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Drug Resistance in Eukaryotic Microorganisms

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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 20th 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 public¹.

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 profiles².
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 infection³. 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 falsified⁴. 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*⁵, mirroring the situation with antibiotic exposure in
105 veterinary contexts for bacterial infections, where environmental contamination
106 generates significant regulatory concern⁶.

107 A further similarity between bacterial and eukaryotic microbial pathogens is the
108 phenomenon of persister populations⁷. 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 review⁸). 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 communities⁹⁻¹¹. 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 bacteria¹² but is a phenomenon recently
129 characterised in *P. falciparum*¹³. Furthermore, environmental signals may induce
130 persistence such as the nutrient starvation typically encountered by *C. albicans* in
131 biofilms^{9,14}.

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 system¹⁵. 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 pathogens¹⁶. 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 EC₅₀-values without providing the Hill slope
169 of the growth inhibition curve or calculating the EC₉₀ value. It is perfectly possible to
170 obtain a resistant line with an identical EC₅₀ 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^{17,18} to limit the emergence and spread of artemisinin-
193 resistant malaria, and for trypanosomes where nifurtimox/eflornithine combination
194 therapy¹⁹ 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 emergence²⁰. 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 infection²¹. 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 immunosuppression²². 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 efficacy²³.

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 1934²⁴. 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 Africa²⁵. Whilst disputed by
250 some²⁶ the leading candidate for resistance to CQ (CQR) is PfCRT (P. falciparum CQR
251 transporter)²⁷. However, despite reports that PfCRT functions as a chloride channel, a
252 proton pump, an activator of Na⁺/H⁺ exchangers or a cation channel, the physiological
253 function of PfCRT remains unclear²⁸. 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 2008²⁹
262 characterised by a failure to rapidly clear parasites in patients around the Thai-
263 Cambodian border^{30,31}. Resistant parasites were characterized by transcriptomics³²,
264 large scale whole genome sequencing (WGS) of clinical isolates^{33,34} and classical
265 generation of resistant mutants by in vitro culture followed by WGS³⁵. 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^{36,37}. 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 resistance²⁹. 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 resistance²⁹. 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 Asia³⁸.

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)³⁹. Experimental
285 overexpression of PI3K results in enhanced artemisinin resistance and PI3P levels are
286 predictive of resistance to artemisinin³⁹. 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 resistance³². Worryingly, resistance to
289 some of the various ACT regimens (involving lumefantrine and amodiaquine and
290 PfCRT) is becoming evident⁴⁰⁻⁴³. 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*⁴⁴. 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 barrier², 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 problem⁴⁴. 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, trypanothione⁴⁵, to form a cyclic complex known as MeIT⁴⁶. 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 Sudan². 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^{47,48}. 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)⁴⁹, with a recent report indicating that
320 pentamidine binds and inhibits the transporter and is then internalised via
321 endocytosis⁵⁰.

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 phase⁵¹ and benznidazole also eliminates parasitaemia in the indeterminate
331 and chronic phases of the disease^{52,53}. 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-up⁵³. 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^{52,54,55}.

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^{56,57}. Treatment varies according to geographical
346 location, the immune status and other co-morbidities of the patient, and the disease
347 classification⁵⁸.

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 regions⁵⁹. 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 hypothesis⁶⁰. 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*⁶¹, 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*⁶². A retrospective clinico-epidemiological study identified a trend towards
362 increased treatment failure in arsenic exposed patients, but failed to reach statistical
363 significance⁶³.

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^V to Sb^{III} has been reported in

370 resistant leishmania amastigotes⁶⁴ and two candidate “antimony reductases”
371 identified, although genetic^{65,66} and proteomic studies^{67,68} have not identified any
372 changes in either TDR1⁶⁹ or ArsC⁷⁰. The mechanism of uptake of Sb^V is not known,
373 but modulation of expression of aquaglyceroporin 1 (AQP1) affects Sb^{III} susceptibility⁷¹⁻
374 ⁷³. AQP1 copy number and expression levels correlate with susceptibility to Sb^{III} in
375 some, but not all, clinical isolates^{74,75}. However, interpretation of this observation is
376 complicated by the fact that AQP1 is located on chromosome 31, which is frequently
377 trisomic or tetrasomic⁷⁶ in these mosaic aneuploid parasites⁷⁷. Upregulation of
378 trypanothione and ancillary biosynthetic pathways has also been observed in
379 genomic^{65,66} and metabolomic^{78,79} studies. MRPA is responsible for ATP-dependent
380 efflux of Sb^{III} as a thiol conjugate into membrane vesicles⁸⁰ and a homodimeric ABC
381 half-transporter (ABC14) is one possible candidate for efflux across the plasma
382 membrane⁸¹.

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^{82,83}, 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^{84,85}.

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^{86,87}. 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 carcinoma⁸⁸. 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^{89,90} as well as strains with cross
415 resistance to azoles and echinocandins suggesting worrisome multi-drug resistance
416 (MDR) phenotypes in medically important fungi⁹¹. 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 dispersal⁹²⁻⁹⁵. The prevalence of these alleles is increasing in Europe and now
420 in other parts of the world^{90,96,97}. In *Candida* mutants harbouring azole resistance have
421 a fitness deficit⁹⁸; 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 sensitivity⁹⁹. 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^{100,101}. 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 ¹⁰²). 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 identified¹⁰³⁻¹⁰⁶, whilst
505 genome-wide RNAi screens allow the mapping of resistance pathways^{107,108}, and
506 overexpression libraries¹⁰⁹ 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 emergence¹¹⁰. Combined with the improved sensitivity and resolution of
511 metabolomics analysis¹¹¹, 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 transmitted¹¹². 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*¹¹³ or the application of
542 vector control measures such as insecticide impregnated bed nets¹¹⁴, peri-domestic
543 and indoor residual insecticide spraying^{114,115}, tsetse traps¹¹⁶, or improved housing¹¹⁷.
544 Sterile insect release is also a route to limiting the vector population and so restricting
545 disease spread^{118,119}. 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^{120,121}. These social responses can control
548 parasite density or the development of transmission stages¹²²⁻¹²⁴, 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

599

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600

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

				Melarsoprol (i.v.)
American trypanosomiasis	kinetoplastid	Triatomine bugs	<i>Trypanosoma cruzi</i>	Benznidazole Nifurtimox
Leishmaniasis	kinetoplastid	Phlebotomine sandflies	Visceral disease <i>Leishmania donovani</i> <i>L. infantum</i> Mucocutaneous disease <i>L. braziliensis</i> <i>L. panamensis</i> Cutaneous disease, e.g. <i>L. major</i> <i>L. tropica</i> <i>L. mexicana</i> <i>L. amazonensis</i>	Visceral disease: 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) Mucocutaneous: SSG (systemic) Cutaneous: 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

^a Second line treatment options are given in parentheses
Data from WHO ^{58,125-127} and other sources ^{57,128,129}

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

Pathogen	Drug and date of resistance reported	Drug class and Mode of action	Resistance mechanism
Plasmodium	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 ¹³⁰ .	K76T ²⁷ mutation in a Digestive Vacuole-sited, ATP-dependent, 10 transmembrane domain transporter PfCRT (P. falciparum CQR transporter) ²⁷ ; a range of more than 30 different mutations might interact epistatically ¹³¹⁻¹³³ . These stimulate the active efflux of CQ by mutant PfCRT or the passive efflux of diprotonated CQ ¹³³ . 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 ¹³⁴ . 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 ¹³⁵ . 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 ¹³⁶). The mutation N326D confers increased resistance to the antimalarial amodiaquine ⁴⁰
	Mefloquine 1982 (Thailand)	<i>Quinoline-4-methanol</i> Blockade of haemozoin formation and binding to phospholipids	PfMDR1 is associated with mefloquine resistance ¹³⁷ but may also modulate CQR through compensatory mutations that counteract PfCRT mutations that compromise parasite fitness ¹³⁸ .
	Artesunate Dihydroartemisinin Artemether	<i>Sesquiterpene lactone endoperoxides.</i>	Dormancy resulting in an extended ring stage phase of development in the erythrocyte promotes resistance ³⁰⁻³² .

2008 ²⁹ (SE Asia)	Form a carbon-centred free radical or reactive electrophilic intermediate that alkylates a number of malaria proteins ¹³⁹ after activation by haem or free iron.	Multiple independent mutations in a gene encoding a Kelch propeller protein (Kelch 13) confer resistance ³³⁻³⁷ . 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 ³³
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 ¹⁴⁰ . 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 ¹⁴¹ Increased GTP-cyclohydrolase (CNVs) enhances folate biosynthesis compensating for loss of fitness ¹⁴¹
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 ¹⁴² .
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 ¹⁴³
Suramin	<i>Naphthylamine trisulfonic acid</i>	Laboratory-generated resistance mediated through the silencing of invariant surface glycoprotein (ISG75), the AP1 adaptin complex,

African Trypanosomes		Mode of action unknown	lysosomal proteases and major lysosomal transmembrane protein, as well as spermidine and N-acetylglucosamine biosynthesis ¹⁰⁸ .
	Pentamidine	<i>Diamidine</i>	Resistance is associated with loss of uptake on the P2 adenine/adenosine transporter ¹⁴⁴ , (AT1) ¹⁴⁵ .
	Clinical resistance is not significant.	Mode of action unknown	Cross-resistance between melaminophenyl arsenicals and diamidines is mediated by aquaglyceroporin 2 (AQP2) ¹⁴⁶ . A chimeric AQP2/AQP3 gene is associated with cross resistance to melarsoprol and pentamidine in laboratory-generated ^{149,146,147} and clinical isolates ^{148,149}
	Melarsoprol	<i>Trivalent melaminophenyl arsenical.</i>	Resistance is associated with loss of uptake on the P2 adenine/adenosine transporter ^{144,145} . A non-functional mutant has been identified in melarsoprol-resistant field isolates ¹⁵⁰ .
	Treatment failures have been reported in the Democratic Republic of Congo, Uganda, Angola and Sudan ²	Forms a cyclic complex with trypanothione known as MeIT ⁴⁶ . 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 ^{151,152} . 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> ² .	
	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 ¹⁰⁸ . NTR is also the key resistance determinant in laboratory-generated lines ^{156,157} 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) ¹⁵³ to		

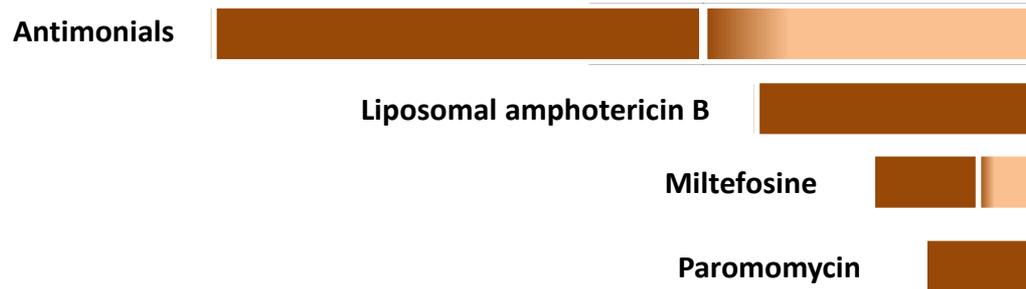
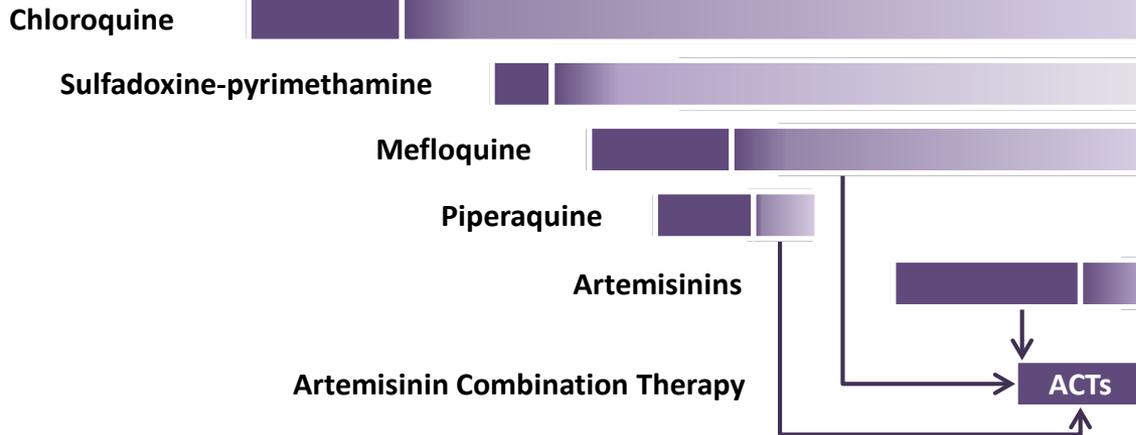
	eflornithine [NECT])	form highly reactive drug metabolites ¹⁵⁴ that kill trypanosomes via unknown mechanisms ¹⁵⁵ .	
South American Trypanosomes	Benznidazole, Nifurtimox (natural resistance in some <i>T. cruzi</i> isolates)	<i>Nitroheterocyclics</i> Benznidazole is activated by mitochondrial NTR ^{153,158} to form electrophilic drug metabolites ^{159,160}	Drug efflux via an ABCG-like transporter ¹⁶¹ The NAD(P)H flavin oxidoreductase (old yellow enzyme) is downregulated in resistant lines ^{162,163} . However, this enzyme does not reduce benznidazole and only reduces nifurtimox under anaerobic conditions ¹⁶⁴ .
Visceral Leishmaniasis	Sodium stibogluconate, Meglumine antimonite 1990s widespread resistance in India and Nepal. Not widespread in Sub-Saharan Africa or Brazil	<i>Pentavalent antimonials</i> Sb ^V is reduced to Sb ^{III} to attack intracellular amastigotes. Likely to bind multiple targets including trypanothione reductase ^{165,166} , tryparedoxin peroxidase ¹⁶⁷ and CCHC Zinc finger proteins ¹⁶⁶ .	Selection for resistance to trivalent arsenic results in cross-resistance to trivalent antimony in vitro ⁶¹ , and in vivo ⁶² . Resistance is multifactorial through several mechanisms: <ul style="list-style-type: none"> • Decreased reduction of Sb^V to Sb^{III} • Sb^{III} is taken up via an aquaglyceroporin⁷³ and modulation of expression of aquaglyceroporin 1 affects Sb^{III} susceptibility⁷¹⁻⁷³. • Elevated Intracellular trypanothione levels¹⁶⁸ or increased biosynthetic potential^{165,66,78,79}. • Increased levels of tryparedoxin peroxidase confer resistance to Sb^{III}¹⁶⁹ and are found in clinical resistant isolates¹⁶⁷ • MRPA (also known as PgpA or ABCC3), a member of the ATP-binding cassette (ABC) transporters, is amplified in some resistant lines¹⁷⁰⁻¹⁷² and sequesters Sb^{III} in an intracellular vacuolar compartment close to the flagellar pocket⁸⁰. • chaperones and stress related proteins are upregulated^{167,68}, potentially reducing or repairing cellular damage induced by antimonials¹⁷³

	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 ¹⁷⁴ .
	Miltefosine 2012 (Indian subcontinent)	<i>Alkylphosphocholine</i> Miltefosine significantly perturbs lipid metabolism ¹⁷⁵⁻¹⁷⁷ , but the targets and precise mechanism of action are not fully understood ¹⁷⁸	Resistance involves either: loss-of-function mutations or under-expression of an aminophospholipid translocase (LdMT) ¹⁷⁹⁻¹⁸¹ or its regulatory subunit LdRos3 ¹⁸² ; or drug efflux by ABC transporters ^{183,184} . Laboratory-generated resistant lines show alterations in lipid metabolism and gene expression ^{85,185} , but WGS in another study identified mutations only in the miltefosine transporter, pyridoxal kinase and an α -adaptin-like protein ¹⁷⁶ .
	Amphotericin B (deoxycholate or liposomal formulation)	<i>Polyene macrolide antibiotics</i> See below	No significant clinical resistance reported
Fungi	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 ¹⁸⁶ . Binding to ergosterol might contribute to its mode of action ¹⁸⁷ .

	<p>Fluconazole, Itraconazole, Voriconazole, Posaconazole, Ravuconazole, Isavuconazole</p>	<p><i>Azoles;</i></p> <p>Bind haem-groups and inhibit the P450-mediated 14α-demethylation (Erg11p or Cyp51p) of lanosterol in the ergosterol biosynthetic pathway. Leads to impaired membrane permeability, membrane protein action and cell wall synthesis¹⁸⁸.</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 genes¹⁸⁹⁻¹⁹¹. 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^{192,193}. 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>¹⁹⁴⁻¹⁹⁶.</p> <p>Interference with RNA polymerase II interacting Mediator-complex can re-sensitize Pdr1 dependent regulation of drug efflux pumps¹⁹⁷</p> <p>Chaperone Hsp90 can mitigate against stress induced damage¹⁹⁸ 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 β-1,3-glucan synthase Fks cell membrane proteins, disrupting cell wall integrity.</p>	<p>Resistance through point mutations in two major hotspots in the β-1,3 glucan synthase genes <i>FKS1</i> - and, in <i>C. glabrata</i>, <i>FKS2</i>^{86,101,199}, these reducing drug binding^{57,181,182}.</p> <p>Upregulation of cell wall chitin can protect cell wall damage¹⁸⁴⁻¹⁸⁶. Hsp90 chaperone can mitigate against stress induced damage¹⁷⁰</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 ²⁰⁰ . Their impact is lessened by the use of 5FC in combination therapy.
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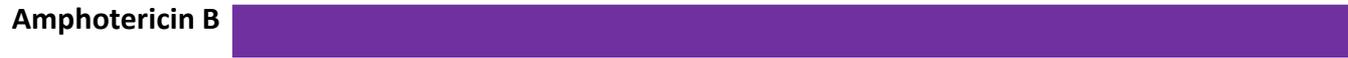


Malaria (*P. falciparum*)

HAT (*T. b. gambiense*)

VL (Asia)

B



Polyenes

Azoles

Echinocandins



