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Fairlamb, Alan H.; Gow, Neil A. R.; Matthews, Keith R.; P. Waters, Andrew

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## Drug Resistance in Eukaryotic Microorganisms

Alan Fairlamb<sup>1</sup>, Neil A. R. Gow<sup>2</sup>, Keith R. Matthews<sup>3\*</sup>, Andrew P. Waters<sup>4</sup>

Notes: All authors contributed equally to the preparation of the manuscript. The individual affiliation of each author is given below; the authors comprise members of the consortium of Wellcome Trust funded Centres of *Infectious Disease Research in Scotland* (IDRIS).

\*Corresponding author: Email: [keith.matthews@ed.ac.uk](mailto:keith.matthews@ed.ac.uk); Phone: +44-131-651-3639; Fax: +44-131-651-3670

### Author affiliations

1. Dundee Drug Discovery Unit; Biological Chemistry and Drug Discovery, College of Life Sciences, University of Dundee, Dundee; UK
2. Aberdeen Fungal Group, Wellcome Trust Strategic Award in Medical Mycology and Fungal Immunology, School of Medical Sciences Institute of Medical Sciences Foresterhill University of Aberdeen, Aberdeen AB25 2ZD; UK
3. Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh; Edinburgh, UK
4. Wellcome Trust Centre for Molecular Parasitology, Institute of Infection, Immunity and Inflammation, College of Medical and Veterinary Life Sciences, University of Glasgow; Glasgow, G12 8TA, UK.

34 **Eukaryotic microbial pathogens are major contributors to illness and death**  
35 **globally but much of their impact can be controlled by drug therapy. However,**  
36 **as with prokaryotic microbes, the emergence of drug resistance has threatened**  
37 **these treatment efforts. Here, we discuss the challenges posed by eukaryotic**  
38 **microbial pathogens and how these are similar to, or differ from, the challenges**  
39 **of prokaryotic antibiotic resistance. The therapies used for several major**  
40 **eukaryotic microbes are then detailed and the mechanisms that they have**  
41 **evolved to overcome these described. The rapid emergence of resistance and**  
42 **the restricted pipeline of new drug therapies pose significant risks to global**  
43 **health and are particularly acute in the developing world. Nonetheless, we detail**  
44 **how an integration of new technology, biological understanding, epidemiology**  
45 **and evolutionary analysis can help sustain existing therapies, anticipate the**  
46 **emergence of resistance or optimise the deployment of new therapies.**

47

48 The identification and use of antibiotics presents one of the great medical  
49 achievements of the 20<sup>th</sup> Century, saving countless lives by controlling the risk of  
50 infection from contagion, after injury, surgery or in immunosuppressed individuals.  
51 However, in only 80 years since the introduction of penicillin, resistance to a broad  
52 range of antibiotic drugs has become widespread, with the compounded risk from  
53 multi-drug resistant bacterial infections severely limiting treatment options. This has  
54 created justified concern and global attention, not only in the medical community but  
55 also at Government level, in the media and the public<sup>1</sup>.

56 Whilst predominantly applied to control prokaryotic microbial infections, the  
57 threat of disease from eukaryotic microbes has also been contained by therapeutic  
58 drugs - preventing or controlling disease caused by eukaryotic parasites and fungi in  
59 both a human and animal health setting. These represent some of the most important  
60 disease-causing agents (Table 1), particularly in the tropics where the distribution of  
61 the pathogen is frequently linked to the distribution of the arthropods that act as  
62 disease vectors. Such vector-borne parasites include malaria (*Plasmodium* spp.) and  
63 kinetoplastid parasites (*Trypanosoma cruzi*, causing Chagas' disease; *Trypanosoma*  
64 *brucei gambiense* and *T. b. rhodesiense* causing human African trypanosomiasis  
65 (HAT), and 17 *Leishmania* spp. causing a variety of cutaneous and visceral diseases).  
66 Other clinically important protozoan parasite species not considered in this review are  
67 transmitted either orally (*Toxoplasma*, *Giardia* and *Entamoeba*) or venereally  
68 (*Trichomonas*). Distinct from the many obligate eukaryotic unicellular parasites,  
69 opportunistic fungal pathogens are global in distribution and include *Candida*,  
70 *Aspergillus* spp., *Cryptococcus* and *Pneumocystis* spp.

71 The control of these eukaryotic pathogens has often involved therapies  
72 predating the use of penicillin and in some cases with unacceptable toxicity profiles<sup>2</sup>.  
73 Nonetheless, as with the rise of antimicrobial resistance in bacteria, resistance has or  
74 is emerging in the therapies targeting these eukaryotic microbes, with potentially  
75 devastating consequences for exposed populations. This, however, has received far  
76 less attention despite some commonality in its underlying causes. In this perspective,  
77 we detail how the control of eukaryotic microbes poses both similar and distinct  
78 challenges to that of bacterial pathogens, the drugs used to combat these pathogens  
79 and the resistance mechanisms they are evolving. Finally we discuss how the latest  
80 methodological approaches can anticipate the emergence of drug resistance and  
81 support the development of new therapeutic approaches, either through the  
82 development of new drugs, the maintenance of existing therapies or through the use  
83 of alternative approaches to limit the spread of drug resistance.

84

#### 85 **Common challenges for the control of prokaryotic and eukaryotic microbial** 86 **pathogens.**

87

88 The challenges in the control of eukaryotic microbial pathogens share many similarities  
89 with bacterial infections. Both replicate more rapidly than their hosts, such that  
90 resistance can be selected within a relatively short timescale within a treated host  
91 population. This is exacerbated by inappropriate treatment profiles, leading to  
92 subcurative exposure in the context of infection<sup>3</sup>. Problems of sub optimal dosing are  
93 particularly acute when applied to tropical parasites. For example, for antimalarials, up  
94 to 35% of drugs may be of poor quality, have poor packaging and labelling or be  
95 falsified<sup>4</sup>. With lower than optimal concentrations of the active agent, this rapidly  
96 selects resistance in exposed populations, as does underdosing resulting from self-  
97 prescription. Where zoonoses are concerned, such as with African trypanosomes,  
98 parasite selection in livestock populations treated with trypanocides in a context where  
99 there is poor supply chain management, fraudulent provision or cost barriers to optimal  
100 dosing, can also lead to resistance emergence. This represents a significant threat  
101 where up to 50 million doses of trypanocides are used in sub-Saharan livestock  
102 annually, mainly as a preventative, and trypanocides represent 45% of animal health  
103 costs. Agricultural use of fungicides might also contribute to the selection of azole  
104 resistant *Aspergillus fumigatus*<sup>5</sup>, mirroring the situation with antibiotic exposure in  
105 veterinary contexts for bacterial infections, where environmental contamination  
106 generates significant regulatory concern<sup>6</sup>.

107           A further similarity between bacterial and eukaryotic microbial pathogens is the  
108 phenomenon of persister populations<sup>7</sup>. This is the survival of a fraction of the  
109 population of pathogens following exposure to a chemotherapeutic agent (or vaccine).  
110 These can then re-establish patent infection whilst remaining drug sensitive (see  
111 review<sup>8</sup>). The state of persistence is not heritable and resistance is not due to genetic  
112 alterations directly linked to rendering a drug ineffective. Rather, persistence is a  
113 physiologically active state involving pathogen response to the assault which is  
114 initiated upon demand. Persistence ensures incidental survival but does not future-  
115 proof a pathogen as genetically heritable resistance would. However, the combination  
116 of persisters and sub-optimal drug dosage might form an enhanced reservoir for the  
117 emergence of resistance and may even provide a population pre-disposed to evolve  
118 resistance more readily. An example of this relating to parasite dormancy is the  
119 resistance of *Plasmodium falciparum* to artemisinin (and other antimalarials such as  
120 mefloquine, atovaquone), which was first characterised by degrees of persistence  
121 followed by the emergence of genomic changes now causally associated with  
122 resistance (see below). Similarly, fungal infections (e.g. *C. albicans*) associated with  
123 biofilms are a good example of persister populations analogous to those in bacterial  
124 communities<sup>9-11</sup>. The duration of persistence can range from days (*P. falciparum*) to  
125 lifelong (e.g. *C. albicans*). Mechanisms of persistence vary – they may emerge  
126 spontaneously possibly through stochastic changes in gene expression that prepare a  
127 population of pathogens for survival in varying environmental conditions (“bet  
128 hedging”). This is best described in bacteria<sup>12</sup> but is a phenomenon recently  
129 characterised in *P. falciparum*<sup>13</sup>. Furthermore, environmental signals may induce  
130 persistence such as the nutrient starvation typically encountered by *C. albicans* in  
131 biofilms<sup>9,14</sup>.

132

133 **Distinct challenges for the control of prokaryotic and eukaryotic microbial**  
134 **pathogens.**

135

136 Although bacterial and eukaryotic microbes share common features with respect to  
137 their responses to drug exposure, there are also differences that particularly challenge  
138 the control of eukaryotic pathogens. First, eukaryotic microbes are more similar to their  
139 hosts than prokaryotic pathogens in terms of their biochemistry and metabolism,  
140 genetic composition, cell architecture and biology. Consequently, drugs targeting  
141 eukaryotic microbes must focus on differences from the eukaryotic norm, or particular  
142 specialisms of each pathogen group. This restricts the cross-specificity of drugs, such  
143 that there are distinctions in sensitivity between different apicomplexans (malaria,

144 toxoplasma) or between the evolutionarily divergent trypanosomes, *T. brucei* spp. and  
145 *T. cruzi*. Comprising a different evolutionary kingdom, fungi have many differences  
146 from other eukaryotic microbial pathogens, again necessitating drugs to be developed  
147 for, and targeted to, a particular pathogen. This increases the challenges for drug  
148 development and inevitably constricts the new drug pipeline.

149         Second, many eukaryotic microbial pathogens have evolved a parasitic life  
150 style distinct from the opportunistic infections characteristic of most bacterial  
151 pathogens (but also fungi). The evolution of parasitism is often accompanied by the  
152 development of sophisticated immune evasion mechanisms, which promotes the  
153 impact of persister phenotypes described earlier. Specifically, bacteriostatic drugs can  
154 operate to clear infection in concert with the immune system<sup>15</sup>. However, drugs that  
155 generate cytostatic rather than cytocidal responses in infection with an  
156 immunosuppressive parasite can lead to recrudescence upon the removal of drug  
157 exposure. This, in turn, can predispose the population to the selection for drug  
158 resistance. Similarly the adaptation to an intracellular life style or particular body niche  
159 can protect parasites from drug exposure, a feature shared with some bacterial  
160 pathogens that have evolved to survive in cells rather than systemically (*Legionella*,  
161 *Mycobacteria*).

162         A third challenge relates to the clinical diagnosis and the screening for drug  
163 resistance in eukaryotic microbial pathogens<sup>16</sup>. In bacterial infections, screening for  
164 the sensitivity to antibiotics is straightforward and routine. In contrast, eukaryotic  
165 parasites can require highly-specialised growth media and considerable growth  
166 periods to determine their susceptibility or otherwise to potential drug therapies. Also,  
167 unlike bacterial susceptibility testing where a Minimum Inhibitory Concentration (MIC)  
168 is determined, most parasitologists report EC<sub>50</sub>-values without providing the Hill slope  
169 of the growth inhibition curve or calculating the EC<sub>90</sub> value. It is perfectly possible to  
170 obtain a resistant line with an identical EC<sub>50</sub> to the susceptible isolate, yet that is still  
171 resistant due to a shallower Hill slope. As a consequence clinical diagnosis and the  
172 selection of the appropriate clinical management can be slow, or practically impossible  
173 in the context of all but the most specialised laboratories.

174         A fourth distinction from common bacterial infections is the economic challenge  
175 of treating diseases of the developing world. Diseases such as malaria,  
176 trypanosomiasis, leishmaniasis and cryptococcosis are common in the poorest parts  
177 of the world where the economic capacity to develop or deliver treatments are very  
178 limited and restricted to philanthropic and charitable donations, or the concerted  
179 actions of multi-Government agencies. This makes the threat of drug resistance even  
180 more acute, because there is not the financial incentive to develop new drugs to

181 replace those to which resistance emerges. Nonetheless, certain major  
182 pharmaceutical companies are increasingly engaged in Public Private Partnerships  
183 providing access to chemical compound collections and other resources to discover  
184 and develop new drugs for neglected tropical diseases. Excellent examples of this  
185 collaborative spirit include the Medicines for Malaria Venture (<http://www.mmv.org/>),  
186 the Drugs for Neglected Diseases initiative (<http://www.dndi.org/>) and the Tres Cantos  
187 Open Lab Foundation (<http://www.openlabfoundation.org/>).

188         One route to limit the impact of drug resistance has been the exploitation of  
189 combination therapies for parasitic infections. This approach has proved useful for  
190 cancer therapy as well as for the treatment of TB, leprosy and viral infections such as  
191 HIV. It has also been encouraged for parasitic infections, for example through  
192 artemisinin combination therapy<sup>17,18</sup> to limit the emergence and spread of artemisinin-  
193 resistant malaria, and for trypanosomes where nifurtimox/eflornithine combination  
194 therapy<sup>19</sup> is proving more robust than eflornithine-based therapy alone. However,  
195 combination therapies for parasitic diseases require the availability of more than one  
196 effective drug or drug class, which is not always the case. Moreover, combination  
197 therapies have been often embraced only when resistance is already detected to one  
198 of the front line monotherapies, allowing multidrug resistant parasites to be selected.  
199 Here, the use of drug combinations with different pharmacokinetics in plasma, as with  
200 artemisinin and piperazine, can limit resistance emergence<sup>20</sup>. However, the cost of  
201 drugs for many parasites of the developing world can generate geographical  
202 discrepancy in the use of mono and combination therapies. Here, the efficacy of  
203 combination therapies can be threatened by ingression of resistant parasites selected  
204 under monotherapy.

205         The final challenge for eukaryotic microbes that differs from many prokaryotic  
206 and viral pathogens has been the failure to formulate and use effective vaccines to  
207 prevent infection<sup>21</sup>. Malaria research has focused intensively on vaccine development  
208 without transformative success, whereas for African trypanosomes the immune  
209 evasion mechanism employed by the parasite (antigenic variation) effectively renders  
210 vaccine approaches impossible. Other kinetoplastids have also proved challenging to  
211 produce safe effective vaccines, despite the widespread early use of 'leishmanization'  
212 for the cutaneous form of leishmaniasis, which has the risk of virulence in some  
213 individuals and immunosuppression<sup>22</sup>. Fungal pathogens have their greatest impact  
214 in immunocompromised individuals rendering vaccines potentially less useful. At  
215 present there are no licenced fungal vaccines; nonetheless, there are promising  
216 developments for adhesion-like substance 3 (Als3) and secreted aspartic protease 2

217 (Sap2) based vaccines, although concerns have been raised over their univalency and  
218 the potential for *C. albicans* to circumvent their efficacy<sup>23</sup>.

219

220 ***Drugs used against different eukaryotic microbes and examples of the***  
221 ***resistance mechanisms against them***

222

223 Throughout evolution microorganisms have evolved numerous strategies to counteract  
224 cellular toxicity induced by diverse chemical stresses (xenobiotics, metals, reactive  
225 oxygen and reactive nitrogen species, etc). Many of these generic defences have  
226 been co-opted for drug resistance. Figure 1 summarises the major therapeutic agents  
227 used to target malaria, kinetoplastid parasites and fungi, highlighting the dates of  
228 introduction and the appearance of resistance for each. The principal methods of  
229 resistance (Figure 2) involve either reduction of the free drug level at the target site of  
230 action, alterations in the drug target reducing its drug binding affinity or over-expression  
231 of the target restoring its essential function. In the case of inhibition of a metabolic  
232 pathway, the essential end-product can be produced either by induction of an  
233 alternative pathway or by upregulation of a salvage pathway in order to obtain an  
234 essential metabolite from the host. Downstream consequences of target inhibition  
235 include damage to DNA, proteins and lipids such that upregulation of repair pathways  
236 can also contribute to resistance. Unlike bacteria, acquisition of resistance genes by  
237 lateral gene transfer on plasmids has not been observed for protozoan parasites or  
238 fungal pathogens. In Table 2 we summarise the drugs used to treat eukaryotic  
239 microbial pathogens, their mode of action and mechanisms of resistance where  
240 known. Below, we highlight specific examples where drug resistance or the threat of  
241 resistance challenges current control efforts.

242

243 *Malaria:*

244 The most successful antimalarial in history to date has been chloroquine (CQ), a 4-  
245 aminoquinoline derivative of quinine (itself the world's first mass-distributed  
246 antimalarial) and first synthesized in 1934<sup>24</sup>. CQ was cheap and remained effective  
247 for decades. However, due to massive overuse and suboptimal compliance, resistance  
248 to chloroquine emerged in Southeast Asia in 1957 and in South America in 1960, and-  
249 by the mid 1980's- it was barely possible to use even in Africa<sup>25</sup>. Whilst disputed by  
250 some<sup>26</sup> the leading candidate for resistance to CQ (CQR) is PfCRT (P. falciparum CQR  
251 transporter)<sup>27</sup>. However, despite reports that PfCRT functions as a chloride channel, a  
252 proton pump, an activator of Na<sup>+</sup>/H<sup>+</sup> exchangers or a cation channel, the physiological  
253 function of PfCRT remains unclear<sup>28</sup>. Nonetheless, PfCRT is central to much



254 antimalarial resistance, the precise profile of which is modulated by associated  
255 mutations in other genes.

256 Artemisinin and its derivatives are fast acting but short-lived antimalarials that  
257 have been globally successful. In particular artemisinin-based combination therapies  
258 (ACTs, e.g. artemether-lumefantrine, artesunate-amodiaquine, and  
259 dihydroartemisinin-piperaquine) were recommended by the WHO in 2001 to ensure  
260 high cure rates of falciparum malaria and to reduce the spread of drug resistance to  
261 other front line drugs. However, clinical resistance was confirmed in 2008<sup>29</sup>  
262 characterised by a failure to rapidly clear parasites in patients around the Thai-  
263 Cambodian border<sup>30,31</sup>. Resistant parasites were characterized by transcriptomics<sup>32</sup>,  
264 large scale whole genome sequencing (WGS) of clinical isolates<sup>33,34</sup> and classical  
265 generation of resistant mutants by in vitro culture followed by WGS<sup>35</sup>. This pinpointed  
266 multiple independent mutations in a gene encoding a Kelch propeller protein (Kelch  
267 13) which was then causally linked to resistance by reverse genetics<sup>36,37</sup>. Large-scale  
268 genomic epidemiological evidence suggests that artemisinin resistance is not as  
269 straightforward as the simple acquisition of mutations in kelch13. Indeed,  
270 nonsynonymous mutations in ferredoxin, apicoplast ribosomal protein S10, multidrug  
271 resistance protein 2 and the chloroquine resistance transporter (PfCRT) also showed  
272 strong associations with artemisinin resistance<sup>29</sup>. These mutations appear to act as  
273 markers of a genetic landscape upon which artemisinin resistance-conferring  
274 kelch13 mutations are more likely to occur. These landscape mutations also correlate  
275 with the current geographical limits of artemisinin resistance<sup>29</sup>. This concept is further  
276 supported by additional genomic epidemiological evidence that demonstrates many of  
277 the 20 or so mutations in kelch13 that have been implicated in the SE Asian  
278 manifestation of artemisinin resistance are also found in African PF isolates. However,  
279 these mutations are present at no greater frequency in the African strains than other  
280 PF genes indicating a lack of selective pressure in that continent and that these strains  
281 lack the enabling genetic background observed in SE Asia<sup>38</sup>.

282 Kelch propeller domain proteins are subcellular organisers of multiprotein  
283 complexes and indeed artemisinin resistance associated mutation of Kelch 13 results  
284 in its enhanced association with phosphatidylinositol-3-Kinase (PI3K)<sup>39</sup>. Experimental  
285 overexpression of PI3K results in enhanced artemisinin resistance and PI3P levels are  
286 predictive of resistance to artemisinin<sup>39</sup>. In addition, upregulation of the chaperonin  
287 complexes, PROSC and TRiC, involved in the unfolded protein stress response in  
288 other eukaryotes, may contribute to artemisinin resistance<sup>32</sup>. Worryingly, resistance to  
289 some of the various ACT regimens (involving lumefantrine and amodiaquine and  
290 PfCRT) is becoming evident<sup>40-43</sup>. However the framework for the rapid evaluation of  
291 genome evolution in the face of drugs is in place and will hopefully swiftly indicate any  
292 further potential mechanisms.

293

294

295 *Human African trypanosomiasis:*

296 The vast majority of reported cases of HAT are caused by *T. b. gambiense*, with less  
297 than 2% caused by *T. b. rhodesiense*<sup>44</sup>. Treatment involves either pentamidine or  
298 suramin for stage 1 infection (before CNS involvement) whereas melarsoprol,  
299 eflornithine or nifurtimox/eflornithine combination therapy are used once the parasite  
300 crosses the blood-brain barrier<sup>2</sup>, the latter combination therapy reducing the duration  
301 of treatment regimens. Given the limited chemotherapeutic options for the treatment  
302 of HAT (Table 2), drug resistance could seriously compromise efforts to eliminate this  
303 epidemic disease as a public health problem<sup>44</sup>. Fortunately, resistance emergence for  
304 pentamidine has not been significant, despite continuous use of pentamidine since the  
305 1940s, including a mass chemoprophylactic campaign in the 1950s in the then Belgian  
306 Congo. However, cross resistance to pentamidine and melarsoprol, used for stage 2  
307 of infection, is frequently observed. Melarsoprol is a trivalent melaminophenyl arsenical  
308 which has a propensity to react covalently with vicinal dithiols, including the parasite-  
309 specific dithiol, trypanothione<sup>45</sup>, to form a cyclic complex known as MeIT<sup>46</sup>. Melarsoprol  
310 has a high incidence of severe (lethal) toxicities and high rates of treatment failures  
311 have been reported in the Democratic Republic of Congo, Uganda, Angola and  
312 Sudan<sup>2</sup>. Although therapeutic failure does not necessarily equate with drug resistance,  
313 it appears that the high relapse rate in northwest Uganda is associated with reduced  
314 susceptibility to melarsoprol<sup>47,48</sup>. The recent report that the aquaglyceroporin AQP2  
315 appears to function as a transporter for large drugs such as pentamidine and  
316 melarsoprol was surprising given that Aquaglyceroporins are channels facilitating the  
317 passive transport of water and small neutral molecules across cell membranes.  
318 Nonetheless, there is strong evidence that AQP2 is indeed synonymous with the high  
319 affinity pentamidine transporter (HAPT1)<sup>49</sup>, with a recent report indicating that  
320 pentamidine binds and inhibits the transporter and is then internalised via  
321 endocytosis<sup>50</sup>.

322

### 323 *Chagas' disease:*

324 For *Trypanosoma cruzi*, an intracellular parasite with a wide tissue tropism, infection  
325 has three phases: an acute phase associated with high parasitaemia; an asymptomatic  
326 (indeterminate) phase lasting anywhere between 10-30 years, where parasitaemia is  
327 controlled by the immune response; and a chronic phase in about 30-40% of patients  
328 characterised by either cardiac disease or digestive disease (mega-oesophagus and  
329 mega-colon). For treatment, benznidazole and nifurtimox have significant activity in  
330 the acute phase<sup>51</sup> and benznidazole also eliminates parasitaemia in the indeterminate  
331 and chronic phases of the disease<sup>52,53</sup>. However, a large multi-centre, randomized trial  
332 of benznidazole for chronic Chagas' cardiomyopathy failed to significantly reduce

333 cardiac clinical deterioration through 5 years follow-up<sup>53</sup>. Whether this is due to  
334 differences in drug susceptibility, pharmacokinetic/pharmacodynamic issues or the  
335 pathophysiology of the disease is not known. The results of two recent clinical trials  
336 with azole ergosterol inhibitors, posaconazole and E1224 (a pro-drug of ravuconazole)  
337 have been equally disappointing<sup>52,54,55</sup>.

338

339

340 *Visceral leishmaniasis:*

341 Treatment of visceral leishmaniasis (VL), cutaneous and mucocutaneous  
342 leishmaniasis is limited to four main drugs: pentavalent antimonial complexes (sodium  
343 stibogluconate and meglumine antimonate); amphotericin B (as deoxycholate or  
344 liposomal formulations); the aminoglycoside paromomycin; and the  
345 alkylphosphocholine miltefosine<sup>56,57</sup>. Treatment varies according to geographical  
346 location, the immune status and other co-morbidities of the patient, and the disease  
347 classification<sup>58</sup>.

348 Of these treatments, widespread resistance to antimonial drugs is specific to  
349 Southern Asia and not in Sub-Saharan Africa or Brazil. Indeed, antimonial drugs are  
350 not recommended in India or Nepal due to treatment failures commencing in the 1990s  
351 and now reported to be as high as 60% in some regions<sup>59</sup>. This has been attributed to  
352 inappropriate treatment in an unregulated private health system or to the use  
353 substandard antimonial drugs. However, Southern Asia is the only region where  
354 arsenic exposure and widespread antimonial resistance co-exist. Thus, environmental  
355 pollution and exposure of patients to arsenic in food and drinking water was proposed  
356 as an alternative hypothesis<sup>60</sup>. Arsenic and antimony are both metalloids and selection  
357 of leishmania parasites for resistance to trivalent arsenic results in cross-resistance to  
358 trivalent antimony *in vitro*<sup>61</sup>, but its physiological relevance was uncertain. Chronic  
359 exposure of infected mice to arsenic in drinking water at environmentally relevant  
360 levels demonstrated that it is possible to generate resistance to pentavalent antimony  
361 *in vivo*<sup>62</sup>. A retrospective clinico-epidemiological study identified a trend towards  
362 increased treatment failure in arsenic exposed patients, but failed to reach statistical  
363 significance<sup>63</sup>.

364

365 Resistance to antimonials is multifactorial and most of the mechanisms shown in  
366 Figure 2 have been implicated in *Leishmania*. Studies on experimental and clinical  
367 resistant isolates strongly support the hypothesis that trypanothione plays a pivotal role  
368 in antimonial resistance. However, none of the following mechanisms are universal in  
369 resistant isolates. Decreased biological reduction of Sb<sup>V</sup> to Sb<sup>III</sup> has been reported in

370 resistant leishmania amastigotes<sup>64</sup> and two candidate “antimony reductases”  
371 identified, although genetic<sup>65,66</sup> and proteomic studies<sup>67,68</sup> have not identified any  
372 changes in either TDR1<sup>69</sup> or ArsC<sup>70</sup>. The mechanism of uptake of Sb<sup>V</sup> is not known,  
373 but modulation of expression of aquaglyceroporin 1 (AQP1) affects Sb<sup>III</sup> susceptibility<sup>71-</sup>  
374 <sup>73</sup>. AQP1 copy number and expression levels correlate with susceptibility to Sb<sup>III</sup> in  
375 some, but not all, clinical isolates<sup>74,75</sup>. However, interpretation of this observation is  
376 complicated by the fact that AQP1 is located on chromosome 31, which is frequently  
377 trisomic or tetrasomic<sup>76</sup> in these mosaic aneuploid parasites<sup>77</sup>. Upregulation of  
378 trypanothione and ancillary biosynthetic pathways has also been observed in  
379 genomic<sup>65,66</sup> and metabolomic<sup>78,79</sup> studies. MRPA is responsible for ATP-dependent  
380 efflux of Sb<sup>III</sup> as a thiol conjugate into membrane vesicles<sup>80</sup> and a homodimeric ABC  
381 half-transporter (ABC14) is one possible candidate for efflux across the plasma  
382 membrane<sup>81</sup>.

383 Miltefosine, the only oral treatment for VL, was first approved for use in India in  
384 2002. However, a decade on there is an increasing rate of clinical relapse<sup>82,83</sup>, which  
385 threatens to undermine the Kala-Azar Elimination Program in the Indian subcontinent.  
386 Stable resistance is readily generated in the laboratory with no cross-resistance to  
387 other anti-leishmanial drugs<sup>84,85</sup>.

388

389 *Fungi:*

390 Several classes of antifungals are used clinically (Table 1, Table 2) – each with very  
391 different drug resistance profiles. The oldest antifungals are the polyene macrolide  
392 antibiotics, exemplified by amphotericin B, which remains a front-line choice of a broad  
393 spectrum agent for fungal infections of unknown aetiology. Amphotericin deoxycholate  
394 has significant nephrotoxicity which is significantly ameliorated in lipid carrier  
395 formulations such as AmBisome, which also has potent anti-*Leishmania* activity. As  
396 with other eukaryotic pathogens, resistance to antifungal drugs has become an  
397 increasing important clinical problem<sup>86,87</sup>. A few recognised cases exist of inherent  
398 resistance of specific fungi to specific antifungals, but mostly resistance is due to  
399 induced changes and mutations.

400 The imidazoles and more modern triazoles (collectively known as the “azoles”)  
401 constitute the main class of antifungals used in the treatment of infections. Various  
402 modifications of the triazole ring have generated a series of antifungals including  
403 fluconazole (used mainly in the treatment of *Candida* infections), and itraconazole,  
404 voriconazole, posaconazole, ravuconazole and the recently licenced isavuconazole  
405 which have improved activity against *Aspergillus* and filamentous fungal species.  
406 These compounds have important differences in antifungal potencies, spectrum of

407 activities, bioavailability, drug interactions and toxic potential. For example, some  
408 patients treated with voriconazole suffer from photosensitivity and an elevated risk of  
409 skin carcinoma<sup>88</sup>. Other sterol inhibitors include the allylamines squalene epoxidase  
410 inhibitors and phenylmorpholine Erg24 D14 reductase and Erg2 D8-D7 isomerase  
411 inhibitors that are used topically against dermatophytic infections for which clinical  
412 resistance is low.

413 Although some fungi such as *Candida krusei* are inherently azole resistant,  
414 multiply triazole resistant strains are now emerging<sup>89,90</sup> as well as strains with cross  
415 resistance to azoles and echinocandins suggesting worrisome multi-drug resistance  
416 (MDR) phenotypes in medically important fungi<sup>91</sup>. A threat from multi-azole resistant  
417 strains of *A. fumigatus* may have arisen under the selective pressure of agricultural  
418 azole fungicides and subsequent transmission of azole resistant strains to the clinic by  
419 spore dispersal<sup>92-95</sup>. The prevalence of these alleles is increasing in Europe and now  
420 in other parts of the world<sup>90,96,97</sup>. In *Candida* mutants harbouring azole resistance have  
421 a fitness deficit<sup>98</sup>; however, MDR strains of *Aspergillus* do not seem to have  
422 significantly decreased fitness implying they may become stably represented in the  
423 environment.

424 The most recently developed major class of antifungal are the echinocandin  
425 antibiotics of which caspofungin, micafungin and anidulafungin are used clinically.  
426 These have similar pharmacokinetic properties although a new echinocandin (CD101-  
427 formerly Biofungin) is in clinical trials and has improved stability *in vivo* and requires  
428 less frequent i.v. dosing. Echinocandins are fungicidal against *Candida* species and  
429 fungistatic or fungicidal against *Aspergillus* causing hyphal or bud tip lysis but they are  
430 not efficacious against *Pneumocystis jiroveci* and some other species.

431 Hsp90-mediated changes in drug tolerance have also been implicated in  
432 determining echinocandin sensitivity<sup>99</sup>. Recently, multi-drug azole/ echinocandin  
433 resistance has been identified in fungi and this is particularly frequent in strains of  
434 *C. glabrata* which is common in patients with haematological malignancies and solid  
435 tumours<sup>100,101</sup>. These MDR strains of *C. glabrata* become reliant on i.v. amphotericin  
436 treatment, and since this agent has poor penetration into urine such infections are  
437 essentially untreatable.

438  
439

#### 440 ***Outstanding challenges and future prospects***

441

442 This review began by highlighting the similarities and differences between drug  
443 resistance emergence in prokaryotic and eukaryotic microbes. The control of the

444 emergence of drug resistance for eukaryotic microbial pathogens also has similarities  
445 and distinctions from prokaryotic drug resistance, and our challenge for the future is to  
446 ensure best practice is employed for both groups. One effective mechanism to control  
447 drug resistance spread in bacterial pathogens is the application of appropriate  
448 antibiotic stewardship, applying the right drug at the right dose, at the right time, for the  
449 right duration. This approach operates effectively where there is well-regulated  
450 healthcare, effective and rapid screening, a selection of available drugs as contingency  
451 and the necessary education and engagement between the patient and healthcare  
452 provider. Moreover, bacterial drug resistance is a global phenomenon where  
453 resistance selected through poor stewardship in one geographical area may be  
454 contained by stringent practices in other areas, or combatted by an investment in new  
455 pharmaceutical development in wealthy countries. These containment measures are  
456 inevitably less effective where primary care is limited or too expensive, education is  
457 lacking or where the diseases involved do not have direct impact in the developed  
458 world. In consequence, the limitation of many eukaryotic pathogens to the poorer parts  
459 of the world makes a co-ordinated response to resistance emergence more difficult to  
460 achieve.

461         The drivers of resistance emergence are also more difficult to mitigate for many  
462 eukaryotic pathogens. As highlighted earlier, drug provenance and effective delivery  
463 is a significant challenge in the developing world. The latter is a particular challenge  
464 for prospective mass drug administration programmes where delivery to a population  
465 on a broad or local scale, if incomplete, can counteract its intention to contain the  
466 spread of existing resistance in target regions. A further complication in low and  
467 middle-income countries is the effects of co-infection or malnutrition in populations  
468 treated with drugs targeting a particular pathogen (discussed in <sup>102</sup>). Notably, the  
469 pharmacokinetic behaviour of drugs in malnourished individuals may be variable and  
470 unpredictable leading to inadvertent under-dosing, driving resistance emergence.  
471 When combined with immunosuppression induced by many parasites, or the hospital-  
472 induced immunosuppression of patients that become susceptible to fungal infection,  
473 drug concentrations that would clear infections in the context of a robust immune  
474 system may fall short in its absence. The ecological balance between distinct  
475 pathogens in patients with coinfections can also lead to unanticipated consequences,  
476 where the removal of one pathogen can create a niche exploited by a distinct pathogen  
477 or where the normal interactions between pathogens with each other, and with the  
478 immune system, is perturbed with drug pressure. The resistance mechanisms selected  
479 in drug treated populations can also alter pathogen phenotypes with the risk of  
480 enhanced virulence.

481           Although the factors that drive drug resistance are well known, it remains  
482 essential to identify when drug resistance arises and to respond rapidly and effectively.  
483 As with health care, surveillance is a key challenge for diseases in the developing  
484 world, where populations may be inaccessible, reluctant to engage or where treatment  
485 failure can have multiple causes beyond the emergence of drug resistance. Moreover,  
486 resistance can show considerable variation amongst populations or in different  
487 geographical settings. Here, accurate and rapid detection is critical to understand  
488 resistance epidemiology and thereby the best treatment to deliver, but this can be  
489 difficult to achieve. Despite this, developments in field PCR assays and next generation  
490 sequencing permit the sensitive identification and tracking of emergent resistance,  
491 allowing earlier control responses than could be previously achieved. Hence, an  
492 integration of improved therapeutic delivery and treatment monitoring are critical  
493 control points to reduce resistance emergence, in tandem with the discovery of the  
494 relevant resistance mechanisms and the search for new drug therapies. These  
495 combined approaches span from the individual scientific researcher to clinician, to  
496 health agency, to government and population, which must be well-integrated, and alert,  
497 with effective and rapid communication between distinct levels to allow appropriate  
498 responses to be put into action if needed.

499           Fortunately, whilst drug resistance is emerging in many eukaryotic microbial  
500 pathogens, new tools and methodologies are being developed to (i) predict resistance  
501 mechanisms, (ii) to identify modes of drug action and potential escape pathways and  
502 (iii) to understand pathogen biochemistry as a means to discover new potential  
503 therapies. With respect to drug resistance, the advent of cost-effective and rapid  
504 genome resequencing allows signatures of selection to be identified<sup>103-106</sup>, whilst  
505 genome-wide RNAi screens allow the mapping of resistance pathways<sup>107,108</sup>, and  
506 overexpression libraries<sup>109</sup> can assist with drug target deconvolution through selective  
507 screens. These genetic tools are complemented by improvements in proteomics such  
508 that adaptations accompanying drug resistance can be pinpointed, providing  
509 information on resistance mechanisms, and potential diagnostic tools to detect  
510 resistance emergence<sup>110</sup>. Combined with the improved sensitivity and resolution of  
511 metabolomics analysis<sup>111</sup>, biochemical pathways can also be mapped in the context of  
512 drug exposure, allowing bypass mechanisms to be highlighted, if present. These each  
513 provide the essential early warning systems necessary to identify and combat the  
514 spread of drug resistance. Furthermore, certain combination therapies might offer  
515 novel transmission blocking strategies: very recently resistance to the antimalarial  
516 atovaquone, a component (with proguanil) of the widely used and successful treatment  
517 marketed as Malarone, has been further characterised. Resistance mutations that

518 appear during the target blood stage infection localise to the mitochondrial protein  
519 cytochrome b, one of the few proteins encoded by the highly reduced *Plasmodium*  
520 mitochondrial genome. All atovaquone resistance mutations examined generate a  
521 deficient mitochondrion and a parasite that, whilst viable in the blood, is incapable of  
522 development in the mosquito and thereby cannot be transmitted<sup>112</sup>. Thus despite the  
523 fact that resistance to atovaquone might arise repeatedly, each incident is isolated.  
524 Drugs that target cytochrome b could form part of combination therapies that are self-  
525 limiting in terms of spread of drug resistance and may delay any transmission of  
526 resistance that arises to the drug it is partnered with.

527

### 528 **Concluding remarks**

529

530 Drug resistance in eukaryotic microbes is an increasing global problem that threatens  
531 the advances in healthcare over the last 50 years. This mirrors the situation for  
532 bacterial and viral pathogens but is particularly acute given the abundance of  
533 eukaryotic pathogens in the poorest regions of the world. These countries have the  
534 least capacity to respond to resistance emergence through the development of new  
535 drugs vaccines and diagnostics, whilst developed countries lack financial incentives to  
536 assist. Nonetheless, there are opportunities to respond to this threat due to the distinct  
537 biology of many major eukaryotic pathogens and the discoveries made in basic  
538 research focused on their biology. Furthermore, many eukaryotic microbes are  
539 arthropod-borne diseases, such that targeting transmission can be a route to pathogen  
540 control not available for opportunistic pathogens. This can take the form of  
541 transmission-blocking vaccines or drugs targeting *Plasmodium*<sup>113</sup> or the application of  
542 vector control measures such as insecticide impregnated bed nets<sup>114</sup>, peri-domestic  
543 and indoor residual insecticide spraying<sup>114,115</sup>, tsetse traps<sup>116</sup>, or improved housing<sup>117</sup>.  
544 Sterile insect release is also a route to limiting the vector population and so restricting  
545 disease spread<sup>118,119</sup>. Eukaryotic microbes have also, like some bacterial pathogens,  
546 been found to show co-operative and social behaviours to optimise their establishment  
547 and transmission in their hosts or vectors<sup>120,121</sup>. These social responses can control  
548 parasite density or the development of transmission stages<sup>122-124</sup>, such that blocking or  
549 mimicking signals for communication or their transduction pathways provides new  
550 routes to limit the impact of the pathogens using strategies that might be less  
551 susceptible to resistance emergence.

552 Whether or not new targets or new approaches can be identified, there is a real  
553 need to optimise the delivery and deployment of drugs. Control of drug quality,  
554 distribution and supply of cost-effective drugs is crucial. Also the application of both



555 epidemiological modelling and evolutionary theory to guide drug treatment policies is  
556 important in prolonging the life span of drugs and thereby maximising the return on the  
557 considerable cost associated with developing and introducing a new drug. Targeted  
558 therapy as opposed to mass drug administration is key to limiting the emergence of  
559 resistance, or containing resistance when it is detected. This requires an integration of  
560 epidemiology, diagnosis, detection and supply chain control as well as investment in a  
561 pipeline of new therapeutics ready to be deployed when resistance inevitably emerges.  
562 Only through slowing resistance emergence and accelerating new drug discovery will  
563 the control successes achieved against eukaryotic microbial pathogens be sustained.  
564

565 **Table 1.**  
566 **Diseases caused by eukaryotic microbes, their vectors and front-line treatment**  
567 **options.** Several of the parasitic pathogens are arthropod-transmitted, and in these  
568 cases the responsible vector is shown. Fungal pathogens are predominantly  
569 opportunistic.

570

571 **Table 2**  
572 **Modes of action and mechanisms of drug resistance in eukaryotic microbes**

573

574 **Figure Legends**

575

576 **Figure 1 Timelines for emergence of drug resistance in parasitic diseases (A)**  
577 **and Fungi (B).** The darker bar represents the time from first widespread clinical use  
578 to the first year drug resistance was suspected or confirmed. The shading indicates  
579 that certain drugs are still in use for particular indications or in specific geographical  
580 locations. Abbreviations: S-P, sulfadoxine-pyrimethamine; PPQ, piperazine; ACTs,  
581 artemisinin combination therapies; NECT, nifurtimox eflornithine combination therapy;  
582 L-AMB, liposomal amphotericin B; MLT, miltefosine. For fungal pathogens, *insensitive*  
583 *or resistant strains have been identified shortly after the introduction of all of the major*  
584 *classes of antifungal agents. In the case of amphotericin B, there remains very little*  
585 *resistance – and differences in sensitivity mainly reflect the relative inherent sensitivity*  
586 *of different species to this agent.*

587

588

589 **Figure 2 Molecular mechanisms of drug-resistance.**

590 Eukaryotic microbial pathogens can exhibit drug resistance through reducing the  
591 overall intracellular concentration of the drug (less uptake, more efflux), by inactivating  
592 or failing to activate the drug, or by sequestering the drug away from its target.  
593 Resistance can also be mediated by reducing affinity of the drug for the target by  
594 mutation or by reducing the drug effect by overexpression of the target. Salvage and  
595 by-pass pathways can also lower the overall impact of the drug action, as can the  
596 activation of pathways in order to repair any damage caused.

597

598

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**Table 1. Diseases caused by eukaryotic microbes, their vectors and front-line treatment options**

Disease	Pathogen group	Vector	Pathogen	Front-line treatments <sup>a</sup>
Malaria	apicomplexan	Anopheline mosquitoes	<i>Plasmodium falciparum</i>	<p><b>Uncomplicated <i>P. falciparum</i> malaria:</b> Artemisinin combination therapies (ACTs)</p> <ul style="list-style-type: none"> <li>• Artemether + lumefantrine</li> <li>• Artesunate + amodiaquine</li> <li>• Artesunate + mefloquine</li> <li>• Dihydroartemisinin + piperazine</li> <li>• Artesunate + sulfadoxine + pyrimethamine</li> </ul> <p><b>Severe malaria:</b> Parenteral (or rectal, children &lt; 6 years) artesunate followed by oral ACT (i.m. artemether or i.m. quinine if artesunate unavailable)</p>
			<i>P. vivax</i> , <i>P. ovale</i> , <i>P. malariae</i> or <i>P. knowlesi</i>	<p><b>Blood stage infections:</b> Chloroquine (except in areas of chloroquine resistance) ACTs (except pregnant women and infants &lt; 6 months) <b>Radical cure of liver (hypnozoite) infection:</b> Primaquine (close medical supervision with G6PD-deficient patients)</p>
African trypanosomiasis	kinetoplastid	Tsetse flies	<i>Trypanosoma brucei gambiense</i> (chronic form)	<p><b>Haemolymphatic stage</b> (no CNS involvement): Pentamidine (i.m.) <b>CNS stage:</b> Nifurtimox (oral) / eflornithine (i.v.) combination therapy (NECT) (Melarsoprol if NECT unavailable)</p>
			<i>T. b. rhodesiense</i> (acute form)	<p><b>Haemolymphatic stage</b> (no CNS involvement): Suramin (i.v.) <b>CNS stage:</b></p>

				Melarsoprol (i.v.)
American trypanosomiasis	kinetoplastid	Triatomine bugs	<i>Trypanosoma cruzi</i>	Benznidazole Nifurtimox
Leishmaniasis	kinetoplastid	Phlebotomine sandflies	<b>Visceral disease</b> <i>Leishmania donovani</i> <i>L. infantum</i> <b>Mucocutaneous disease</b> <i>L. braziliensis</i> <i>L. panamensis</i> <b>Cutaneous disease,</b> e.g. <i>L. major</i> <i>L. tropica</i> <i>L. mexicana</i> <i>L. amazonensis</i>	<b>Visceral disease:</b> Amphotericin B (as liposomal or deoxycholate complex, i.v.) Miltefosine (oral, contraindicated in pregnancy) Paromomycin (i.m.) Sodium stibogluconate (SSG) or meglumine antimonate, parenteral (except India and Nepal) SSG plus paromomycin (East Africa) <b>Mucocutaneous:</b> SSG (systemic) <b>Cutaneous:</b> SSG (intralesional) Paromomycin (ointment) Miltefosine
Invasive Candidiasis	fungal	opportunistic	<i>Candida albicans</i> <i>Candida glabrata</i> <i>Candida parapsilosis</i>	Echinocandins, Fluconazole, Liposomal Amphotericin B
Aspergillosis	fungal	opportunistic	<i>Aspergillus fumigatus</i>	Voriconazole (Amphotericin B formulations; caspofungin; micafungin; posaconazole; itraconazole)
Pneumocystis pneumonia	fungal	opportunistic	<i>Pneumocystis carinii</i>	Sulfamethoxazole-Trimethoprim (clindamycin-primaquine)
Cryptococcal meningitis	fungal	opportunistic	<i>Cryptococcus neoformans</i>	Amphotericin B plus flucytosine Amphotericin B plus fluconazole

<sup>a</sup> Second line treatment options are given in parentheses  
Data from WHO <sup>58,125-127</sup> and other sources <sup>57,128,129</sup>

**Table 2. Modes of action and mechanisms of drug resistance in eukaryotic microbes**

<b>Pathogen</b>	<b>Drug and date of resistance reported</b>	<b>Drug class and Mode of action</b>	<b>Resistance mechanism</b>
<b>Plasmodium</b>	Chloroquine (CQ)  1957 (SE Asia) 1960 (S America) Mid 1980s (Africa)	<i>4-Aminoquinoline</i>  Chloroquine interferes with the detoxification of haem into chemically inert haemozoin resulting in accumulation of toxic CQ ferric haem complex and subsequent parasite lysis <sup>130</sup> .	K76T <sup>27</sup> mutation in a Digestive Vacuole-sited, ATP-dependent, 10 transmembrane domain transporter PfCRT (P. falciparum CQR transporter) <sup>27</sup> ; a range of more than 30 different mutations might interact epistatically <sup>131-133</sup> . These stimulate the active efflux of CQ by mutant PfCRT or the passive efflux of diprotonated CQ <sup>133</sup> .  Other genes contributing to resistance include: the P multidrug resistance transporter 1 (PfMDR1) homologue; multipass transmembrane transporter CG2; and PfNHE1 and a sodium hydrogen antiporter also associated with quinine resistance <sup>134</sup> . The specific genetic background of the parasite and the range of mutations in genes other than PfCRT are also key to the manifestation of CQR <sup>135</sup> .  An independent mutation in PfCRT (C350R) can reverse CQR and also increase susceptibility of the parasite to other antimalarials (mefloquine, quinine and lumefantrine but not piperiquine <sup>136</sup> ). The mutation N326D confers increased resistance to the antimalarial amodiaquine <sup>40</sup>
	Mefloquine  1982 (Thailand)	<i>Quinoline-4-methanol</i>  Blockade of haemozoin formation and binding to phospholipids	PfMDR1 is associated with mefloquine resistance <sup>137</sup> but may also modulate CQR through compensatory mutations that counteract PfCRT mutations that compromise parasite fitness <sup>138</sup> .
	Artesunate Dihydroartemisinin Artemether	<i>Sesquiterpene lactone endoperoxides.</i>	Dormancy resulting in an extended ring stage phase of development in the erythrocyte promotes resistance <sup>30-32</sup> .

	2008 <sup>29</sup> (SE Asia)	Form a carbon-centred free radical or reactive electrophilic intermediate that alkylates a number of malaria proteins <sup>139</sup> after activation by haem or free iron.	Multiple independent mutations in a gene encoding a Kelch propeller protein (Kelch 13) confer resistance <sup>33-37</sup> . This results in its enhanced association with phosphatidylinositol-3-Kinase (PI3K), which is subsequently under-ubiquitinated and accumulates along with its lipid product, phosphatidylinositol-3-phosphate (PI3P).  The specific genetic background of the parasite and the range of mutations in genes other than <i>kelch13</i> may also be key to the manifestation of resistance to artemisinin <sup>33</sup>
	Sulfadoxine / Pyrimethamine  1967 (Thailand); 1980s (Africa)	<i>Antifols.</i>  Sulfadoxine – inhibition of dihydropteroate synthase (DHPS) Pyrimethamine – inhibition of dihydrofolate reductase (DHFR) Synergistic effect on thymidylate synthesis	Decreased affinity of both drugs for their respective targets.  Resistance to sulfadoxine involves DHPS point mutations., DHPS variant A437G confers moderate resistance, with the additional mutations S436F plus A613S conferring a high level resistance <sup>140</sup> .  Pyrimethamine clinical resistance involves DHFR point mutations at S108N in Africa and SE Asia. Additional mutations that confer high level resistance are N51I and C59R <sup>141</sup>  Increased GTP-cyclohydrolase (CNVs) enhances folate biosynthesis compensating for loss of fitness <sup>141</sup>
	Proguanil	DHFR inhibitor	High level resistance to cycloguanil (a metabolite of proguanil) involves DHFR mutation of serine 108 to threonine. The triple mutations (C59R, S108N and I164L) confer cross resistance to both pyrimethamine and cycloguanil <sup>142</sup> .
	Atovaquone (in combination with proguanil for prophylaxis or treatment)	Cytochrome b inhibitor	Effective resistance to atovaquone involves one of a range of mutations in <i>cyt b</i> most commonly Y268S. Other mutations associated with such resistance include I258M, Y268C, M133I and V259L <sup>143</sup>
	Suramin	<i>Naphthylamine trisulfonic acid</i>	Laboratory-generated resistance mediated through the silencing of invariant surface glycoprotein (ISG75), the AP1 adaptin complex,

<b>African Trypanosomes</b>		Mode of action unknown	lysosomal proteases and major lysosomal transmembrane protein, as well as spermidine and N-acetylglucosamine biosynthesis <sup>108</sup> .
	Pentamidine	<i>Diamidine</i>	Resistance is associated with loss of uptake on the P2 adenine/adenosine transporter <sup>144</sup> , (AT1) <sup>145</sup> .
	Clinical resistance is not significant.	Mode of action unknown	Cross-resistance between melaminophenyl arsenicals and diamidines is mediated by aquaglyceroporin 2 (AQP2) <sup>146</sup> . A chimeric AQP2/AQP3 gene is associated with cross resistance to melarsoprol and pentamidine in laboratory-generated <sup>149,146,147</sup> and clinical isolates <sup>148,149</sup>
	Melarsoprol	<i>Trivalent melaminophenyl arsenical.</i>	Resistance is associated with loss of uptake on the P2 adenine/adenosine transporter <sup>144,145</sup> . A non-functional mutant has been identified in melarsoprol-resistant field isolates <sup>150</sup> .
	Treatment failures have been reported in the Democratic Republic of Congo, Uganda, Angola and Sudan <sup>2</sup>	Forms a cyclic complex with trypanothione known as MeIT <sup>46</sup> . Inhibits trypanothione reductase and no doubt other targets.	See also AQP in pentamidine section.
Eflornithine (difluoromethyl-ornithine)	<i>Fluorinated amino acid.</i>	Laboratory-generated resistance is due to loss of a non-essential amino acid transporter <sup>151,152</sup> . There is no detected resistance in <i>T. b. gambiense</i> , but there is inherent resistance in some clinical isolates of <i>T. b. rhodesiense</i> <sup>2</sup> .	
	Mechanism-based inhibitor of ornithine decarboxylase, required for biosynthesis of polyamines and trypanothione.		
Nifurtimox	<i>Nitrofurans</i>	A genome-scale RNA interference screen identified NTR and a number of other genes possibly associated with NTR function <sup>108</sup> . NTR is also the key resistance determinant in laboratory-generated lines <sup>156,157</sup> showing cross resistance to fexinidazole an oral nitro-imidazole currently undergoing Phase II/III clinical trials for HAT.	
(poor efficacy as monotherapy; used in combination therapy with	Prodrug activated by an oxygen-insensitive mitochondrial nitroreductase (NTR) <sup>153</sup> to		

	eflornithine [NECT])	form highly reactive drug metabolites <sup>154</sup> that kill trypanosomes via unknown mechanisms <sup>155</sup> .	
<b>South American Trypanosomes</b>	Benznidazole, Nifurtimox  (natural resistance in some <i>T. cruzi</i> isolates)	<i>Nitroheterocyclics</i>  Benznidazole is activated by mitochondrial NTR <sup>153,158</sup> to form electrophilic drug metabolites <sup>159,160</sup>	Drug efflux via an ABCG-like transporter <sup>161</sup>  The NAD(P)H flavin oxidoreductase (old yellow enzyme) is downregulated in resistant lines <sup>162,163</sup> . However, this enzyme does not reduce benznidazole and only reduces nifurtimox under anaerobic conditions <sup>164</sup> .
<b>Visceral Leishmaniasis</b>	Sodium stibogluconate, Meglumine antimonite  1990s widespread resistance in India and Nepal. Not widespread in Sub-Saharan Africa or Brazil	<i>Pentavalent antimonials</i>  Sb <sup>V</sup> is reduced to Sb <sup>III</sup> to attack intracellular amastigotes. Likely to bind multiple targets including trypanothione reductase <sup>165,166</sup> , tryparedoxin peroxidase <sup>167</sup> and CCHC Zinc finger proteins <sup>166</sup> .	Selection for resistance to trivalent arsenic results in cross-resistance to trivalent antimony in vitro <sup>61</sup> , and in vivo <sup>62</sup> . Resistance is multifactorial through several mechanisms: <ul style="list-style-type: none"> <li>• Decreased reduction of Sb<sup>V</sup> to Sb<sup>III</sup></li> <li>• Sb<sup>III</sup> is taken up via an aquaglyceroporin<sup>73</sup> and modulation of expression of aquaglyceroporin 1 affects Sb<sup>III</sup> susceptibility<sup>71-73</sup>.</li> <li>• Elevated Intracellular trypanothione levels<sup>168</sup> or increased biosynthetic potential<sup>165,66,78,79</sup>.</li> <li>• Increased levels of tryparedoxin peroxidase confer resistance to Sb<sup>III</sup><sup>169</sup> and are found in clinical resistant isolates<sup>167</sup></li> <li>• MRPA (also known as PgpA or ABCC3), a member of the ATP-binding cassette (ABC) transporters, is amplified in some resistant lines<sup>170-172</sup> and sequesters Sb<sup>III</sup> in an intracellular vacuolar compartment close to the flagellar pocket<sup>80</sup>.</li> <li>• chaperones and stress related proteins are upregulated<sup>167,68</sup>, potentially reducing or repairing cellular damage induced by antimonials<sup>173</sup></li> </ul>

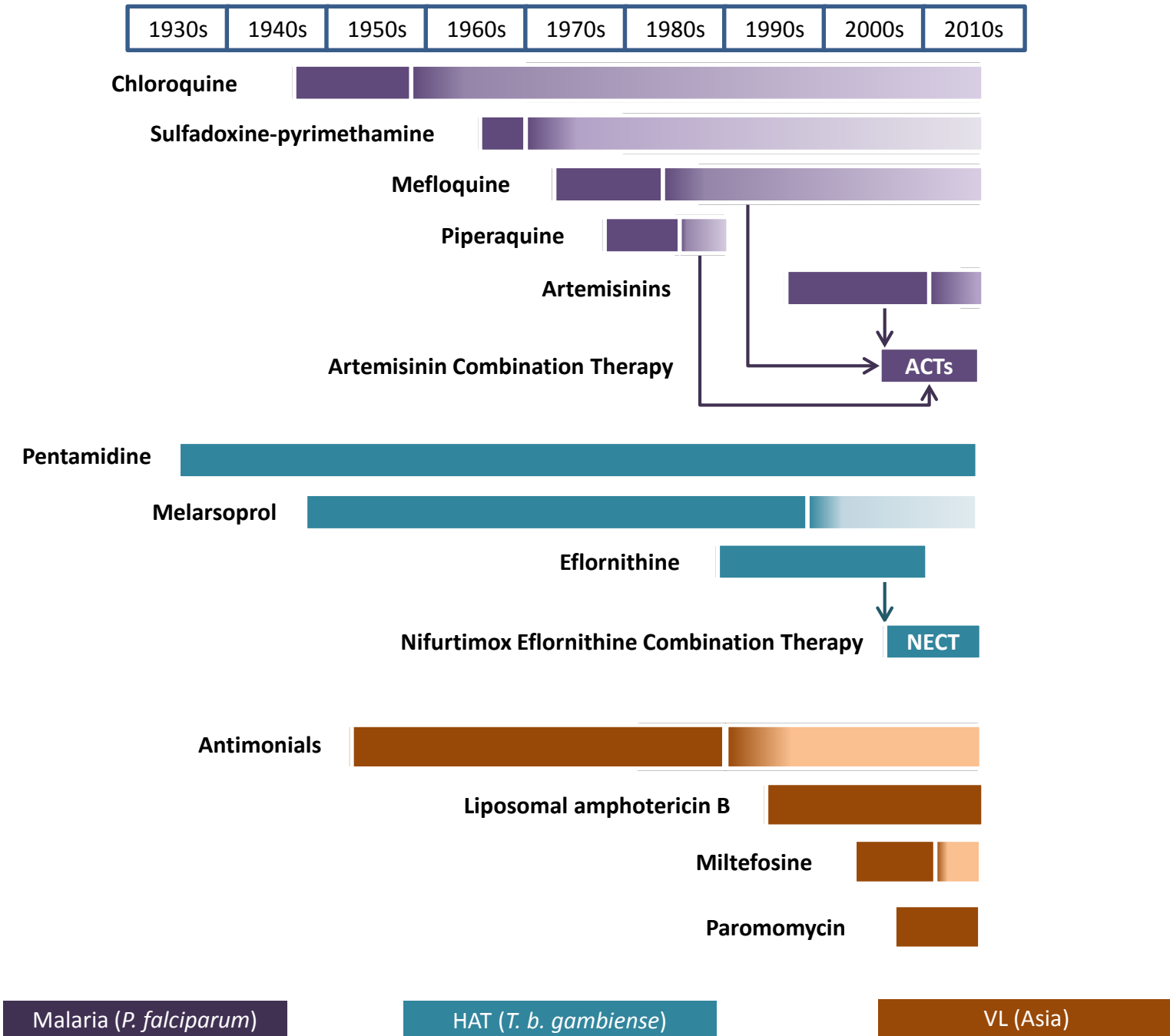
	Paromomycin	<i>Aminoglycoside</i>  Inhibition of protein synthesis	Added to WHO essential medicines list in 2007. No significant clinical resistance. Laboratory-derived resistant lines show decreased drug uptake and increased expression of ribosomal proteins <sup>174</sup> .
	Miltefosine  2012 (Indian subcontinent)	<i>Alkylphosphocholine</i>  Miltefosine significantly perturbs lipid metabolism <sup>175-177</sup> , but the targets and precise mechanism of action are not fully understood <sup>178</sup>	Resistance involves either: loss-of-function mutations or under-expression of an aminophospholipid translocase (LdMT) <sup>179-181</sup> or its regulatory subunit LdRos3 <sup>182</sup> ; or drug efflux by ABC transporters <sup>183,184</sup> . Laboratory-generated resistant lines show alterations in lipid metabolism and gene expression <sup>85,185</sup> , but WGS in another study identified mutations only in the miltefosine transporter, pyridoxal kinase and an $\alpha$ -adaptin-like protein <sup>176</sup> .
	Amphotericin B (deoxycholate or liposomal formulation)	<i>Polyene macrolide antibiotics</i>  See below	No significant clinical resistance reported
<b>Fungi</b>	Amphotericin B, amphotericin deoxycholate	<i>Polyene macrolide antibiotics;</i>  Binds ergosterol more avidly than human cholesterol disrupting the semipermeable membrane causing leakage of essential metabolites and the collapse of electrochemical gradients. Binding of low density lipoprotein receptors and amphotericin-mediated oxidative damage may also contribute.	Laboratory mutants with lower ergosterol content are less sensitive to amphotericin B, but are rare clinically. <i>Aspergillus terreus</i> is intrinsically less amphotericin sensitive but resistant strains have a normal ergosterol content suggesting that membrane permeability may not be the only mechanism of amphotericin action <sup>186</sup> . Binding to ergosterol might contribute to its mode of action <sup>187</sup> .

<p>Fluconazole, Itraconazole, Voriconazole, Posaconazole, Ravuconazole, Isavuconazole</p>	<p><i>Azoles;</i></p> <p>Bind haem-groups and inhibit the P450-mediated 14<math>\alpha</math>-demethylation (Erg11p or Cyp51p) of lanosterol in the ergosterol biosynthetic pathway. Leads to impaired membrane permeability, membrane protein action and cell wall synthesis<sup>188</sup>.</p>	<p>Resistance involves the overexpression of drug efflux pumps and point mutations in the target <i>ERG11 / CYP51A</i> gene product, along with promoter mutations in these genes<sup>189-191</sup>. Changes in the levels of three main efflux pumps Cdr1, Cdr2 and Mdr1 and mutations in the genes encoding the Tac1, Upc2, Pdr1 and Mrr1 transcription factors required for efflux pump upregulation, represent major causes of decreased drug sensitivity<sup>192,193</sup>. This type of azole resistance can be exacerbated by isochromosome formation and aneuploidy which can increase the copy number of key resistance genes such as <i>ERG11</i> and <i>TAC1</i><sup>194-196</sup>.</p> <p>Interference with RNA polymerase II interacting Mediator-complex can re-sensitize Pdr1 dependent regulation of drug efflux pumps<sup>197</sup></p> <p>Chaperone Hsp90 can mitigate against stress induced damage<sup>198</sup> and also contribute to multidrug resistance with Echinocandins.</p> <p>TR34/L98H and the more recently identified TR46/Y121F/T289A alleles that confer clinical azole resistance are likely to have arisen from environmentally generated mutations</p>
<p>Caspofungin, Micafungin, Anidulafungin, Cd101 (formerly biofungin)</p>	<p><i>Echinocandins;</i></p> <p>Cyclic hexapeptides with an antifungal bioactive lipid side chain that binds the fungal specific <math>\beta</math>-1,3-glucan synthase Fks cell membrane proteins, disrupting cell wall integrity.</p>	<p>Resistance through point mutations in two major hotspots in the <math>\beta</math>-1,3 glucan synthase genes <i>FKS1</i> - and, in <i>C. glabrata</i>, <i>FKS2</i><sup>86,101,199</sup>, these reducing drug binding<sup>57,181,182</sup>.</p> <p>Upregulation of cell wall chitin can protect cell wall damage<sup>184-186</sup>. Hsp90 chaperone can mitigate against stress induced damage<sup>170</sup></p>
<p>Flucytosine (5-fluorocytosine)</p>	<p><i>Fluoropyrimidines;</i> converted to 5-fluorouracil</p>	<p>Resistance results from mutations in the genes encoding cytosine permease transporter, cytosine deaminase, which converts 5-FC to 5-</p>



		by cytosine deaminase which becomes incorporated into RNA resulting in inhibition of DNA synthesis.	fluorouracil or the uracil phosphoribosyl transferase required to convert 5-fluorocytosine into a substrate for nucleic acid synthesis <sup>200</sup> . Their impact is lessened by the use of 5FC in combination therapy.
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# A



# B



