HIV-1 protease inhibitors for treatment of visceral leishmaniasis in HIV-co-infected individuals

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The global prevalence of HIV is a major challenge for control of visceral leishmaniasis, a disseminated protozoan infection. In some east African regions, up to 40% of patients with visceral leishmaniasis are co-infected with HIV. Management of visceral leishmaniasis in such patients is complicated by treatment failures and relapses, even while patients are receiving standard antiretroviral therapy. In-vitro studies have consistently documented an inhibitory effect of specific HIV-1 protease inhibitors on leishmania parasites, and the underlying mechanism is partly explained. With the global scaling up of HIV treatment, HIV-1 protease inhibitors are increasingly becoming available for second-line HIV treatment in regions where visceral leishmaniasis and HIV are endemic. However, additional research is needed before HIV-1 protease inhibitors can be taken forward for clinical use against visceral leishmaniasis in HIV-infected patients. Since the effect of protease inhibitors against *Leishmania* species was generally observed at high drug concentrations, efficacy and dose–response relationships should be studied in animals before these drugs are used in clinical trials. More extensive studies of all available HIV protease inhibitors are needed, including investigation of drug interactions and emergence of drug-resistant parasites. In addition to exploring the full potential of current HIV-1 protease inhibitors against visceral leishmaniasis, leishmania-specific protease inhibitors should be developed.

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Introduction

Visceral leishmaniasis, also called kala-azar, is a vector-borne disseminated protozoan infection caused by species of the *Leishmania donovani* complex, which mainly target tissue macrophages. Characteristics of the disease include chronic fever, hepatosplenomegaly, and pancytopenia. Without treatment, overt disease is universally lethal.[1] The zoonotic form, for which dogs are the main reservoir, is mainly prevalent in the Mediterranean basin and in South America, and is caused by *Leishmania infantum*. The anthroponotic form is caused by *L donovani*, and is prevalent in the Indian subcontinent and east Africa.[1,2] Worldwide, an estimated 300 000 cases of visceral leishmaniasis occur each year, with around 30 000 cases in east Africa, mainly in Sudan and Ethiopia.[3] Most leishmania infections in immunocompetent hosts remain asymptomatic, with only a small proportion leading to disease (visceral leishmaniasis).

HIV has been identified as one of the emerging challenges for control of visceral leishmaniasis.[4] HIV co-infection substantially increases the risk of progression from asymptomatic leishmania infection to active disease, and visceral leishmaniasis accelerates HIV disease progression. Prevalence of HIV has fuelled the re-emergence of visceral leishmaniasis in Europe, and the problem is particularly severe in areas of east Africa, such as Ethiopia, where up to 40% of patients with visceral leishmaniasis are co-infected with HIV.[4]

Treatment of visceral leishmaniasis–HIV co-infection in east Africa is very challenging, compounded by high mortality, low cure rates, and increased drug toxicity.[4,5] Few therapeutic options exist and current treatment recommendations are not ideal;[4,6,7] treatment with liposomal amphotericin B in co-infected individuals has been disappointing, with roughly 16% of primary visceral leishmaniasis cases and 56% of relapse cases showing parasitological failure (parasites persist in tissue) despite treatment up to a total dose of 30 mg/kg.[7] Even when apparent parasitological clearance is achieved and antiretroviral treatment is started, up to 60% of individuals relapse within 1 year, irrespective of the type of visceral leishmaniasis drugs used.[8] In areas with zoonotic transmission of visceral leishmaniasis, WHO recommends secondary prophylaxis against visceral leishmaniasis among individuals co-infected with HIV; however, this recommendation is less clear when visceral leishmaniasis transmission is anthroponotic.[4] In this situation, long-term administration of visceral leishmaniasis drugs to prevent relapse (secondary prophylaxis) might increase the risk of emergence and spread of drug-resistant parasites, which could compromise effective treatment of visceral leishmaniasis in immunocompetent individuals. With regard to HIV treatment response among patients with both visceral leishmaniasis and HIV, CD4 cell-count recovery is generally poor even when viral suppression is achieved. Overall, these patients respond poorly to visceral leishmaniasis treatment and standard HIV treatment,[4] and new approaches are needed.

Combining different antileishmanial drugs (combination therapy) for individuals with both visceral leishmaniasis and HIV has been proposed, but no trial data are currently available.[2] A clinical trial investigating monthly intravenous pentamidine as secondary prophylaxis for visceral leishmaniasis is ongoing in Ethiopia (NCT01360762). An unexplored approach to achieve better treatment success for visceral leishmaniasis and reduce relapse rates is the optimisation of HIV antiretroviral therapy. Several lines of evidence suggest that HIV-1 protease inhibitors might directly exert antiparasitic effects,[9] including against leishmania parasites.[4,10–12] Protease-inhibitor activity was seen when tested against antimony-resistant field isolates of

L donovani and against strains isolated from HIV patients.[12] With the scaling up of antiretroviral therapy in low-income and middle-income countries, HIV-1 protease inhibitors are increasingly available and are used by national treatment programmes for second-line HIV treatment in regions with endemic visceral leishmaniasis and HIV. The finding that HIV-1 protease inhibitors are clinically effective against visceral leishmaniasis would greatly facilitate implementation of these drugs for treatment of visceral leishmaniasis in co-infected individuals in these regions. A randomised trial in Uganda[13] recently provided proof of concept for the use of HIV-1 protease inhibitors for control of parasitic diseases in resource-constrained settings; a significant decrease in malaria incidence was reported for HIV-infected children initiated on protease-inhibitor-based antiretroviral therapy, compared with those given non-nucleoside reverse transcription inhibitor (NNRTI)-based antiretroviral therapy. A similar strategy could consist of replacing NNRTIs with HIV-1 protease inhibitors in first-line antiretroviral therapy regimens in regions where visceral leishmaniasis is endemic. A strong evidence base and adequate risk assessment are needed before such strategies can be taken forward for clinical use against visceral leishmaniasis. In view of the potential role of HIV-1 protease inhibitors for treatment and control of visceral leishmaniasis in HIV-co-infected individuals in low-income and middle-income countries, in this Review we critically evaluate the experimental evidence for use of HIV-1 protease inhibitors against leishmaniasis, and give an overview of clinical experience with protease-inhibitor-based antiretroviral therapy in visceral leishmaniasis–HIV co-infection. Additionally, we highlight key knowledge gaps and propose future research areas.

***Panel 1:* Overview of approved HIV-1 protease inhibitors**

First-generation HIV-1 protease inhibitors: nelfinavir, indinavir, ritonavir, saquinavir

- High pill burden and low tolerance, mainly replaced by newer protease inhibitors in clinical practice
- Ritonavir mainly used as a component of boosted protease inhibitors
- Nelfinavir not on the market

Second-generation HIV-1 protease inhibitors: lopinavir, atazanavir, amprenavir, fosamprenavir

- Increased potency and tolerance
- Lopinavir plus ritonavir: available as a heat-stable, fixed-dose combination; currently the only coformulated protease inhibitor; available and recommended by WHO (since 2003) for second-line antiretroviral therapy in low-income and middle-income countries
- Atazanavir plus ritonavir is recommended by WHO as an alternative for second-line antiretroviral therapy in low-income and middle-income countries
- Fosamprenavir (prodrug of amprenavir) is preferred over amprenavir

Third-generation HIV-1 protease inhibitors: darunavir, tipranavir

- High genetic barrier, retain activity against viruses resistant to older generation protease inhibitors
- Darunavir is tolerated very well, tipranavir is reserved for treatment-experienced patients
- Darunavir and ritonavir are recommended by WHO for third-line antiretroviral therapy in low-income and middle-income countries

Protease inhibitors in HIV therapy

HIV-1 protease inhibitors are small molecules that inhibit HIV-1 replication by actively competing for the binding site of the viral protease enzyme.[14] The first HIV-1 protease inhibitors were developed in the mid-1990s and approved for clinical practice by 1995. So far, ten such drugs have been approved for HIV treatment by the US Food and Drug Administration, broadly divided into first, second, and third generations, with progressive improvements in terms of potency and genetic barrier, dosing schedule, or toxic effects (panel 1).[15,16] As a group, protease inhibitors share common side-effects. Gastrointestinal intolerance is the most common short-term side-effect, and is generally most pronounced with first-generation protease inhibitors. Long-term adverse events include metabolic abnormalities (dyslipidaemia, hyperglycaemia, and body-fat distribution), and are less common with more recent protease inhibitors. All HIV-1 protease inhibitors undergo substantial hepatic metabolism and have the potential to interact with concomitantly prescribed drugs, mediated through hepatic cytochromal enzymes. Protease inhibitors are now mainly given in a boosted format, whereby the active component is given with a low dose of ritonavir, which inhibits hepatic cytochromal enzymes, to increase concentration of the active drug. In general, boosted protease inhibitors are more potent with a better pharmacokinetic profile, have more favourable dosing schedules, and are less likely to lead to HIV resistance in the event of suboptimum adherence.[15,16] Although HIV-1 is capable of developing resistance to all drugs used to treat it, HIV-1 protease inhibitors are among the drugs with the highest genetic barrier, requiring several virus mutations for resistance.

Protease inhibitors form the backbone of combination antiretroviral therapy, combining high potency with well-defined adverse events and an acceptable safety record. Although recommended for first-line treatment in high-income countries, HIV-1 protease inhibitors are reserved for second-line treatment in low-income and middle-income countries, according to WHO guidelines, mainly because of cost.[17,18] One of the most commonly prescribed and extensively studied protease inhibitors is boosted lopinavir (lopinavir plus ritonavir), which has been in use for more than 10 years and has a

favourable toxicity profile.[19] Since 2003, WHO has recommended boosted lopinavir for second-line treatment in resource-constrained settings, with NNRTI-based antiretroviral therapy recommended for first-line treatment.[17,18,20] As a result, boosted lopinavir is now readily available for HIV treatment in most regions with endemic visceral leishmaniasis. Boosted lopinavir is coformulated, heat-stable, does not require refrigeration, and generic drugs have been developed. Price reductions have been applied to several protease inhibitors for use in low-income countries in the past few years, and for such countries, boosted lopinavir can be obtained at a cost of around US$1 per day. The latest WHO guidelines recommend boosted darunavir for use in third-line treatment regimens and boosted atazanavir as an alternative protease inhibitor for second-line treatment.[18] Compared with boosted lopinavir, atazanavir is better tolerated, less expensive, and simpler to give (one tablet per day compared with two tablets twice a day for lopinavir).

Protease inhibitors and visceral leishmaniasis

Direct effects

The first in-vitro study of HIV-1 protease inhibitor activity against *Leishmania* species, in 2005, assessed the effect of indinavir and saquinavir on *L infantum* and *L major* promastigote growth (tables 1 and 2);[11] both HIV-1 protease inhibitors showed dose-dependent leishmanicidal activity (dose range 6·25–50 μM/L). The half maximum inhibitory concentrations (IC_{50}) of indinavir and saquinavir on *L major* were 8·3 μM/L and 7·0 μM/L, respectively. The inhibitory effect of the drugs on *L infantum* was less evident, ranging from 5 to 34% inhibition in a dose-dependent manner, with an IC_{50} greater than 50 μM/L. Higher drug tolerance of promastigotes compared with amastigotes has been noted for some antileishmanial drugs, such as antimonials.[26] Trudel and colleagues[12] showed an absence of inhibition of HIV-1 protease inhibitors (nelfinavir, ritonavir, and saquinavir) on *L infantum* promastigotes, but a clear effect on axenic and intracellular amastigotes. Protease inhibitor activity has been reported against a field isolate of *L donovani* with resistance to antimonials, and in an HIV co-infection model (table 1). The Drugs for Neglected Diseases initiative (DNDi) further investigated the activity of lopinavir and ritonavir on *L donovani* in the macrophage model. Lopinavir was inactive (IC_{50} >16 μM/L; higher concentrations showed cytotoxic effects on the host cell) and ritonavir showed limited activity in the assay (table 1; J-R Ioset, DNDi, personal communication). Several subsequent studies, discussed below, attempted to elucidate the mechanism behind the direct inhibitory effects of HIV-1 protease inhibitors on different *Leishmania* species.

For more on the **Drugs for Neglected Diseases initiative** see http://www.dndi.org/

	HIV-1 protease inhibitor	Drug concentration; inhibition in parasite growth (%)			Comments
		Promastigotes	Axenic amastigotes	Intracellular amastigotes	
L infantum[11]	Indinavir	50 μM/L; 29%	..	..	First study showing inhibitory effect of HIV-1 protease inhibitors on promastigotes
	Saquinavir	50 μM/L; 34%	..	..	
L infantum[12]	Nelfinavir	25 μM/L; NSI	25 μM/L; 77%	25 μM/L; 95%	Using home-made luciferase assay, nelfinavir showed the strongest effect
	Ritonavir	25 μM/L; NSI	25 μM/L; 83%	25 μM/L; 51%	
	Saquinavir	25 μM/L; NSI	25 μM/L; NSI	25 μM/L; 64%	Intracellular assay of MDM and promastigotes; inhibitory effect also seen with THP-1 cell line, HIV-co-infected MDMs, and SbV-resistant *L donovani* field strain
L donovani (SbV-resistant field strain)[12]	Nelfinavir	25 μM/L; NSI	..	25 μM/L; 92%	
	Ritonavir	25 μM/L; NSI	..	25 μM/L; 53%	
	Saquinavir	25 μM/L; NSI	..	25 μM/L; 50%	
L infantum[10]	Nelfinavir	14–27 μM/L; 50%	..	15 μM/L; 46%	Intracellular assay of U1 cell line (stably HIV-infected human monocytic cells)
	Saquinavir	51–64 μM/L; 50%	..	25 μM/L; 51%	
L donovani[10]	Nelfinavir	14 μM/L; 50%	..	..	Inhibitory effect on *L infantum* aspartyl protease activity: IC_{50} for nelfinavir of 22·8 μM/L; IC_{50} for saquinavir of 55·2 μM/L
	Saquinavir	52 μM/L; 50%	..	..	
L pifanoi[10]	Nelfinavir	..	10 μM/L; 50%	..	Microscopic changes: binucleate and multinucleate cells in *L infantum* promastigotes
	Saquinavir	..	15 μM/L; 50%	..	
L donovani field strain (*L infantum*)[21]	Nelfinavir	..	6·25 μM/L; 21%	..	Mechanistic study showing protease-inhibitor-induced oxidative stress in the parasite, leading to DNA degradation and apoptosis
	Nelfinavir	..	12·5 μM/L; 33%	..	
*L donovani**	Lopinavir	..	..	16 μM/L; <50%	IC_{50} for lopinavir of >16 μM/L; IC_{50} for ritonavir of 26 μM/L
	Ritonavir	..	..	26 μM/L; 50%	
L chagasi[22]					No or minimum effect with saquinavir apart from 62% inhibition of *L donovani*
Reference strain	Nelfinavir	25 μM/L; 96·2%	..	..	
Patients with HIV					Inhibitory effect on parasite aspartyl protease activity: no effect with saquinavir (1–10 μM/L); ≥50% inhibition (all strains) with nelfinavir (10 μM/L)
No antiretroviral therapy	Nelfinavir	25 μM/L; 96·6%	..	..	
Antiretroviral therapy without protease inhibitor	Nelfinavir	25 μM/L; 96·3%	..	..	
Antiretroviral therapy with protease inhibitor	Nelfinavir	25 μM/L; 0%	..	..	
L donovani	Nelfinavir	25 μM/L; 94%	..	..	

SbV=sodium stibogluconate. NSI=no significant inhibition. MDM=monocyte-derived macrophages. IC_{50}=half maximum inhibitory concentration. *Unpublished data from the Drugs for Neglected Diseases Initiative.

***Table 1*: Effect of HIV-1 protease inhibitors against *Leishmania* spp parasites associated with visceral leishmaniasis**

	HIV-1 protease inhibitor	Drug concentration; inhibition in parasite growth (%)*		Comments
		Promastigotes	Intracellular amastigotes	
L major[11]	Indinavir	8·3 μM/L; 50%	..	Effect on *L infantum*, assessed in parallel, was less pronounced (table 1)
	Saquinavir	7·0 μM/L; 50%	..	
L mexicana[10]	Nelfinavir	10–12 μM/L; 50%	10·5 μM/L; 74%	Intracellular test using murine macrophage cells; effect on *L infantum* was less pronounced (table 1)
	Saquinavir	39–42 μM/L; 50%	10·0 μM/L; 43%	
L amazonensis[23]	Nelfinavir	15·1 μM/L; 50%	25 μM; 70%†	Intracellular assay with murine macrophages
	Lopinavir	16·5 μM/L; 50%	6·25 μM/L; 70%†	Inhibition of leishmania aspartyl peptidase (protease inhibitors at 0·1–10 μM/L): lopinavir > nelfinavir > amprenavir
	Amprenavir	62·0 μM/L; 50%	12·5 μM/L; 40%†	
	Saquinavir	500 μM/L; <50%	..	Limited effect of indinavir or saquinavir on promastigotes
	Indinavir	500 μM/L; <50%	..	
L amazonensis[24]	Indinavir	100 μM/L; 50%	25 μM/L; 56·8%	IC_{50} for atanazavir of 266–400 μM/L (promastigotes)
	Ritonavir	40 μM/L; 50%	12·5 μM/L; 26·3%	Study in mice: lesion improvement seen in the third week with indinavir and in the fifth week with ritonavir
	Atanazavir	266 μM/L; 50%	..	
L braziliensis[24]	Indinavir	400 μM/L; 50%	25 μM/L; 37·8%	
	Ritonavir	2·3 μM/L; 50%	12·5 μM/L; 27·6%	
	Atanazavir	400 μM/L; 50%	..	
L major[22]	Nelfinavir	25 μM/L; 50%	..	No or minimum effect with saquinavir
L amazonensis	Nelfinavir	25 μM/L; 95·5%	..	Effect on parasite aspartyl protease activity: 50% or higher inhibition with nelfinavir 10 μM/L (all strains)
L braziliensis	Nelfinavir	25 μM/L; 95·6%	..	
L major[22]	Saquinavir	25 μM/L; 21·2%	..	
L amazonensis	Saquinavir	25 μM/L; 5·4%	..	
L braziliensis	Saquinavir	25 μM/L; 13·6%	..	
L major[25]	See comments	..	..	Effect of protease inhibitors assessed through inhibition of *L major* Ddi1 orthologue (aspartic protease) in yeast, and associated changes in protein secretion IC_{50} values: nelfinavir 0·44 μM/L; saquinavir 2·8 μM/L; tipranavir 2·9 μM/L; indinavir 3·0 μM/L; amprenavir 5·7 μM/L; lopinavir 56 μM/L; ritonavir 380 μM/L

IC_{50}=half maximum inhibitory concentration. *No evidence exists for effects on axenic amastigotes. †Approximate values.

***Table 2*: Effect of HIV-1 protease inhibitors against *Leishmania* spp parasites associated with cutaneous leishmaniasis**

Proteases are highly conserved proteins involved in many biological processes in human beings and infectious agents. The aspartic protease (or peptidase) family has been further divided into groups A1, A2, and A3.[27] Drugs targeting human and non-human aspartic proteases have been designed, with the HIV-1 protease inhibitors the best-known example. At therapeutic doses (micromolar range) of HIV-1 protease inhibitors, the aspartic proteases (A1 family) of *Candida albicans* and *Plasmodium* spp can be inhibited.[27] The only aspartic protease present in leishmania parasites seems to be a retroviral-like protease in the A2 family.[28] Several protease inhibitors (lopinavir, nelfinavir, indinavir, saquinavir, and amprenavir) induced antiparasitic effects in *L amazonensis* amastigotes compatible with autophagy and apoptosis.[23] Ultrastructural changes in exposed promastigotes included cytoplasm shrinkage, mitochondrial swelling, and chromatin condensation, and parasite aspartyl peptidase activity was decreased upon exposure to HIV protease inhibitors (table 2).[23] It was postulated that HIV-1 protease inhibitors might (at least partly) exert their effect by direct interaction with parasite aspartyl peptidase.[23] The investigators also noted an increase in expression of other peptidases associated with parasite virulence (cysteine peptidase and gp63); this increased expression might reflect compensatory mechanisms and should be taken into account in further risk assessment of protease inhibitors, because it could select more virulent parasites. In a subsequent study, an effect of nelfinavir and saquinavir on several *Leishmania* species was shown, including *L infantum* and *L mexicana*; an inhibitory effect was also seen on intracellular infection, including in human cells stably infected with HIV-1.[10] On the basis of the observed inhibition of parasite aspartyl peptidase activity by HIV-1 protease inhibitors, inhibition of this parasite enzyme was proposed as the mechanism of action.

The potential risk of emergence of leishmania resistance against protease inhibitors was highlighted in a recent study, comparing the effect of nelfinavir and saquinavir on the growth of *L infantum* promastigotes isolated from HIV-positive patients.[22] Whereas nelfinavir exerted strong inhibitory effects on strains isolated from patients not receiving treatment or on a treatment regimen without protease inhibitors, no effect was observed on a strain isolated from a patient on antiretroviral therapy that included protease inhibitors (table 1). This strain, exposed to HIV-1 protease inhibitors in vivo, showed substantially less aspartyl peptidase activity compared with other strains. After successive rounds of in-vitro passage under protease inhibitor pressure, a further decrease in overall aspartic peptidase activity was seen. The investigators

suggested that parasites exposed to HIV-1 protease inhibitors could undergo metabolic changes that affect susceptibility to the drugs.[22]

Other researchers have studied the effect of HIV-1 protease inhibitors on the parasite protease by building on identification of an orthologue of the yeast Ddi1 protein as the only member of the aspartic protease family in leishmania parasites.[28] Ddi1 proteins are highly conserved within eukaryotes, suggesting a key biological role, and have structural similarity to retroviral proteases.[29] White and colleagues[25] studied how HIV-1 protease inhibitors affected protein secretion in knockout yeast cells complemented with the *L major* Ddi1 orthologue. The protease inhibitors studied were nelfinavir, lopinavir, ritonavir, saquinavir, indinavir, amprenavir, and tipranavir. The strongest effect was seen with nelfinavir, and the weakest effects with lopinavir and ritonavir (table 2). The researchers suggested that inhibition of the leishmania Ddi1 orthologue might be the mechanism by which HIV-1 protease inhibitors mediate their antiparasitic effect. A recent study has provided additional evidence that the Ddi1-like protein in leishmania constitutes a functional aspartyl protease, at least in *L major*; the enzyme showed optimum activity in acid conditions and could be inhibited by nelfinavir.[30]

Kumar and colleagues[21] investigated the underlying mechanism of HIV protease inhibitor activity against leishmania by use of axenic *L donovani* amastigotes obtained from field isolates in India, including an antimonial-resistant strain. Their data suggested that nelfinavir induces oxidative stress in leishmania amastigotes, leading to caspase-independent apoptosis, in which DNA is degraded by mitochondrial endonuclease G. This process could largely be prevented by pretreatment with an antioxidant. Nelfinavir-resistant parasites were readily obtained (after eight passages) by culturing parasites at step-wise increased protease inhibitor concentrations. Further study is needed to verify if protease inhibitor resistance can emerge quickly in all *Leishmania* species, or whether this observation might be related to the metabolic adaptations pre-existing in antimonial-resistant strains that are abundant in India.[31,32] A recent study[33] showed that some anti-leishmanial drugs might induce or undergo cross-resistance with other drugs with the same mechanism of action. If the effect of HIV protease inhibitors on leishmania is indeed mediated through induction of oxidative stress, cross-resistance with drugs such as antimonials could be an issue.[33,34] Kumar and colleagues'[21] results showing activity of HIV protease inhibitors on an antimonial-resistant strain of leishmania argue against cross-resistance, but it merits further study.

In addition to a direct antiviral effect, several studies have suggested that HIV-1 protease inhibitors exhibit inhibitory effects on human proteasomes.[35] Proteasomes are large, non-lysosomal, multisubunit protease complexes with an important role in cell differentiation and replication. Proteasomes with molecular similarity are present in several protozoans, including *Leishmania* species,[36,37] and have a key biological role in proteolysis and host–parasite interaction. Lactacystin, a specific proteasome inhibitor, has a negative effect on leishmania promastigotes replication.[36] Whether HIV-1 protease inhibitors exert their effect though inhibition of the parasite proteasome has not been explored.

Indirect effects

In addition to the direct effects of HIV-1 protease inhibitors on parasites, indirect effects through the host cell might also be important. Protease inhibitors affect several cellular pathways, including antigen processing and presentation, proteasome activity, apoptosis, and T-cell responses.[35,38–43] Both inhibitory and stimulatory effects have been observed. However, with the complex and only partly understood immunopathogenesis of leishmania infection, even more so in the context of HIV infection, predicting how the observed effects might affect parasite growth and replication is difficult. Whether the effects of protease inhibitors on host cells contribute to the observed inhibition of leishmania growth and replication in experimental set-ups has not been well studied. A recent study in mice reported on HIV-1 protease inhibitor activity against *Leishmania* species associated with cutaneous leishmaniasis (table 2).[24] Lesion improvement was seen in week 3 after indinavir, and in week 5 after ritonavir treatment.

Several studies, including laboratory investigations and large randomised trials, suggest enhanced CD4 cell recovery with antiretroviral therapy based on protease inhibitor compared with NNRTI-based, therapy.[41,44] Such immunological effects could be particularly relevant clinically in visceral leishmaniasis–HIV co-infection, since CD4 cell recovery is thought to be a key determinant of treatment outcome and prevention of visceral leishmaniasis relapse in co-infected patients, irrespective of the type of antileishmanial drug used.[4] However, enhanced CD4 recovery is usually modest and has not been reported in other studies of protease inhibitors.[45]

Clinical data

HIV protease inhibitors have been used extensively for HIV treatment in visceral leishmaniasis–HIV co-infection in visceral leishmaniasis endemic areas of Europe since the mid-1990s.[4] Although the sharp decline in visceral leishmaniasis in Europe concurrent with the introduction of protease-inhibitor-based antiretroviral therapy has been partly linked to the effect of protease inhibitors on leishmania,[9] this conclusion is based on retrospective studies of observational data. Comparison was mainly done with patients who were not on antiretroviral therapy, or who were on monotherapy or dual therapy. Good comparison data with patients on NNRTI-based, highly active antiretroviral therapy are lacking.[46–50] Clinical relevance of the inhibitory effect of

HIV protease inhibitors on leishmania parasites seen in in-vitro studies has never been appropriately assessed. Some investigators argue that, on the basis of results from laboratory studies, the inhibitory effects of HIV protease inhibitors on other protozoa (eg, plasmodium and cryptosporidium) and yeast (candida) could partly explain the substantial reduction in opportunistic infections noted at the time of introduction of protease-inhibitor-based antiretroviral therapy.[9,16,51] In a small, randomised trial comparing protease-inhibitor-based versus NNRTI-based antiretroviral therapy in HIV-infected individuals, protease inhibitors led to clear inhibition of aspartic protease secretion of *Candida*, associated with clinical resolution of oral candidiasis.[52] Recent observations that boosted lopinavir can reduce clinical malaria in Ugandan children supports the notion that HIV-1 protease inhibitors could exert clinically relevant antiprotozoal effects.[13] These findings were not confirmed in a multicountry study involving adults with HIV infection;[53,54] however, the study consisted of a secondary analysis of HIV trial data in adults. By contrast, the Ugandan study was specifically designed to assess the effect of HIV-1 protease inhibitors on malaria in children. The Ugandan study documented drug interactions between antimalarials and HIV protease inhibitors, with increased concentrations of lumefantrine associated with protease inhibitor use. Studies of drug interactions between antileishmanial drugs and antiretrovirals, particularly HIV-1 protease inhibitors, are lacking. In view of the prevalence of tuberculosis infection in east Africa, studies of protease inhibitor interactions with antituberculosis drugs might also be warranted. In conclusion, there is substantial experimental evidence of inhibitory effects of HIV-1 protease inhibitors on several infectious pathogens; however, most of these effects have not been assessed in rigorous clinical studies, so the clinical relevance of these observations is unclear.

Panel 2: **Current evidence of antileishmanial effect of HIV-1 protease inhibitors**

- Inhibitory effect of HIV-1 protease inhibitors on intracellular infection shown for several *Leishmania* species and strains, including field strains with resistance against pentavalent antimonials; inhibitory effect also observed in an HIV-co-infection model
- No convincing evidence of the clinical relevance of these antileishmanial effects
- Mechanism of inhibitory effect partly explained and includes inhibition of parasite peptidase, induction of oxidative stress, mitochondrial dysfunction, and apoptosis; more studies on mechanism necessary, particularly on host cellular mechanisms
- Antileishmanial effect is observed at low micromolar doses, whereas antiviral effect occurs in the nanomolar range; with current drug dosing recommendations for antiretroviral therapy, the lower micromolar range is reached
- Nelfinavir-resistant strains were readily induced in an experimental set-up, at subtherapeutic protease inhibitor concentrations
- Increased expression of peptidases related to stress and virulence seen in *L amazonensis* in experimental settings
- Lopinavir was effective against *L amazonensis*, but showed only limited activity against *L donovani* in the macrophage model

Some studies have suggested an increased risk of immune reconstitution inflammatory syndrome associated with protease inhibitors in patients with both tuberculosis and HIV. This theory might merit assessement when treating patients with both visceral leishmaniasis and HIV, although data suggest that immune reconstitution inflammatory syndrome associated with visceral leishmaniasis is very scarce and not associated with complications such as those seen with tuberculosis co-infection.[4]

Key questions and knowledge gaps

Although some evidence supports the use of boosted lopinavir or other HIV-1 protease inhibitors for the treatment of visceral leishmaniasis in HIV-co-infected patients (panel 2), clinical evidence is lacking. A few key concerns need to be highlighted. The antileishmanial effect of HIV-1 protease inhibitors in laboratory studies is observed at doses around 1000 times higher (micromolar range) than doses required for the antiviral effect (nanomolar range). Detailed evaluation of the efficacy and dose–response relation of HIV protease inhibitors should be done in animal studies to help define whether antileishmanial effects could be expected in human beings at clinically achievable doses. However, drugs such as miltefosine and antimonials have proven highly effective in clinical use despite limited effects in the macrophage model (at the micromolar range).[26] For *Plasmodium* spp and *C albicans*, some evidence suggests that the inhibitory effects of HIV-1 protease inhibitors observed at the micromolar range in an experimental setting can yield clinical effects. The concentration of protease inhibitors within macrophages in patients receiving antiretroviral therapy is not well known; assessment of patients receiving such treatment according to current dosing recommendations shows that protease inhibitors are present in plasma in the lower micromolar range.

With the availability of boosted lopinavir in most of the countries where visceral leishmaniasis is endemic, including east Africa, studies assessing the effect of lopinavir on *L donovani* are of key interest—unfortunately, such studies are scarce. Extensive screening of a broad range of HIV-1 protease inhibitors (particularly second and third generations) needs to be done. No studies so far have assessed atazanivir activity against *L donovani*; atazanivir is a protease inhibitor recommended by WHO as an alternative for second-line antiretroviral therapy in low-income and middle-income countries, which is increasingly available worldwide. More extensive assessment of the underlying mechanisms of action of protease inhibitors against leishmania parasites is also needed. If antileishmanial activity of specific protease inhibitors could be consistently shown at clinically achievable concentrations, particularly when tested on intracellular stages, these drugs could be taken forward for further development in animal studies.

Adverse events and toxic effects associated with HIV-1 protease inhibitors have been extensively documented in

human beings; however, their effect on parasites should be further elucidated before widespread clinical use against visceral leishmaniasis. Recent data have highlighted the potential of leishmania parasites to adapt to drug pressure. With antimonials, resistance has been associated with increased parasite virulence and resistance to oxidative stress.[31,32] This observation of increased expression of virulence factors upon exposure to a protease inhibitor requires further follow-up. Rapid emergence of drug resistance or associated changes in parasite virulence could be an issue if only borderline therapeutic drug concentrations are reached with clinical use of protease inhibitors, or during periods of suboptimum antiretroviral treatment.

Potential clinical scenarios

If HIV protease inhibitors are effective at clinically achievable concentrations in animal studies, their antileishmanial effect could be investigated in clinical scenarios (figure). Currently, no HIV-1 protease inhibitors are recommended for clinical use in visceral leishmaniasis, but should be seen as potential candidates pending further experimental and clinical studies. First, standard antileishmanial treatment could be combined with protease-inhibitor-based rather than NNRTI-based antiretroviral therapy, to improve initial treatment response by adding another compound with antileishmanial effects. Additionally, protease inhibitors could act as secondary prophylaxis to prevent relapse of visceral leishmaniasis. Combination with another antileishmanial drug to prevent resistance might also be indicated. Alternatively, these drugs could prove an invaluable option for patients who are clinically and parasitologically unresponsive, even to extended treatment courses of antileishmanials. Such patients currently have no therapeutic options and face a dire prognosis. In this scenario, HIV protease inhibitors—as part of antiretroviral therapy—could act as maintenance therapy, with the aim to curtail parasite replication and increase survival pending immunological recovery. Rapid emergence of protease-inhibitor-resistant parasites in patients with overt parasite replication is a potential risk that has to be considered.

Finally, protease inhibitors might also be valuable as primary prophylaxis for HIV-infected individuals with asymptomatic leishmania infection who are at risk of developing visceral leishmaniasis. Use of protease inhibitors in this scenario could be restricted to high-risk patients (advanced HIV infection with low CD4 counts), or to those with indications of ongoing parasite replication. Such pre-emptive therapeutic approaches in HIV-infected individuals are feasible and potentially effective, as shown in individuals screened for cryptococcal infection.[55] Such screening relies on a new, highly sensitive urine antigen test and entails extended treatment with oral drugs for individuals with asymptomatic cryptococcal infection (positive urine antigen test). Development of an improved leishmania antigen urine test would be essential for such an approach.[56] For all indications mentioned above, protease inhibitors could be replaced by NNRTIs once CD4 cell counts have increased sufficiently; such an approach might optimise the cost-effectiveness of protease inhibitor use, in view of their high cost.

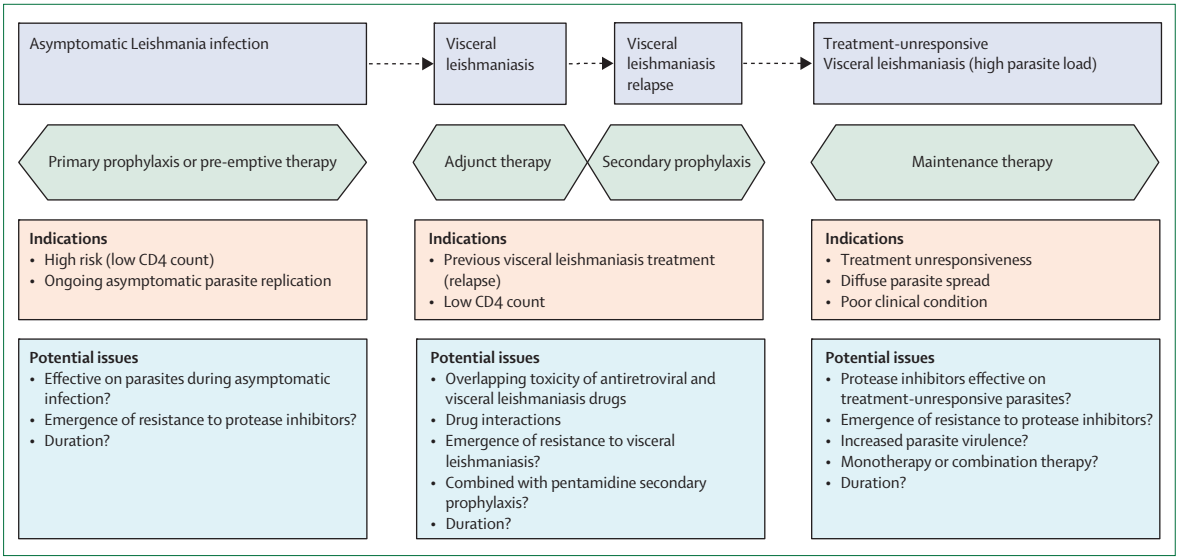

***Figure*: Stages of co-infection with visceral leishmaniasis and HIV and potential use of HIV-1 protease inhibitors**

HIV-infected individuals co-infected with the leishmania parasite have an increased risk of progression to visceral leishmaniasis. Treatment of visceral leishmaniasis is complicated by high rates of treatment failure and relapse. Individuals co-infected with HIV can develop unresponsiveness to repeated visceral leishmaniasis treatment, with persistent diffuse parasite spread. Protease inhibitors given at any stage of infection should be combined with two other antiretroviral drugs, as is standard for HIV treatment. HIV-1 protease inhibitors are not currently recommended as treatment for visceral leishmaniasis in HIV co-infection, but should be seen as a potential therapeutic approach pending further experimental and clinical studies.

Search strategy and selection criteria

Articles cited in this Review were obtained through searches of PubMed and Medline with terms "visceral leishmaniasis", "cutaneous leishmaniasis", "leishmaniasis", "kala azar", "hiv", "protease", "peptidase", "proteinase", "treatment", "therapy", "therapeutic use", "antiretroviral treatment", "efficacy", and "outcome". We did not limit our search by language, and the last search was done on Nov 15, 2012. Reference lists of selected articles were searched to identify other relevant publications. Clinical trial websites were verified. Abstracts of recent international conferences on infectious diseases were also reviewed. Review papers were cited if they provided comprehensive overviews beyond the scope of this Review.

Clinical use of HIV protease inhibitors against visceral leishmaniasis should be investigated in view of the key role of these drugs in HIV treatment. The advantages and disadvantages of short-term versus long-term use of HIV-1 protease inhibitors to improve management of visceral leishmaniasis in individuals with HIV co-infection should be assessed. In most countries, patients with co-infection are a small fraction of the overall visceral leishmaniasis burden (eg, in Sudan and the Indian subcontinent) or of the total population with HIV (eg, in Ethiopia). Therefore, at the national level, treatment of co-infection with HIV-1 protease inhibitors would probably involve several tens to several hundreds of patients per year, depending on the indications used. The higher drug cost of HIV-1 protease inhibitors compared with standard treatment (NNRTIs) might be offset by improved treatment outcomes with regard to visceral leishmaniasis and reduced need for treatment and hospitalisation.

The aims of research and development should be to design and screen new compounds specifically targeting the leishmanial aspartic protease, and should be extended to other proteases. Serine and cysteine proteases have already been explored as drug targets for several neglected tropical diseases, including leishmaniasis, with some compounds in preclinical development.[57–59]

Conclusions

HIV-1 protease inhibitors are the backbone of second-line HIV therapy in low-income and middle-income countries. In-vitro studies have consistently documented the effect of HIV-1 protease inhibitors on *Leishmania* spp parasites, and the underlying mechanism is at least partly explained. These results argue for broader use of boosted lopinavir or other protease inhibitors in regions with endemic visceral leishmaniasis, to improve management of visceral leishmaniasis in HIV co-infected individuals. However, additional research is needed before these drugs can be taken forward for clinical use in this context. Because the effect of protease inhibitors against visceral leishmaniasis was generally observed at high drug concentrations, efficacy and dose–response relations should be studied in animals before assessment in clinical trials. More extensive studies with all currently available HIV protease inhibitors should be done. Drug interactions and emergence of drug-resistant parasites should be carefully studied. With the lack of effective visceral leishmaniasis treatment options in HIV-infected individuals in east Africa, and the wide availability of HIV-1 protease inhibitors, the full potential of these drugs should be explored. Specific aspartate protease inhibitors should also be developed.

Contributors

JvG developed the concept of the review, did the literature search, provided figure, tables, and panels, and wrote the first draft. ED, RL-V, EZ, LL, MB, J-CD, and AH reviewed subsequent drafts and provided specific input. All authors read and approved the final manuscript.

Conflicts of interest

We declare that we have no conflicts of interest.

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References

1 van Griensven J, Diro E. Visceral leishmaniasis. *Infect Dis Clin North Am* 2012; **26**: 309–22.
2 van Griensven J, Balasegaram M, Meheus F, et al. Combination therapy for visceral leishmaniasis. *Lancet Infect Dis* 2010; **10**: 184–94.
3 Alvar J, Velez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. *PloS One* 2012; **7**: e35671.
4 Alvar J, Aparicio P, Aseffa A, et al. The relationship between leishmaniasis and AIDS: the second 10 years. *Clin Microbiol Rev* 2008; **21**: 334–59.
5 Ritmeijer K, Dejenie A, Assefa Y, et al. A comparison of miltefosine and sodium stibogluconate for treatment of visceral leishmaniasis in an Ethiopian population with high prevalence of HIV infection. *Clin Infect Dis* 2006; **43**: 357–64.
6 Sinha PK, van Griensven J, Pandey K, et al. Liposomal amphotericin B for visceral leishmaniasis in human immunodeficiency virus-coinfected patients: 2-year treatment outcomes in Bihar, India. *Clin Infect Dis* 2011; **53**: e91–98.
7 Ritmeijer K, Ter Horst R, Chane S, et al. Limited effectiveness of high-dose liposomal amphotericin B (AmBisome) for treatment of visceral leishmaniasis in an Ethiopian population with high HIV prevalence. *Clin Infect Dis* 2011; **53**: e152–58.
8 Ter Horst R, Collin SM, Ritmeijer K, Bogale A, Davidson RN. Concordant HIV infection and visceral leishmaniasis in Ethiopia: the influence of antiretroviral treatment and other factors on outcome. *Clin Infect Dis* 2008; **46**: 1702–09.
9 Pozio E, Morales MA. The impact of HIV-protease inhibitors on opportunistic parasites. *Trends Parasitol* 2005; **21**: 58–63.
10 Valdivieso E, Rangel A, Moreno J, et al. Effects of HIV aspartyl-proteinase inhibitors on *Leishmania* sp. *Exp Parasitol* 2010; **126**: 557–63.
11 Savoia D, Allice T, Tovo PA. Antileishmanial activity of HIV protease inhibitors. *Int J Antimicrob Agents* 2005; **26**: 92–94.
12 Trudel N, Garg R, Messier N, Sundar S, Ouellette M, Tremblay MJ. Intracellular survival of *Leishmania* species that cause visceral leishmaniasis is significantly reduced by HIV-1 protease inhibitors. *J Infect Dis* 2008; **198**: 1292–99.
13 Achan J, Kahuru A, IkileziG, et al. Significant reduction in risk of malaria among HIV+ children receiving lopinavir/ritonavir-based ART compared to NNRTI-based ART, a randomized open-label trial. 19th Conference on Retroviruses and Opportunistic Infections (CROI); Seattle, WA, USA; March 5–8, 2012. Abstract 26.
14 De Clercq E. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. *Int J Antimicrob Agents* 2009; **33**: 307–20.
15 Wensing AM, van Maarseveen NM, Nijhuis M. Fifteen years of HIV protease inhibitors: raising the barrier to resistance. *Antiviral Res* 2010; **85**: 59–74.

16 Mastrolorenzo A, Rusconi S, Scozzafava A, Barbaro G, Supuran CT. Inhibitors of HIV-1 protease: current state of the art 10 years after their introduction. From antiretroviral drugs to antifungal, antibacterial and antitumor agents based on aspartic protease inhibitors. *Curr Med Chem* 2007; **14**: 2734–48.

17 WHO. Antiretroviral therapy for HIV infection in adults and adolescents: recommendations for a public health approach, 2006 Revision. Geneva: World Health Organization, 2006.

18 WHO. Antiretroviral therapy for HIV infection in adults and adolescents: recommendations for a public health approach, 2010 Revision. Geneva: World Health Organization, 2010.

19 Oldfield V, Plosker GL. Lopinavir/ritonavir: a review of its use in the management of HIV infection. *Drugs* 2006; **66**: 1275–99.

20 WHO. Scaling up antiretroviral therapy in resource-limited settings: treatment guidelines for a public health approach, 2003 revision. Geneva: World Health Organization, 2004.

21 Kumar P, Lodge R, Trudel N, Ouellet M, Ouellette M, Tremblay MJ. Nelfinavir, an HIV-1 protease inhibitor, induces oxidative stress-mediated, caspase-independent apoptosis in leishmania amastigotes. *PLoS Negl Trop Dis* 2010; **4**: e642.

22 Santos LO, Vitorio BS, Branquinha MH, e Silva CMP, Santos ALS, d'Avila-Levy CM. Nelfinavir is effective in inhibiting the multiplication and aspartic peptidase activity of *Leishmania* species, including strains obtained from HIV-positive patients. *J Antimicrob Chemother* 2012; published online Oct 28. DOI:10.1093/jac/dks410.

23 Santos LO, Marinho FA, Altoe EF. HIV aspartyl peptidase inhibitors interfere with cellular proliferation, ultrastructure and macrophage infection of *Leishmania amazonensis*. *PLoS One* 2009; **4**: e4918.

24 Demarchi IG, Silveira TG, Ferreira IC, Lonardoni MV. Effect of HIV protease inhibitors on New World Leishmania. *Parasitol Int* 2012; **61:** 538–44.

25 White RE, Powell DJ, Berry C. HIV proteinase inhibitors target the Ddi1-like protein of leishmania parasites. *FASEB J* 2011; **25**: 1729–36.

26 Vermeersch M, da Luz RI, Tote K, Timmermans JP, Cos P, Maes L. In vitro susceptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. *Antimicrob Agents Chemother* 2009; **53**: 3855–59.

27 Dash C, Kulkarni A, Dunn B, Rao M. Aspartic peptidase inhibitors: implications in drug development. *Crit Rev Biochem Mol Biol* 2003; **38**: 89–119.

28 Krylov DM, Koonin EV. A novel family of predicted retroviral-like aspartyl proteases with a possible key role in eukaryotic cell cycle control. *Curr Biol* 2001; **11**: 584–87.

29 Sirkis R, Gerst JE, Fass D. Ddi1, a eukaryotic protein with the retroviral protease fold. *J Mol Biol* 2006; **364**: 376–87.

30 Perteguer MJ, Gomez-Puertas P, Canavate C, Dagger F, Garate T, Valdivieso E. Ddi1-like protein from *Leishmania major* is an active aspartyl proteinase. *Cell Stress Chaperones* 2012; published online Aug 30. DOI:10.1007/s12192-012-0368-9.

31 Vanaerschot M, Maes I, Ouakad M, et al. Linking in vitro and in vivo survival of clinical *Leishmania donovani* strains. *PLoS One* 2010; **5**: e12211.

32 Decuypere S, Vanaerschot M, Brunker K, et al. Molecular mechanisms of drug resistance in natural leishmania populations vary with genetic background. *PLoS Negl Trop Dis* 2012; **6**: e1514.

33 Moreira W, Leprohon P, Ouellette M. Tolerance of drug-induced cell death favours the acquisition of multidrug resistance in leishmania. *Cell Death Dis* 2011; **2:** e201–08.

34 Den Boer M, Rivas L, Alvar J. Co-infection with HIV. In: Ponte-Sucre A, Diaz E, Padron-Nieves M, eds. Drug resistance in leishmania parasites. Consequences, molecular mechanisms and possible treatments. Vienna: Springer, 2012: 167–82.

35 Piccinini M, Rinaudo MT, Chiapello N, et al. The human 26S proteasome is a target of antiretroviral agents. *AIDS* 2002; **16**: 693–700.

36 Robertson CD. The *Leishmania mexicana* proteasome. *Mol Biochem Parasitol* 1999; **103**: 49–60.

37 Christensen CB, Jorgensen L, Jensen AT, et al. Molecular characterization of a *Leishmania donovanii* cDNA clone with similarity to human 20S proteasome a-type subunit. *Biochim Biophys Acta* 2000; **1500**: 77–87.

38 Andre P, Groettrup M, Klenerman P, et al. An inhibitor of HIV-1 protease modulates proteasome activity, antigen presentation, and T cell responses. *Proc Natl Acad Sci USA* 1998; **95**: 13120–24.

39 Deeks SG. Protease inhibitors as immunomodulatory drugs for HIV infection. *Clin Pharmacol Ther* 2007; **82**: 248–50.

40 Vlahakis SR, Bennett SA, Whitehead SN, Badley AD. HIV protease inhibitors modulate apoptosis signaling in vitro and in vivo. *Apoptosis* 2007; **12**: 969–77.

41 Vlahakis SR, Bren GD, Algeciras-Schimnich A, et al. Flying in the face of resistance: antiviral-independent benefit of HIV protease inhibitors on T-cell survival. *Clin Pharmacol Ther* 2007; **82**: 294–99.

42 Gruber A, Wheat JC, Kuhen KL, Looney DJ, Wong-Staal F. Differential effects of HIV-1 protease inhibitors on dendritic cell immunophenotype and function. *J Biol Chem* 2001; **276**: 47840–43.

43 Bugelski PJ, Kaplan JM, Hart TK, et al. Effect of a human immunodeficiency virus protease inhibitor on human monocyte function. *AIDS Res Hum Retroviruses* 1992; **8**: 1951–58.

44 Riddler SA, Haubrich R, DiRienzo AG, et al. Class-sparing regimens for initial treatment of HIV-1 infection. *N Engl J Med* 2008; **358**: 2095–106.

45 MacArthur RD, Novak RM, Peng G, et al. A comparison of three highly active antiretroviral treatment strategies consisting of non-nucleoside reverse transcriptase inhibitors, protease inhibitors, or both in the presence of nucleoside reverse transcriptase inhibitors as initial therapy (CPCRA 058 FIRST Study): a long-term randomised trial. *Lancet* 2006; **368**: 2125–35.

46 Del Giudice P, Mary-Krause M, Pradier C, et al. Impact of highly active antiretroviral therapy on the incidence of visceral leishmaniasis in a French cohort of patients infected with human immunodeficiency virus. *J Infect Dis* 2002; **186**: 1366–70.

47 Villanueva JL, Alarcon A, Bernabeu-Wittel M, et al. Prospective evaluation and follow-up of European patients with visceral leishmaniasis and HIV-1 coinfection in the era of highly active antiretroviral therapy. *Eur J Clin Microbiol Infect Dis* 2000, **19**: 798–901.

48 De La Rosa R, Pineda JA, Delgado J, et al. Influence of highly active antiretroviral therapy on the outcome of subclinical visceral leishmaniasis in human immunodeficiency virus-infected patients. *Clin Infect Dis* 2001; **32**: 633–35.

49 de La Rosa R, Pineda JA, Delgado J, et al. Incidence of and risk factors for symptomatic visceral leishmaniasis among human immunodeficiency virus type 1-infected patients from Spain in the era of highly active antiretroviral therapy. *J Clin Microbiol* 2002; **40**: 762–67.

50 Casado JL, Lopez-Velez R, Pintado V, et al. Relapsing visceral leishmaniasis in HIV-infected patients undergoing successful protease inhibitor therapy. *Eur J Clin Microbiol Infect Dis* 2001; **20**: 202–5.

51 Alfonso Y, Monzote L. HIV protease inhibitors: effect on the opportunistic protozoan parasites. *Open Med Chem J* 2011; **5**: 40–50.

52 Cassone A, Tacconelli E, De Bernardis F, et al. Antiretroviral therapy with protease inhibitors has an early, immune reconstitution-independent beneficial effect on candida virulence and oral candidiasis in human immunodeficiency virus-infected subjects. *J Infect Dis* 2002; **185**: 188–95.

53 Skinner-Adams TS, Butterworth AS, Porter KA, et al. The frequency of malaria is similar among women receiving either lopinavir/ritonavir or nevirapine-based antiretroviral treatment. *PLoS One* 2012; **7**: e34399.

54 Porter KA, Cole SR, Eron JJ, et al. HIV-1 protease inhibitors and clinical malaria: a secondary analysis of the AIDS Clinical Trials Group A5208 study. *Antimicrob Agents Chemother* 2012; **56**: 995–1000.

55 Rajasingham R, Meya DB, Boulware DR. Integrating cryptococcal antigen screening and pre-emptive treatment into routine HIV care. *J Acquir Immune Defic Syndr* 2012; **59**: e85–91.

56 Hommel M, Sarkari B, Carney J, Chance ML. Katex for the diagnosis of human visceral leishmaniasis. *Med Trop* 2001; **61**: 503–5.

57 McKerrow JH, Rosenthal PJ, Swenerton R, Doyle P. Development of protease inhibitors for protozoan infections. *Curr Opin Infect Dis* 2008; **21**: 668–72.

58 Silva-Lopez RE, Morgado-Diaz JA, Chavez MA, Giovanni-De-Simone S. Effects of serine protease inhibitors on viability and morphology of *Leishmania* (*Leishmania*) *amazonensis* promastigotes. *Parasitol Res* 2007; **101**: 1627–35.

59 Williams RA, Tetley L, Mottram JC, Coombs GH. Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*. *Mol Microbiol* 2006; **61**: 655–74.