Antifungal resistance in *Aspergillus fumigatus*

Dr Lily Novak Frazer and Dr Caroline Moore
University of Manchester at the Manchester Academic Health & Science Centre and the Mycology Reference Centre, National Aspergillosis Centre, University Hospital of South Manchester Foundation Trust, Manchester
Content

- Antifungal drugs, use and activity
- Global resistance problem
- How triazole resistance develops
- Resistance mechanisms
- Scale of the problem in the UK
- Different methods for monitoring resistance
- How to monitor resistance using pyrosequencing
- How this test may improve patient care
- Future developments
# Antifungal drugs for Aspergillus

<table>
<thead>
<tr>
<th>Class</th>
<th>Drug</th>
<th>Route of administration</th>
<th>Indication with respect to Aspergillus diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triazole</td>
<td>Itraconazole</td>
<td>Intravenous/oral</td>
<td>Treatment of chronic Aspergillus diseases</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Salvage therapy</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>Intravenous/oral</td>
<td>Primary therapy of invasive aspergillosis (IA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Salvage therapy</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>Oral</td>
<td>Prophylaxis of invasive fungal disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Salvage therapy</td>
</tr>
<tr>
<td></td>
<td>Isavuconazole</td>
<td>Intravenous/oral</td>
<td>Primary therapy of invasive aspergillosis (IA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>as an alternative choice for voriconazole</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Salvage therapy</td>
</tr>
<tr>
<td>Polyene</td>
<td>Lipid formulations of amphotericin b</td>
<td>Intravenous</td>
<td>Primary therapy of invasive aspergillosis (IA)</td>
</tr>
<tr>
<td>Echinocandin</td>
<td>Caspofungin</td>
<td>Intravenous</td>
<td>Prophylaxis of refractory invasive fungal disease</td>
</tr>
<tr>
<td></td>
<td>Anidulafungin</td>
<td>Intravenous</td>
<td>Salvage therapy</td>
</tr>
<tr>
<td></td>
<td>Micafungin</td>
<td>Intravenous</td>
<td></td>
</tr>
</tbody>
</table>


Resistance – a global problem

Current global prevalence of azole resistance is estimated at between 0.3 and 28%
Development of resistance

Echinocandins inhibit glucan synthase responsible of β-(1,3)-glucan synthesis

Nucleosides inhibit nucleic acid synthesis

Ergosterol

Polyenes bind to ergosterol

Azoles inhibit CYP-450 enzyme responsible for ergosterol synthesis

Mannoproteins

Phospholipid bilayer of fungal cell membrane

β-(1,6)-glucan

β-(1,3)-glucan

Caspofungin
Anidulafungin
Micafungin

Nystatin
Amphotericin B

Fluconazole
Itraconazole
Voriconazole
Posaconazole
Isavuconazole

5-flucytocine
Terbinafine

Courtesy Prof MD Richardson
Mechanisms of triazole resistance

Resistance mechanisms in *Aspergillus fumigatus*:

- Target enzyme of tri-azoles: lanosterol 14α-demethylase, *cyp51A*
- Target pathway: ergosterol biosynthesis, resulting in ergosterol depletion and accumulation of toxic sterols
- Mutations in target gene result in decreased drug binding and effectiveness

Other mechanisms:
- Overexpression of efflux pumps which clear the drug
- Mutations in gene transcription (e.g. *Hap*, Aft1) leading to overexpression of *cyp51A*
- Unknown…

Resistance markers in *cyp51A*

*Aspergillus fumigatus cyp51A*

- **TR34 plus L98H**: pan-azole R
  - TR34/L98I/Q/R/Y: ITR R

- **TR46 plus Y121F and T289A**: pan-azole R

- **G54E/K**: ITR R
  - G54R/V/W: ITR R and PSC R

- **M220I/V**: ITR R
  - M220R/K/T/W: ITR R and PSC R

**ITR** = itraconazole
**VOR** = voriconazole
**PSC** = posaconazole


cyp51A mutations in Europe

Prospetive multicentre international surveillance study in which a total of 3,788 Aspergillus isolates were screened in 22 centres from 19 countries.

Prevalence of 3.2%azole-resistance in A. fumigatus isolates in a period of 8 months to 1 year.


<table>
<thead>
<tr>
<th>Country</th>
<th>No. azole-resistant isolates, n = 47</th>
<th>TR₃₄/L98H or TR₄₆/Y121F/T289A mechanism (no. isolates)</th>
<th>Other mutations (no. isolates)</th>
<th>No. isolates without cyp51A -mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>2</td>
<td>TR₃₄/L98H (2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Belgium</td>
<td>8</td>
<td>TR₃₄/L98H (7)</td>
<td>F46Y/M172G (1)</td>
<td>0</td>
</tr>
<tr>
<td>Denmark</td>
<td>6</td>
<td>TR₃₄/L98H (4)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>France</td>
<td>4</td>
<td>TR₃₄/L98H (1)</td>
<td>G54W (1)</td>
<td>2</td>
</tr>
<tr>
<td>Italy</td>
<td>5</td>
<td>TR₃₄/L98H (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>The Netherlands</td>
<td>7</td>
<td>TR₃₄/L98H (4), TR₄₆/Y121F/T289A (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spain</td>
<td>1</td>
<td>No isolates</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sweden</td>
<td>1</td>
<td>No isolates</td>
<td>F46Y/M172G</td>
<td>0</td>
</tr>
<tr>
<td>Resistant isolates, %</td>
<td>100</td>
<td>55.3</td>
<td>29.8</td>
<td>14.9</td>
</tr>
</tbody>
</table>
Patient case 1

- Male, early forties, admitted to Burns Centre in April 2016 following self-inflicted burns (44% total body surface area)
- Works in UK marble plant, resizing imported marble from Spain and Italy
- Last travel to Spain was ~3 months prior, no history of prior azole use
- Prior to day 47, 12 respiratory samples: all negative for fungi
- **Day 47 – Aspergillus fumigatus** isolated from non-directed BAL – resistant to itraconazole, voriconazole, posaconazole and isavuconazole; also on days 53, 57, 69 and 74
- Isolates from days 47 and 57: sequencing revealed a TR46 repeat insertion, and also mutations Y121F and T289A
- All **A. fumigatus** isolates from air samples were susceptible to all azoles

- The first case of a pan-azole resistant **A. fumigatus cyp51A TR46/Y121F/T289A mutant in the UK**
The National Aspergillosis Centre

• 457 referrals, 111 new cases in 2015/16
• Chronic Pulmonary Aspergillosis (CPA) patients, ~10-15% annual mortality
• 346 additional referrals in 2015/16:
  Allergic Bronchopulmonary Aspergillosis (ABPA)
  Severe Asthma with Fungal Sensitisation (SAFS)
  Cystic Fibrosis (CF)
  Fewer cases of invasive aspergillosis (IA)
  Rhinosinusitis and Aspergillus bronchitis
• Globally: 100,000 IA, 3 million CPA, 7.5 million allergic
Scale of the problem in the UK*

Since 2007: patients monitored
2009 – 112
2011 – 136
2013 – 218

Resistance levels stabilised to 18-20%
Increased voriconazole and itraconazole resistance in 2012-13

*National Aspergillosis Centre
Monitoring infection & resistance

Sputum
BAL
blood

* High volume culture

→

No growth?
Typically only 2.1 patients of 10 000 admissions grow *Aspergillus fumigatus*
HVC: 50-70% culture negative

→

EUCAST: Antifungal drug susceptibility testing plate
All 4 azoles
Amphotericin b
Micafungin

→

Susceptibility results: S, I, R
Guidance for clinicians

→

Patient responds to recommended treatment

→

Pro-active therapeutic drug monitoring


Monitoring infection & resistance

- Sputum BAL blood
- DNA extraction 4-6 h
- ASPERGILLUS qPCR (quantitative PCR, 2 h)

  → PCR negative – other cause?
  → PCR positive
    - Patient responds to recommended treatment
    - PCR positive >> Patient does not respond
      - Pro-active therapeutic drug monitoring
Monitoring resistance: the demand*

Monthly:

- 80-90 positive cultures - susceptibility testing
- 250-300 respiratory samples are culture negative > processed by qPCR
- A quarter of PCR samples (60-75) test positive for *Aspergillus* spp.
- Aim: minimum of 600 samples per annum monitored for resistance (two thirds)
- Future: process all new patients at diagnosis

*National Aspergillosis Centre
Monitoring resistance

- VIPcheck™ azole resistance detection
- Pathonostica AsperGenius® PCR
- In-house qPCRs
- Sanger sequencing
Monitoring resistance by pyrosequencing

- Discovered in 1990s, up to 150 base pyrosequencing
- DNA extract directly from patient sample, polymerase chain reaction (PCR) (6h)
- PCR and pyrosequencing, time to result: 6h
- Determination of mutations in *cyp51A* associated with azole resistance:
  
  TR34/L98H, TR46/Y121/T289, G54, M220

**Funding:** January 2016  
**Dedicated personnel:** July 2016  
**First patient samples processed:** December 2016
Pyrosequencing in a nutshell (1)

First polymerase chain reaction (PCR):
Amplify *Aspergillus fumigatus* cyp51A gene from the patient sample.

EXAMPLE: Section of the *cyp51A* gene containing the **Met220** amino acid sequence

ATCGATGAAGGGTTCAT**GTGCATGCTAGATATC**

Second PCR: Amplify short sections of the *cyp51A* gene with biotinylated primers. This enables purification of the single strands of DNA of interest.

Pyrosequencing
Pyrosequencing in a nutshell (2)

All components included to make an exact copy of the patient *cyp51A* sequence.

Components are added one at a time and in a known sequence so that they can be monitored and checked by the software.

Software then compares the new, patient pyrosequence to the normal *cyp51A* sequence: mismatches (or mutations) can be identified by sequence comparison.
Pyrosequencing output

CAT-AAAA

normal

M220K >> ITR/PSC R

CTT-AAAA

L98H >> ITR/VOR R

normal

CTC

normal

CAC
Can we improve patient care?

- Assess whether therapy failure is associated with a $cyp51A$ mutation
- Predict whether a patient may fail therapy by detecting a $cyp51A$ mutation
- Alternative azole therapy
e.g. G54R/V/W ITR R / PSC R
e.g. M220I/V ITR R
- Alternative therapy if tri-azole resistance is detected
e.g. M220R/K ITR/PSC R, VOR elevated MICs
- Combination therapies?
- Surgery
Patient case 2

- Female, mid sixties, first diagnosed with CPA in 2010, prescribed itraconazole in August
- TDM demonstrates maintenance of high serum itraconazole levels
- Switched to voriconazole in 2012
- Susceptibility testing of *A. fumigatus* isolates reveals:
  - April 2012: resistant to itraconazole and voriconazole, susceptible to posaconazole
  - June 2012: resistant to itraconazole, voriconazole, posaconazole intermediate
  - August 2012: resistant to itraconazole, voriconazole, and posaconazole
- Continued sampling: no growth in culture but PCR positive, *Aspergillus fumigatus species complex* confirmed by sequencing
- Surgery suggested: left upper lobectomy in February 2014, full recovery, no symptoms
- Fungal cultures are negative, GM negative, PCRs negative
- Discharged from service in March 2015
Future Prospects: resistance monitoring

Other resistance mechanisms in Aspergillus:

- Increased expression resulting in decreased cytosolic drug levels and stress response proteins
  - Efflux pumps (MDR1 or CDR1/2)
  - ATP-binding cassette transporters
  - Other regulatory elements, e.g. SrbA

- Other pyrosequencing targets: expression changes leading to resistance but via nucleotide substitutions
  - *HapE* (P88L), transcription factor complex subunit
  - Presence of Aft1 transposon (inserted 370 bp upstream of the *cyp51A* start codon)

- Resistance to other antifungal drugs, in other fungi/yeasts, bacteria/antibiotics

Summary

- Antifungal resistance is on the rise in the UK and globally
- Early and pro-active monitoring of triazole resistance can improve:
  - Patient outcome > the right drug, right time
  - Patient well-being and experience
  - Antifungal stewardship
- Save costs
Acknowledgements

• Staff in the:
• Mycology Reference Centre Manchester
• National Aspergillosis Centre
• Profs Denning and Richardson
• NHS England