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Drug resistance in protozoan parasites

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As with all other anti-infectives (antibiotics, anti-viral drugs, and anthelminthics), the limited arsenal of anti-protozoal drugs is being depleted by a combination of two factors: increasing drug resistance and the failure to replace old and often shamefully inadequate drugs, including those compromised by (cross)-resistance, through the development of new anti-parasitics. Both factors are equally to blame: a leaking bathtub may have plenty of water if the tap is left open; if not, it will soon be empty. Here, I will reflect on the factors that contribute to the drug resistance emergency that is unfolding around us, specifically resistance in protozoan parasites.

Infections with protozoan pathogens will be with us for the foreseeable future. There is still no effective malaria vaccine despite enormous investment in money and effort over many years. Leishmaniasis is still spreading, including in Southern Europe. At least 100 million women worldwide suffer infection with the sexually transmitted *Trichomonas vaginalis*. Chagas Disease (American trypanosomiasis) affects communities from Texas to Argentina. Sleeping sickness (human African trypanosomiasis) remains a scourge in sub-Saharan Africa. Billions of people are infected with *Toxoplasma gondii*. Then, there are *Cryptosporidium* spp., *Entamoeba histolytica*, and *Giardia* spp. — but you get the idea.

In almost all cases, we rely on treatment (no vaccines) with a few old drugs that would never pass safety evaluation if entered into trials now. Despite the clear clinical need, there is in fact very little serious new drug development going on for these protozoan infections, and what little there is, is driven by private organisations including the Medicines for Malaria Venture (MMV), the Drugs for Neglected Diseases initiative (DNDi), the Wellcome Trust (in part through its support of the Drug Development Unit at the University of Dundee), the Bill and Melinda Gates Foundation [in part through the Consortium for Parasitic Drug Development (CPDD)], and the Institute for OneWorld Health rather than governments or the private sector. These organisations do phenomenally good work with limited resources and have taken multiple new drugs or formulations into clinical use or trials. However, their budgets clearly fall far short of the level of effort that is needed to tackle all these neglected protozoan diseases. The investment discrepancy with welfare diseases such as obesity, atherosclerosis, diabetes, and some forms of cancer does not need further elaboration.

Moreover, while malaria and the kinetoplastid diseases have a champion in MMV and DNDi, respectively, there is no such champion for trichomoniasis, cryptosporidiosis, giardiasis, toxoplasmosis, etc. And that is not mentioning important protozoan animal infections such as nagana (e.g. cattle; *Trypanosoma congolense*, *T. brucei*, and *T. vivax*), surra (e.g. camels, buffalo; *T. evansi*), babesiosis (cattle, dogs; *Babesia bovis*, *B. canis*), dourine (horses; *T. equiperdum*), theileriosis (cattle, sheep, goats; *Theileria annulata*, *Theileria parva*), and leishmaniosis (canines; *Leishmania* spp.), for which usually very few treatment options exist. For the veterinary applications, again, little drug development is taking place, as the problems are mostly those of tropical countries and poor farming communities and herdsmen, and this does not fit the necessarily profit-driven drug discovery model, nor does it translate into easy logistics of delivery and administration.

The inevitable consequence is the use, for decades, of the same old treatments, often inexpertly, predictably giving rise to the drug resistance that follows close behind. So far, so obvious; but is resistance so inevitable that we should just shrug and move on? After a century of chemotherapy, are there any

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lessons learned and steps that can be taken to minimise the impact of (early onset) drug resistance in protozoan disease?

Actually, the best strategy is to avoid over-reliance on chemotherapy in the first place — anything that reduces transmission rates brings the disease burden down. Good sanitation reduces transmission of waterborne parasites such as *Giardia*, *Entamoeba*, and *Cryptosporidium*, just as condoms prevent trichomoniasis. Clearly, effective vaccines would be our best chance of actually eradicating specific diseases, but none are likely to be approved any time soon. While a commercial vaccine for canine leishmaniasis is now available, it appears to be more effective in preventing disease progression than infection rates [1], and one study concluded that a simple anti-flea collar on the dog prevented infection much more effectively [2]. Similarly, insecticide-treated bed nets have saved many people from malaria [3]; housing improvements, insecticide and the screening of blood donors greatly reduced transmission of Chagas disease [4]; and mathematical modelling shows that spraying cattle with insecticide is much more effective against tsetse-transmitted trypanosomiasis than chemotherapy [5], especially when combined with scent-baited tsetse traps [6] or insecticide-treated tsetse 'targets' that can be as small as 0.06 m^2 to be effective [7]. One form of tsetse control that has attracted much attention is the use of sterile insect technology (SIT), which eliminated the insects from the island of Zanzibar [8], but this is a very expensive option, and the release of large numbers of (sterile) tsetse risks a dramatic increase in transmission in the short term. Moreover, reinvasion by wild-type flies from adjourning areas would be impossible to prevent on much of the African continent. An example of an alternative to vector control is the use of trypanotolerant indigenous cattle, but these breeds usually have much lower food production value than imported ones, and can still carry and transmit the parasite, and may therefore perpetuate the problem by masking it. Indeed, there is a strong case to be made to also treat asymptomatic infections for two main reasons: continued transmission, and the risk that the infection will not remain asymptomatic. For *T. b. gambiense* sleeping sickness, asymptomatic carriers present a serious obstacle to eradication [9]; however, even if they could be identified, the treatment of healthy carriers raises ethical issues, especially with the currently available drugs and their well-documented side effects.

Vector control, repellents and barriers such as bed nets are all important in reducing transmission but more so for some protozoan diseases than others; *Toxoplasma* and *Giardia*, to name just two human parasites, are not transmitted by insect vectors, nor is dourine, which is caused by *T. equiperdum*. Moreover, full eradication by vector/transmission control is at best a long-term prospect, and meanwhile infected patients and animals must be effectively treated. Given that no new treatments will be introduced for most of these infections, we must make the best possible use of the current pharmacopoeia and not lose any to drug resistance. One good strategy is to go all-in with a full eradication programme of active case finding, mass treatment, diverse and aggressive vector control measures and the monitoring of treatment outcomes, with a second-line treatment option for the cases of drug failure – and see it through to the end. This does not allow for resistance to take hold and spread, picking off one valuable (near-irreplaceable) drug at the time. Of course, this requires a very high level of funding and organisation, and if not successfully implemented, the mass treatment programme can lead exactly to early-onset resistance as was arguably the case with chloroquine and malaria. Such an approach is likely to work, but this ideal scenario is probably unrealistic given current realities such as the lack of political leadership, prioritisation and funding for the control of the diseases of the poor.

Is drug resistance really inevitable, or is it possible to develop drugs to which the parasites simply cannot develop resistance? It is worth exploring this question, especially at this time, when confidence in antibiotics is fast disappearing because of rapidly adapting pathogens. However, antibiotics are generally products produced by an organism to defend themselves against (other) bacteria, which means that bacteria have already been exposed to them for periods on an evolutionary scale, achieving an ecological balance. In other words, antibiotic resistance adaptations are as much part of the natural world as are the antibiotics themselves. But, is the situation any different for eukaryotic parasites and the anti-parasitic drugs, which are, with a few exceptions such as tetracycline for malaria, not natural compounds? Not fundamentally, no: protozoan parasites still adapt, and have become resistant to many standard treatments. Common adaptations include: target enzyme mutations reducing interactions with the drug (antifolates in malaria [10]); reduced drug uptake (diminazene aceturate in *T. b. brucei* [11] or antimonials in *L. donovani* [12]); up-regulation of a metabolic bypass (methotrexate in *Leishmania* [13]); failing to activate a prodrug (nifurtimox in *T. cruzi* [14]); increased drug efflux (chloroquine in malaria [15]); and even the failure to produce the target (amphotericin B in *Leishmania* [16]). The genetic mechanisms of resistance include gene deletions [17], point mutations in targets [10] or transporters (enabling [15] or disabling [18]), copy number variations [19,20], base pair insertions/deletions causing frame shifts in the target gene [12] and the formation of chimeric genes through recombination [21].

Despite the plethora of mechanisms by which protozoa can acquire drug resistance, for some drugs this happens much faster than for others. In many cases, a single mutation or gene deletion is sufficient for a loss of sensitivity, sometimes followed by secondary mutations to give higher levels of resistance [10,18]. Whereas a target protein is of course usually essential, active-site mutations that selectively lose affinity for an inhibitor, yet retain sufficient functionality, are certainly possible. In the case of a transporter mutation, the transporter is usually not essential, because of redundancy [22] or its substrate is non-essential to the cell [23], and in such cases the mutation can be disabling to the transport of both the physiological substrate and the drug [17,18,21]. In other cases, however, resistance does not come about so easily. The drug may not have a single protein target that can be mutated but may bind instead to DNA as intercalator or minor groove binder (e.g. phenanthridines, diamidines [24–26]), or e.g. to haem (chloroquine [27]), or be more generally cytotoxic, with multiple targets (polypharmacology), as may be the case for heavy metal drugs (arsenicals, antimonials) and suramin [28], in which case selectivity depends mostly on selective entry into the parasite rather than into the host cells, through unique transporters or other uptake mechanisms [20,28,29].

In some such cases, resistance still occurs, through mutation or deletion of these transporters [20,29], but in the case of pentamidine, for instance, clinical resistance for sleeping sickness has not been reported despite being virtually the only drug used against early-stage *T. b. gambiense* sleeping sickness since the mid-1930s, including population-scale mass treatment campaigns [30], although various levels of resistance can be induced *in vitro* [31]. The lack (of reports) of clinical resistance, in this case, can be traced to the fact that pentamidine is taken up by three different transport mechanisms, at least two of which, the aminopurine transporter AT1 and the High Affinity Pentamidine Transporter, HAPT1 (now identified as an aquaglyceroporin, TbAQP2 [21]), are highly efficient, allowing a fast accumulation of the drug [32]; moreover, the drug action becomes irreversible after only a brief exposure [33], giving little scope for resistance to develop. Recently, AQP2-mutated *T. b. gambiense* strains have been isolated from sleeping sickness patients in the Democratic Republic of Congo and from South Sudan, who relapsed after melarsoprol treatment; these isolates were verified to be significantly resistant to both melarsoprol and pentamidine *in vitro* [34]. However, it is of note that the relapses were after treatment with melarsoprol (which is also a substrate of both AT1 and AQP2 [21]), not pentamidine. As explained by Graf et al. [34], the pharmacokinetics of pentamidine (but not of melarsoprol), especially the high peak plasma concentration and long clearance time, may prevent treatment failure even though the rate by which the drug enters the parasite is reduced dramatically, leading to a significant *in vitro* resistance phenotype. Similarly, there have been no reports of resistance to suramin, still in use for *T. b. rhodesiense* sleeping sickness after 100 years, and this is believed to be linked to the drug being internalised by endocytosis, which is uniquely fast in bloodstream trypanosomes, after binding to the invariant surface glycoprotein ISG75, of which there are multiple paralogues [28].

Interestingly, the known protozoan 'drug transporters' are mostly members of highly conserved gene families, and often involved in nutrient uptake or other household functions. For example, in *Leishmania* the miltefosine transporter is a phospholipid translocase [35] and methotrexate is taken up by a folate transporter [13]; the *T. brucei* carrier for eflornithine is an amino acid transporter [17], while pentamidine and melarsoprol are taken up by an aminopurine transporter and an aquaglyceroporin [18,21]. Thus, it is often hard to predict what might be a (potential) drug transporter in a protozoan: they are hidden in plain sight, masked by their innocuous roles. Conversely, creative use of the uniqueness of protozoan transporters from conserved families can be exploited for the selective targeting of therapeutic agents [36,37].

Thus, we see that polypharmacology, combined with an uptake mechanism that is not easily disabled by mutations in a single Open Reading Frame (ORF), can prolong a drug's lifespan to (most of) a century. This point can be illustrated with our work on curcumin and a series of analogues that display *in vitro* activity against *T. brucei* species, with an EC_{50} value of 53 nM for the most potent analogue, AS-HK014, compared with 2.7 μM for curcumin itself [38]. We were unable to induce any level of resistance against curcumin *in vitro*, although we easily induced a 50-fold level of resistance to the analogue, which brought it to the exact same level as curcumin, which we could not then surpass [39]. Curcumin is believed to diffuse across the membrane and indeed act *on* the membrane [40], and we certainly were unable to measure any saturable uptake using ^{3}H-curcumin (unpublished data). The failure to induce curcumin resistance is consistent with the current model that attributes the many biological actions of curcumin to polypharmacology [41] and a capacity to interact with membrane proteins [40]. In contrast, the activity of analogue AS-HK014 was well defined (reaction with cellular thiols leading to depletion of trypanothione and glutathione [39]), and resistance developed rapidly.

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A more important example, of course, is the essential antimalarial drug artemisinin; it is estimated that widespread resistance to this drug, as is now spreading in South-East Asia [42], will result in well over 100 000 additional malaria deaths and US$385M/year in additional loss of economic activity [43]. As artemisinin appears to act, when activated by haem, through highly promiscuous covalent binding to many cellular targets in the parasite [44], resistance through target mutations/gene deletion would appear impossible, and no transporter mutations have been found. Indeed, as noted by Wang et al. [45] no *Plasmodium falciparum* strain has so far been found to be insensitive to artemisinin in a standard *in vitro* protocol. Yet treatment failure with artemisinin combination therapy (ACT) is undoubtedly real, and associated with mutations in a single gene, *kelch13* (K13) [42,46], which is neither an artemisinin target nor a transporter [45]. However, drug resistance is not an all-or-nothing proposition, and the answer to the Sphinx's riddle is that the parasites remain sensitive but that the parasite clearance time is significantly increased in the adapted strains [42]. The K13 mutations have the effect of shortening the duration of the trophozoite stage, during which the parasite is particularly susceptible to artemisinin, and lengthening the duration of the ring stage that is relatively insensitive due its low haem content [47]. This adaptation of intra-erythrocyte development is yet another unimagined way by which parasites can develop clinical resistance, but the clinical resistance would probably not have taken hold if the ACT partner drugs had not suffered from resistance first. It is the function of these partner drugs to 'mop up' the parasites that survive the relatively short exposure to artemisinin, caused by the rapid clearance of the drug.

We thus see that combination chemotherapy as in ACT, or nifurtimox/eflornithine in late-stage sleeping sickness, can keep resistance effectively at bay, unless the parasite can either develop transmissible resistance to one of the component drugs separately (which is not true combination therapy) or if resistance to one of the drugs in the combination already exists in the pathogen population. In other words, it may be a really counterproductive idea to introduce a combination in order to try to 'salvage' a drug *after* resistance has been reported, because that may lead to the functional loss of both components in the combined formulation. This is not a trivial concern, as we truly cannot lose any of the main anti-protozoal drugs, and should feature in current discussions about the potential introduction of combinations against visceral leishmaniasis. Resistance against all the major anti-leishmanial drugs has been reported [16,48,49] and, alarmingly, resistance against several combinations can be induced readily *in vitro* [50].

In conclusion, we see that it is extremely difficult to prevent the onset, or spread of drug resistance and that, therefore, the reality is that we are and will continue to lose life-saving treatments. While more can be done on the side of prevention, vector control, sanitation, etc., the only way to prevent a catastrophic inability to treat protozoan disease is by investing in new treatments. For that, genuine, long-term partners with very deep pockets are eagerly sought.

Abbreviations

ACT, artemisinin combination therapy; DNDi, Drugs for Neglected Diseases initiative; MMV, Medicines for Malaria Venture.

Competing Interests

The Author declares that there are no competing interests associated with this manuscript.

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