

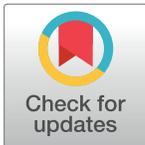
PEARLS

Joining forces: Leveraging novel combination therapies to combat infections with eukaryotic pathogens

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Introduction

Rising drug resistance, in pathogenic parasites and fungi, has become a major public health threat (reviewed in [1]). In endemic areas, substantial morbidity and mortality are caused by drug-resistant (DR) species. Resistant pathogens may also complicate treatments for noncommunicable conditions such as cancer chemotherapy and organ transplantation. **The problem of drug-resistance is heightened by a lack of investment in novel antibiotic drug discovery by the pharmaceutical industry, in part, because there is a low rate of return for such drugs, compared with those used to treat chronic illnesses.** While the development of new antimicrobial drugs is one approach in countering the DR phenotype, the **discovery of useful drug combinations** represents an alternative tactic that may expedite alternate clinical treatments.

Using 2 or more drugs simultaneously **does not guarantee** that efficacy will exceed single-agent treatments. **Pairwise drug interactions can be classified as either antagonistic, additive, or synergistic.** Antagonism occurs when 2 drugs inhibit the therapeutic effects of each other. Additivism occurs when 2 drugs cause the combined predicted effect for both individual drugs. **Synergism occurs when the combination of drugs exceeds** the predicted effect of the combination [2]. **Additionally, an inert agent, without an individual effect, can potentiate the action of a second drug** [3]. Combination treatment regimens are often determined empirically but are **based on a mathematical understanding** of pairwise relationships, which is often calculated through isobologram, combination index, and/or curve shift analyses (Fig 1). In designing combination therapies, **synergism is an important goal as it has the highest potential to lower off-target effects by decreasing doses (synergistic potency) and to improve clinical outcome by increasing desired effect (synergistic efficacy)** [4].

Many combination therapies are used clinically to treat infections caused by eukaryotic pathogens, including giardiasis, cryptococcal meningitis, malaria, and trypanosomiasis [5,6]. In particular, **combinations of either 2 artemisinin-derived compounds or atovaquone with proguanil** have been instrumental in combatting DR malaria strains in endemic areas [7]. Furthermore, the trypanosomiasis combination therapy, elflornithine and nifurtimox, required only 8 years of development to reach clinical approval, which demonstrates the potential for rapid treatment development from combining drugs [8]. In light of this, the purpose of this article is to highlight advances (≤ 5 years) in identifying new combination therapies for DR parasites and fungi. **Table 1** summarizes the combinations discussed and includes information about whether the combinations were found to be additive (+) or synergistic (*) and whether the combinations were assessed *in vitro* (^a), *in vivo* in animal models (^b), or in human patients (^c).

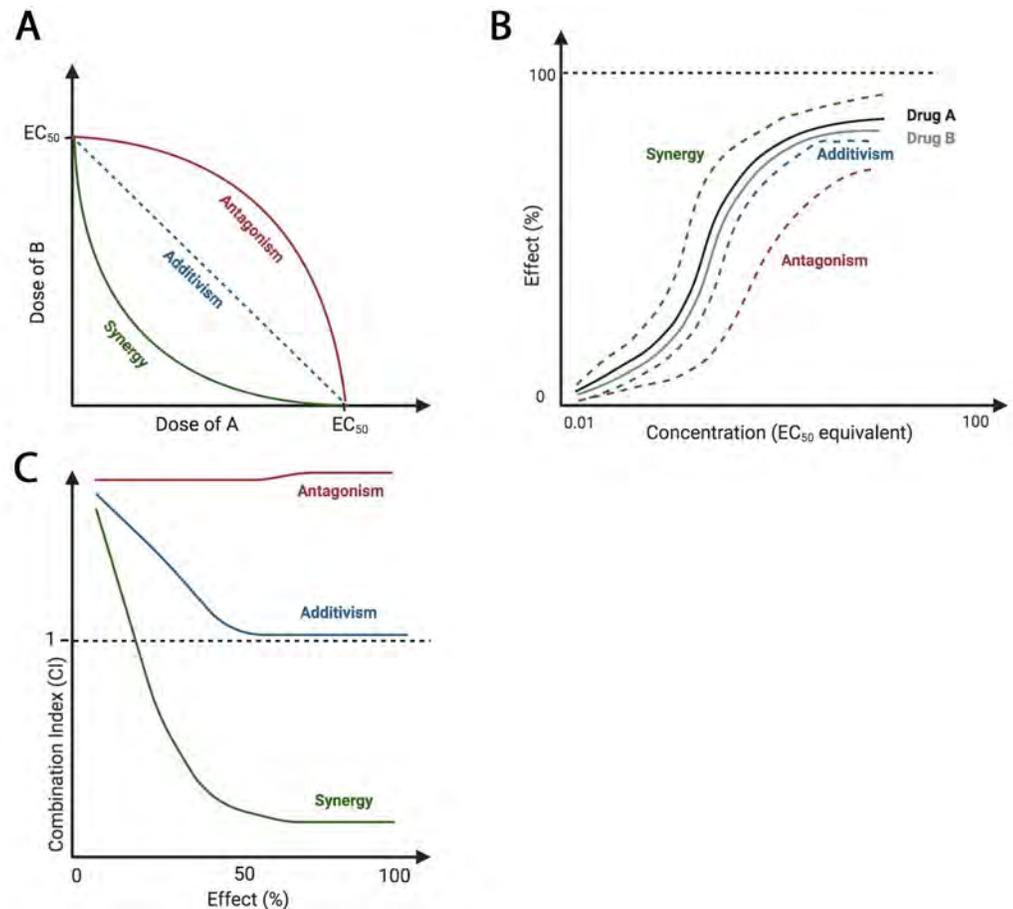


Fig 1. Theoretical isobologram, curve shift, and combination index analyses of drug–drug interactions. Antagonistic relationships require increased dosage of drugs for the same individual effects, additive effects require the same dosage of drugs for the same individual effects, and synergistic interactions require decreased dosage of drugs for the same individual effects. (A) Isoboles connect the points of EC_{50} of each drug, A and B. If the drugs are synergistic, the EC_{50} of agent A in the presence of B should be lower than that of agent A alone. (B) Curve shift analysis reveals a leftward shift, indicating synergy, or a rightward shift, indicating antagonism. This corresponds to changes of EC_{50} . (C) CI values less than, equal to, or greater than 1 indicate synergy, additivism, or antagonism, respectively. Images created with BioRender.com. CI, combination index; EC_{50} , half maximal effective concentration.

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Because the combinations reviewed here have been newly discovered, the majority have not yet been tested clinically.

Conventional antimicrobial and antifungal agents—Better together

Combining conventional drugs that target eukaryotic pathogens has resulted in improved inhibition of pathogen growth. Furthermore, some antimicrobials that target prokaryotes have secondary antiparasitic targets and potentiate the action of standard antiprotozoal drugs. For example, neuroinvasive *Toxoplasma gondii* was successfully countered with a combination of clindamycin and azithromycin, both of which inhibit protein synthesis in bacteria. Although the antiparasitic mechanism is unclear, clindamycin effectively crosses the blood–brain barrier and accumulates in tissues with high parasitic loads [9].

Diminazene aceturate is a DNA-binding drug commonly used to treat canine babesiosis [10], but it has not been widely used in humans due to its narrow therapeutic window.

Table 1. Additive and synergistic drug combination strategies assessed against multiple eukaryotic pathogens.

	Combination 1	Combination 2	Combination 3	Combination 4	Combination 5
EXTRACELLULAR PATHOGENS					
Amoebozoa					
<i>Acanthamoeba castellanii</i>	+Simvastatin, voriconazole ^a [23]	-	-	-	-
<i>Blastocystis</i> sp.	*Atorvastatin, metronidazole ^b [26]	-	-	-	-
<i>Entamoeba histolytica</i>	*Simvastatin, lonafarnib ^a [30]	*Lonafarnib, metronidazole ^a [30]	-	-	-
<i>Naegleria fowleri</i>	*Auranofin, amphotericin B ^a [53]	-	-	-	-
Diplomonads					
<i>Giardia intestinalis</i>	*Paromomycin, bacitracin ^c [5]	*Nitroimidazoles, albendazole ^c [16–18]	*Nitroimidazoles, paromomycin ^c [16–18]	*Nitroimidazoles, mepacrine ^c [16–18]	-
Fungi					
<i>Aspergillus fumigatus</i>	+Posaconazole, clofazamine ^a [15]	+Caspofungin, clofazamine ^a [15]	-	-	-
<i>Candida albicans</i>	+Fluconazole, clofazamine ^a [15]	+Caspofungin, clofazamine ^b [15]	*Amiodarone, caspofungin ^a [15]	*Itraconazole, <i>Mentha × peperita</i> extracts ^a [40]	-
<i>Cryptococcus neoformans</i>	+Fluconazole, amphotericin B ^b [19]	*Amphotericin B, <i>Ocimum basilicum</i> extracts ^a [42]	*Fluconazole, tomatidine ^a [15]	*Itraconazole, <i>Mentha × peperita</i> extracts ^a [40]	-
<i>Fonsecaea monophora</i>	*Terbinafine, amphotericin B ^a [20]	-	-	-	-
Helminths					
<i>Echinococcus granulosus</i>	+Albendazole, 5-fluorouracil ^b [36]	+Albendazole, <i>Zataria microflora</i> extracts ^b [41]	+Albendazole, metformin ^b [52]	+Albendazole, <i>Sophora moorcroftiana</i> alkaloids ^b [48]	-
Trypanosomes					
<i>Trypanosoma brucei</i>	+Temozolomide, elflornithine ^a [8]	+Temozolomide, melarsoprol ^a [8]	-	-	-
<i>Trypanosoma congolense</i>	* <i>Anogeissus leiocarpus</i> , <i>Khaya senegalensis</i> extracts, potash ^b [47]	-	-	-	-
INTRACELLULAR PATHOGENS					
Apicomplexans					
<i>Babesia</i> sp.	+Diminazene aceturate, clofazamine ^b [11]	*Atovaquone, eflornithine ^b [32]	+Diminazene aceturate, synthetic chalcones ^b [38]	*Atovaquone, hydroxyurea ^a [32]	-
<i>Plasmodium falciparum</i>	*B02, artemisinin ^a [34]	+Prochlorperazine, chloroquine ^a [60]	-	-	-
<i>Plasmodium vivax</i>	+Chlorpheniramine, mefloquine ^b [59]	-	-	-	-
<i>Toxoplasma gondii</i>	+Clindamycin, azithromycin ^c [9]	*Ketotifen, pyrimethamine ^b [51]	*Simvastatin, pyrimethamine ^a [27]	*Atorvastatin, C7S ^b [25]	-
Trypanosomes					
<i>Leishmania amazonensis</i>	+Tamoxifen, amphotericin B ^b [37]	-	-	-	-
<i>Leishmania donovani</i>	+Lovastatin, chromium chloride ^a [28]	-	-	-	-
<i>Leishmania infantum</i>	*Nelfinavir, amphotericin B ^b [49]	+Lopinavir, miltefosine ^b [50]	*Sorafenib, auranofin, lopinivir ^a [62]	-	-
<i>Leishmania major</i>	+Paromomycin, 4-aminoquinoline ^b [14]	* <i>Moringa oleifera</i> extracts, amphotericin B ^a [43]	-	-	-
<i>Leishmania martiniquensis</i>	*Allicin, amphotericin B ^a [44]	-	-	-	-

(Continued)

Table 1. (Continued)

	Combination 1	Combination 2	Combination 3	Combination 4	Combination 5
<i>Leishmania mexicana</i>	⁺ Paromomycin, 4-aminoquinoline ^b [14]	-	-	-	-
<i>Trypanosoma cruzi</i>	[*] Clofazamine, benznidazole ^a [12]	⁺ <i>Chlamydomonas reinhardtii</i> extracts, nifurtimox ^a [45]	[*] Clomipramine, benznidazole ^b [58]	⁺ Amiodarone, benznidazole ^a [54]	[*] <i>Lippia alba</i> limonene and citral ^a [46]

Combinations marked with (+) are additive, and combinations marked with (*) are synergistic.

^a Assessed *in vitro*.

^b Assessed *in vivo* in animal models.

^c Assessed *in vivo* in human patients.

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However, combination therapy, using diminizene acetate and clofazimine, an antimycobacterial drug, lowered the dose of diminizene acetate that produced the same *in vivo* outcome as higher doses of diminizene acetate in a mouse model of *Babesia* infection [11]. Both drugs bind to regions of DNA that possess a high content of adenine and thymine residues (AT-rich). AT-rich DNA is common in *Babesia* sp., and when constrained by the drugs, this DNA cannot be replicated, causing cell cycle arrest. Because clofazimine can lower the effective dose of diminizene acetate, it may provide a larger therapeutic window for treating human babesiosis. Clofazimine also inhibits trypanosomal proline transporters and enhances retention of benznidazole, commonly used to treat *Trypanosoma cruzi* infections [12].

Paromomycin is the conventional treatment for cutaneous leishmaniasis. 4-Aminoquinoline chloroquine is often used in combination with doxycycline to treat Q fever, which is caused by intracellular infections with the bacterium, *Coxiella burnetii* [13]. Together, paromomycin and 4-aminoquinoline synergized in the treatment against *Leishmania major* and *L. mexicana* [14]. Both intracellular *Leishmania* amastigotes and *C. burnetii* bacteria induce acidification of macrophages, which decreases the therapeutic activity of conventional drugs. Therefore, alkalization of macrophages, by 4-aminoquinoline chloroquine, may have enhanced the cytotoxic mechanisms of doxycycline and paromomycin. Interestingly, clofazimine can also potentiate many antifungal drugs, including fluconazole, posaconazole, and caspofungin, to inhibit *Candida* sp. and *Aspergillus fumigatus* [15]. Clofazimine elicits cell membrane stress and activates protein kinase C-like 1 (Pkc1) signaling, which is also activated by fluconazole and caspofungin in *Candida albicans* [15]. Similarly, posaconazole and caspofungin each synergized with clofazimine to decrease the hyphal growth of *A. fumigatus* [15].

Combinations with 2 conventional antimicrobial agents have also been used to treat *Giardia intestinalis* infections. Successful combinations include paromomycin with bacitracin zinc, and nitroimidazole-derived drugs with either paromomycin, albendazole, or mepacrine. These combinations have been successful in human patients according to clinical reports and are extensively reviewed elsewhere [16–18]. However, these treatments have not yet been evaluated in formal clinical trials.

Combining multiple antifungals has also proven effective against many pathogens. Amphotericin B is used as a conventional treatment for several fungal infections. This drug interferes with ion transport and membrane polarization, leading to high host toxicity. Additionally, amphotericin B interferes with membrane permeability by binding to ergosterol, a pathogen-specific lipid that regulates outer membrane integrity. Fluconazole, a broad-spectrum antifungal, inhibits ergosterol synthesis; this drug synergized with amphotericin B by preventing the replacement of amphotericin B-bound ergosterol in *Cryptococcus neoformans* membranes [19]. Terbinafine, the conventional drug treatment for chromoblastomycosis, causes

accumulation of an ergosterol intermediate that increases membrane permeability. Amphotericin B and terbinafine synergized through rapid membrane alterations, leading to enhanced lysis of melanized *Fonsecaea monophora*, one of the causes of chromoblastomycosis [20].

Statins—Harnessing pleiotropic effects

Statins, such as simvastatin, atorvastatin, and lovastatin, are a class of drugs that inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase (HMGR) activity and are routinely used to treat hypercholesterolemia. HMGR catalyzes the biosynthesis of many sterols through the conversion of HMG-CoA into 1-mevalonate, a sterol precursor [21,22]. 1-Mevalonate also functions as a biochemical precursor of isoprenoid groups, which serve as posttranslational prenylations on many proteins. Statins are versatile because they have large therapeutic windows. Many parasites scavenge sterols from the host in addition to synthesizing sterols themselves. Therefore, combining multiple compounds that limit sterol synthesis in either pathogen or host is predicted to be synergistic.

Combining simvastatin with the antifungal drug, voriconazole, was effective in targeting 2 different biochemical steps in *Acanthamoeba* ergosterol synthesis [23]. Simvastatin blocked amebic HMG-CoA, while the antifungal drug voriconazole blocked 14 α -demethylase, an enzyme responsible for assembling ergosterols [24]. In a mouse model of *T. gondii* infection, strong synergism was observed between atorvastatin and C7S, a bisphosphonate that also functions as an inhibitor of parasitic isoprenoid synthesis [25].

Statins have been shown to be particularly useful against intracellular parasites because the drugs can alter host cell membranes and modulate host immunity. Atorvastatin enhances host cellular membrane integrity during *Blastocystis* infection, preventing protozoan invasion and leaving parasites vulnerable to metronidazole [26]. Similarly, simvastatin decreased *T. gondii* tachyzoite adhesion and invasion, allowing a conventional anti-toxoplasmosis drug, pyrimethamine, to kill exposed parasites in the bloodstream [27]. Statins may enhance oxidative stress, part of the host immune response. For example, combining lovastatin with the antileishmanial, chromium chloride, produced 2 varieties of reactive oxygen species in parasitic cells, which induced apoptosis in *Leishmania donovani* amastigotes [28].

Statins can pleiotropically inhibit protein prenylation in a number of conserved protein families, including those in the Ras superfamily of G-proteins. Farnesyltransferase is an essential enzyme that catalyzes the posttranslational attachment of farnesyl groups on proteins. Combination therapy with simvastatin and lonafarnib, an antineoplastic that inhibits farnesyltransferase [29], was synergistic against *Entamoeba histolytica* [30]. This finding was unanticipated because genome data suggest that *E. histolytica* lacks HMGR. Thus, the therapeutic efficacy of simvastatin may be due to pleiotropic effects in the isoprenoid biosynthesis pathway. Combination therapy with the standard antiprotozoal, metronidazole, and lonafarnib was also synergistic against *E. histolytica* [30]. This outcome may have been due to lonafarnib-induced membrane destabilization, caused by impaired protein prenylation, which, in turn, intensified oxidative damage caused by metronidazole.

Antineoplastics—DNA damage and membrane transport disruption

Many antineoplastic drugs inhibit unrestrained tumor cell proliferation by targeting DNA or DNA repair. Such drugs may potentiate conventional antimicrobials that function by damaging DNA. The antiparasitic drug, atovaquone, which induces double-stranded DNA breaks [31], synergized with either hydroxyurea or eflornithine, 2 antineoplastic agents, to block multiplication and differentiation of *Babesia* parasites [32]. *Trypanosoma brucei* growth was inhibited by combining the antineoplastic agent, temozolomide, with either eflornithine or

melarsoprol, both standard medications used for treatment of sleeping sickness. Temozolomide, developed for glioblastoma, is a DNA-damaging agent that crosses the blood–brain barrier. Thus, it is particularly advantageous for cases with brain migration of trypanosomes [8]. Temozolomide was shown to trigger trypanosomal cell cycle arrest through DNA damage, limiting proliferation and increasing parasitic susceptibility to standard treatments. Similarly, B02, an inhibitor of DNA repair [33], synergistically enhanced the DNA damaging activity of artemisinin and chloroquine in *Plasmodium falciparum* [34]. *Echinococcus* cell division was limited by the DNA damaging antiparasitic drug, albendazole [35], and the antineoplastic, 5-fluorouracil, a nucleoside analog that is incorporated into DNA and produces toxic metabolites [36]. Tamoxifen, commonly used to inhibit human estrogen receptors, increases membrane depolarization in *Leishmania amazonensis* and may also depolarize mitochondrial membranes, leading to synergy with amphotericin B [37].

Natural compounds—Expanding the toolbox of traditional medicine

Combination methodology is commonplace within traditional medicine because raw plant material contains bioactive compounds with unknown mechanisms. **Chalcones** are flavonoids that are plant-derived phenolic compounds. Synthetic chalcones inhibit glucose metabolism of *Babesia* and reduce the toxicity of diminazene aceturate through an unknown mechanism [38]. **Tomatidine, an alkaloid originating from tomato** (*Solanum lycopersicum*) that is conventionally used to inhibit skeletal muscle atrophy, **synergized with fluconazole to reduce the growth of *C. albicans*** [15]. This alkaloid inhibits sterol methyltransferase and sterol reductase, members of the ergosterol synthesis pathway in several fungal species [39]. Similarly, peppermint (*Mentha × peperita*) phenolic extracts inhibit ergosterol synthesis and increase intracellular acidification by blocking plasma membrane ATPases; these extracts synergized with itraconazole against *C. albicans* and *C. neoformans* [40]. Albendazole activates macrophages and impairs motility of *Echinococcus* cells, and both activities can be improved with *Zataria multiflora* phenolic extracts. *Z. multiflora* extracts increased membrane permeability and altered parasite motility [41].

Additionally, amphotericin B synergizes with many phenolic plant extracts to inhibit the growth of several pathogens. Two models have been proposed for phenolic extract synergy with amphotericin B: enhanced lipophilic transport that increases drug permeation and inhibition of outer membrane ergosterol exchange with intercellular membranes. For example, phenolic extracts from basil (*Ocimum basilicum*) synergized with amphotericin B to inhibit the growth of *C. neoformans* through increased lipophilic transport of drug [42]. Similarly, *Moringa oleifera*, a medicinal plant, also produced phenols that increased lipophilic transport of amphotericin B into *L. major* parasites [43]. The garlic-derived compound, allicin, inhibited outer membrane ergosterol exchange with vacuole membranes and increased the rate of amphotericin B interference with membrane ergosterol in *Leishmania martiniquensis* [44]. Phenolic compounds may also bind intracellular proteins and increase oxidative stress in *L. martiniquensis*, enhancing the antiparasitic activity of amphotericin B [44].

Other non-phenolic–derived compounds can enhance standard antimicrobial treatments. Microalgae extracts from *Chlamydomonas reinhardtii* inhibited intracellular invasion of *T. cruzi*, potentiating the action of nifurtimox, a conventional treatment only active against bloodstream trypomastigotes [45]. Alternatively, natural compounds may produce synergy through immune system stimulation and protection from drug-induced host toxicity. Benzimidazole induces oxidative stress that damages both *T. cruzi* and host cells; however, supplementation with *Lippia alba*–derived terpenes inhibited host inflammation and may have induced additional parasite-specific oxidative stress [46]. *Trypanosoma congolense*, the major agent of

cattle trypanosomiasis that also serves as a model organism for human *T. brucei* infection, exhibited impaired motility in a mouse model of infection caused by a combination of extracts from *Anogeissus leiocarpus* and *Khaya senegalensis* along with potash salts, resulting in decreased parasitemia [47]. Albendazole treatment of *Echinococcus* infections can also be enhanced by a combination treatment with *Sophora moorcroftiana* alkaloids, which increase host complement activation [48].

Surprisingly successful combinations—Repurposed drugs

Drug screening has revealed unexpected agents with antiparasitic activity. Furthermore, clinicians have observed improved outcomes from patients with protozoan infections already taking chemotherapeutics for unrelated conditions. Patients infected with *L. infantum* and taking nelfinavir, an antiretroviral for treatment of concurrent HIV, had better outcomes than HIV-negative patients taking only amphotericin B [49]. *L. infantum* infections were also cleared more quickly when treated with a combination of an antiviral, lopinavir, and an antileishmanial, miltefosine [50]. Upon further investigation, it was shown that both antiretrovirals prevented intracellular multiplication of amastigotes [49] and altered parasite membrane lipid composition [50], leading to a reduced parasite load and synergy with conventional antiparasitic drugs. Likewise, ketotifen, a tricyclic antihistamine, prevented host cell invasion by *T. gondii* through stabilization of host cell membrane. This drug also inhibits intracellular parasite multiplication, which increases the percentage of bloodstream parasites that are vulnerable to pyrimethamine [51]. Interestingly, ketotifen may also reduce host cell inflammation, rendering infections less harmful [51]. Finally, metformin, a hydrophilic antihyperglycemic that also prevents parasitic proliferation, improved conventional albendazole treatment of echinococcosis resulting in reduced parasite load [52].

Several repurposed drugs may increase the delivery of conventional drugs to target tissues. Auranofin, an anti-arthritic drug, synergized with conventional amphotericin B to inhibit growth of *Naegleria fowleri* [53]. Auranofin impairs the *N. fowleri* oxidative stress response by inhibiting both selenoprotein synthesis and thioreductase activity. It also may improve drug delivery of amphotericin B across the blood–brain barrier, which might be advantageous for treating neuroinvasive *N. fowleri* infections [53]. Conventional treatment of *T. cruzi* with benznidazole was enhanced with amiodarone, a cardiac antiarrhythmic drug often prescribed for symptomatic treatment of invasive cardiac cases of Chagas disease. Both drugs were shown to have distinct targets in *T. cruzi* and to reach therapeutic concentrations in myocardial tissue. Benznidazole caused oxidative stress and amiodarone inhibited parasitic ergosterol biosynthesis, and the combination decreased parasitic cell counts *in vitro* more effectively than single-drug treatment [54]. Amiodarone also impairs ion transport in *C. albicans* [55] and has synergy with caspofungin inhibition of $\beta(1,3)$ -glucan synthase, leading to decreased cell wall integrity and limited fungal growth [15].

Many repurposed drugs also exhibit binding to parasite-specific proteins. Clomipramine, a tricyclic antidepressant, binds trypanothione reductases and mitochondrial membranes in many trypanosomatida, including *L. amazonensis* [56], *T. brucei* [57], and *T. cruzi* [58]. Trypanothione reductase is a protein that is critical in the antioxidant response of *T. cruzi*; thus, oxidative stress, inflicted by benznidazole, may be intensified by an inhibited antioxidant response mediated by clomipramine [58]. The antipsychotic drug, prochlorperazine [59], and the antihistamine, chlorpheniramine [60], both potentiated chloroquine and mefloquine by binding parasite-specific efflux pumps and ATP-binding cassette (ABC) transporters in *Plasmodium vivax* [59] or *P. falciparum* [60]. This, in turn, forced the retention of the conventional antimalarial drugs.

Future perspectives—Nearly endless combinations

Combination therapies have been reported for most infections with eukaryotic pathogens. An abundance of studies in the last 5 years demonstrates that there are many viable avenues including those that use antibiotic/antibiotic combinations and antibiotic/nonantibiotic combinations. Notably, such combination regimens are not without risk. Potential disadvantages include the possibility of increased side effects coupled with a difficulty in discerning the source of side effects, increased toxicity, incompatible pharmacokinetics, and increased cost [7,61]. Conversely, many of these shortcomings could be offset by the potential that combination therapies may result in lowering the effective dosage of each drug in the combination and/or shortening drug treatment regimens.

Capitalizing on the wide availability of data and the variety of drug mechanisms may also yield promising 3-way combinations in the future. For example, *L. infantum* parasites have been more successfully eliminated with a combination of sorafenib, a kinase inhibitor used to treat kidney cancer, lopinavir, an antiretroviral, and auranofin, an inflammation reducing anti-arthritis, than when treated with these agents alone [62]. Pairwise combinations of these 3 drugs were additive, but the combination of all 3 produced synergy. Furthermore, the complete cure of DR giardiasis, using paromomycin, albendazole, and metronidazole, has been reported [17]. Overall, the lack of development of novel drugs, the consistent resistance acquisition caused by drug monotherapies, and the preliminary success of many of the approaches described here makes the combination approach vital in the war against DR eukaryotic pathogens.

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References

1. Tanwar J, Das S, Fatima Z, Hameed S. Multidrug resistance: an emerging crisis. *Interdiscip Perspect Infect Dis*. 2014; 2014:541340. <https://doi.org/10.1155/2014/541340> PMID: 25140175
2. Chan GKY, Wilson S, Schmidt S, Moffat JG. Unlocking the potential of high-throughput drug combination assays using acoustic dispensing. *J Lab Autom*. 2016; 21:125–32. <https://doi.org/10.1177/2211068215593759> PMID: 26160862
3. Li R, Chen J, Cesario TC, Wang X, Yuan JS, Rentzepis PM. Synergistic reaction of silver nitrate, silver nanoparticles, and methylene blue against bacteria. *Proc Natl Acad Sci U S A*. 2016; 113:13612–7. <https://doi.org/10.1073/pnas.1611193113> PMID: 27849602
4. Meyer CT, Wooten DJ, Paudel BB, Bauer J, Hardeman KN, Westover D, et al. Quantifying drug combination synergy along potency and efficacy axes. *Cell Syst*. 2019; 8: 97–108.e16. <https://doi.org/10.1016/j.cels.2019.01.003> PMID: 30797775
5. Brouwer AE, Rajanuwong A, Chierakul W, Griffin GE, Larsen RA, White NJ, et al. Combination antifungal therapies for HIV-associated cryptococcal meningitis: a randomized trial. *Lancet*. 2004; 363:1764–7. [https://doi.org/10.1016/S0140-6736\(04\)16301-0](https://doi.org/10.1016/S0140-6736(04)16301-0) PMID: 15172774
6. Nash TE, Ohl CA, Thomas E, Subramanian G, Keiser P, Moore TA. Treatment of patients with refractory giardiasis. *Clin Infect Dis*. 2001; 33:22–8. <https://doi.org/10.1086/320886> PMID: 11389490
7. Martinelli A, Moreira R, Cravo P. Malaria combination therapies: advantages and shortcomings. *Mini Rev Med Chem*. 2008; 8:201–12. <https://doi.org/10.2174/138955708783744092> PMID: 18336340
8. Steverding D, Rushworth SA. Front-line glioblastoma chemotherapeutic temozolomide is toxic to *Trypanosoma brucei* and potently enhances melarsoprol and eflornithine. *Exp Parasitol*. 2017; 178:45–50. <https://doi.org/10.1016/j.exppara.2017.05.006> PMID: 28552794
9. Shiojiri D, Kinai E, Teruya K, Kikuchi Y, Oka S. Combination of clindamycin and azithromycin as alternative treatment for *Toxoplasma gondii* encephalitis. *Emerg Infect Dis*. 2019; 25:841–3. <https://doi.org/10.3201/eid2504.181689> PMID: 30882331

10. Rajapakshage BKW, Yamasaki M, Hwang S, Sasaki N, Murakami M, Tamura Y, et al. Involvement of mitochondrial genes of *Babesia gibsoni* in resistance to diminazene aceturate. *J Vet Med Sci*. 2012; 74:1139–48. <https://doi.org/10.1292/jvms.12-0056> PMID: 22673639
11. Tuvshintulga B, Abou-Laila M, Sivakumar T, Tayebwa DS, Gantuya S, Naranbaatar K, et al. Chemotherapeutic efficacies of a clofazimine and diminazene aceturate combination against piroplasm parasites and their AT-rich DNA-binding activity on *Babesia bovis*. *Sci Rep*. 2017; 7:13888. <https://doi.org/10.1038/s41598-017-14304-0> PMID: 29066849
12. Sayé M, Gauna L, Valera-Vera E, Reigada C, Miranda MR, Pereira CA. Crystal violet structural analogues identified by in silico drug repositioning present anti-*Trypanosoma cruzi* activity through inhibition of proline transporter TcAAAP069. *PLoS Negl Trop Dis*. 2020; 14:e0007481. <https://doi.org/10.1371/journal.pntd.0007481> PMID: 31961864
13. Kersh GJ. Antimicrobial therapies for Q fever. *Expert Rev Anti-Infect Ther*. 2013; 11:1207–14. <https://doi.org/10.1586/14787210.2013.840534> PMID: 24073941
14. Wijnant GJ, Van Bocxlaer K, Yardley V, Murdan S, Croft SL. Efficacy of paromomycin-chloroquine combination therapy in experimental cutaneous leishmaniasis. *Antimicrob Agents Chemother*. 2017; 61:e00358–17. <https://doi.org/10.1128/AAC.00358-17> PMID: 28607026
15. Robbins N, Spitzer M, Yu T, Cerone RP, Averette AK, Bahn Y, et al. An antifungal combination matrix identifies a rich pool of adjuvant molecules that enhance drug activity against diverse fungal pathogens. *Cell Rep*. 2015; 13:1481–92. <https://doi.org/10.1016/j.celrep.2015.10.018> PMID: 26549450
16. Escobedo AA, Lalle M, Hrastnik NI, Rodriguez-Morales AJ, Castro-Sanchez E, Cimerman S, et al. Combination therapy in the management of giardiasis: what laboratory and clinical studies tell us so far. *Acta Trop*. 2016; 162:196–205. <https://doi.org/10.1016/j.actatropica.2016.06.026> PMID: 27349189
17. Carter ER, Nabarro LE, Hedley L, Chiodini PL. Nitroimidazole-refractory giardiasis: a growing problem requiring rational solutions. *Clin Microbiol Infect*. 2017; 24:37–42. <https://doi.org/10.1016/j.cmi.2017.05.028> PMID: 28624613
18. Lalle M, Hanevik K. Treatment-refractory giardiasis: challenges and solutions. *Infect Drug Resist*. 2018; 11:1921–33. <https://doi.org/10.2147/IDR.S141468> PMID: 30498364
19. Khan AA, Alanazi AM, Jabeen M, Khan S, Malik A. Additive potential of combination therapy against cryptococcosis employing a novel amphotericin B and fluconazole loaded dual delivery system. *Eur J Pharm Sci*. 2018; 119:171–8. <https://doi.org/10.1016/j.ejps.2018.04.015> PMID: 29653177
20. Deng S, Lei W, de Hoog GS, Yang L, Vitale RG, Rafati H, et al. Combination of amphotericin B and terbinafine against melanized fungi associated with chromoblastomycosis. *Antimicrob Agents Chemother*. 2018; 62:e00270–18. <https://doi.org/10.1128/AAC.00270-18> PMID: 29581111
21. Edwards PA, Ericsson J. Sterols and isoprenoids: signaling molecules derived from the cholesterol biosynthetic pathway. *Annu Rev Biochem*. 1999; 68:157–85. <https://doi.org/10.1146/annurev.biochem.68.1.157> PMID: 10872447
22. Gazzero P, Proto MC, Gangemi G, Malfitano AM, Ciaglia E, Pisanti S, et al. Pharmacological actions of statins: a critical appraisal in the management of cancer. *Pharmacol Rev*. 2012; 64:102–46. <https://doi.org/10.1124/pr.111.004994> PMID: 22106090
23. Sifaoui I, Martín-Navarro CM, López-Arencibia A, Reyes-Battle M, Valladares B, Piñero JE, et al. Optimized combinations of statins and azoles against *Acanthamoeba* trophozoites and cysts *in vitro*. *Asian Pac J Trop Med*. 2019; 12:283–7.
24. Thomson S, Rice CA, Zhang T, Edrada-Ebel R, Henriquez FL, Roberts CW. Characterisation of sterol biosynthesis and validation of 14 α -demethylase as a drug target in *Acanthamoeba*. *Sci Rep*. 2017; 7:8247. <https://doi.org/10.1038/s41598-017-07495-z> PMID: 28811501
25. Li Z, Li C, Szajnman SH, Rodriguez JB, Moreno SN. Synergistic activity between statins and bisphosphonates against acute experimental toxoplasmosis. *Antimicrob Agents Chemother*. 2017; 61:e02628–16. <https://doi.org/10.1128/AAC.02628-16> PMID: 28559264
26. Basyoni MM, Fouad SA, Amer MF, Amer AF, Ismail DI. Atorvastatin: *in vivo* synergy with metronidazole as anti-*Blastocystis* therapy. *Korean J Parasitol*. 2018; 56:105–12. <https://doi.org/10.3347/kjp.2018.56.2.105> PMID: 29742864
27. Sanfelice RA, da Silva SS, Bosqui LR, Machado LF, Miranda-Sapla MM, Panagio LA, et al. Pravastatin and simvastatin pretreatment in combination with pyrimethamine and sulfadiazine reduces infection process of *Toxoplasma gondii* tachyzoites (RH strain) in HeLa cells. *Acta Parasitol*. 2019; 64:612–6. <https://doi.org/10.2478/s11686-019-00076-2> PMID: 31286354
28. Verma AK, Laha B, Pandey M, Pal U, Ghosh M. Cholesterol-lowering drug, in combination with chromium chloride, induces early apoptotic signals in intracellular *L. donovani* amastigotes, leading to death. *J Biosci*. 2017; 42:427–38. <https://doi.org/10.1007/s12038-017-9690-9> PMID: 29358556

29. Ochocki JD, Distefano MD. Prenyltransferase inhibitors: treating human ailments from cancer to parasitic infections. *Medchemcomm*. 2013; 4:476–92. <https://doi.org/10.1039/C2MD20299A> PMID: [25530833](https://pubmed.ncbi.nlm.nih.gov/25530833/)
30. Probst A, Nguyen TN, El-Sakkary N, Skinner D, Suzuki BM, Buckner FS, et al. Bioactivity of farnesyltransferase inhibitors against *Entamoeba histolytica* and *Schistosoma mansoni*. *Front Cell Infect Microbiol*. 2019; 9:180. <https://doi.org/10.3389/fcimb.2019.00180> PMID: [31192168](https://pubmed.ncbi.nlm.nih.gov/31192168/)
31. Gao X, Liu X, Shan W, Liu Q, Wang C, Zheng J, et al. Anti-malarial atovaquone exhibits anti-tumor effects by inducing DNA damage in hepatocellular carcinoma. *Am J Cancer Res*. 2018; 8:1697–711. PMID: [30323964](https://pubmed.ncbi.nlm.nih.gov/30323964/)
32. El-Saber Batiha G, Magdy-Beshbishy A, Adeyemi SO, Nadwa E, Rashwan E, Yokoyama N, et al. Safety and efficacy of hydroxyurea and eflornithine against most blood parasites *Babesia* and *Theileria*. *PLoS ONE*. 2020; 15:e0228996. <https://doi.org/10.1371/journal.pone.0228996> PMID: [32053698](https://pubmed.ncbi.nlm.nih.gov/32053698/)
33. Huang F, Mazin AV. A small molecule inhibitor of human RAD51 potentiates breast cancer cell killing by therapeutic agents in mouse xenografts. *PLoS ONE*. 2014; 9:e100993. <https://doi.org/10.1371/journal.pone.0100993> PMID: [24971740](https://pubmed.ncbi.nlm.nih.gov/24971740/)
34. Vidyam P, Dutta D, Sutram N, Bhattacharyya S, Bhattacharyya MK. A small-molecule inhibitor of the DNA recombinase Rad51 from *Plasmodium falciparum* synergizes with the antimalarial drugs artemisinin and chloroquine. *J Biol Chem*. 2019; 294:8171–83. <https://doi.org/10.1074/jbc.RA118.005009> PMID: [30936202](https://pubmed.ncbi.nlm.nih.gov/30936202/)
35. Castro L, Kwiecinski MR, Ourique F, Parisotto EB, Grinevicius V, Correia J, et al. Albendazole as a promising molecule for tumor control. *Redox Biol*. 2016; 10:90–9. <https://doi.org/10.1016/j.redox.2016.09.013> PMID: [27710854](https://pubmed.ncbi.nlm.nih.gov/27710854/)
36. Pense PE, Elissondo N, Gambino G, Gamboa GU, Benoit J, Elissondo MC. Experimental cystic echinococcosis therapy: *in vitro* and *in vivo* combined 5-fluorouracil/albendazole treatment. *Vet Parasitol*. 2017; 245:62–70. <https://doi.org/10.1016/j.vetpar.2017.08.011> PMID: [28969840](https://pubmed.ncbi.nlm.nih.gov/28969840/)
37. Trinconi CT, Reimão JQ, Yokoyama-Yasunaka JK, Miguel DC, Uliana SRB. Combination therapy with tamoxifen and amphotericin B in experimental cutaneous leishmaniasis. *Antimicrob Agents Chemother*. 2014; 58:2608–13. <https://doi.org/10.1128/AAC.01315-13> PMID: [24550333](https://pubmed.ncbi.nlm.nih.gov/24550333/)
38. Batiha GE, Beshbishy AM, Tayebwa DS, Adeyemi OS, Shaheen H, Yokoyama N, et al. The effects of trans-chalcone and chalcone 4 hydrate on the growth of *Babesia* and *Theileria*. *PLoS Negl Trop Dis*. 2019; 13:e0007030. <https://doi.org/10.1371/journal.pntd.0007030> PMID: [31125333](https://pubmed.ncbi.nlm.nih.gov/31125333/)
39. Dorsaz S, Snäkä T, Favre-Godal Q, Maudens P, Boulens N, Furrer P, et al. Identification and mode of action of a plant natural product targeting human fungal pathogens. *Antimicrob Agents Chemother*. 2017; 61: e00829–17. <https://doi.org/10.1128/AAC.00829-17> PMID: [28674054](https://pubmed.ncbi.nlm.nih.gov/28674054/)
40. Tullio VC, Roana J, Scalas D, Mandras N. Evaluation of the antifungal activity of *Mentha x piperita* (Lamiaceae) of Pancalieri (Turin, Italy) essential oil and its synergistic interaction with azoles. *Molecules*. 2019; 24:3148. <https://doi.org/10.3390/molecules24173148> PMID: [31470602](https://pubmed.ncbi.nlm.nih.gov/31470602/)
41. Moazeni M, Borji H, Saboor DM. Enhancement of the therapeutic effect of albendazole on cystic echinococcosis using a herbal product. *J Investig Surg*. 2019; 32:103–10. <https://doi.org/10.1080/08941939.2017.1380089> PMID: [28990848](https://pubmed.ncbi.nlm.nih.gov/28990848/)
42. Cardoso NN, Alviano CS, Blank AF, Arrigoni-Blank MDF, Romanos MTV, Cunha MML, et al. Anti-cryptococcal activity of ethanol crude extract and hexane fraction from *Ocimum basilicum* var. Maria bonita: mechanisms of action and synergism with amphotericin B and *Ocimum basilicum* essential oil. *Pharm Biol*. 2017; 55:1380–8. <https://doi.org/10.1080/13880209.2017.1302483> PMID: [28317465](https://pubmed.ncbi.nlm.nih.gov/28317465/)
43. Hammi KM, Essid R, Tabbene O, Elkahoui S, Majdoub H, Ksouri R. Antileishmanial activity of *Moringa oleifera* leaf extracts and potential synergy with amphotericin B. *S Afr J Bot*. 2020; 129:67–73.
44. Intakhan N, Chanmol W, Somboon P, Bates MD, Yardley V, Bates PA, et al. Antileishmanial activity and synergistic effects of amphotericin B deoxycholate with allicin and andrographolide against *Leishmania martiniquensis* *in vitro*. *Pathogens*. 2020; 9:49. <https://doi.org/10.3390/pathogens9010049> PMID: [31936536](https://pubmed.ncbi.nlm.nih.gov/31936536/)
45. Veas R, Rojas-Pirela M, Castillo C, Olea-Azar C, Moncada M, Ulloa P, et al. Microalgae extracts: Potential anti-*Trypanosoma cruzi* agents? *Biomed Pharmacother*. 2020; 127:110178. <https://doi.org/10.1016/j.biopha.2020.110178> PMID: [32371317](https://pubmed.ncbi.nlm.nih.gov/32371317/)
46. Moreno ÉM, Leal SM, Stashenko EE, García LT. Induction of programmed cell death in *Trypanosoma cruzi* by *Lippia alba* essential oils and their major and synergistic terpenes (citral, limonene and caryophyllene oxide). *BMC Complement Altern Med*. 2018; 18:225. <https://doi.org/10.1186/s12906-018-2293-7> PMID: [30053848](https://pubmed.ncbi.nlm.nih.gov/30053848/)
47. Tauheed AM, Mamman M, Ahmed A, Suleiman MM, Balogun EO. *In vitro* and *in vivo* antitrypanosomal efficacy of combination therapy of *Anogeissus leiocarpus*, *Khaya senegalensis* and potash *J Ethnopharmacol*. 2020; 258:112805. <https://doi.org/10.1016/j.jep.2020.112805> PMID: [32243988](https://pubmed.ncbi.nlm.nih.gov/32243988/)

48. Zhang F, Hu C, Cheng S, Wang S, Li B, Cao D, et al. The investigation of the effect and mechanism of *Sophora moorcroftiana* alkaloids in combination with albendazole on echinococcosis in an experimental rats model. *Evid Based Complement Alternat Med*. 2018; 2018:3523126. <https://doi.org/10.1155/2018/3523126> PMID: 29785189
49. Valdivieso E, Mejías F, Carrillo E, Sánchez C, Moreno J. Potentiation of the leishmanicidal activity of nelfinavir in combination with miltefosine or amphotericin B. *Int J Antimicrob Agents*. 2018; 52:682–7. <https://doi.org/10.1016/j.ijantimicag.2018.06.016> PMID: 29969693
50. Rebello KM, Andrade-Neto VV, Gomes CRB, de Souza MVN, Branquinha MH, Santos ALS, et al. Miltefosine-lopinavir combination therapy against *Leishmania infantum* infection: *in vitro* and *in vivo* approaches. *Front Cell Infect Microbiol*. 2019; 9:229. <https://doi.org/10.3389/fcimb.2019.00229> PMID: 31316919
51. Montazeri M, Rezaei K, Ebrahimzadeh MA, Sharif M, Sarvi S, Ahmadpour E, et al. Survey on synergism effect of ketotifen in combination with pyrimethamine in treatment of acute murine toxoplasmosis. *Trop Med Health*. 2017; 45:39. <https://doi.org/10.1186/s41182-017-0079-0> PMID: 29200930
52. Loos JA, Dávila VA, Rodrigues CR, Petrigh R, Zoppi JA, Crocenzi FA, et al. Metformin exhibits preventive and therapeutic efficacy against experimental cystic echinococcosis. *PLoS Negl Trop Dis*. 2017; 11:e0005370. <https://doi.org/10.1371/journal.pntd.0005370> PMID: 28182659
53. Escrig JI, Hahn HJ, Debnath A. Activity of auranofin against multiple genotypes of *Naegleria fowleri* and its synergistic effect with amphotericin B *in vitro*. *ACS Chem Neurosci*. 2020; 11 (16):2464–71. <https://doi.org/10.1021/acscchemneuro.0c00165> PMID: 32392039
54. Lourenço AM, Faccini CC, de Jesus-Costa CA, Mendes GB, Fragata Filho AA. Evaluation of *in vitro* anti-*Trypanosoma cruzi* activity of medications benznidazole, amiodarone hydrochloride, and their combination. *Rev Soc Bras Med Trop*. 2018; 51:52–6. <https://doi.org/10.1590/0037-8682-0285-2017> PMID: 29513842
55. Liu S, Yue L, Gu W, Li X, Zhang L, Sun S. Synergistic effect of fluconazole and calcium channel blockers against resistant *Candida albicans*. *PLoS ONE*. 2016; 17:e0150859. <https://doi.org/10.1371/journal.pone.0150859> PMID: 26986478
56. da Silva-Rodrigues JH, Miranda N, Volpato H, Ueda-Nakamura T, Nakamura CV. The antidepressant clomipramine induces programmed cell death in *Leishmania amazonensis* through a mitochondrial pathway. *Parasitol Res*. 2019; 118:977–89. <https://doi.org/10.1007/s00436-018-06200-x> PMID: 30694414
57. O'Sullivan MC, Durham TB, Valdes HE, Dauer KL, Karney NJ, Forrestel AC, et al. Dibenzosuberyl substituted polyamines and analogs of clomipramine as effective inhibitors of trypanothione reductase; molecular docking, and assessment of trypanocidal activities. *Bioorg Med Chem*. 2015; 23:996–1010. <https://doi.org/10.1016/j.bmc.2015.01.018> PMID: 25661449
58. García MC, Ponce NE, Sanmarco LM, Manzo RH, Jimenez-Kairuz AF, Aoki MP. Clomipramine and benznidazole act synergistically and ameliorate the outcome of experimental Chagas disease. *Antimicrob Agents Chemother*. 2016; 60:3700–8. <https://doi.org/10.1128/AAC.00404-16> PMID: 27067322
59. Obaldia N 3rd, Milhous WK, Kyle DE. Reversal of chloroquine resistance of *Plasmodium vivax* in Aotus monkeys. *Antimicrob Agents Chemother*. 2018; 62:e00582–18. <https://doi.org/10.1128/AAC.00582-18> PMID: 29941642
60. Nakornchai S, Konthiang P. Potentiation of antimalarial drug action by chlorpheniramine against multi-drug-resistant *Plasmodium falciparum* *in vitro*. *Parasitol Int*. 2006; 55:195–9.
61. Gintjee TJ, Donnelley MA, Thompson GR. Aspiring Antifungals: Review of Current Antifungal Pipeline Developments. *J Fungi (Basel)*. 2020; 6:28. <https://doi.org/10.3390/jof6010028> PMID: 32106450
62. Abou-EI-Naga IF, Mady RF, Mogahed NMFH. *In vitro* effectivity of three approved drugs and their synergistic interaction against *Leishmania infantum*. *Biomedica*. 2020; 40:89–101. <https://doi.org/10.7705/biomedica.4891> PMID: 32463611