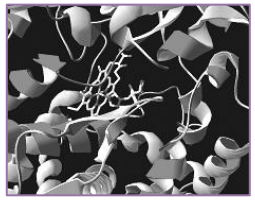
Percentage of patients with azole-resistant *A. fumigatus* strains in Manchester, UK and Nijmegen, the Netherlands (1998–2007).



Verweij et al. DRU *12 (2009) 141*

Known azole resistance mechanisms

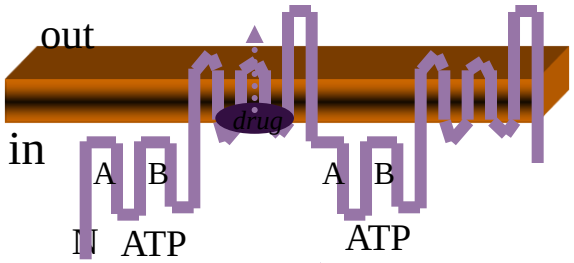
I. Alteration of Drug Target



Erg11 *Candida* spp.
Erg5 Candida spp.
Erg11 *Cr. neoformans*
Cyp51A *Aspergillus* spp.

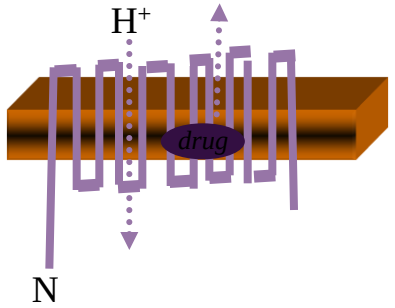
II. Over-expression of Efflux Transporters

ABC



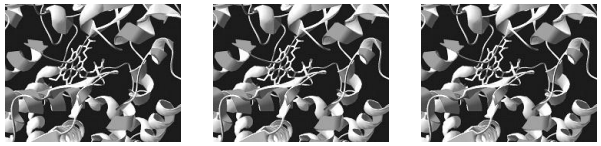
Cdr1, *C. albicans*
Cdr1 *C. glabrata*
Pdh1 *C. glabrata*
Mdr1,2,4 *A. fumigatus*
Afr1 *C. neoformans*

MFS



Mdr1 *C. albicans*
Flu1 *C. albicans*
Mcm1 *C. albicans*
Mdr3 *A. fumigatus*

III. Over-expression of Target Site



Erg11 *Candida spp.*
Cyp51A *A. fumigatus*

IV. Chromosomal duplications

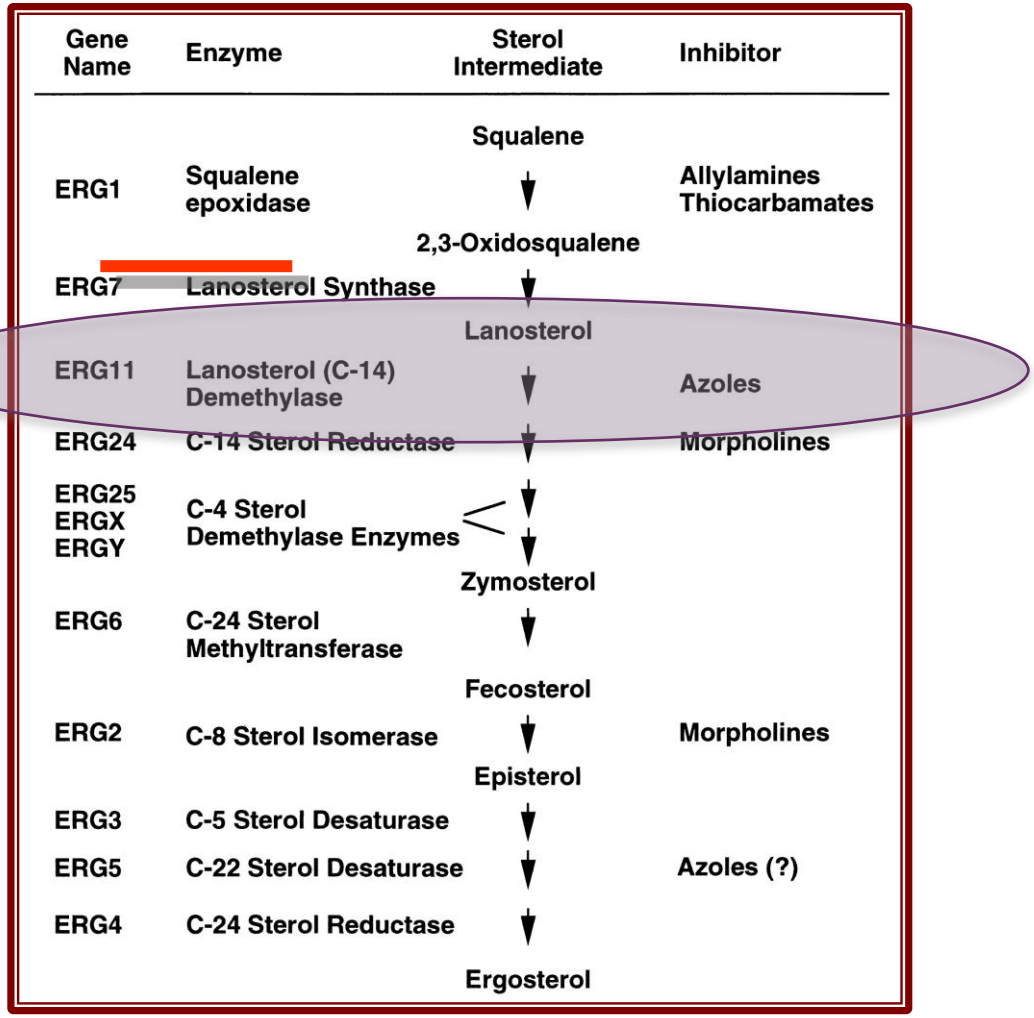
C. albicans

V. Transcription factors

Drug pumps
Erg 11

Prominent amino acid substitutions in the target site, lanosterol demethylase encoded by Cyp51A, account for >80% of acquired resistance in *A. fumigatus*.

***Aspergillu* spp. :**
Cyp51A
Cyp51B



Ergosterol Biosynthesis: Azole Inhibition

Influence of most prominent azole-resistant Cyp51A genotypes on susceptibility of *A. fumigatus* to highly active triazole antifungal agents

Genotype	MIC (mg/L) Phenotype			Reference[a]
	ITR	VOR	POS	
L98H+TR	>8	0.5 to >8	0.5–4	Mellado et al. (2007), Hodiamont et al. (2009), Van der Linden et al. (2009)
G54	>8	0.125–1	1 to >8	Chen et al. (2005), Mann et al. (2003), Diaz-Guerra et al. (2003), Nascimento et al. (2003), Howard et al. (2006c), Manavathu et al. (2003)
G138	>8	2 to >8	1 to >8	[Howard et al., 2006b] and [Howard et al., 2006c], Manavathu et al. (2003)
M220	>8	0.5–4	0.125 to >8	Mellado et al. (2004), Chen et al. (2005), Diaz-Guerra et al. (2003), Howard et al. (2006c)
G448	>8	>8	0.5–2	Manavathu et al. (2003)

Environment acquired

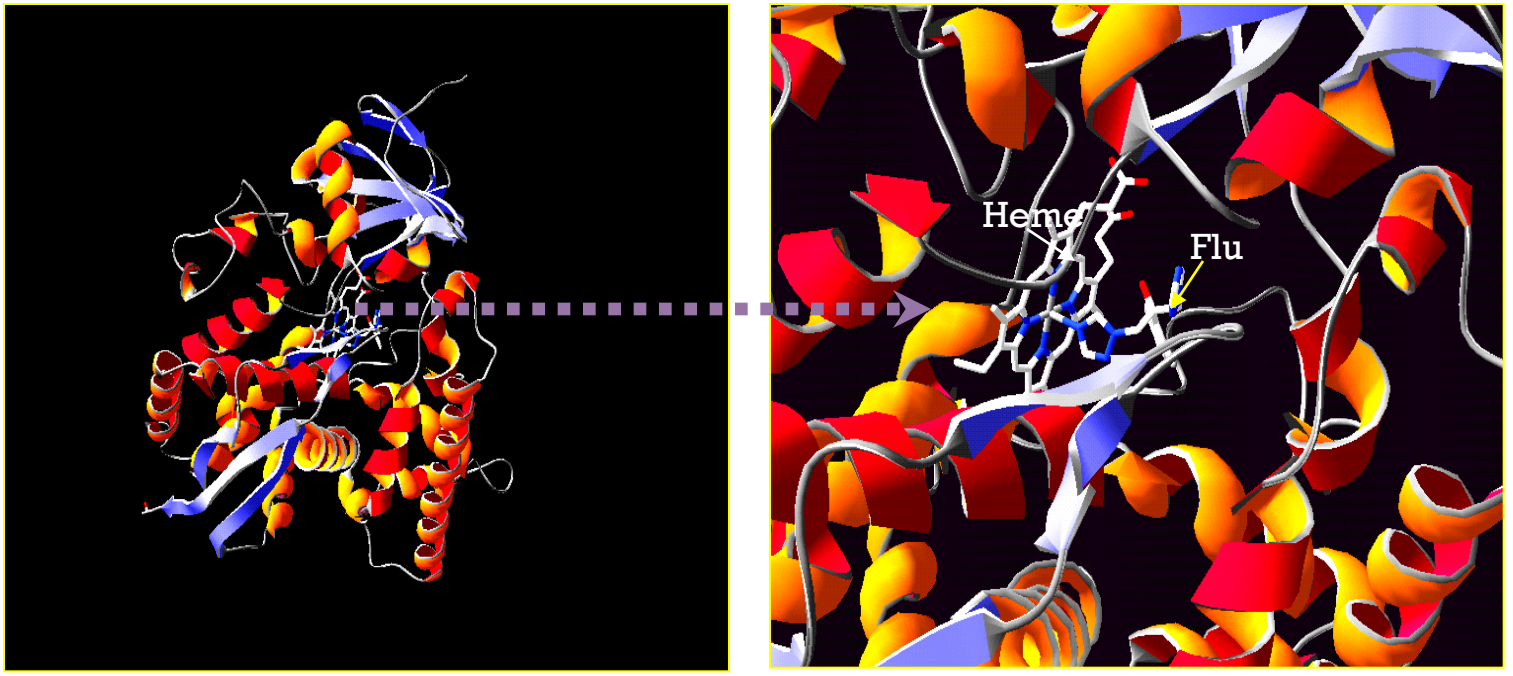
Acquired during therapy

Verweij et al. DRU *12 (2009) 141*

Reported Cyp51A amino acid substitutions and associated cross-resistance patterns in azole-resistant RMLM *Aspergillus fumigatus* isolates

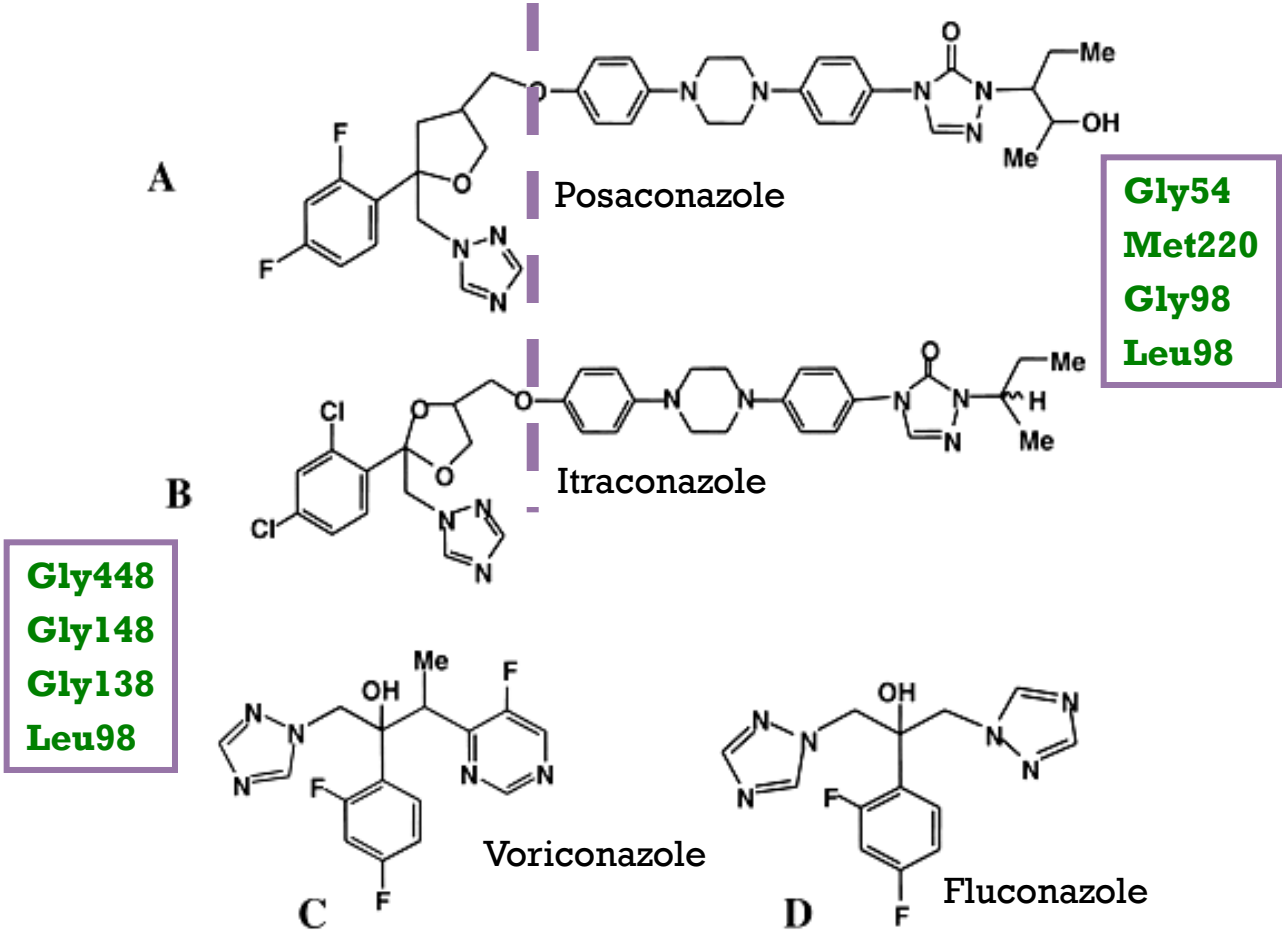
Cyp51A	Patients	Isolates	AA subst	MIC, mg/L Itra	Vori	Posa
F46‡	3	4	Y	>8	2–4	0.125–0.5
G54	4	5	E, R, V	>8	0.125–1	1–>8
L98+TR	2	2	H	>8	8	1–2
G138	1	10	C	>8	8–>8	2–>8
H147§	1	1	Y	>8	>8	0.5
M172‡	3	4	V	>8	2–4	0.125–0.5
P216	1	1	L	>8	1	1
M220	3	4	K, T	>8	1–4	0.5–>8
N248‡	1	1	T	>8	2	0.25
D255‡	1	1	E	>8	2	0.25
E427‡	4	5	G, K	>8	2–4	0.125–0.5
Y431	1	1	C	>8	4	1
G434	1	1	C	>8	4	1
G448	2	2	S	>8	>8	0.5–1
No subst	2	3	NA	>8	2–8	0.25–1

Target site mutations alter the binding of azoles to the active site where it coordinates the heme iron as sixth ligand.

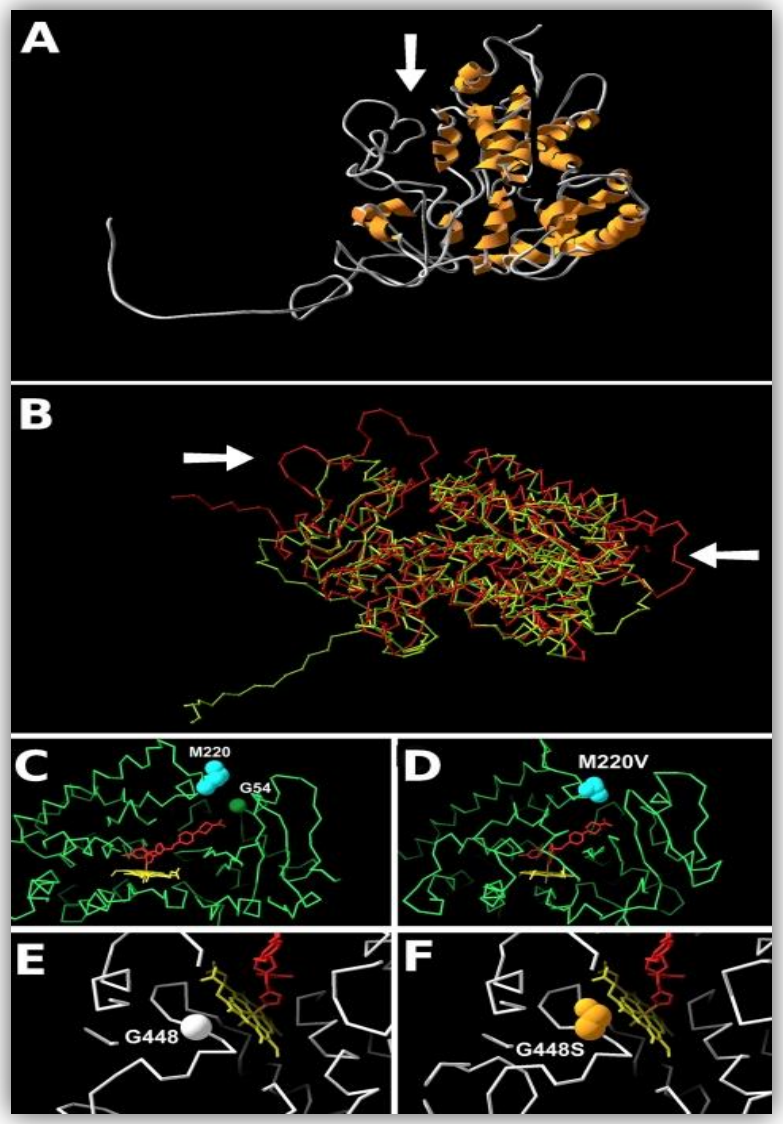


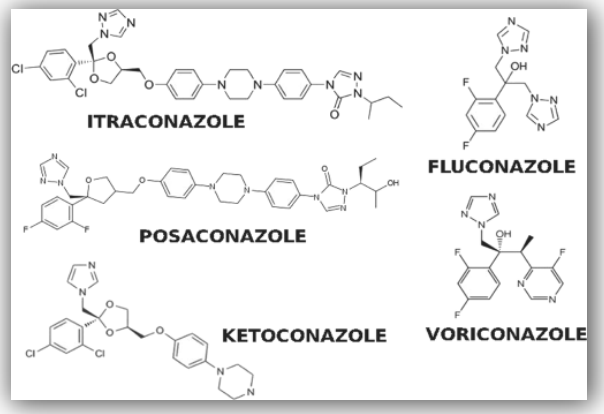
Podust et al. 2001 PNAS

Different spectrum of mutations identified for triazole drugs consistent with chemical structure



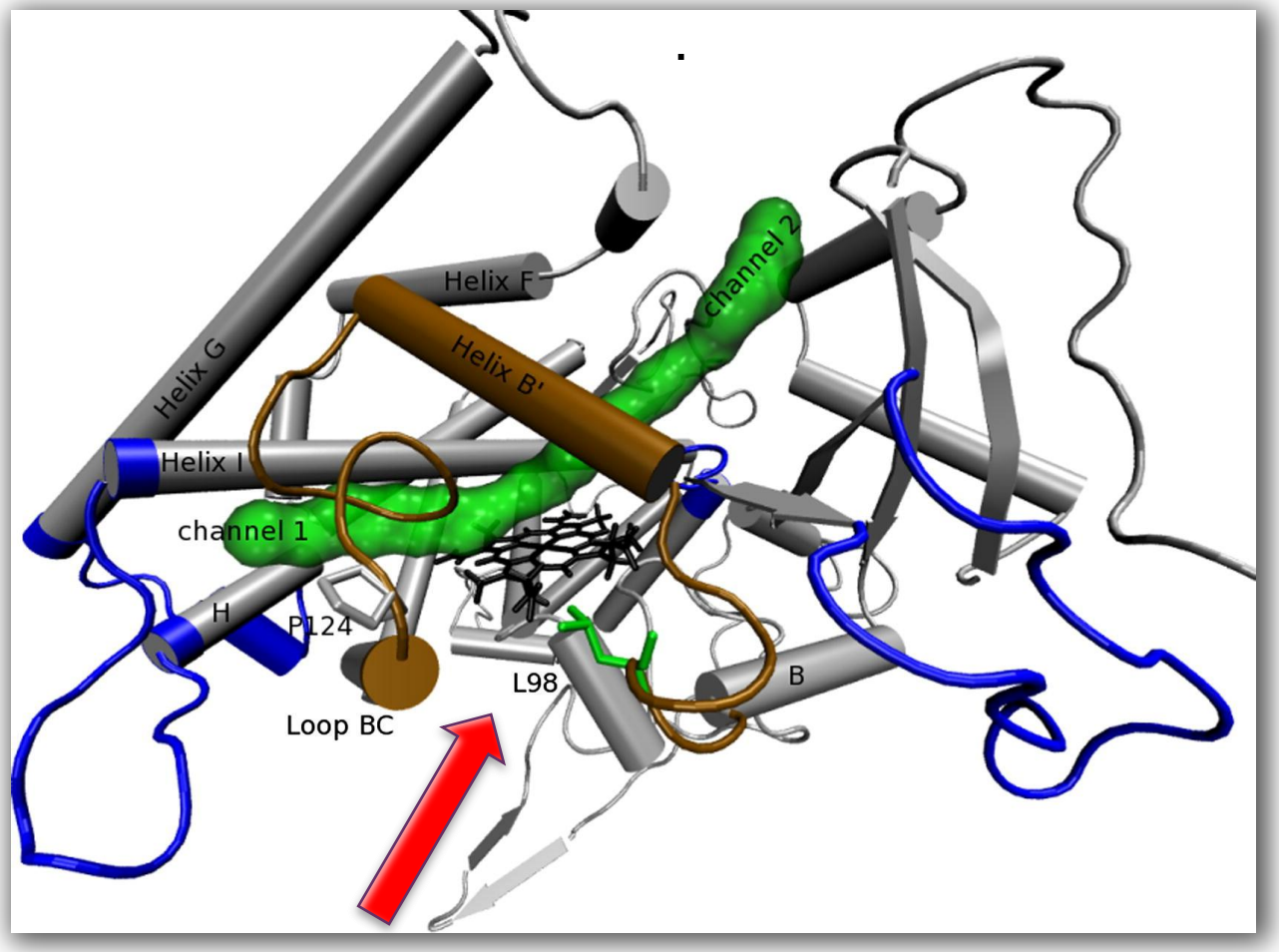
Models of prominent CYP51A and azole resistance-associated amino acid variants.





Fraczek , Bromley Bowyer 2011 AAC

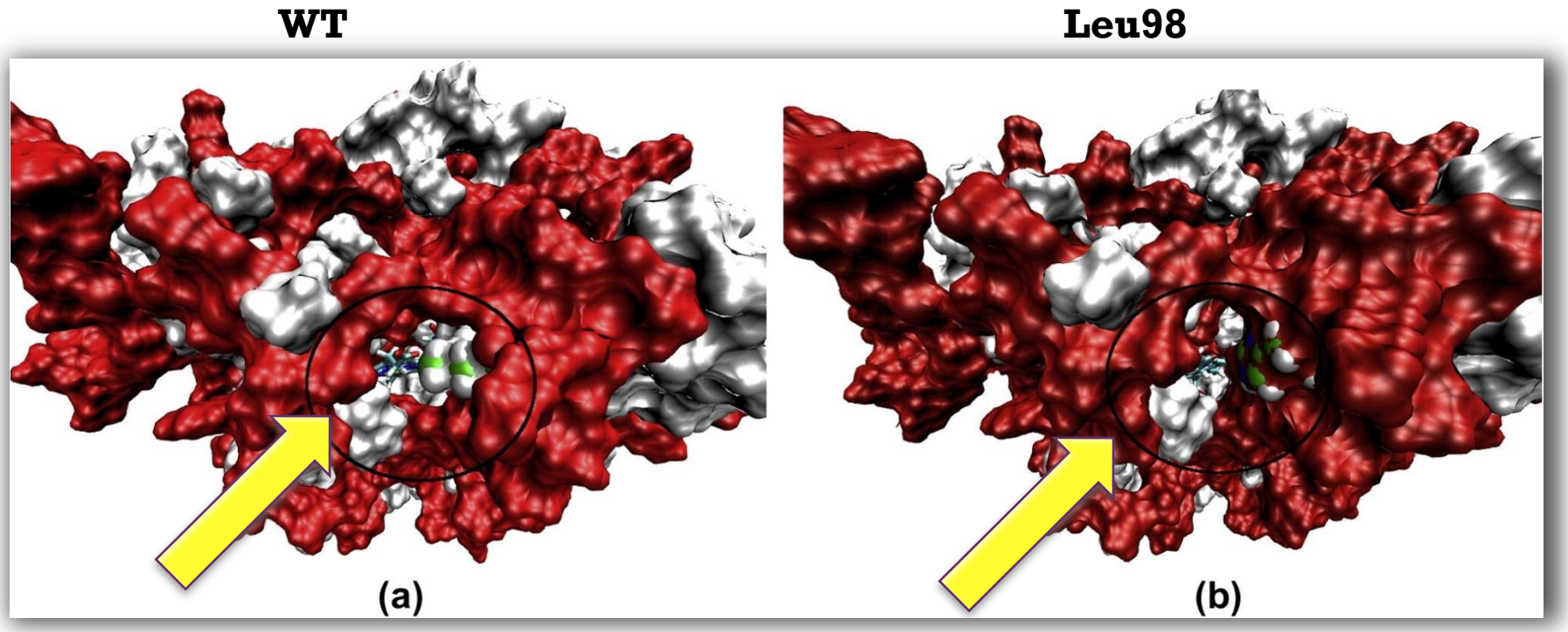
Model of CYP51A with Leu98 highlighted.



Heme cofactor is in black, the two channels are filled with green

Snelders et al. 2010 AAC

L98H changes relative hydrophobicity of local environment



Surface representation of hydrophobic (white) versus hydrophilic (red) residues of the CYP51A protein in wild type situation (a) and mutated situation (b).

Snelders FGB 2011

MIC of mutant *A. fumigatus* strains with different cyp51A amino acid substitutions at Leu98

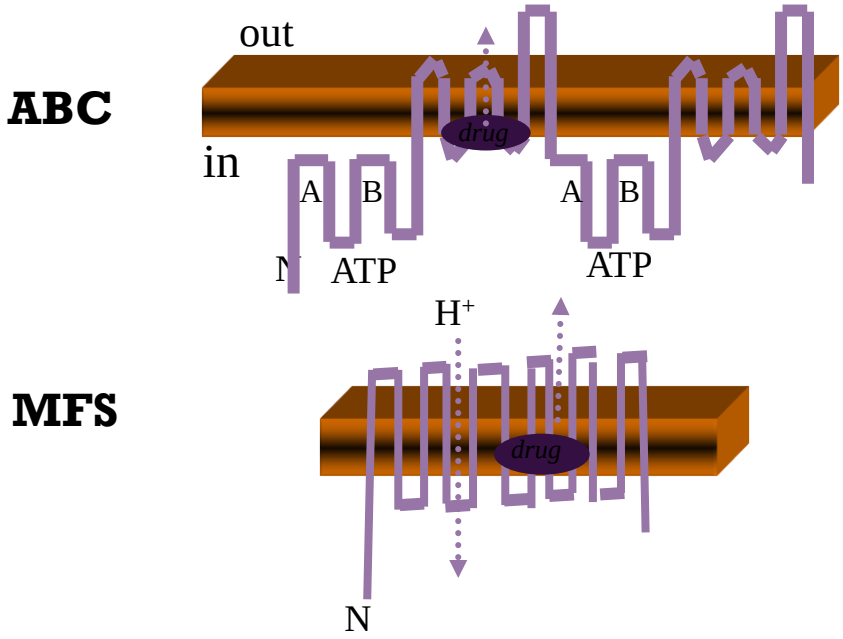
EUCAST	TR	Gene	MIC (mg/ml) 48 h ITC	VCZ	POS
akuBKU80 recipient strain			0.5	0.25	0.25
akuBKU80 cassette	-		0.5	0.25	0.25
akuBKU80-G54W	-	G54W	>16	0.25	>16
akuBKU80-TR	**+**	**–**	**1**	**2**	**0.5**
akuBKU80-L98H	**-**	**L98H**	**1**	**1**	**0.5**
akuBKU80-TR L98H	**+**	**L98H**	**>16**	**2**	**0.5**
akuBKU80-TR L98R	**+**	**L98R**	**>16**	**2**	**0.5**
akuBKU80-TR L98Y	**+**	**L98Y**	**>16**	**1**	**0.5**
akuBKU80-TR L98Q	**+**	**L98Q**	**>16**	**2**	**0.5**
akuBKU80-TR L98I	**+**	**L98I**	**>16**	**1**	**0.5**

TR: 34 bp tandem repeat in the promoter region,

Promoter modification (TR) in conjunction with changes in Cyp51A at Leu98 are required to confer resistance

Snelders FGB 2011

Do drug transporters play a role in resistance?



MFS and ABC transporters in various fungal genomes

	A. fumigatus	***A. nidulans***	***A. oryzae***	*S. cerevisiae*	*S. pombe*	*C. albicans*
Genome size (Mb)	**32**	**31**	**36.7**	13	14	13
Total ORFs	**10,003**	**10,003**	**14,063**	6,309	4,916	9168
MFS total	**278**	**357**	**508**	84	57	71
Drug:H$^+$ (12 spanner)	**64**	**76**	**125**	14	20	
Drug:H$^+$ (14 spanner)	**32**	**41**	**65**	10	6	
ABC total	**49**	**47**	**72**	24	10	28
Multidrug permease	**35**	**34**	**57**	13	1	

Numerous putative transporters are known, but only a small number: 3 ABC (MDR1;2; 4) and 1 MFS (MDR3) contribute to resistance phenotypes

Cellular regulation of drug pumps and/or drug target (Cyp51A) leading to resistance

C. albicans

- **Tac1 controls CDR1 expression (Coste et al. 2004 Eukaryot. Cell)**
- **Mrr1 controls MDR1 expression (Morschhäuser et al. 2007 PLoS Pathog)**
- **Upc2/Ecm22 induce expression of Erg genes upon sterol depletion (Silver, Olivder and White 2004 Eukar. Cell)**
- **PDR1 upregulates *C. glabrata* including *CDR1* and *PDH1*. Mitochondrial loss is an activator of PDR1 (Vermitsky et al. 2004 AAC; 2006 Mol. Microbiol)**

A. fumigatus

- Rocha et al. 2007. AzRF1 (Cys6 TF) confers triazole resistance.. target is unknown (Rocha AAC 2007)
- SREBP **(Blosser and Cramer, AAC 2012)**
- Others?
- Up-regulation of Cyp51A (TR/Leu98) and others (sa Silva AAC 2004; Nascimento et al. 2003; Arendrup e al. PLoS One 2010; Albarrag et al. AAC 2011)

M. Bromley et al. AAA2012 **Poster 20**

The Systems Biology of Azole Resistance in *Aspergillus fumigatus*

Methods: Multi-omic approach:

- Illumina sequencing of Itr resistant isolates;
- RNAseq +/- drug
- Satn transposon mutagenesis
- HTP gene KOs.

Results:

- 9 novel genes/pathways;
- 2 new transporters
- Up-regulation of Cyp51B

What about other prominent mechanisms of reduced susceptibility?

- Biofilms: drug pumps, gluan and nucleic acid sink for azoles; sterol regulation for high resistance (Mukheriee et al. 2003 Infect. Immun; da Silva Ferrieira et al. AAC (2004); Rajendran et al. AAC 2011; Rajendran et al. AAA 2012 Poster 14)

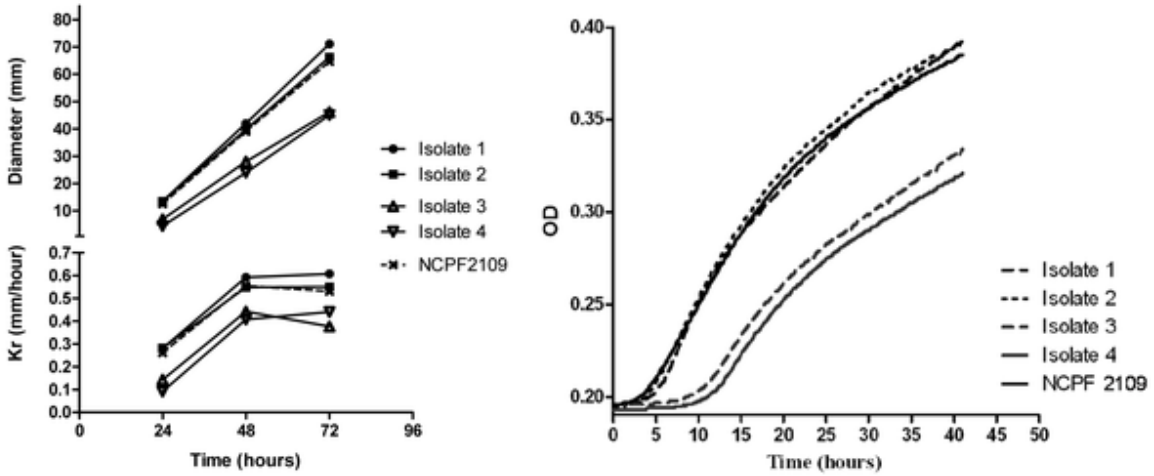
- HSP90/Calcineurin inhibitors (cyclosporin A and FK-506) (Cowen Science 2005; Eukar. Cell 2006; Lamoth et al. AAA 2012 Poster132)

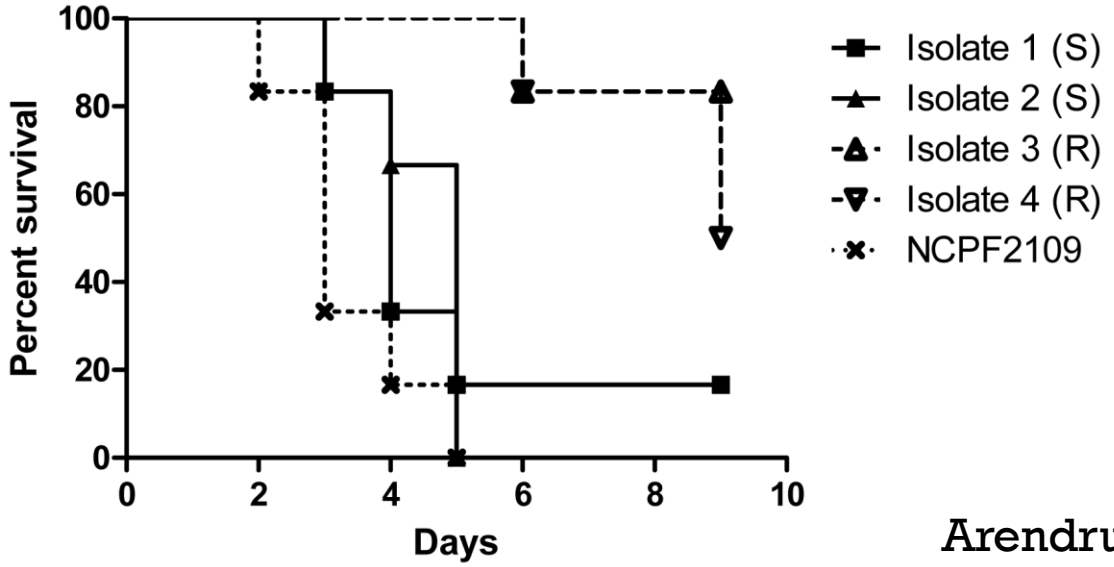
Not relevant in Aspergillus

Isochromosome ([i(5L)] i), two left arms of chromosome 5, amplifyies ERG11 and TAC1 (Selmecki , Forche and Berman 2006 Science; Selmecki et al. 2008 Mol. Miobiol.)

Is resistance self-limiting?

Development of azole resistance in *A. fumigatus* during azole therapy is associated with reduced virulence.





Arendrup PLoS One.(2010)

Limited spectrum of mutations in Cyp51A associated with multi-azole resistance is ideal for molecular assessment of drug resistance

Rapid diagnosis of azole-resistant aspergillosis by direct PCR using tissue specimens.

van der Linden JW, Snelders E, Arends JP, Daenen SM, Melchers WJ, Verweij PE. J Clin Microbiol. 2010 Apr;48(4):1478-80.

Garcia-Effron et al. JCM 2008;Balashov et al. JCM 2005

High-frequency triazole resistance found In nonculturable Aspergillus fumigatus from lungs of patients with chronic fungal disease.

Denning DW, Park S, Lass-Florl C, Fraczek MG, Kirwan M, Gore R, Smith J, Bueid A, Moore CB, Bowyer P, Perlin DS. Clin Infect Dis. (2011) 52(9):1123-9;

Table 1
Aspergillus culture, qPCR and *A. fumigatus* resistance mutation detection in 4 study populations.

Laboratory result	ABPA	CPA	IPA	Normals
Culture positive for *Aspergillus* spp.	0/19	7/42 (16.7%)	20/22 (90.9%)	0/11
Culture positive for *A. fumigatus*	0/19	7/42 (16.7%)	10/22 (45.5%)	0/11
qRT PCR positive for *Aspergillus* spp	15/19 (78.9%)	30/42 (71.4%)	21/22 (95.5%)	4/11 (36.4%)
A. fumigatus CYP51A mutation detected directly from qPCR positive sample	6/8 (75%)	12/24 (50%)	NT[a]	NT[a]

ABPA = allergic bronchopulmonary aspergillosis; CPA = chronic pulmonary aspergillosis; IPA = invasive pulmonary aspergillosis
a = Not tested (insufficient sample remaining)

Molecular probing improves detection sensitivity with a simultaneous assessment of resistance. Is resistance more prevalent than currently reported?

Denning et al. CID 2011

Will the global spread of resistant isolates render azole drugs useless?

Future Microbiol. 2011 Mar;6(3):335-47.
Azole resistance in Aspergillus fumigatus: a new challenge in the management of invasive aspergillosis?

Snelders E, Melchers WJ, Verweij PE.

Future Microbiol. 2011 Nov;6(11):1229-32.
Azole resistance in Aspergillus: a growing public health menace.

Denning DW, Perlin DS.

Open Questions

- Is there a fitness cost to resistance?
- Why is acquired resistance found readily with chronic disease but rarely with acute infections?
- Is resistance strictly related to time in presence of drug? Does the drug matter?
- Does stress (host pressure) promote resistance?
- Will the global spread of the TR/L98 resistance mechanism render existing azole drugs useless?

PERLIN Lab: Resistance Group

Ayiasha Pratt | Steven Park | Cristina Jiménez-Ortigosa | Padmaja Paderu | Yanan Zhao

Former Members

Guillermo Garcia-Effron
Ana Alastruey-Izquierdo
Steven Cagas
Sergey Balashov

Collaborators

David Denning Manchester
Sue Howard Manchester
Maiken Arendrup SSI

Eveline Snelders Nijmegen

Supported by NIH/NIAID