Protocol

This trial protocol has been provided by the authors to give readers additional information about their work.

Protocol for: Le T, Van Kinh N, Cuc NTK, et al. A trial of itraconazole or amphotericin B for HIV-associated talaromycosis. N Engl J Med 2017;376:2329-40. DOI: 10.1056/NEJMoa1613306

A Randomized, Open-Label, Comparative Study of the Effectiveness of Itraconazole versus Amphotericin B in the Induction Treatment of Penicilliosis in HIV-Infected Adults (IVAP)

This supplement contains the following items:

Contents

Section 1 – IVAP original protocol

A Randomized, Open-Label, Comparative Study of the Effectiveness of Itraconazole versus Amphotericin B in the Induction Treatment of Penicilliosis in HIV-Infected Adults

Itraconazole versus Amphotericin B for the Treatment of Penicilliosis

Lead Principal Investigator (Lead PI):

Nguyen Van Kinh, MD PhD
National Hospital for Tropical Diseases, Hanoi

Thuy Le, MD
Oxford University Clinical Research Unit, Viet Nam
University of Hawaii at Manoa, Honolulu, Hawaii USA

Principal Investigator (PI):

Ngo T Kim Cuc, MD
Hospital for Tropical Diseases, Ho Chi Minh City

Pham Thanh Thuy, MD
Bach Mai hospital, Ha Noi

Doan T Hong Hanh, MD
Vietnam-Sweden Uong Bi Hospital, Quang Ninh

Pham Hanh Phuc, MD
Viet Tiep General Hospital, Hai Phong

Co-Investigators:

Nguyen Tran Chinh, MD PhD
Hospital for Tropical Diseases, Ho Chi Minh City

Nguyen Huu Chi, MD
Hospital for Tropical Diseases, Ho Chi Minh City

Vo Minh Quang, MD
Hospital for Tropical Diseases, Ho Chi Minh City

Nguyen Tien Lam, MD
National Hospital for Tropical Diseases, Hanoi

Cao Thi Thanh Thuy, MD
National Hospital for Tropical Diseases, Hanoi

Hoang Dang Mich, Associate Professor, PhD, MD
Viet Tiep General Hospital, Hai Phong

Pham Si Lam, MD
Oxford Univerity Clinical Research Unit, Vietnam

Jeremy Day, MD
Oxford University Clinical Research Unit, Vietnam

Marcel Wolbers, PhD
Oxford University Clinical Research Unit, Vietnam

Heiman Wertheim, MD PhD
Oxford University Clinical Research Unit in Ha Noi

Mattias Larsson, MD
Oxford University Clinical Research Unit in Ha Noi

Alastair Gray, Prof
Health Economic Research Centre at Oxford University, Oxford, UK

Cecilia Shikuma, MD
Hawaii Center of AIDS, Honolulu, Hawaii, USA

Jeremy Farrar, MD PhD
Oxford University Clinical Research Unit, Viet Nam

Study statistician:
Marcel Wolbers, PhD.
Oxford University Clinical Research Unit, Viet Nam

Study Sponsor:
University of Oxford

Study Sites:
Hospital for Tropical Diseases, Ho Chi Minh City
190 Ben Ham Tu, District 5
Ho Chi Minh City, Viet Nam

National Hospital for Tropical Diseases, Hanoi
78 Giai Phong Street
Hanoi, Viet Nam

Bach Mai Hospital, Hanoi
78 Giai Phong Street
Hanoi, Viet Nam

Vietnam-Sweden Uong Bi Hospital, Quang Ninh

Thanh Son, Uong Bi, Quang Ninh, Viet Nam

Viet Tiep General Hospital
1 Nha Thuong, Le Chan, Hai Phong, Viet Nam

Study Funders:
The Department of International Development, UK
The Wellcome Trust, UK
The Medical Research Council, UK

Précis

Penicillium marneffei is an emerging endemic pathogenic fungus that can cause a fatal systemic mycosis in patients infected with human immunodeficiency virus (HIV) in areas of Southeast Asia. The mortality rate is close to 100% when diagnosis and treatment are delayed [1]. Since the HIV/AIDS pandemic arrived in Southeast Asia and since the first case of penicilliosis reported in Thailand in 1988, penicilliosis has become one of the most serious and common AIDS-defining illnesses in this region [2]. Increasingly immunocompromised patients in other parts of the world where the disease is not endemic are diagnosed with penicilliosis after traveling to Southeast Asia and the illness has been reported either in patients with recent or very remote travel histories to these areas [3-10].

Despite being one of the most common and fatal opportunistic infection in HIV-infected patients in Southeast Asia for nearly two decades, there has been a complete lack of clinical trials on the treatment of penicilliosis. Treatment choices therefore must be based upon data from case series and non-comparative studies. The most objective evidence came from a study by Supparatpinyo et al. who described treatment responses (defined by absence of fungal growth and resolution of clinical signs and symptoms) in a series of 80 HIV-infected patients with disseminated penicilliosis. Antifungal choices were at the discretion of clinicians without prior knowledge of antifungal susceptibility testing. Response rates were 77% for amphotericin B, 75% for itraconazole, and 36% for fluconazole [1]. A few years later the same group described a case series of 74 HIV-infected patients with penicilliosis treated with intravenous amphotericin B 0.6 mg/kg/day for 2 weeks followed by oral itraconazole 400 mg/day for 10 weeks [11]. The treatment response rate (defined by negative blood culture and resolution of fever and skin lesions at the end of 12 weeks therapy) was 97%. Remarkably there was only one death. Unfortunately this has not been the experience in Vietnam and elsewhere in Southeast Asia. The basis for choosing intravenous amphotericin B for initial therapy followed by oral itraconazole as maintenance therapy and the reported treatment success rate need to be subjected to clinical trials rather than be accepted currently as the “standard of care”.

Amphotericin B is an expensive drug for most patients at risk of penicilliosis. The need for intravenous access and side effect monitoring requires hospitalization, which adds to the cost burden of patients. By comparison, oral itraconazole is more tolerable and is readily available at a fraction of the price. Itraconazole has been shown to be at least as efficacious and is better tolerated compared to amphotericin B in the empirical treatment of febrile neutropenia [12]. Further, itraconazole (in various formulations) has been shown in case series to effectively treat other serious systemic fungal infections such as invasive aspergillosis, coccidioidomycosis, cryptococcosis, and blastomycosis [13-21]. For this reason physicians in Thailand, Burma, India, and Vietnam often use itraconazole alone in patients who either cannot afford amphotericin B therapy or are able to be treated as outpatient and anecdotally report comparable success rates compared to amphotericin B (personal communications: Nicolas White, MD., Former Director of Wellcome Trust Mahidol University Oxford in Thailand; Nguyen Huu Chi, MD., Director of HIV for inpatients at the Hospital for Tropical Diseases (HTD); and Vo Minh Quang, MD. Director of HTD’s outpatient HIV clinic). Indeed, Ranjana et al. recently reported a success rate of 97% using itraconazole alone at 400 mg/d for 3-4 weeks from India (n=50) [22].

The vast majority of patients with penicilliosis are able to take oral medication. The capsule formulation of itraconazole is the only formulation widely available in pharmacies across Asia. Itraconazole oral suspension was developed (co-formulated with cyclodextrin) to improve the

bioavailability of the capsule formulation, resulting in 30% increase in the area under the curve (AUC) [23]. This formulation however is not widely available and is associated with nausea due to cyclodextrin's osmotic effect, which may affect compliance and potentially be counter-productive in the goal to improve bioavailability [24].

We aim to conduct a randomized, open-label, comparative non-inferiority trial of the efficacy and safety of itraconazole versus amphotericin B for the acute-phase treatment of penicilliosis. If our hypothesis is correct, that itraconazole is at least as effective as amphotericin B, it becomes difficult to justify using amphotericin B in most areas of Southeast Asia where cost has a major role in the therapeutic decision process. However if our hypothesis is incorrect, that amphotericin B is found to be more effective than itraconazole, then there will be empirical evidence for Ministries of Health and policy makers across Asia to make amphotericin B more widely available and affordable. This study provides opportunities to investigate the microbiologic and pharmacokinetic basis for observed efficacies from the 2 antifungal regimens. The questions whether time to negative fungal blood culture and/or whether early fungicidal activities do correlate with treatment outcomes are relevant both to clinicians as well as clinical trial investigators studying fungal diseases. Population kinetic models for the 2 antifungal drugs will be constructed and pharmacokinetic variables such as peak/trough serum drug concentration, area under the curve in a drug concentration versus time analysis, and drug minimal inhibitory concentration (MIC) will be correlated with microbiological and treatment outcomes. These results will further implement treatment strategies for this infection.

1 Background

Introduction

Penicillium marneffei is an emerging endemic pathogenic fungus that can cause a fatal systemic mycosis in patients infected with human immunodeficiency virus (HIV) and advanced acquired immunodeficiency syndrome (AIDS) in areas of Southeast Asia. The mortality rate is close to 100% if left untreated or when diagnosis and treatment are delayed [1]. Since the first case of disseminated penicilliosis was reported in an HIV-positive patient in Thailand in 1988, penicilliosis has become the third most common AIDS-defining illness (after tuberculosis and cryptococcosis) in Northern Thailand [2]. Penicilliosis has been reported from Northeast India across Myanmar, Thailand, Cambodia, Viet Nam, Taiwan, Hong Kong, southern China to Malaysia and Indonesia [25]. Increasingly immunocompromised patients in other parts of the world where the disease is not endemic are diagnosed with penicilliosis after traveling to Southeast Asia, and the illness has been reported either in patients with recent or very remote travel histories to these areas [3-5].

Epidemiology

Penicillium marneffei was first isolated by Segretain from hepatic lesions of a captive bamboo rat (*Rhizomys sinensis*) used for experimental infections at the Pasteur Institute in Dalat, Vietnam in 1956. The bamboo rat died spontaneously from the reticuloendothelial mycosis [26]. The fungus was named *Penicillium marneffei* in honor of Hubert Marneffei, Director of the Pasteur Institute of Vietnam. Human penicilliosis was first described by Segretain himself after pricking his own finger with a needle filled with *P. marneffei* used to inoculate hamsters [27]. He developed a small nodule at the site of inoculation with maxillary lymphadenopathy. The infection was cured by 30 days of oral nystatin. Fourteen years later Di Salvo reported the first disseminated penicilliosis in 1973 in a US missionary with Hodgkin's disease who lived in South Carolina and had traveled through Southeast Asia [28]. The patient had recurrent hemoptysis and underwent pneumonectomy. Pathology showed granuloma with yeast-like cells on tissue sections, and *P. marneffei* grew on culture. The same year 5 more cases were reported from Bangkok, Thailand. The rarity of human penicilliosis changed when the HIV pandemic arrived in Southeast Asia. In 1988, cases of *P. marneffei* infection were first being observed in patients with advanced AIDS. *P. marneffei* has now become the third most common AIDS-defining illness (after tuberculosis and cryptococcosis) in Northern Thailand [2].

The only known natural hosts are bamboo rats (*Rhizomys* and *Cannomys* species) and humans [29-32]. *P. marneffei* can be isolated from the soil around bamboo rats' burrows, though only rarely from other environmental sources [33]. The exact route of acquisition in humans is unknown but it is thought unlikely to be from direct contact with the rodents and presumed to be via inhalation and, rarely, inoculation [34]. In Thailand human infection is seasonal – particularly coinciding with rainy seasons – and has been associated with soil exposure [34, 35]. There is no evidence of person-to-person spread. Infections have been described solely in those exposed in Asia except for one case in an HIV-infected African male with no such travel history [36]. It has become the third most common HIV-related opportunistic infection in Southeast Asia – accounting for 15% of all HIV-related illness in Northern Thailand [2], affecting 10% of the AIDS patients in Hong Kong [37], and is the second most common single pathogen isolated from blood cultures in the Hospital for Tropical Diseases (HTD), Ho Chi Minh City, Viet Nam after *Cryptococcus neoformans*. Patients with cellular immune deficiency are at risk for this

disease. Patients with advanced AIDS tend to develop disseminated disease at CD4 count <50 cells//µL. Despite more than a decade of research efforts, the natural reservoir and vehicle of transmission of *P. marneffei*, whether it is a zoonosis or a sapronosis, remains perplexing.

Microbiology

P. marneffei is the only known *Penicillium* species that exhibits temperature-dependent dimorphic feature. At 25^{o}C the fungus grows as mycelia (a mold) with septate hyphae that bear conidiophores and conidia (similar to *Aspergillus* spp), producing a deep wine red, water-soluble pigment that diffuses into the Sabouraud agar medium. This feature is similar to other 220 Penicillium species; of those 8 species are known to be pathogenic. At 37^{o}C on artificial medium or in human tissue, *P. marneffei* converts to yeast-like spherical that multiplies by binary fission and not budding. The fission yeast cells represent the parasitic form of *P. marneffei*. This form is seen in the intracellular infection of the macrophages. The mold to yeast transformation or phase transition, which is thermally regulated, is a diagnostic characteristic of *P. marneffei* and is thought to be the key factor in its' virulence.

Clinical Features

Patients with penicilliosis have various manifestations and degrees of severity. Common clinical presentations include fever, fatigue, weight loss, non productive cough, generalized lymphadenopathy, hepatosplenomegaly, and characteristic skin lesions [1, 11, 38]. CD4 count at presentation is generally less than 50/mm^3. Blood culture is positive in about 88% of patient while skin lesions are present in 85% of patients in one series [11]. Skin lesions tend to be papules with central necrosis, generally referred to as "molluscum-like" lesions on face, neck, oral mucosa, upper more than lower extremities and trunk. The skin lesions are very similar to those seen in disseminated cryptococcosis, and concomitant cryptococcosis (5% in one study in Thailand) and other opportunistic infections are not un-common in patients with penicilliosis. The most common laboratory abnormality is anemia. 76% of patients have hemoglobin level of 10 g/dl or less, but it was not possible to unequivocally attribute anemia to *P. marneffei* alone in patients with late stage HIV. Other reported manifestations include ulcerated oral mucosal lesions [39], consolidated pneumonia or pulmonary nodule [40], hepatic penicilliosis without any skin lesion [41], pericarditis, osteoarticular lesions of ribs, long bones, skull, lumbar vertebrae, scapula, and temporomandibular region [42, 43].

Laboratory Diagnosis

Laboratory diagnosis is currently based on direct microscopic identification of the fungus with confirmation by culture, though there has been increasing interest in the use of immunodiagnostics and molecular assays.

1.1.1 Microscopy & Culture

Microscopically *P. marneffei* can be seen as oval or round intracellular and extracellular yeasts in biopsies of cutaneous lesions, bone marrow, lymph node, liver and blood smear using Wright, Wright-Giemsa, or Gomori-Grocott methenamine (GMS) stains. More rarely, the infection has been diagnosed directly from sputum, pleural fluid, cerebro-spinal fluid, pericardium, stool, urine and fine needle aspirates of lymph nodes [2, 44, 45]. *P. marneffei* has characteristic central septate or cross-wall formation that is essentially diagnostic. The differential diagnosis of such

intracellular yeasts include histoplasmosis (which also has similar clinical presentations), cryptococcosis (which is associated with more neurological symptoms and less respiratory involvement, lymphadenopathy and hepatosplenomegaly), and *Candida glabrata* [46, 47].

Unlike many other endemic dimorphic fungi, *P. marneffei* grows readily in standard media and Sabouraud dextrose agar and can take up to 4-14 days. The classical culture characteristics of thermal dimorphism and the production of red pigment are easily demonstrated. Bone marrow, blood, and biopsies of skin lesions all have high culture yield (100%, 76%, and 90% respectively) [48].

1.1.2 Immunodiagnosis

Various methods have been developed assessing host antibody production (such as immunoblot, indirect fluorescent antibody test [IFAT], latex agglutination, and enzyme-linked immunosorbent assay [ELISA]); however they have so far been studied on only small numbers of patients or there have been issues with sensitivity and specificity [49-51]. There has been recent interest in detecting circulating galactomannan. The *Penicillium* galactomannan has considerable homology to that of *Aspergillus* and commercial assays for the detection of the latter have recently been investigated in *P. marneffei* infection. Sera from 11 of 15 culture confirmed penicilliosis cases were positive though 9% of HIV positive controls were apparent false positives [52].

1.1.3 Urinary Antigen Assay

An ELISA test for detection of *P. marneffei* antigen in urine has been developed and prospectively evaluated in 33 HIV-positive Thai patients with culture-confirmed *P. marneffei* and 248 patients with other diagnoses [53]. This ELISA detected *P. marneffei* antigen in the urine samples of all 33 (100%) patients with penicilliosis with a median titer of 1:20,480. *P. marneffei* was not detected in 94% of samples from healthy volunteer; however it was detected in 27% of 248 urine samples from inpatients with diagnoses other than penicilliosis (include cryptococcosis, melioidosis, and other bacteria septicemia). Sensitivity and specificity for this assay to detect penicilliosis at a cut off titer of 1:40 was 97% and 98% with the positive predictive value of 84.2% and negative predictive value of 99.7%.

The same polyclonal hyperimmune IgG was used to develop a simplified dot blot ELISA and a latex agglutination test for detecting *P. marneffei* antigenuria and prospectively evaluated in urine specimens from 37 patients with culture proven penicilliosis and 300 controls (52 healthy and 248 hospitalized patients without penicilliosis). The sensitivities for ELISA, dot blot ELISA, and agglutination test were 97.3%, 94.6%, and 100% respectively; specificities were 98%, 97.3%, and 99.3%, respectively. Of these 3 promising tests, the agglutination test seems to be the simplest, most rapid and robust and needs to be validated in larger prospective cohort studies for both diagnostic purpose and for use as a surrogate marker of treatment response and treatment relapse.

1.1.4 Molecular Diagnosis

Polymerase chain reaction (PCR) assays, detecting fungal DNA in blood samples, have been developed. High sensitivity and specificity have been reported. However the protocols remain labor (and equipment) intensive and they have yet to enter routine clinical practice [54].

Treatment

Disseminated penicilliosis has a high mortality if untreated. All 9 patients who were not treated died from disseminated disease in an early series [1]. In vitro *P. marneffei* is highly sensitive to itraconazole, ketoconazole, miconazole, voriconazole, terbinafine, and 5-flourocytosine - intermediately sensitive to amphotericin B but largely resistant to fluconazole [1, 55-58]. No clear data are presently available for the echinocandins, though they may work poorly against the pathogenic yeast phase [59].

1.1.5 Acute infection

There have been no comparative trials on the acute treatment of penicilliosis, and thus treatment choices must be based upon data from case series and in vitro data on antifungal sensitivities. An early case series of 80 consecutive HIV positive Thai patients with penicilliosis described responses to treatment with amphotericin B, itraconazole, or fluconazole. In addition, 30 isolates underwent antifungal sensitivity testing. The failure rates (defined as persistent fungemia, clinical deterioration, or lack of clinical improvement) were 22.8%, 25%, 63.6%, and 100% for amphotericin B, itraconazole, fluconazole, and no treatment respectively. Treatment choice was at the discretion of the attending physician without knowledge of the minimum inhibitory concentration (MIC) of antifungal drugs for the isolates. Consistent with the poorer response to fluconazole, there were consistently higher in vitro MICs for this drug (73% of isolates were classified as borderline susceptible or resistant). 41% of isolates tested for amphotericin B susceptibility were classified as only moderately sensitive or resistant, but despite this the *Penicillium*-attributable death rate was low (12.8%) in patients receiving amphotericin B. All isolates were sensitive to 5-fluorocytosine [1].

1.1.6 Amphotericin B therapy

A subsequent series described 74 HIV patients with disseminated penicilliosis treated with amphotericin B 0.6 mg/kg/day for 2 weeks followed by itraconazole 400 mg/day for 10 weeks [4]. All patients received cotrimoxazole as primary prophylaxis for *Pneumocystis jirovecii*. Remarkably there were no deaths in the study. The treatment success rate (defined by negative blood culture and resolution of fever and skin lesions at the end of 12 weeks therapy) was 97%. It is not clear from the report how this treatment strategy was chosen and the basis for the high success rate compared to early trials. Nevertheless, this treatment regimen has become the “standard of care”.

Unfortunately amphotericin B is a prohibitively expensive drug for most patients at risk of penicilliosis, and the requirement for hospitalization adds to the cost burden to patients. For this reason, physicians in Thailand, Burma, India, and Vietnam in practice use itraconazole alone in patients who either cannot afford amphotericin B therapy or who are clinically stable enough to be treated as outpatient and report comparable success rates compared to amphotericin B (personal communications: Nicolas White, MD. former Director of Wellcome Trust Mahidol University Oxford in Thailand, Nguyen Huu Chi, MD. Former director of HIV inpatient at Hospital for Tropical Diseases (HTD), and Vo Minh Quang, MD. Director of outpatient HIV clinic at HTD).

1.1.7 Itraconazole therapy

In a small case series of 10 HIV-infected Thai patients with penicilliosis who were treated with itraconazole 400 mg/day monotherapy for 2 months, two patients died while on therapy; the

other 8 achieved clinical improvement, but the mean duration to culture negative was unacceptably long at 57 days [60]. A more recent study from India described successful treatment with itraconazole 400 mg/day for 3-4 weeks with a remarkable success rate of 97% (N=40 patients) [22]. However if the number of loss to follow up (N=10) is stringently considered as failure, the success rate is reduced to 78%. Oral itraconazole has been shown to be at least as efficacous and have less side effects compared to amphotericin B in empirical treatment of febrile neutropenia [12]. Further, itraconazole has been shown in case series to effectively treat other serious systemic fungal infections such as invasive aspergillosis, coccidioidomycosis, cryptococcosis, and blastomycosis [13-21]. Unfortunately treatment response rates for different drugs cannot be compared across different studies that employ different study designs and study endpoints.

1.1.8 Secondary prophylaxis

Before the widespread introduction of highly active antiretroviral therapy (HAART) it was recognized that disease relapse rate after initial treatment success is as high as 57% with the median relapse time of 24 weeks [11]. A subsequent randomized, double-blind, placebo-controlled study of itraconazole secondary prophylaxis (200 mg once/day) was discontinued early as all relapses were within the placebo arm [61]. Long term maintenance therapy with itraconazole has since been adopted.

1.1.9 Discontinuation of secondary prophylaxis

Several reports have investigated the discontinuation of itraconazole secondary prophylaxis after immune reconstitution from HAART. However, all have been retrospective observational studies. There were no relapses after itraconazole discontinuation in 33 patients with a CD4 lymphocyte count >100/μL for >6 months who were followed for a median time of 18 months, nor in another study on those stabilized on HAART which unfortunately did not specify CD4 counts [62, 63]. One relapse was described in a series of 19 patients who discontinued prophylaxis at a median CD4 lymphocyte count of 95/μL (18 patients had a CD4 count <200/μL and ten <100/μL) equating to a relapse rate of 1.72/100 patient-years [64]. It therefore appears reasonable to discontinue secondary prophylaxis after significant immune restoration from antiretrovirals, though exact criteria need to be established in larger, prospective, randomized studies.

1.1.10 Primary prophylaxis

The potential for primary prophylaxis for fungal opportunistic infection in advanced HIV patients has been explored with a randomized placebo-controlled double-blinded study of itraconazole (200 mg/day) in those with CD4 lymphocyte counts <200/μL [65]. There was a significant decrease in the incidence of both cryptococcosis and penicilliosis in the intervention group (principally in those with CD4 count <100/μL); however there was no survival advantage to being on itraconazole (though the study was not powered for this end-point). This intervention has not been adopted in clinical practice.

Immune Responses

The mechanism of host-fungus interaction and host immune response to *P. marneffei* are not completely understood. Infection is presumably via inhalation of conidia from the environment;

although this has never been definitely shown. Phagocytic cells are likely the primary line of host defense against this fungus. *P. marneffei* conidia are able to recognize fibronectin and bind to laminin via a sialic acid-specific lectin [66]. This may play an important role in the attachment of conidia to bronchoalveolar epithelia before ingestion by host mononuclear phagocytes. Studies in mouse model have shown that *P. marneffei* can be cleared within 2 to 3 weeks in healthy hosts, whereas in nude mice or in T-cell-depleted mice, *P. marneffei* infection is fatal, demonstrating that T cells, and CD4+ T cells in particular, are necessary for clearing this fungal infection in mice [67]. Recently by use of an in vitro analysis of a sublethal *P. marneffei* infection in BALB/c mice, it was demonstrated that protective immunity follows a Th1 response, with high levels of interleukin-12, IFN-γ, and TNF-α being developed [68]. This finding is consistent with the general knowledge that a Th1 response plays a crucial role in host resistance to intracellular pathogens such as mycobacteria infections and infections with other fungi.

Circulating human monocytes have been shown to respond to *P. marneffei* conidia with an oxidative burst which was significantly enhanced by a macrophage colony-stimulating factor [69]. Human neutrophils are found to have antifungal activity against the yeast form of *P. marneffei* but not the conidia. This activity was mediated by exocytosis of the granular cytolytic molecules from neutrophils rather than by oxygen radical-dependent mechanisms [70].

Molecular Epidemiology

Modern molecular methods such as multilocus genotypes have provided opportunities to identify isolates of a similar or identical genetic background that are derived from a common infective population, to describe the hierarchical organization of population structure, to identify the reproductive mode and to provide information on the deeper phylogenetic and evolutionary history of the pathogen [71]. Until recently, molecular approaches to typing *P. marneffei* have relied on surveying the genome by using methods that randomly sample for genetic variation.

1.1.11 Restriction fragment length polymorphism

A group from Thailand has used HaeIII digests of genomic DNA to search for restriction fragment length polymorphisms (RFLPs) in order to differentiate *P. marneffei* isolates from Chiang Mai region [72]. The 22 human isolates in their study were classified into 2 DNA types (type I, 73%; type II, 27%). Another group study of 20 *P. marneffei* isolates from Taiwanese patients that used the same restriction digestion assayuncovered the same 2 HaeIII RFLP patterns that had been found in Thailand with the same frequencies. However, the use of randomly amplified polymorphic DNA (RAPD) assays yielded 8 different RAPD patterns, suggested that there was greater genetic diversity than had been uncovered by the RFLP assay [73].

1.1.12 Pulse-field gel electrophoresis

A separate study used pulsed-field gel electrophoresis of 69 *P. marneffei* isolates from several regions of Thailand using restriction enzyme NotI revealed 2 macro-restriction patterns (MPI and MPII) that could be grouped into 9 sub-types, yielding 54 genotypes in total [74]. Another assay using the tetranucleotide repeat primer $(GACA)_4$ and the phage M13 core sequence identified 4 genotypes that varied in frequency between northern and southern Thailand [55]. However there has been no correlation between the restriction patterns from various *P. marneffei* isolates and geographic regions or clinical phenotypes. The drawbacks of these

typing systems are the low discriminatory power due to small numbers of alleles, the reproducibility of RAPD and macrorestriction profiles between laboratories, and variation within alleles.

1.1.13 Mutilocus sequence typing (MLST)

Recently, sequence-specific assays of genetic variation in the *P. marneffei* genome have been developed to address the above drawbacks. These are the multilocus sequence typing (MLST) and mutilocus microsatellite typing (MLMT). MLST characterizes isolates by sequencing housekeeping genes (usually seven), and is becoming the technique of choice for bacterial species and *Candida albicans* [75]. The alleles present at each locus are combined into a mutilocus sequence type, which is deposited in a species-specific online database held at http://www.mlst.net/. However, the use of MLST is limited when it is unclear whether the species being typed (*P. marneffei* in this case) contain insufficient genetic variation in the house keeping loci to discriminate between isolates.

1.1.14 Multilocus microsatellite typing (MLMT)

MLMT was designed to circumvent the problem of low levels of genetic variation. It targets loci that contain di-, tri-, or tetranucleotide repeats. These repeats (or microsatellites) are more highly variable than housekeeping loci due to the accumulation of length polymorphisms as a consequence of slippage by DNA polymerase during genome replication [76, 77]. The alleles at each locus are scored by electrophoresing PCR-amplified loci through an automated sequencer, typing the length polymorphisms, and then combining the alleles from each locus into a mutilocus microsatellite types that can be used to query online databases held at http://www.mutilocus.net/. The resulting outputs from these queries can be used to analyze the population genetic structure of the organism or to test epidemiological hypotheses.

Fisher et al. [76] screened 1.7 Mb of *P. marneffei* genome sequence for microsatellite motifs, using all possible permutations of di-, tri-, and tetranucleotide motifs with a minimum repeat number of six. This research resulted in 30 dinucleotide, 14 trinucleotide, and 5 tetranucleotide repeats being discovered. However, a similar study on the same genome sequence by Lasker and Ran [78] uncovered only 3 microsatellites. It is unclear why there is such a discrepancy, although the software used in the later study excluded tri- and tetra nucleotide repeats. Of the 49 loci identified by Fisher et al [76], 24 were chosen and amplified as multiplex PCRs in four groups of six loci and used to type a panel of 29 clinical and bamboo rat isolates chosen from across the endemic range of *P. marneffei* [25, 31, 76]. Of the 24 loci, 23 were amplifiable and 21 were polymorphic with between 2 and 14 alleles present at each locus, comprising 19 unique microsatellites in total. Clustering of isolates based on the microsatellite genetic distance D_1 [79] showed that isolates occur within 2 geographically separated clades that account for 26% of the total observed genetic diversity [25]. The “eastern” clade contained isolates from mainland China, Hong Kong, Indonesia, and Vietnam, while the “western” clade contained isolates from Thailand and India, showing that *P. marneffei* has a geographic component to its population genetic structure. A study over a smaller geographical scale in Manipur, India showed that while the microsatellites of isolates were identical within bamboo plantations, they were dissimilar between bamboo plantations [31]. This finding suggests that the population genetic structure of *P. marneffei* may in fact be partitioned over local, as well as large, geographical scales, although further studies are necessary to confirm the generality of this finding.

These molecular methods, particularly the highly discriminatory MLMT techniques provide unique means to screen samples from human clinical populations, from bamboo rat populations, and environmental sources from different geographical areas and to identify the natural cycle of infection by *P. marneffei* in nature.

Pharmacokinetics - Pharmacodynamics of Itraconazole and Amphotericin B

1.1.15 Itraconazole spectrum of activity and mechanism of action

Itraconazole is a triazole compound that has in general broader spectrum of antifungal activity than other azole antifungals, from activity against mucocutaneous candidiasis, dermatomycosis, to deep mycoses including aspergillosis, candidiasis, cryptococcosis, histoplasmosis, and several endemic mycoses such as paracoccidioidomycosis, chromoblastomycosis, and penicilliosis. Itraconazole, like other azoles, has 3 nitrogen atoms in its azole ring which might improve tissue penetration, prolong half-life, and increase specificity for fungal enzymes [80]. The nitrogen atoms interact with the heme iron of the fungal cytochrome P450 3A (CYP3A), inhibiting the function of lanosine 14α-demethylase which converts lanosterol to ergosterol, the main sterol in the fungal cell membrane. This inhibits replication and promotes cell death, or in the case of yeast cells of *Candida albicans*, transformation into hypothetically invasive hyphae [81]. Itraconazole has little effect on mammalian cytochome P450 enzymes even at high concentrations or on the sterol and steroid pathways of the human pituitary-adrenal-testicular axis [82]. Resistance to azole antifungals rarely develops and appears to be a problem mainly with fluconazole in HIV-positive subjects [81, 82].

1.1.16 Pharmacokinetics of itraconazole

Plasma level of itraconazole can be measured either by high performance liquid chromatography (HPLC) or by bioassay. HPLC has a high specificity and sensitivity (2 ng/mL plasma) and has been used in most pharmacokinetic studies [83]. The absolute bioavailability of oral itraconazole is 55% (±15%). Oral itraconazole should be administered with food since the bioavailability is reduced by 40% when it is administered under fasting condition [84]. The bioavailability of itraconazole is reduced by 50% when administered with H_2 blocker [85]. Since the bioavailability of oral itraconazole is affected by gastric acidity, acid-reducing drugs (H2 blockers, proton pump inhibitors) should be administered at least 2 hours after administration of itraconazole. Itraconazole is highly lipophilic, is strongly protein binding (99.8%), and has a high tissue penetration. Body fluids such as cerebrospinal fluid (CSF), eye fluid and saliva contain low to non-detectable amounts of itraconazole, whereas in many organs and tissues the concentrations exceed the corresponding plasma levels by a factor of 1.5 to 20 [86].

Metabolism of itraconazole is extensive in the liver, and excretion of inactive metabolites occurs primarily in the urine and feces. Dosing of oral itraconazole does not need to be adjusted for renal insufficiency. A hepatic metabolite, hydroxyitraconazole, is bioactive and has activity similar to that of the parent compound [87]. Because of the high volume distribution of itraconazole, oral or intravenous loading doses are needed to reach protective level quickly especially when given for treatment of systemic mycosis. It is recommended that 600 mg/day in two divided doses for 3 days is used for oral loading dose, and 400 mg/day in 2 divided doses for 2 days is used for intravenous loading doses.

1.1.17 Itraconazole formulations

Oral itraconazole suspension and intravenous formulations have recently been developed to circumvent the variation in serum concentrations of itraconazole capsules. In general the oral suspension (with cyclodextrin) preparation is more readily absorbed than the tablets, resulting in roughly a 30% larger AUC than with the tablet preparation. Peak serum concentration at steady state, after the oral solution at a dose of 200 mg twice daily, ranged from 513 to 2,278 ng/L with a median concentration of 1,326 ng/L. In contrast, the peak serum concentration at steady state after administration of the capsule formulation at the same dose ranged from 297 to 1,609 ng/L with a median value of 741 ng/L [88]. Opposite to the tablet formulation, the absorption of the liquid suspension is enhanced when it is taken in a fasted state and has a more predictable absorption. Nausea is more common with the liquid formulation due to the osmotic effects of cyclodextrin. This may affect compliance and is potentially counter-productive in the goal to improve bioavailability.

The same vehicle (cyclodextrin) is used to solubilise the IV formulation as the oral solution. This vehicle is known to accumulate in patients with impaired renal function and therefore, use of the intravenous preparation is limited to patients with a creatinine clearance >30 mL/min and is usually reserved for patients with severe infections who are intolerant of amphotericin B. The intravenous formulation is no longer manufactured in the United States but is available in some other countries.

1.1.18 Pharmacodynamics of itraconazole

The concentration-effect relationship for any systemic antifungal agent remains a controversial issue. Historically the target plasma level for itraconazole has been estimated at 250 ng/mL (by HPLC) based on the in vitro IC_{90} (the concentration needed to achieve 90% reduction in replication) [89, 90]. Numerous itraconazole concentration-effect studies have been undertaken and each has demonstrated a link to drug efficacy [15, 17, 91]. A similar relationship for toxicity has not been identified. The pharmacodynamic efficacy investigations include both preclinical animal model and clinical trials using itraconazole both as prophylaxis to prevent the development of invasive fungal disease and as treatment of invasive fungal diseases. In a group of 21 patients with invasive aspergillosis, mean itraconazole concentration in responders was 6.5 mg/L and 4.2 mg/L in nonresponders (based on a microbiologic assay) [17]. A similar quantitative relationship was observed in a group of patients with nonmeningeal coccidioidomycosis. In this cohort of 39 patients, itraconazole concentrations measured by bioassay were 6.5 ± 4.2 mg/L in the 28 patients who had a clinical response and 4.0 ± 3.2 mg/L in 11 nonresponders [91]. In another study of 25 patients with HIV and cryptococcal meningitis, trough itraconazole concentrations exceeding 1 mg/L was observed in the group of patients with 100% response rate; whereas trough concentrations below 1 mg/L was observed in the group of patients with a 66% response rate [15].

In regards to investigations of itraconazole use as prophylaxis to prevent the development of invasive fungal disease, the relationship is similar to that observed in treatment studies; however, the concentrations associated with effective disease prevention is two to fourfold lower than that shown necessary for fungal disease treatment [92-94]

1.1.19 Clinical experiences with itraconazole for prophylaxis and treatment of invasive fungal diseases

In clinical trials, itraconazole oral solution (5 mg/kg/day) was more effective at preventing systemic fungal infection in patients with hematological malignancy than placebo, fluconazole suspension (100 mg/day), oral amphotericin B (2 g/kg/day) and was highly effective at preventing fungal infections in liver transplant recipients [13, 13, 95]. There were no unexpected AEs with the itraconazole oral solution in any of these trials. In a randomized clinical trial, intravenous itraconazole solution is at least as effective as intravenous amphotericin B in the empirical treatment of neutropenic patients with systemic fungal infections, and drug-related AEs are more frequent in patients treated with amphotericin B [12]. Itraconazole has been successfully used to treat a variety of invasive fungal infections including invasive aspergillosis, coccidioidomycosis, cryptococcosis, and blastomycosis in case series [13-21]. However, both the lack of direct systematic comparative studies and the reported variable bioavailability of the tablet formation of itraconazole have contributed to the slow coming of this drug.

1.1.20 Amphotericin B introduction

Amphotericin B is a polyene antibiotic first isolated in 1955 from *Streptomyces nodosus*. It is a broad antimycotic agent and a highly antiparasitic agent. After 5 decades of experiences and the births of newer antifungal drug classes, amphotericin B remains the agent of choice for many invasive fungal infections. Amphotericin B has a broad spectrum of action that includes most of the major fungal pathogens of man. This drug binds to the membrane sterols of fungal cells, causing impairment of their barrier function and loss of cell constituents. Metabolic disruption and cell death are consequent upon membrane alterations.

1.1.21 Amphotericin formulations

The most important drawback to the formulation of amphotericin B is that it is scarcely soluble in water. The reference conventional formulation Fungizone® which was a mixture with deoxycholate was developed for intravenous administration; unfortunately this formulation is nephrotoxic. Second generationamphotericin B formulations which depend on different lipid-carrier systems were developed in the 1990s to circumvent this side effect. These are Abelcet® (ABLC), AmBisome® (L-AmB) and Amphotec® (ABCD). Abelcet® is a formulation with 2 phospholipids in a 1:1 drug-to-lipid molar ratio, has a better therapeudic index and lower risk of renal disorders at a dosage of 1-5 mg/kg/day. Amphotec® is a formulation with cholesterol sulfate in equimolar concentrations, has similar antifungal efficacy as Fungizone® but less cytotoxic and hemolytic. AmBisome® formulation is integrated into small unilamellar liposomes and is superior to Fungizone® in bioavailability and side effects. Ostrosky-Zeichner et al have summarized 10 major controlled clinical studies and concluded that no study has ever shown a lipid new amphotericin B formulation to be less effective than Fungizone®, and some studies show strong evidence that the new formulations may be more effective and consistently less toxic than Fungizone®. In resource rich countries, these new formulations are used more commonly as their lower rate of side effects are usually considered to outweigh their high costs and to afford the use of higher doses [96].

1.1.22 Amphotericin B pharmacokinetics

Due to its low solubility amphotericin B gastrointestinal uptake of oral formulation is minimal, and IV infusion remains the route of choice. Amphotericin B is extensively bound to plasma proteins (~95%) by ß-lipoproteins, albumin, and α_1-acid glycoprotein [97]. Amphotericin B is highly amphipathic in nature (being both hydrophilic and hydrophobic). In water it forms a

mixture of water-soluble monomers and oligomers with insoluble aggregates [96]. Different aggregation states can be present in the same formulation, the proportions of each association form has been shown to depend on the interaction between amphotericin B and solvents such as amphotericin B concentrations [98], the medium in which the drug is dispersed [99], the action of surfactants and serum albumin [100, 101], or the temperature they have been exposed to. The various aggregation states of amphotericin B may interact with membrane sterol in different ways to induce changes in cell membrane, and may have different impacts on amphotericin efficacy and toxicity.

1.1.23 Comparison of amphotericin B and itraconazole in empirical treatment of invasive fungal infection

In an open, randomized, controlled, multicenter trial, powered for equivalence, involving 60 oncology centers in 10 countries evaluated 384 neutropenic patients with cancer who had persistent fever that did not respond to antibiotic therapy, itraconazole and amphotericin B have at least equivalent efficacy, and itraconazole is associated with significantly less toxicity than amphotericin B [12]. In another open, randomized controlled study evaluated 162 patients with underlying hematological malignancy and febrile neutropenia, significantly fewer itraconazole patients discontinued treatment due to any AE(22.2 vs. 56.8% AMB [amphotericin B]; $p < 0.0001$). The main reason for discontinuation was a rise in serum creatinine (1.2% itraconazole vs. 23.5% AMB). Intention-to-treat (ITT) analysis showed favorable efficacy for itraconazole: response and success rate were both significantly higher than for AMB (61.7 vs. 42% and 70.4 vs. 49.3%, both $p < 0.0001$). Treatment failure was markedly reduced in itraconazole patients (25.9 vs. 43.2%), largely due to the better tolerability [102]. Another study from Korea compared the efficacy and tolerability of the two drugs as an empirical antifungal agent in 96 patients with febrile neutropenia. The overall success rates were 47.9% for itraconazole and 43.8% for amphotericin B deoxycholate (% difference: 4.1% [95% confidence interval for the difference: -15.8 to 24]), which fulfilled the statistical criteria for the non-inferiority of itraconazole. The proportions of patients who survived for at least seven days after discontinuation of therapy or who were prematurely discontinued from the study were not significantly different between the two groups. The rates of breakthrough fungal infections and resolution of fever during neutropenia were similar in both groups. More patients who received amphotericin B deoxycholate developed nephrotoxicity, hypokalemia or infusion-related events than did those patients who received itraconazole (nephrotoxicity: 16.7% vs. 1.8%, hypokalemia: 66.7% vs. 24.6%, and infusion-related events: 41.7% vs. 3.5%, respectively) [103].

2 Study Objectives

Primary Objective

To compare the efficacy of Itraconazole and amphotericin B in the acute-phase treatment of penicilliosis as assessed by the absolute risk of death during the first 2 weeks of therapy.

Secondary Objectives

1. Determine overall survival until week 24

2. Determine time to treatment success (defined by absence of fungal growth in follow up culture, temperature <38ºC for 3 days, and complete resolution of skin lesions or lesions in the final stages of healing as judged by treating clinicians)
3. Determine relapse-free survival until week 24 of therapy (i.e., time to the first treatment relapse or death). Relapse is defined as recurrence of culture-confirmed penicilliosis after achieving treatment success at week 12
4. Determine time to culture sterilization
5. Determine the rate of early antifungicidal activities as assessed by the decrease in colony forming unit (CFU) count per mL of blood in serial blood samples
6. Determine safety and tolerability as assessed by Grade 3 and Grade 4 adverse events (AEs) and serious adverse events (SAEs)
7. Identify baseline clinical, microbiological, and/or laboratory predictors of outcome
8. Develop population pharmacokinetic (PK) models of amphotericin B and itraconazole in HIV-infected patients to characterize the absorption, distribution, and clearance, and identify the sources of variance in pharmacokinetic parameters. Correlate PK variables to fungal clearance, early antifungicidal activity, and treatment outcomes.
9. Study the epidemiology of *P. marneffei* infection, focusing on finding the natural reservoir and vehicle of transmission of *P. marneffei*. A simultaneous case-control study will be performed to identify exposure risk factor/s for the development of penicilliosis in age, sex, CD4 or WHO-disease-stage matched HIV-infected patients with and without penicilliosis. Detailed exposure histories related to living and working environment (proximity/exposure to any body of water, tropical plants/trees, soil, domestic/farm/wild animals, types of raw/rarely cooked foods consumed, injection drug use history/practices, type/seasonality of jobs, current/past specific activities most days) will be investigated. Global positioning system (GPS) mapping technology will be used to characterize the geo-spatial distribution of cases and controls (Appendix D)
10. Investigate the molecular epidemiology of *P. marneffei* infection using a number of cutting-edge molecular technologies including highly discriminatory mutilocus microsatetlite typing (MLMT) and correlate the identified genotypes with clinical and geo-spatial epidemiology data (appendix E)
11. Evaluate an ELISA assay to detect *P. marneffei* urinary antigen for diagnostic accuracy of penicilliosis and as a surrogate marker for microbiological and clinical outcomes (Appendix F)
12. Determine the cost effectiveness of treating the acute-phase of penicilliosis with itraconazole versus amphotericin B (Appendix G)
13. Characterize the incidence, clinical features and outcome of patients who develop penicillium associated immune reconstitution disease (IRD) (Appendix H)

3 Study Plans

Study Designs and Overview

This study is a randomized, open-label, comparative, multi-center trial with the following treatment groups:

Group 1: intravenous amphotericin B 0.7 mg/kg/day x 2 wks
Group 2: oral itraconazole 400 mg/day x 2 weeks (including 600 mg/day x first 3 days for loading)

After the 2-week acute-phase therapy, all patients will continue on to the maintenance-phase therapy with oral itraconazole 400 mg/day x 10 weeks, followed by the suppressive-phase therapy with itraconazole 200 mg/day until CD4 count rises above 100 for 6 months on antiretroviral therapy (ART) for HIV.

Randomization will be 1:1 and stratified for the study site. Patients will be followed until 6 months post randomization.

Study Size

Planned enrollment of 440 subjects total

Study Duration

Study enrollment will anticipate to begin in 2012 and to end when 440 subjects are enrolled and have been followed for at least 6 months. This is anticipated to occur over 4 years.

Study Population

3.1.1 Screening Criteria

1. HIV positive

AND

2. Age ≥18 year

AND

3. Clinicians suspect penicilliosis illness in a patient with typical umbilicated skin lesions or a combination of the following features without skin lesions: fever, malaise, enlarged lymph nodes, hepatomegaly and/or splenomegaly, cough and/or respiratory complaints, gastrointestinal complaints, anemia, thrombocytopenia, elevated AST, and ALT.

3.1.2 Screening Procedure

Subjects that meet the above criteria will be invited to participate in the study. If the subjects agree to participate and sign the informed consent form, they will undergo the following screening procedures.

- Blood samples for blood culture and routine hematology, chemistry, and liver function tests
- Skin lesion scrapping for direct microscopy and culture as deemed appropriate by treating clinicians
- Urine sample to rule out pregnancy in females
- Peripheral lymph node aspiration if lymph node size is >1cm
- Bone marrow aspiration only if deemed appropriate by attending physicians
- HIV testing (in accordance with Vietnam MOH guidelines on diagnosis and treatment of HIV/AIDS dated Aug 2009) if not already done

During the screening period (while awaiting culture results), subjects will be treated as clinically indicated, by best medical practice. If empiric antifungal is deemed appropriate, patients will be randomized to receive either amphotericin B or itraconazole. If culture does not subsequently confirm the diagnosis of penicilliosis, subjects will be withdrawn from the trial. After signing the informed consent form, subjects that had culture confirmed penicilliosis at an outside hospital will not require a repeat culture part of the screening step and be directly evaluated with the other inclusion and exclusion criteria.

After screening results are available, eligibility for this treatment protocol will be assessed by the following inclusion and exclusion criteria:

3.1.3 Inclusion Criteria

1. HIV positive

AND

2. Age ≥18 year

AND

3. Syndrome consistent with penicilliosis (primary or relapse) PLUS culture-confirmed diagnosis of penicilliosis (from blood, skin lesion scrapping, lymph node or bone marrow biopsy).

3.1.4 Exclusion Criteria (any of the following):

1. Age <18
2. Pregnancy or urine β-hCG positive
3. History of allergy or severe reaction to either itraconazole or amphotericin B
4. Central nervous system involvement (assessed clinically and by evidence of inflammation and/or infection in the CSF)
5. Use of the following prohibited drugs: phenytoin, barbiturates, carbamazepine, rifampin, isoniazid, H2 blocker, HMG-CoA reductase inhibitors, cisapride, terfenadine, midazolam, dihydropyridine Ca channel blocker, cyclosporine, cyclophosphamide, tacrolimus, digoxin, quinidine, ergot derivatives, pimozide, coumadin, or investigational drugs.
6. Baseline AST or ALT >5 times the upper limit of normal
7. Absolute neutrophil count < 500 cells/μL
8. Creatinine clearance of <30 by Cockcroft-Gault formula or on hemodialysis
9. Concurrent diagnosis of cryptococcal meningitis or active tuberculosis (as amphotericin B is the treatment of choice for cryptococcal meningitis, and tuberculosis treatment with INH and Rifampin is contraindicated when used with itraconazole)
10. Current treatment with an antifungal drug for confirmed or suspected penicilliosis for >48 hours

The reasons why patients who meet the screening criteria but are later excluded from the study will be recorded in a separate patient log.

Estimating creatinine clearance (mL/min)
Cockcroft and Gault equation:
CrCl = (140 - age) x weight(Kg) / (Cr(mg%) x 72) for males (x 0.85 for females)

(If unit for Cr is mmol/L, convert to mg% by Cr x 0.01)

Nornal range: Male = 90-140 ml/minute, Female = 85-135 ml/minute

Randomization

Randomization will be 1:1 and stratification by study site. Each site will have a separate randomization list to ensure the 1:1 ratio of the treatment arms at each site. In addition, to

ensure that the 1:1 ratio can be approximately obtained at any time during the study, the randomization list at each site is further divided into even block sizes of 4-10 patients, and within each randomization block, treatment allocation is maintained at 1:1.
A computer-generated randomization list will be produced by a study pharmacist with no clinical involvement in the trial. This list will then be incorporated into a web based program. This program can be assessed 24 hours/day with secured log in by study personnel from each centre. When a patient is enrolled to the study, an authorized study staff will enter patient details (patient ID, year of birth and patient initials) into the system to obtain the treatment allocation for that patient based on the randomization list. All transactions on the web server will be intermediately logged, unchangeable and auditable.

Criteria for Evaluation

3.1.5 Primary Endpoint

Absolute risk of death during the first 2 weeks after randomization

3.1.6 Secondary Endpoints

3.1.6.1Clinical endpoints

- Overall survival until week 24
- Time to treatment success (defined by absence of fungal growth in follow up culture, temperature <38ºC for 3 days, and complete resolution of lesions or lesions in the final stage of healing as judged by treating clinicians)
- Relapse-free survival until week 24 of therapy (i.e., time from treatment success to the first treatment relapse or death). (Relapse is defined as recurrence of culture-confirmed penicilliosis after achieving treatment success at week 12)
- Deaths from penicilliosis until week 24 (causes of death will be determined by investigators)
- Time to change of therapy from assigned study therapy
- Total number of patients with Grade 3 and Grade 4 AEs and SAEs, and the cumulative incidence of Grade 3 and Grade 4 AEs and SAEs, associated with cessation of randomly assigned therapy between treatment arms
- Antifungal medication adherence
- Incidence of Immune Reconstitution Diseases

3.1.6.2Microbiological endpoints

- Time to blood culture sterilization
- Rate of early fungicidal activity as determined by serial blood samplings during therapy and measured by the decrease in log colony forming units per mL of blood (CFUs/mL)
- Frequency and patterns of itraconazole and amphotericin B resistance emergence

3.1.6.3Pharmacological endpoints

- Antifungal concentration time curves
- Maximum antifungal concentrations/MIC, area under the curve (AUC) of antifungals/MIC over time

3.1.6.4Serological endpoints

- Time to *P. marneffei* urinary antigen clearance
- Rate of decrease in *P. marneffei* urinary antigen titers

Statistical Considerations

3.1.7 Analysis of the primary endpoint and overall survival

This is a non-inferiority trial with a non-inferiority margin of Δ=10%; i.e., the aim is to prove that the absolute risks of death during the first 2 weeks of treatment in the two treatment arms differ by less than 10% (at worst) in favour of amphotericin B. Two-week mortality estimates will be based on the Kaplan-Meier method. Patients lost to follow-up before the week 2 assessments will be treated as censored. Based on these estimates and corresponding standard errors (calculated according to Greenwood's formula), a two-sided 95% confidence interval (CI) for the difference in the absolute risks of death will be calculated. If the CI excludes differences of 10% or more in favour of the amphotericin B arm, the primary objective of the trial will be met.

In addition, we will assess the joint effect of treatment assignment and the baseline covariates age, sex, injection drug use, ART naïve/experienced, and presence of fungemia on the primary endpoint. This adjusted analysis will be based on logistic regression. As we expect only few patients lost to follow-up during the first 2 weeks, these patients will be removed from the adjusted analysis.

In a second step, we will analyze overall survival, i.e., time to death during the entire follow-up period of 24 weeks. Overall survival will be summarized by Kaplan-Meier curves and the 2 arms will be compared with a Cox proportional hazards regression model with treatment as the only covariate. In addition, an adjusted analysis will be performed using the Cox model and the same baseline covariates as listed above.

Potential heterogeneity of the treatment effect will be explored in the following pre-defined subgroups:

- Injection drug use (yes vs no)
- ART status (naive vs experienced)
- Presence of fungemia (yes vs no)
- Baseline CD4 count

3.1.8 Analysis of secondary endpoints

Time to treatment success**:** The cumulative proportion of patients achieving treatment success over time will be summarized with the cumulative incidence function, which takes the competing risk of prior death into account. Comparison between the two arms will be based on the Fine and Gray model with treatment as the only covariate. An adjusted analysis including the same covariates as for the analysis of overall survival described above will also be conducted.

Relapse-free survival until week 24 of therapy (i.e., time from treatment success to the first treatment relapse or death): Relapse free survival in both arms will be summarized using Kaplan-Meier curves.

Other time-to-event endpoints**:** Will be summarized using Kaplan-Meier curves (in case they include death in the endpoint) or cumulative incidence functions (otherwise, to take into account the competing risk of death). In addition, they will be modeled with (cause-specific) Cox proportional hazards models including the same covariates as for the analysis of overall survival.

Adverse events: Frequency tables and listings of Grade 3 and 4 AEs, SAEs, and AEs leading to discontinuation of the randomized treatment will be produced. The overall frequency of each of these types of adverse events will be compared between the 2 arms with Fisher's exact test.

3.1.9 Analysis of populations

The primary analysis will be based on the full analysis population including all randomized patients following an intention-to-treat principle, but excluding subjects without microbiological confirmed penicilliosis. As the analysis of non-inferiority trials on the full analysis set is not necessarily conservative, the analysis of the primary endpoint will be repeated on the per-protocol population. This population excludes patients if they meet any exclusion criteria while in the study, are not treated according to the randomized treatment arm, or lost to follow-up before day 14.

3.1.10 Sample size calculation

The inpatient mortality rate of patients with HIV-associated penicilliosis at HTD in 2009 was 10% [5]. Not all of these patients received antifungal treatment before death. On the other hand, this rate did not include out-of-hospital deaths. In considering these opposing factors we estimate that the mortality in both treatment arms will be approximately 10% with a plausible range of 5-15%.

The primary aim of this trial is to demonstrate non-inferiority of itraconazole compared to amphotericin B treatment with respect to overall mortality at the end of 2 week induction therapy. The sample size calculation is based on an assumed mortality rate of 15% in both arms, a non-inferiority margin of 10% and a one-sided significance level of 2.5%. Based on these assumption, a total sample size of 400 patients will guarantee a power of 80% to show non-inferiority or, equivalently, that the two-sided 95% confidence interval for the difference in mortality between the two arm excludes an excess mortality of 10% or more in favour of amphotericin B therapy. We expect that the combined proportion of losses to follow-up and major protocol violations will be no more than 10%. To account for this, a total of **440 patients** (220 per treatment arm) will be randomized in this trial.

3.1.11 Justification of the non-inferiority margin

Given that without proper treatment, penicilliosis has almost a 100% mortality rate, a non-inferiority margin of 10% and a "cure" rate of at least 85% for patients receiving amphotericin B

would allow us to prove that itraconazole retains at least 88% of the benefits of amphotericin B over placebo. A non-inferiority margin of 10% may seem large given that the primary outcome is mortality. We nevertheless regard it as acceptable due to the following reasons:

First, it should be highlighted that the 10% excess mortality for itraconazole refers to a worst-case scenario, i.e. the degree of inferiority that we aim to exclude with 95% confidence. Our actual best guess of the true mortality difference based on the trial data, i.e. the observed difference, will be much less than 10%. For example, if the observed mortality risk for patients with amphotericin B is 15%, the observed mortality risk for patients with itraconazole must be <18% in order to guarantee that the 95% confidence interval excludes mortality differences of 10% or more.

Second, our sample size calculation is based on a conservative assumption regarding mortality. If the true mortality in both arms is equal but lower than 15%, e.g. 5% or 10%, we will have 80% power to exclude excess mortalities of >6% or >8%, respectively, in the itraconazole arm.

Third, in case itraconazole is substantially inferior to amphotericin B treatment, the trial will also have sufficient power to detect this: If the true mortality in the itraconazole arm is 15% and the true mortality in the amphotericin B arm is 6.5% or less, we will have >80% power that the 95% confidence interval excludes 0, i.e. to confirm a difference between the two arms.

Fourth, the possibility of some excess mortality in the itraconazole arm should be balanced with the unavailability of amphotericin B (particularly in provincial/district hospitals), prohibitive costs, the more complex administration, and the less favourable safety profile..

Finally, practicability and feasibility of the trial must be considered [20]. A non-inferiority margin of 7.5% appears to provide little gain but would lead to a sample size of 792 (80% increase), whereas a margin of 5% would result in a prohibitively large sample size of 1,320 (300% increase).

Subject and Study Modification or Discontinuation

3.1.12 Subject withdrawal/discontinuation

Participants, or their surrogates if the patient is otherwise unable to make informed decisions, can terminate study participation at any point they wish to. If a patient is withdrawn prior to completion of the study, the reason for this decision will be recorded in the case report forms (CRFs). The remaining follow-up evaluation will be conducted if patient consent is obtained.

4 Study Treatment

Overview

This protocol will compare the two current treatment strategies for acute penicilliosis: itraconazole versus amphotericin B followed by itraconazole therapy. There is no placebo arm (i.e. no arm without active drug administered).

Eligible patients will be randomized to receive either:

A. Itraconazole: 400 mg/day in two divided doses for 12 weeks (including 600 mg/day in two divided doses x 3 days for loading).
B. Amphotericin B: 0.7 mg/kg/day IV x 2 weeks, followed by itraconazole 400 mg/day po for 10 weeks.

Products

4.1.1 Itraconazole

Itraconazole capsules (Itranstad) purchased by the trial pharmacist from licensed suppliers in Viet Nam and provided to the study participants free of charge throughout the whole 12 weeks duration of the treatment. Study participants will be transferred to the National HIV/AIDS Treatment Program which provides free opportunistic infection treatment and anti-retroviral therapy (ART) as soon as possible.

4.1.2 Amphotericin B

Amphotericin B intravenous formulation purchased by the trial pharmacist and provided to the study participants free of charge for the 2 week duration of the treatment.

Storage and Handling

The itraconazole capsules will be kept at room temperature (approximately 25ºC or 77ºF). Amphotericin B intravenous formulation will be kept under refrigeration (2-8ºC or 36-46ºF) and not allow to freeze. All medication storage and administration will be regulated through the central pharmacy departments at each study site to ensure good quality and control of medication handling.

Study Drug Dosing

4.1.3 Itraconazole dosing

Because of the high volume distribution of itraconazole, oral loading dose is needed to reach protective level quickly for treatment of systemic mycosis. Note that itraconazole capsules are to be taken only with food and/or an acidic drink (likely cola drink) as its absorption is dependent on gastric pH. Any gastric acid reducing drugs (H2 blocker, proton pump inhibitor) are not allowed, and concomitant therapies with these drugs are exclusion criteria for the trial. If an acid blocking agent needs to be given, H2 blocker is recommended to be used 6 hours before or after administration of oral itraconazole.

Itraconazole oral loading dose: 3 capsules 100 mg po bid (or 600 mg/day) for 3 days, followed by the standard treatment dose of 2 capsules 100 mg po bid (or 400 mg/day) for a total of 12 weeks.

4.1.4 Amphotericin B dosing

Amphotericin B 0.7 mg/kg/day IV x 2 weeks, followed by itraconazole 2 capsules 100 mg po bid (or 400 mg/day) for 10 weeks. A loading dose of itraconazole is not necessary for subjects already on amphotericin B.

Product Administration

The initial dose of both components of the study drug should be given as soon as possible after enrollment and randomization. These can be administered with food or a snack whenever possible. Itraconazole needs to be taken with food or an acidic drink (likely cola drink).

Post Dose Emesis

If emesis occurs within 60 minutes after oral study drug administration, and is thought to be of sufficient volume to evacuate the study drug from the stomach (i.e., 5 cc vomitus probably would not remove the study drug from the stomach), a repeat dose of the study drug should be administered. The maximum number of repeat doses is two (after initial dose) per dosing interval. If all three doses are vomited, this will be recorded and participants will continue with the next scheduled dose. If a patient vomits all given doses within a 24 hour period or if a patient is judged by the treating clinician to be intolerant of oral medication, a nasal gastric tube placement is indicated. Patients who cannot tolerate nasal gastric tube placement will be considered intolerant to treatment, recorded as such and may be switched to appropriate treatment at the discretion of the treating physician.

Concomitant and Prohibited Medications

4.1.5 Prohibited medications

Concomitant administration with cisapride, dofetilide, ergot derivatives, levomethadyl, lovastatin, midazolam, pimozide, quinidine, simvastatin, or triazolam is prohibited during administration of study drug. Rare cases of serious cardiovascular AEs (including death), ventricular tachycardia, and torsade de pointes have been observed due to increased cisapride, pimozide, quinidine, dofetilide or levomethadyl concentrations induced by itraconazole. Concurrent use of these drugs is contraindicated.

4.1.6 Category C drugs with amphotericin B where monitor therapy is recommended

Amphotericin B may enhance the nephrotoxic effect of aminoglycosides and cyclosporine. Corticosteroids (systemic) may enhance the hypokalemic effect of amphotericin B.

4.1.7 Category B drugs with amphotericin B where no action is needed

Amphotericin B may enhance the adverse/toxic effect of Cardiac Glycosides such as Digoxin and neuromuscular-blocking effect of Neuromuscular-Blocking Agents such as Atracurium; Cisatracurium; Doxacurium [Off Market]; Metocurine Iodide; Mivacurium [Off Market]; Pancuronium; Rocuronium; Succinylcholine; Vecuronium.

4.1.8 Anti-pyretic

If an anti-pyretic is needed, acetaminophen / paracetamol is recommended.

4.1.9 Anti-emesis

As itraconazole can cause nausea and vomiting, an anti-emetic may be used for intractable symptoms. The drugs listed in Section 8.1.1 may be considered.

5 Study Procedure

See Appendix B for graphical representations of study assessments and frequency.

Hospitalization

After signing the informed consent form, all enrolled patients will be admitted to the hospital at the participating study site and will remain hospitalized through the first 2 weeks of therapy. Patients who choose to self-discharge before the end of the initial two week treatment will continue to be followed through out-patients visits or at home.

Initial Evaluation

5.1.1 History and physical examination on day 1

Including (but not limited to):

- Presence of symptoms
  - Fever
  - Weight loss
  - Enlarging lymph nodes
  - Fatigue/anorexia
  - Cough and/or shortness of breath
  - Nausea and/or vomiting
  - Skin and/or mucosal lesions
- Development of symptoms listed above
- Epidemiologic factors (only for patients participating in the case control study)
  - Home and work addresses
  - Type/s of work and specific activities at work
  - Specific exposure to soil, location and type of soil
  - Travel history
  - Exposure/contact with bamboo and/or bamboo rats
  - Animals in household (dogs, cats, birds including chickens and ducks, reptiles, pigs, rabbits or other rodents)
  - Animals in surrounding area (yard, farm etc)
  - Illness in animals noted above
  - Live by or close contact with any body of water
  - Exotic food including raw or rarely cooked food
  - Exposure to ill persons with similar symptoms
- Previous peniciliosis history

- HIV history
  - Injection drug use
  - Antiretroviral history and drugs
  - Latest CD4 count if known
- Allergies
- Physical Exam
  - Vital signs and weight
  - Detailed physical examination
- Clinical Data

5.1.2 Admission clinical laboratory tests

At the time of enrollment, the following routine laboratory tests will be performed:

- CBC
- Blood chemistries
- Urine pregnancy test for women at child-bearing age
- Blood culture
- Skin scraping for microscopy and culture
- Lymphnode aspiration for microcopy and culture if >1cm
- Bone marrow aspiration for microcopy and culture as deemed appropriate by the treating clinician
- Sputum microscopy (Zn stain)
- Liver function test: AST, ALT, bilirubin, LDH
- HIV testing (in accordance with Vietnam MOH guidelines on diagnosis and treatment of HIV/AIDS dated Aug 2009) if not already done

5.1.3 Admission research laboratory tests

At the time of enrollment, the following research laboratory tests will be performed:

5.1.3.1Blood draw for fungal colony count

1 mL of blood will be collected prior to the start of antifungal therapy.

5.1.3.2 Blood draw for routine and fungal culture

5 mL of blood will be collected at screening for routine culture (which will also culture *P. marneffei*).

5.1.3.3 Blood draw for PK-PD analyses

2 mL of heparinized blood will be collected prior to the start of antifungal therapy for all patients enrolled at Hospital for Tropical Diseases and National Hospital for Tropical Diseases.

5.1.3.4 Archived whole blood for molecular and serology research

5 mL of blood prior to the start of antifungal will be collected, processed and archived at -70ºC for serological and molecular research purposes as per protocol.

5.1.3.5 Urinary P. marneffei antigen test

20 mL of urine will be collected prior to the start of antifungal and stored at -20ºC.

5.1.3.6 Admission chest X-ray

A chest X-ray will be performed at enrollment.

Interval Assessments

5.1.4 Interval history and physical exam

The following will be performed according to the schedule in Appendix B

- Presence, worsening or improvement of admission symptoms
- Vital signs and weight
- Physical examination
- Signs and symptoms of AEs

5.1.5 Interval clinical laboratory tests

The following will be performed according to the schedule in Appendix B

- CBC with differential
- Blood chemistries including LFTs
- Sputum microscopy (Zn stain) in day 2 and 3

5.1.6 Interval research laboratory tests

5.1.6.1Blood draw for fungal colony count

1 mL of blood will be collected daily during the first week and every other day during 2nd week until *P. marneffei* yeast cells are no longer seen for 2 consecutive days.

5.1.6.2Blood draw for fungal culture

5 mL of blood will be collected forroutine culture every other day during the first 2 weeks until the fungus can no longer be grown from culture for a total incubation time of 14 days. Blood culture will be performed as part of evaluation for disease relapse if patient is symptomatic at week 4, 12 and 24.

5.1.6.3Blood draw for PK-PD analyses

Patients enrolled at Hospital for Tropical Diseases and National Hospital for Tropical Diseases will participate in a population pharmacokinetic (PK) study. In addition, 30 enrolled patients at the Hospital for Tropical Diseases will participate in the intensive PK analysis. The test schedule for these two groups is shown in the table in Appendix C. For the intensive PK study, we will invite enrolled subjects to participate in this sub-study from the 1st day of enrollment on a continuous basis, and this substudy will close when 30 subjects are enrolled. 2 mL of heparinized blood will be collected 15-17 times over 3 days (day 1, 2 and 8) following set time points as outlined in Appendix C. For the population PK study, 2ml of heparinized blood will be collected during randomized time blocks on day 1, 2, 3, 4, 8, 10 and 12 of hospitalization as outlined in Appendix C, when possible at times of routine hospital care in order to minimize blood sampling. After 2 weeks of hospitalization, PK samples will be collected at outpatient follow-up times at month one, three, and six into therapy. It is crucial that exact time of antifungal medication administration and subsequent blood collection times are recorded in the Case Report Forms.

5.1.6.4 Archived whole blood for molecular and serology research

5 mL of blood will be collected, processed and archived at -70ºC for serological and molecular research purposes as per protocol. These samples will be collected every 2 days for the first week, once for the second week, week 4, week 8, week 12 and week 24.

5.1.6.5 IRD blood tests

10mL of blood will be collected at day 6 and at week 4, 8, 12, 16, 20 and 24 to study the incidence and characteristics of *P. marneffei* associated immune reconstitution disease

5.1.6.6 Urinary P. marneffei antigen test

Approximately 20 mL of urine will be collected every other day to evaluate the urinary antigen clearance rate until *P. marneffei* antigen is no longer detected.

Left over whole blood or serum from routine clinical or research laboratory tests will be stored at -70ºC in case there is not enough blood for a particular test, loss of specimen, etc. The schedule of assessments and collection of research samples will not change without Ethical Committee notification and approval.

5.1.6.7 Follow-up chest X-ray

A follow-up X-ray towards the end of treatment would be standard care for persons with pulmonary lesions found at enrollment.

Interval assessments can be done outside of the hospital if required by the patient.

The Pharmacokinetic samples may be sent to Manchester University, UK for analysis. Other research samples may be sent to OUCRU collaborating labs in UK, US, Singapore and Thai Lan for analysis.

Other Samples

If any of the following samples are obtained for clinical indications (or in the course of usual care), a small portion of these samples should be stored at -70ºC for later analyses detailed below.

5.1.7 Bronchial alveolar lavage

5 ml of fluid obtained from the bronchial alveolar lavage should be saved for further analyses.

5.1.8 Cerebral spinal fluid (CSF)

1 ml of fluid obtained from the lumber puncture should be saved for future studies.

5.1.9 Pleural fluid

5 ml of fluid obtained from the thoracentesis should be saved for future studies.

6 Clinical Response Assessments

All enrolled patients will be seen daily both by treating clinicians and study investigators. Daily vital signs and physical exams will be performed. Measures such as temperature, weight, progression or resolution of skin or mucosal lesions, lymphadenopathy, hepatosplenomegaly,

fatigue, cough and/or shortness of breath, nausea, vomiting, abdominal pain, diarrhea etc will be recorded daily.

Clinical response is defined as resolution of fever (temperature <38ºC for 3 consecutive days) and resolution of skin lesions (either completely gone or in the final stage of healing) due to penicilliosis at the end week 12. This response will also be evaluated earlier during therapy at week 2, week 4, and later at week 24.

7 Clinical Failure or Relapse Assessments

7.1 Clinical failure assessments and management

Subjects that meet the following criteria after 7 days of therapy will be classified as a clinical failure:

- Persistent fungal blood culture, OR
- Persistent worsening of fever and/or skin lesions due to penicilliosis, AND
- The treating clinician judge the patient to be failing current therapy

Subjects who meet the clinical failure criteria at day 7 may be switched to other medications at the discretion of the treating clinician according to the best medical practice. The treating clinician will also make the decision regarding need for continued hospitalization beyond the first 2 weeks of therapy.

7.2 Treatment relapse assessments and management

Subjects who meet developed cultural-confirmed penicilliosis after achieving treatment success at 12 weeks (see section 6 above) will be classified as a treatment relapse.

8 Risks

Risk of Amphotericin B

8.1.1 Infusion-related reactions

Infusion-related reactions, particularly nausea and vomiting, are common with amphotericin B administration, usually occurring between 15 minutes to 3 hours following the initiation of the dose. Nausea and vomiting may require the use of a phenothiazine such as promethazine (usual adult dose - 12.5 to 25 mg every 4 to 6 hours via deep IM only) or prochlorperazine (usual adult dose - 10 mg IM or IV or 25 mg PR every 4 to 6 hours).

Phlebitis is a complication that primarily occurs in patients receiving infusions via a small peripheral vein. The addition of hydrocortisone (usual adult dose - 25 mg) or heparin (usual final concentration — 500 to 1000 U/L) to the infusion may lessen infusion-related thrombophlebitis, but are not routinely recommended.

Other ways to minimize amphotericin B-induced thrombophlebitis include:

- Infusion of the drug using a central line or a large peripheral vein via a catheter
- Use of alternating infusion sites

- Avoidance of final amphotericin B infusion concentrations exceeding 0.1 mg/mL
- Avoidance of infusion times of less than four hours

Drug-induced fever, chills, and headache can also be seen. These symptoms can be minimized or prevented by premedication with paracetamol (usual adult dose - 500 to 1000 mg PO every 4 hours) and/or diphenhydramine (usual adult dose — 25 to 50 mg PO or IV). Nonsteroidal anti-inflammatory agents may also be useful in this setting. In a double-blind, placebo-controlled trial, ibuprofen administered 30 minutes prior to amphotericin B deoxycholate reduced the rate of occurrence of chills from 87 percent to 49 percent [104].

8.1.2 Nephrotoxicity

Amphotericin B administration may result in nephrotoxicity. With amphotericin B deoxycholate, a reversible and often transient decline in glomerular filtration rate (GFR) has been described in 5 to 80 percent of patients. The net effect is an elevation (above baseline) in the plasma creatinine concentration. Although more severe renal failure due to amphotericin B alone is uncommon, the risks of such reactions increase with diuretic-induced volume depletion or the concurrent administration of another nephrotoxin such as an aminoglycoside, cyclosporine, or foscarnet.

Volume expansion with intravenous sodium chloride (a practice commonly known as "sodium loading") may ameliorate the decline in GFR; 500 mL of 0.9 percent sodium chloride is typically given prior to the amphotericin B infusion.

8.1.3 Electrolyte abnormalities

Hypokalemia, hypomagnesemia, and hyperchloremic acidosis are reflections of an increase in distal tubular membrane permeability. Many patients require potassium and/or magnesium supplementation during therapy. Correction of hypokalemia may be difficult in patients with persistent hypomagnesemia.

8.1.4 Other reactions

A reversible, normochromic, normocytic anemia occurs in most patients receiving amphotericin B, but the onset may be delayed for as long as 10 weeks after the initiation of therapy. Transfusions are infrequently required.

Severe allergic reactions (including anaphylaxis) are extremely rare but have been reported.

8.1.5 Patient monitoring

Patients receiving amphotericin B intravenously will be monitored clinically for infusion-related reactions following each administration. Measurements of renal function will be performed 3 times in the first week and 2 times in the second week. If the plasma creatinine concentration exceeds 2.5 mg/dL (265 µM /L), amphotericin B will be permanently discontinued, and the subject switched to itraconazole, and these patients will not be analyzed per protocol.

Serum electrolytes (particularly potassium and magnesium) will be assessed at baseline and 3 times in the first week and 2 times in the second week. Complete blood counts will be measured 3 times in the first week and 2 times in the second week of therapy.

Risk of Itraconazole capsule formulation

8.1.6 Hepatotoxicity

Itraconazole has been associated with rare cases of serious hepatotoxicity, including liver failure and death. Some of these cases had neither pre-existing liver disease nor a serious underlying medical condition. If clinical signs or symptoms develop that are consistent with liver disease, treatment will be discontinued and liver function testing performed and monitored. The risks and benefits of itraconazole use will be reassessed.

8.1.7 Other adverse events

Most common: dyspepsia, abdominal pain, nausea, vomiting, constipation, diarrhoea, headache, and dizziness.
Rarely: increase liver enzyme values, some cases of hepatitis and cholestatic jaundice, especially in those treated for more than one month. There have been rare cases of liver failure and death. Heart failure and pulmonary oedema and serious cardiovascular events including arrhythmias and sudden death have been attributed to drug interactions in patients receiving itraconazole. Alopecia, oedema, and hypokalaemia with prolonged use, menstrual disorders, and peripheral neuropathy have been reported in a few patients.

Others: allergic reaction such as pruritus, rash, urticaria, and angioedema; the Stevens-Johnson syndrome.

8.2.3 Post-marketing experience

Worldwide post-marketing experiences with the use of itraconazole include adverse events of gastrointestinal origin, such as dyspepsia, nausea, vomiting, diarrhea, abdominal pain and constipation. Other reported AEs include peripheral edema, congestive heart failure and pulmonary edema, headache, dizziness, peripheral neuropathy, menstrual disorders, reversible increases in hepatic enzymes, hepatitis, liver failure, hypokalemia, hypertriglyceridemia, alopecia, allergic reactions (such as pruritus, rash, urticaria, angioedema, anaphylaxis), Stevens-Johnson syndrome, anaphylactic, anaphylactoid and allergic reactions, photosensitivity and neutropenia. There is limited information on the use of itraconazole during pregnancy. Cases of congenital abnormalities including skeletal, genitourinary tract, cardiovascular and ophthalmic malformations as well as chromosomal and multiple malformations have been reported during post-marketing experience. A causal relationship with itraconazole has not been established.

8.1.8 Patient monitoring

Patients receiving itraconazole will be monitored clinically for evidence of hepatic dysfunction. Liver function tests will be performed 3 times in the first week and 2 times in the second week. If the transaminitis (AST/ALT) exceeds 10 times the upper limit of normal or other laboratory evidence of grade IV hepatic dysfunction while on itraconazole, itraconazole will be discontinued, and the subject switched to amphotericin B, and these patients will not be analyzed per protocol.

Risk of Phlebotomy and of Intravenous Catheter Placement

The primary risks of phlebotomy include local discomfort, occasional bleeding or bruising of the skin at the site of needle puncture, and rarely hematoma, infection or fainting. At the time of enrollment and during study visits, each subject will be asked about participation in other research studies to ensure that blood draws do not exceed the following for all research protocols combined: 450 mL over any 6-week period for adults.

Only subjects who are assigned to the amphotericin B group will have a midline peripheral catheter placed in the arm for amphotericin B infusion. The risks for a peripheral catheter placement are similar to the risks of phlebotomy above, plus a possibility of vein inflammation. These risks are minimized by performance of only experienced medical persons. The study doctors must examine subjects with a catheter every day to look for signs of infection and inflammation and will replace the catheter immediately upon such a concern.

Risk of Diagnosis

The risk associated with the diagnosis of penicilliosis is that the infection is an AIDS- defining illness, thus potentially exposing patients underlying HIV status that will potentially cause social isolation and stigmatism. All information about patients will be kept confidential and will not be shared outside the clinical and research team.

9 Benefit(s)

Benefits of Treatment

The benefit of treatment for penicilliosis is clear as penicilliosis is fatal if not diagnosed and treated. The relative benefit of treatment with itraconazole versus amphotericin B is entirely unknown. It has been shown in case series that treatment with either amphotericin B followed by itraconazole strategy or itraconazole alone strategy are both quite effective. Treatment medications (amphotericin B and/or itraconazole) will be provided by the study for the entire duration of the 3 month treatment, which represents a significant relief of financial burden for patients whose access to this treatment may have otherwise been limited.

Benefit of Diagnosis

The benefit of knowing the diagnosis of penicilliosis is also clear as penicilliosis is fatal if not diagnosed and treated. For the majority of infectious diseases in general, early diagnosis often leads to better treatment outcomes.

10 Alternatives

The alternative to participation in this study is routine standard care by the doctors in the hospital. For confirmed penicilliosis, patients will generally receive an antifungal (amphotericin B or itraconazole) based largely on ability to pay the costs and perhaps disease severity. Patients will have to pay for the cost of drugs and the care for the entire treatment duration. Follow up might not be as stringent compared to patients who participate in this study.

11 Data Management

Source documents will be generated during the study by the site study staff at participating institutions. Source documents include all original recordings of observations or notations of clinical activities, and all reports and records necessary for the evaluation and reconstruction of the clinical trial. Source documents include, but are not limited to, the subject's medical records, research case record forms (paper or electronic), laboratory reports, ECG tracings, x-rays, radiologist's reports, subject's diaries and questionnaires, biopsy reports, ultrasound photographs, progress notes, pharmacy records, and any other similar reports or records of procedures performed during the subject's participation in the study.

Access to applicable source documents will need to be made available for study purposes. The site investigators are responsible for maintaining any source documentation related to the study. Source documentation should support the data collected on the CRF when the CRF is not the original site of recording**,** and must be signed and dated by the person recording and/or reviewing the data. Source documentation must be available for review or audit by the sponsor or designee and any applicable national authorities.

Case Report Forms (CRFs) will be used as a data collection tool. The study team will transfer the information from the source documents onto the CRFs. CRFs may be used as source documents if they are the primary data collection tool for specified data as documented in written standard operating procedures. The site Investigators are responsible for maintaining accurate, complete and up-to-date records and for tracking receipt of CRFs for each participant. These forms are to be completed on an ongoing basis during the course of the study by authorized individuals. All subject CRFs will be reviewed by the designated staff and signed as required.

The CRFs and instructions will be distributed to the site(s) by the Principle Investigator. Data entries on paper CRFs must be completed legibly with pen. Corrections must be made by striking through the incorrect entry with a single line (taking care not to obliterate or render the original entry illegible) and entering the correct information adjacent to the incorrect entry. Corrections to paper CRFs must be initialed and dated by the person making the correction. All CRFs should be reviewed by the designated study staff and signed as required with written or electronic signature, as appropriate.

Selected study members will be trained by a Data Manager on how to enter all clinical data as source information, from the CRFs and from laboratory source documents into a internet-based computerized data entry system called CliRes. This is a single computerized data entry that occurs simultaneously as clinical/research data are being collected during the trial as soon as possible after the information is generated. Source documents and electronic data will be verified according to the Trial Monitoring Plan.

12 Monitoring

Study Monitoring

The trial will be conducted in compliance with this protocol, Medical Research Council Guidelines of Good Clinical Practice, International Conference on Harmonization (ICH) Guidelines for Good Clinical Practice (GCP) and all applicable regulatory requirement(s).

As per ICH-GCP 5.18 clinical protocols are required to be adequately monitored by the study sponsor. Monitors will visit the clinical research site to monitor all aspects of the study in accordance with the appropriate regulations and the approved protocol. The objectives of a monitoring visit will be: 1) to verify the existence of signed informed consent documents for each monitored subject; 2) to verify the prompt and accurate recording of all monitored data points, and prompt reporting of all unexpected SAEs; 3) to compare abstracted information with individual subjects' records and source documents (subjects' charts, case report forms, laboratory analyses and test results, physicians' progress notes, nurses' notes, and any other relevant original subject information); and 4) to ensure protection of study subjects, investigators' compliance with the protocol, and completeness and accuracy of study records. The monitors also will inspect the clinical site regulatory files to ensure that regulatory requirements and applicable guidelines are being followed. During the monitoring visits, the investigator (and/or designee) and other study personnel will be available to discuss the study progress and monitoring visit.

Data and Safety Monitoring Plan

An independent data monitoring and ethical committee (DMEC) will oversee the trial. Unexpected serious adverse events will be reported to the DMEC and to the responsible Ethical Committees within ten working days of occurrence.

The DMEC will perform interim analyses after recruitment of 100 patients or after 20 deaths, whichever comes first. The review will include review of summary tables of grade 3 and 4 adverse events, serious adverse events and an analysis of mortality.
Based on these data, the committee will make one of the following recommendations:

- Continue the trial without modification
- Continue the trial with modification
- Discontinue the trial due to safety or other concerns

The DMEC may also suggest discontinuation if the trial results indicate "beyond reasonable doubt" that one of the allocated strategies is better than the other in primary outcome. The Haybittle-Peto boundary, requiring $p<0.001$ at interim analysis to consider stopping for efficacy, should be used as a guidance. However, the DMEC recommendation should not be based purely on statistical tables but also requires clinical judgment.

As the dissemination of preliminary summary data could influence the further conduct of the trial and introduce bias, access to interim data and results will be confidential and strictly limited to the involved statistician and the monitoring board and results (except for the recommendation) will not be communicated to the outside and/or clinical investigators involved in the trial.

Further reviews will be at the discretion of the DMEC or the request of the Trial Steering Committee. All DMEC reports, replies or decisions will be sent to the Trial Steering Committee and the responsible Research Ethical Committees.

13 Definition and Assessment of Adverse Events

Definition of Adverse Events

An adverse event (AE) is any undesirable event that occurs to a study participant during the course of the study whether or not that event is considered related to the study drug. An AE

can, therefore, be any unfavorable and unintended sign (including an abnormal laboratory finding, for example), symptom, or disease temporally associated with the study drug, whether or not considered related to the study drug.
Examples include:

- An increase in severity or frequency of a pre-existing abnormality or disorder (events that are marked by a change from the participant's baseline/entry status)
- All reactions from sensitivity or toxicity to study drug
- Injuries or accidents (e.g., for a fall secondary to dizziness, record "dizziness" as the event and include the information about the fall in the comment/narrative section and information about the injury secondary to the fall as part of the "outcome")
- New clinically significant abnormalities in clinical laboratory values, physiological testing or physical examination.

Stable chronic conditions, such as arthritis, which are present prior to clinical trial entry and do not worsen are not considered AEs and will be documented in the subject's clinical chart as medical history.

Clinical or laboratory events are considered adverse events only if they occur after the first dose of study treatment and before the patient completes trial participation. (See below for reporting of adverse events.)

Definition of Serious Adverse Events

An AE is considered to be "serious" if it results in one of the following outcomes

- Death,
- Life-threatening event (the subject was at immediate risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe),
- Inpatient hospitalization or prolongation of existing hospitalization
- Persistent or significant disability/incapacity (a substantial disruption of a person's ability to conduct normal life functions),
- Congenital anomaly/birth defect
- Important medical event that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the other outcomes listed in the definition above.

An AE needs to meet only one of the above criteria to be considered serious.

Definition of Unexpected Serious Adverse Events

Untoward medical events which fit one or more criteria of SAE above and which are not considered a part of normal clinical progression of disease or expected drug reaction or any event which becomes of concern to the investigators or study doctors during the course of the trial may be reported as a USAE.

Assessment of Adverse Events

All adverse events that occur after the initiation of trial itraconazole or amphotericin B therapy will be graded according to the scale below.

- **Mild:** (Grade 1): Transient or mild symptoms; no limitation in activity; no intervention required. The AE does not interfere with the participant's normal functioning level.

- **Moderate** (Grade 2): Symptom results in mild to moderate limitation in activity; no or minimal intervention required. The AE produces some impairment of functioning, but it is not hazardous to health.

- **Severe** (Grade 3): Symptom results in significant limitation in activity; medical intervention may be required. The AE produces significant impairment of functioning or incapacitation.

- **Life-threatening** (Grade 4): Extreme limitation in activity, significant assistance required; significant medical intervention or therapy required; hospitalization.

[Note: "Life-threatening" as a severity grade is not necessarily the same as "life-threatening" as a "serious" criterion. The former is a "potential" threat to life and the latter is an "immediate" threat to life.]

A laboratory abnormality is an adverse event if it is associated with an intervention. Intervention includes, but is not limited to, discontinuation of treatment, dose reduction/delay, or concomitant treatment. In addition, any medically important laboratory abnormality may be reported as an adverse event at the discretion of the investigator. This would include a laboratory result for which there is no intervention but the abnormal value suggests a disease or organ toxicity. Laboratory events will be graded according to the following criteria:

- Events resulting in severe symptoms, condition or intervention will be classified as Grade 3.
- Events which are deemed to be life-threatening will be classified as Grade 4.

If clinical sequelae are associated with a laboratory abnormality, the diagnosis or medical condition should be reported as the adverse event (e.g., renal failure, hematuria) not the laboratory abnormality (e.g., elevated creatinine, urine RBC increase).

14 Adverse Event Reporting

Since there is extensive experience with both amphotericin B and itraconazole in clinical practice, the fact that evaluation of safety is not a primary objective in this trial, and the fact that both drugs and the dosages used in the protocol are approved by Vietnam Ministry of Health for treatment of penicilliosis, only unexpected Serious Adverse Events (SAEs) which occur at any time during the trial will be reported to the DMEC and Ethical Committees within ten working days of occurrence.

Grade 3 adverse events, grade 4 adverse events and serious adverse events which occur between initial dose of study medication and up to 6 months after initial dose will be recorded in the case report form. These events will be entered into the study database and provided to the DMEC upon safety review as required. Grade 1 and grade 2 adverse events will not be recorded. Events which are not unexpected serious adverse events will not be recorded after 6 months of study participation.

15 Human Subject Protections

Ethical Approval

This protocol, patient information sheet, informed consent document, relevant supporting information will be submitted to the designated Ethical Committee (EC) and must be approved before the study is initiated.

Any amendments must also be approved by the designated EC prior to implementing changes in the study.

The investigators are responsible for keeping the designated EC appraised of the progress of the study as deemed appropriate, but in any case at least once a year.

Compliance with Good Clinical Practice

This study will be conducted in compliance with the conditions stipulated by the Ethical Committee of the Viet Nam Ministry of Health and the Oxford Tropical Research Ethics Committee, Medical Research Council Guidelines of Good Clinical Practice and International Conference on Harmonisation, Good Clinical Practice (ICH/GCP) Guidelines. In addition, all local regulatory requirements will be adhered to, in particular those which afford greater protection to the safety of the trial participants.

Informed Consent

The informed consent for this study will be translated into Vietnamese and must be signed by the study participant or legal representative before participation in the study, including any screening procedures. A copy of the signed consent must be provided to the study participant. Signed consents must remain in each study participants study file, and be available for verification by study monitors at any time.

In the case of illiterate subjects, the consent will be read in Vietnamese to the subjects in the presence of a literate witness who will sign to confirm the accurate reading of the form.

If the subject is too ill to consent, the next of kin may consent for the subject. Once the subject is able, the subject will be consented for continuation in the study.

Separate informed consent forms will be signed for participation in the intensive pharmacokinetic portion of the study. Study sites participating in the case control portion of the study will have appropriate information included in the informed consent form. Participants in the control arm of the case control study will have a separate consent specific only to procedures in that portion of the study.

Rationale for Research Subject Selection

15.1.1 Inclusion of adults male and female age ≥18 years

The study will only include adult patients from both sexes and age ≥18 years as the study sites only treat adult HIV-infected patients. Although in Vietnam a patient is considered an adult at

the physiologic age of ≥15 years, the actual number HIV-infected patients who is at WHO stage IV disease from age 15 to 18 is likely to be too low to justify their inclusion in this protocol.

15.1.2 Justification of Exclusions

The exclusion criteria are primarily to increase subject safety. The exclusion of pregnant women is to minimize any potential thread to the fetus (with itraconazole) and to prevent significant variation in interpretation of PK-PD data. Children <15 years of age are excluded as there are not enough pediatric HIV-infected patients and therefore not enough of those patients with penicilliosis to feasibly set up a study site in a pediatric hospital. Penicilliosis patients with CNS signs/symptoms might have *P. marneffei* CNS infection and should not receive itraconazole as this drug does not penetrate the CNS very well. Patients with transaminases >10 times upper limit of normal should not be on itraconazole. Patients with absolute neutrophil count <500 cells/μL should not be on amphotericin B. Patients with cryptococcal meningitis need to be treated with the current standard of care which is IV amphotericin B. Patients with active TB or being treated for TB with rifampicin should not be on itraconazole because of drug-drug interactions.

Record Retention

The investigator is responsible for retaining all essential documents listed in the ICH Good Clinical Practice Guidelines. All essential documentation for all study subjects are to be maintained by the investigators in a secure storage facility for 15 years according to the requirements of the Viet Nam Ministry of Health. All stored records are to be kept confidential. It is the investigator's responsibility to retain copies of source documents

Storage of Samples

Approximately 15 ml of blood and cultured strains of *P.marneffei* will be stored in the hospital freezer at -70ºC only for the secondary analyses specified in the protocol. In the future, other investigators may wish to study these samples and/or data. In that case, EC approval must be sought prior to any sharing of samples and/or data. Any clinical information shared about the sample would similarly require prior EC approval.

Access to stored samples will be limited using a locked room under the control of Oxford University Clinical Research Unit. Samples and data will be stored using codes (not subjects' names) assigned by the investigators. Only investigators will have access to the samples and data. At the end of the study, samples will continue to be stored indefinitely in the hospital freezer at -70ºC.

Subjects may decide at any point not to have their samples stored. In this case, the principal investigator will destroy all known remaining samples and report what was done to the subject.

Anonymity and Confidentiality

The information obtained during the conduct of this clinical study is confidential. The results of the research study may be published, but patient names or identities will not be revealed. Records will remain confidential. To maintain confidentiality, the principal investigators at each site will keep records in locked cabinets and the results of tests will be coded to prevent association with the subject's names.

Compensation

Monetary reimbursement will be provided in accordance with OUCRU policy for lost time and travel fees incurred to study participants.

The study will cover the costs of the 2 week hospitalization and all related research tests. The study will not cover long term care for disability after hospitalization resulting from the complications of the illness. Reasonable transportation cost for the follow-up visits will also be covered

References

1. Supparatpinyo K, Nelson KE, Merz WG, et al. Response to antifungal therapy by human immunodeficiency virus-infected patients with disseminated penicillium marneffei infections and in vitro susceptibilities of isolates from clinical specimens. Antimicrob Agents Chemother **1993**;37(11):2407-11.

2. Supparatpinyo K, Khamwan C, Baosoung V, Nelson KE, Sirisanthana T. Disseminated penicillium marneffei infection in southeast asia. Lancet **1994**;344(8915):110-3.

3. Antinori S, Gianelli E, Bonaccorso C, et al. Disseminated penicillium marneffei infection in an HIV-positive italian patient and a review of cases reported outside endemic regions. J Travel Med **2006**;13(3):181-8.

4. Filiotou A, Velegraki A, Giannaris M, et al. First case of penicillium marneffei fungemia in greece and strain susceptibility to five licensed systemic antifungal agents and posaconazole. Am J Med Sci **2006**;332(1):43-5.

5. Cristofaro P, Mileno MD. Penicillium marneffei infection in HIV-infected travelers. Aids Alert **2006**;21(12):140-2.

6. Depraetere K, Colebunders R, Ieven M, et al. Two imported cases of penicillium marneffei infection in belgium. Acta Clin Belg **1998**;53(4):255-8.

7. Jones PD, See J. Penicillium marneffei infection in patients infected with human immunodeficiency virus: Late presentation in an area of nonendemicity. Clin Infect Dis **1992**;15(4):744.

8. Julander I, Petrini B. Penicillium marneffei infection in a swedish HIV-infected immunodeficient narcotic addict. Scand J Infect Dis **1997**;29(3):320-2.

9. Nguyen K, Taylor S, Wanger A, Ali A, Rapini RP. A case of penicillium marneffei in a US hospital. J Am Acad Dermatol **2006**;54(4):730-2.

10. Sobottka I, Albrecht H, Mack D, et al. Systemic penicillium marneffei infection in a german AIDS patient. Eur J Clin Microbiol Infect Dis **1996**;15(3):256-9.

11. Sirisanthana T, Supparatpinyo K, Perriens J, Nelson KE. Amphotericin B and itraconazole for treatment of disseminated penicillium marneffei infection in human immunodeficiency virus-infected patients. Clin Infect Dis **1998**;26(5):1107-10.

12. Boogaerts M, Winston DJ, Bow EJ, et al. Intravenous and oral itraconazole versus intravenous amphotericin B deoxycholate as empirical antifungal therapy for persistent fever in neutropenic patients with cancer who are receiving broad-spectrum antibacterial therapy. A randomized, controlled trial. Ann Intern Med **2001**;135(6):412-22.

13. Boogaerts M, Maertens J. Clinical experience with itraconazole in systemic fungal infections. Drugs **2001**;61 Suppl 1:39-47.

14. Caillot D, Bassaris H, McGeer A, et al. Intravenous itraconazole followed by oral itraconazole in the treatment of invasive pulmonary aspergillosis in patients with hematologic malignancies, chronic granulomatous disease, or AIDS. Clin Infect Dis **2001**;33(8):e83-90.

15. Denning DW, Tucker RM, Hanson LH, Hamilton JR, Stevens DA. Itraconazole therapy for cryptococcal meningitis and cryptococcosis. Arch Intern Med **1989**;149(10):2301-8.

16. Denning DW, Tucker RM, Hanson LH, Stevens DA. Itraconazole in opportunistic mycoses: Cryptococcosis and aspergillosis. J Am Acad Dermatol **1990**;23(3 Pt 2):602-7.

17. Denning DW, Tucker RM, Hanson LH, Stevens DA. Treatment of invasive aspergillosis with itraconazole. Am J Med **1989**;86(6 Pt 2):791-800.

18. Diaz M, Puente R, de Hoyos LA, Cruz S. Itraconazole in the treatment of coccidioidomycosis. Chest **1991**;100(3):682-4.

19. Dismukes WE, Bradsher RW,Jr, Cloud GC, et al. Itraconazole therapy for blastomycosis and histoplasmosis. NIAID mycoses study group. Am J Med **1992**;93(5):489-97.

20. Graybill JR, Stevens DA, Galgiani JN, Dismukes WE, Cloud GA. Itraconazole treatment of coccidioidomycosis. NAIAD mycoses study group. Am J Med **1990**;89(3):282-90.

21. Tucker RM, Denning DW, Dupont B, Stevens DA. Itraconazole therapy for chronic coccidioidal meningitis. Ann Intern Med **1990**;112(2):108-12.

22. Ranjana KH, Priyokumar K, Singh TJ, et al. Disseminated penicillium marneffei infection among HIV-infected patients in manipur state, india. J Infect **2002**;45(4):268-71.

23. Barone JA, Moskovitz BL, Guarnieri J, et al. Enhanced bioavailability of itraconazole in hydroxypropyl-beta-cyclodextrin solution versus capsules in healthy volunteers. Antimicrob Agents Chemother **1998**;42(7):1862-5.

24. Saag M. Itraconazole oral solution: Pharmacokinetics and absorption. AIDS Patient Care STDS **1997**;11 Suppl 1:S16-7.

25. Fisher MC, Aanensen D, de Hoog S, Vanittanakom N. Multilocus microsatellite typing system for penicillium marneffei reveals spatially structured populations. J Clin Microbiol **2004**;42(11):5065-9.

26. Capponi M, Sureau P, Segretain G. Penicillose de rhizomys sinensis. Bull Soc Pathol Exot **1956**;49:418-21.

27. SEGRETAIN G. Penicillium marneffei n.sp., agent of a mycosis of the reticuloendothelial system. Mycopathologia **1959**;11:327-53.

28. DiSalvo AF, Fickling AM, Ajello L. Infection caused by penicillium marneffei: Description of first natural infection in man. Am J Clin Pathol **1973**;60(2):259-63.

29. Ajello L, Padhye AA, Sukroongreung S, Nilakul CH, Tantimavanic S. Occurrence of penicillium marneffei infections among wild bamboo rats in thailand. Mycopathologia **1995**;131(1):1-8.

30. Chariyalertsak S, Vanittanakom P, Nelson KE, Sirisanthana T, Vanittanakom N. Rhizomys sumatrensis and cannomys badius, new natural animal hosts of penicillium marneffei. J Med Vet Mycol **1996**;34(2):105-10.

31. Gugnani H, Fisher MC, Paliwal-Johsi A, Vanittanakom N, Singh I, Yadav PS. Role of cannomys badius as a natural animal host of penicillium marneffei in india. J Clin Microbiol **2004**;42(11):5070-5.

32. Deng ZL, Yun M, Ajello L. Human penicilliosis marneffei and its relation to the bamboo rat (rhizomys pruinosus). J Med Vet Mycol **1986**;24(5):383-9.

33. Gugnani HC, Paliwal-Joshi A, Rahman H, et al. Occurrence of pathogenic fungi in soil of burrows of rats and of other sites in bamboo plantations in india and nepal. Mycoses **2007**;50(6):507-11.

34. Chariyalertsak S, Sirisanthana T, Supparatpinyo K, Praparattanapan J, Nelson KE. Case-control study of risk factors for penicillium marneffei infection in human immunodeficiency virus-infected patients in northern thailand. Clin Infect Dis **1997**;24(6):1080-6.

35. Chariyalertsak S, Sirisanthana T, Supparatpinyo K, Nelson KE. Seasonal variation of disseminated penicillium marneffei infections in northern thailand: A clue to the reservoir? J Infect Dis **1996**;173(6):1490-3.

36. Lo Y, Tintelnot K, Lippert U, Hoppe T. Disseminated penicillium marneffei infection in an african AIDS patient. Trans R Soc Trop Med Hyg **2000**;94(2):187.

37. Wong KH, Lee SS. Comparing the first and second hundred AIDS cases in hong kong. Singapore Med J **1998**;39(6):236-40.

38. Wong SS, Wong KH, Hui WT, et al. Differences in clinical and laboratory diagnostic characteristics of penicilliosis marneffei in human immunodeficiency virus (HIV)- and non-HIV-infected patients. J Clin Microbiol **2001**;39(12):4535-40.

39. Chiewchanvit S, Mahanupab P, Hirunsri P, Vanittanakom N. Cutaneous manifestations of disseminated penicillium marneffei mycosis in five HIV-infected patients. Mycoses **1991**;34(5-6):245-9.

40. Cheng NC, Wong WW, Fung CP, Liu CY. Unusual pulmonary manifestations of disseminated penicillium marneffei infection in three AIDS patients. Med Mycol **1998**;36(6):429-32.

41. Kantipong P, Panich V, Pongsurachet V, Watt G. Hepatic penicilliosis in patients without skin lesions. Clin Infect Dis **1998**;26(5):1215-7.

42. Jayanetra P, Nitiyanant P, Ajello L, et al. Penicilliosis marneffei in thailand: Report of five human cases. Am J Trop Med Hyg **1984**;33(4):637-44.

43. Louthrenoo W, Thamprasert K, Sirisanthana T. Osteoarticular penicilliosis marneffei. A report of eight cases and review of the literature. Br J Rheumatol **1994**;33(12):1145-50.

44. Chaiwun B, Khunamornpong S, Sirivanichai C, et al. Lymphadenopathy due to penicillium marneffei infection: Diagnosis by fine needle aspiration cytology. Mod Pathol **2002**;15(9):939-43.

45. Chan YH, Wong KM, Lee KC, et al. Pneumonia and mesenteric lymphadenopathy caused by disseminated penicillium marneffei infection in a cadaveric renal transplant recipient. Transpl Infect Dis **2004**;6(1):28-32.

46. Sun HY, Chen MY, Hsiao CF, Hsieh SM, Hung CC, Chang SC. Endemic fungal infections caused by cryptococcus neoformans and penicillium marneffei in patients infected with human immunodeficiency virus and treated with highly active anti-retroviral therapy. Clin Microbiol Infect **2006**;12(4):381-8.

47. Mootsikapun P, Srikulbutr S. Histoplasmosis and penicilliosis: Comparison of clinical features, laboratory findings and outcome. Int J Infect Dis **2006**;10(1):66-71.

48. Supparatpinyo K, Chiewchanvit S, Hirunsri P, Uthammachai C, Nelson KE, Sirisanthana T. Penicillium marneffei infection in patients infected with human immunodeficiency virus. Clin Infect Dis **1992**;14(4):871-4.

49. Chaiyaroj SC, Chawengkirttikul R, Sirisinha S, Watkins P, Srinoulprasert Y. Antigen detection assay for identification of penicillium marneffei infection. J Clin Microbiol **2003**;41(1):432-4.

50. Panichakul T, Chawengkirttikul R, Chaiyaroj SC, Sirisinha S. Development of a monoclonal antibody-based enzyme-linked immunosorbent assay for the diagnosis of penicillium marneffei infection. Am J Trop Med Hyg **2002**;67(4):443-7.

51. Vanittanakom N, Mekaprateep M, Sittisombut N, et al. Western immunoblot analysis of protein antigens of penicillium marneffei. J Med Vet Mycol **1997**;35(2):123-31.

52. Huang YT, Hung CC, Hsueh PR. Aspergillus galactomannan antigenemia in penicilliosis marneffei. AIDS **2007**;21(14):1990-1.

53. Desakorn V, Simpson AJ, Wuthiekanun V, et al. Development and evaluation of rapid urinary antigen detection tests for diagnosis of penicilliosis marneffei. J Clin Microbiol **2002**;40(9):3179-83.

54. Vanittanakom N, Vanittanakom P, Hay RJ. Rapid identification of penicillium marneffei by PCR-based detection of specific sequences on the rRNA gene. J Clin Microbiol **2002**;40(5):1739-42.

55. Imwidthaya P, Thipsuvan K, Chaiprasert A, Danchaivijitra S, Sutthent R, Jearanaisilavong J. Penicillium marneffei: Types and drug susceptibility. Mycopathologia **2001**;149(3):109-15.

56. Radford SA, Johnson EM, Warnock DW. In vitro studies of activity of voriconazole (UK-109,496), a new triazole antifungal agent, against emerging and less-common mold pathogens. Antimicrob Agents Chemother **1997**;41(4):841-3.

57. Ryder NS. Activity of terbinafine against serious fungal pathogens. Mycoses **1999**;42 Suppl 2:115-9.

58. Sar B, Boy S, Keo C, et al. In vitro antifungal-drug susceptibilities of mycelial and yeast forms of penicillium marneffei isolates in cambodia. J Clin Microbiol **2006**;44(11):4208-10.

59. Nakai T, Uno J, Ikeda F, Tawara S, Nishimura K, Miyaji M. In vitro antifungal activity of micafungin (FK463) against dimorphic fungi: Comparison of yeast-like and mycelial forms. Antimicrob Agents Chemother **2003**;47(4):1376-81.

60. Supparatpinyo K, Chiewchanvit S, Hirunsri P, et al. An efficacy study of itraconazole in the treatment of penicillium marneffei infection. J Med Assoc Thai **1992**;75(12):688-91.

61. Supparatpinyo K, Perriens J, Nelson KE, Sirisanthana T. A controlled trial of itraconazole to prevent relapse of penicillium marneffei infection in patients infected with the human immunodeficiency virus. N Engl J Med **1998**;339(24):1739-43.

62. Chaiwarith R, Charoenyos N, Sirisanthana T, Supparatpinyo K. Discontinuation of secondary prophylaxis against penicilliosis marneffei in AIDS patients after HAART. AIDS **2007**;21(3):365-7.

63. Hung CC, Chen MY, Hsieh SM, Sheng WH, Hsiao CF, Chang SC. Discontinuation of secondary prophylaxis for penicilliosis marneffei in AIDS patients responding to highly active antiretroviral therapy. AIDS **2002**;16(4):672-3.

64. Sun HY, Chen MY, Hsiao CF, Hsieh SM, Hung CC, Chang SC. Endemic fungal infections caused by cryptococcus neoformans and penicillium marneffei in patients infected with human immunodeficiency virus and treated with highly active anti-retroviral therapy. Clin Microbiol Infect **2006**;12(4):381-8.

65. Chariyalertsak S, Supparatpinyo K, Sirisanthana T, Nelson KE. A controlled trial of itraconazole as primary prophylaxis for systemic fungal infections in patients with advanced human immunodeficiency virus infection in thailand. Clin Infect Dis **2002**;34(2):277-84.

66. Hamilton AJ, Jeavons L, Youngchim S, Vanittanakom N. Recognition of fibronectin by penicillium marneffei conidia via a sialic acid-dependent process and its relationship to the interaction between conidia and laminin. Infect Immun **1999**;67(10):5200-5.

67. Kudeken N, Kawakami K, Kusano N, Saito A. Cell-mediated immunity in host resistance against infection caused by penicillium marneffei. J Med Vet Mycol **1996**;34(6):371-8.

68. Sisto F, Miluzio A, Leopardi O, Mirra M, Boelaert JR, Taramelli D. Differential cytokine pattern in the spleens and livers of BALB/c mice infected with penicillium marneffei: Protective role of gamma interferon. Infect Immun **2003**;71(1):465-73.

69. Roilides E, Lyman CA, Sein T, Petraitiene R, Walsh TJ. Macrophage colony-stimulating factor enhances phagocytosis and oxidative burst of mononuclear phagocytes against penicillium marneffei conidia. FEMS Immunol Med Microbiol **2003**;36(1-2):19-26.

70. Kudeken N, Kawakami K, Saito A. Mechanisms of the in vitro fungicidal effects of human neutrophils against penicillium marneffei induced by granulocyte-macrophage colony-stimulating factor (GM-CSF). Clin Exp Immunol **2000**;119(3):472-8.

71. Taylor JW, Geiser DM, Burt A, Koufopanou V. The evolutionary biology and population genetics underlying fungal strain typing. Clin Microbiol Rev **1999**;12(1):126-46.

72. Vanittanakom N, Cooper CR,Jr, Chariyalertsak S, Youngchim S, Nelson KE, Sirisanthana T. Restriction endonuclease analysis of penicillium marneffei. J Clin Microbiol **1996**;34(7):1834-6.

73. Hsueh PR, Teng LJ, Hung CC, et al. Molecular evidence for strain dissemination of penicillium marneffei: An emerging pathogen in taiwan. J Infect Dis **2000**;181(5):1706-12.

74. Trewatcharegon S, Sirisinha S, Romsai A, Eampokalap B, Teanpaisan R, Chaiyaroj SC. Molecular typing of penicillium marneffei isolates from thailand by NotI macrorestriction and pulsed-field gel electrophoresis. J Clin Microbiol **2001**;39(12):4544-8.

75. Maiden MC, Bygraves JA, Feil E, et al. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A **1998**;95(6):3140-5.

76. Fisher MC, DE Hoog S, Akom NV. A highly discriminatory multilocus microsatellite typing (MLMT) system for penicillium marneffei. Mol Ecol Notes **2004**;4(3):515-8.

77. Fisher MC, Koenig G, White TJ, Taylor JW. A test for concordance between the multilocus genealogies of genes and microsatellites in the pathogenic fungus coccidioides immitis. Mol Biol Evol **2000**;17(8):1164-74.

78. Lasker BA. Nucleotide sequence-based analysis for determining the molecular epidemiology of penicillium marneffei. J Clin Microbiol **2006**;44(9):3145-53.

79. Goldstein DB, Ruiz Linares A, Cavalli-Sforza LL, Feldman MW. An evaluation of genetic distances for use with microsatellite loci. Genetics **1995**;139(1):463-71.

80. Zuckerman JM, Tunkel AR. Itraconazole: A new triazole antifungal agent. Infect Control Hosp Epidemiol **1994**;15(6):397-410.

81. Haria M, Bryson HM, Goa KL. Itraconazole. A reappraisal of its pharmacological properties and therapeutic use in the management of superficial fungal infections. Drugs **1996**;51(4):585-620.

82. Van Cauteren H, Heykants J, De Coster R, Cauwenbergh G. Itraconazole: Pharmacologic studies in animals and humans. Rev Infect Dis **1987**;9 Suppl 1:S43-6.

83. Woestenborghs R, Lorreyne W, Heykants J. Determination of itraconazole in plasma and animal tissues by high-performance liquid chromatography. J Chromatogr **1987**;413:332-7.

84. Heykants J, Van Peer A, Van de Velde V, et al. The clinical pharmacokinetics of itraconazole: An overview. Mycoses **1989**;32 Suppl 1:67-87.

85. Lange D, Pavao JH, Wu J, Klausner M. Effect of a cola beverage on the bioavailability of itraconazole in the presence of H2 blockers. J Clin Pharmacol **1997**;37(6):535-40.

86. De Beule K. Itraconazole: Pharmacology, clinical experience and future development. Int J Antimicrob Agents **1996**;6(3):175-81.

87. Odds FC, Bossche HV. Antifungal activity of itraconazole compared with hydroxy-itraconazole in vitro. J Antimicrob Chemother **2000**;45(3):371-3.

88. Cartledge JD, Midgely J, Gazzard BG. Itraconazole solution: Higher serum drug concentrations and better clinical response rates than the capsule formulation in acquired immunodeficiency syndrome patients with candidosis. J Clin Pathol **1997**;50(6):477-80.

89. Van Cutsem J. In-vitro and in-vivo activity of itraconazole. Med Klin (Munich) **1991**;86 Suppl 1:5-8.

90. Van Cutsem J. In vitro antifungal spectrum of itraconazole and treatment of systemic mycoses with old and new antimycotic agents. Chemotherapy **1992**;38 Suppl 1:3-11.

91. Tucker RM, Denning DW, Arathoon EG, Rinaldi MG, Stevens DA. Itraconazole therapy for nonmeningeal coccidioidomycosis: Clinical and laboratory observations. J Am Acad Dermatol **1990**;23(3 Pt 2):593-601.

92. Boogaerts MA, Verhoef GE, Zachee P, Demuynck H, Verbist L, De Beule K. Antifungal prophylaxis with itraconazole in prolonged neutropenia: Correlation with plasma levels. Mycoses **1989**;32 Suppl 1:103-8.

93. Glasmacher A, Hahn C, Leutner C, et al. Breakthrough invasive fungal infections in neutropenic patients after prophylaxis with itraconazole. Mycoses **1999**;42(7-8):443-51.

94. Tricot G, Joosten E, Boogaerts MA, Vande Pitte J, Cauwenbergh G. Ketoconazole vs. itraconazole for antifungal prophylaxis in patients with severe granulocytopenia: Preliminary results of two nonrandomized studies. Rev Infect Dis **1987**;9 Suppl 1:S94-9.

95. Prentice AG, Glasmacher A, Djulbegovic B. In meta-analysis itraconazole is superior to fluconazole for prophylaxis of systemic fungal infection in the treatment of haematological malignancy. Br J Haematol **2006**;132(5):656,8; author reply 658-9.

96. Torrado JJ, Espada R, Ballesteros MP, Torrado-Santiago S. Amphotericin B formulations and drug targeting. J Pharm Sci **2008**;97(7):2405-25.

97. Bennett J. Antimicrobial agents: Antifungal agents. In: Hardman J, Limbird L, eds. The Goodman & Gilman's pharmacological basis of therapeudics. New York: McGraw-Hill, **1995**:1175-790.

98. Kajtar M, Vikmon M, Morlin E, Szejtli J. Aggregation of amphotericin B in the presence of gamma-cyclodextrin. Biopolymers **1989**;28(9):1585-96.

99. Mazerski J, Grzybowska J, Borowski E. Influence of net charge on the aggregation and solubility behaviour of amphotericin B and its derivatives in aqueous media. Eur Biophys J **1990**;18(3):159-64.

100. Tancrede P, Barwicz J, Jutras S, Gruda I. The effect of surfactants on the aggregation state of amphotericin B. Biochim Biophys Acta **1990**;1030(2):289-95.

101. Aramwit P, Yu BG, Lavasanifar A, Samuel J, Kwon GS. The effect of serum albumin on the aggregation state and toxicity of amphotericin B. J Pharm Sci **2000**;89(12):1589-93.

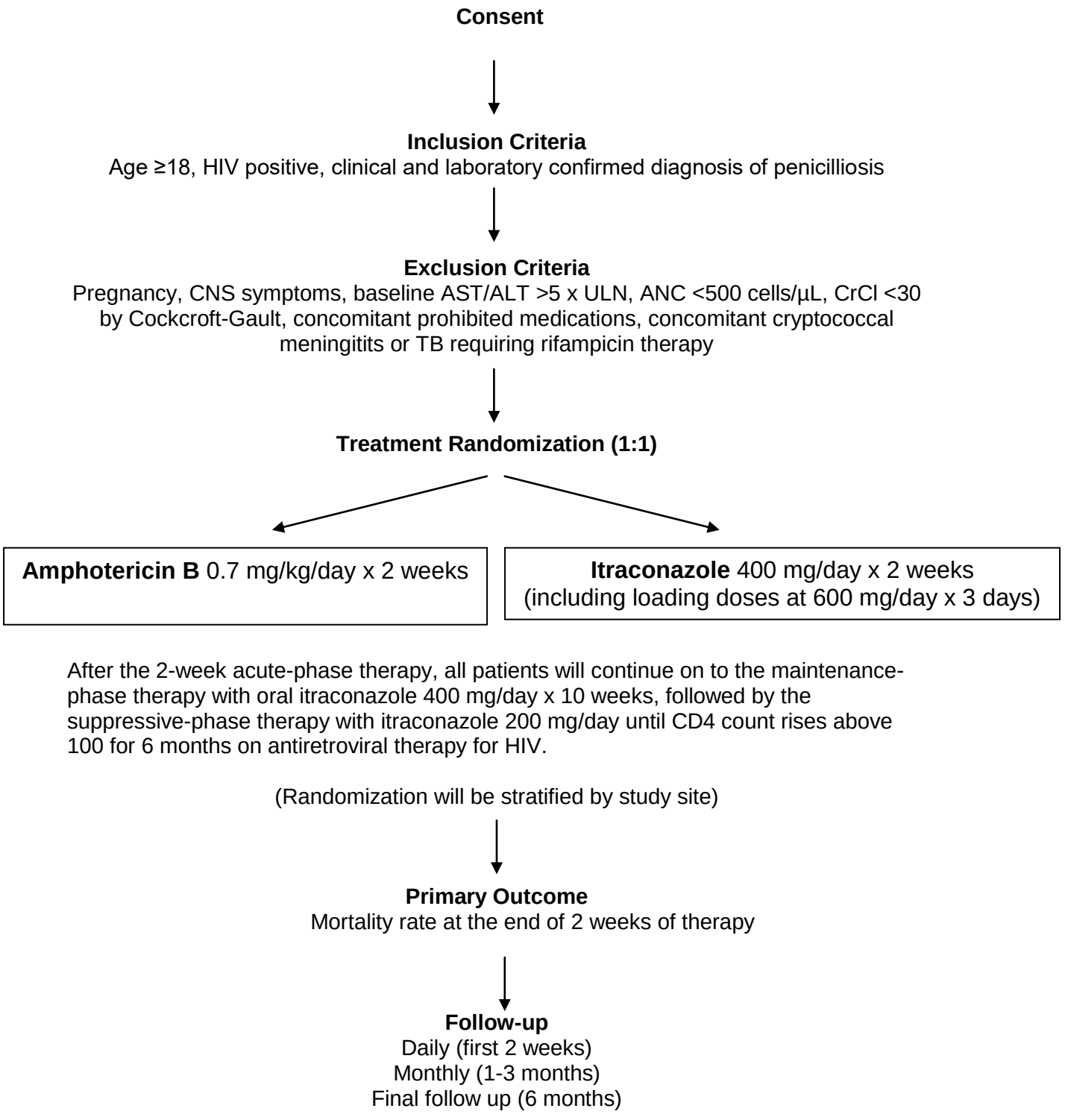
102. Schuler U, Bammer S, Aulitzky WE, et al. Safety and efficacy of itraconazole compared to amphotericin B as empirical antifungal therapy for neutropenic fever in patients with haematological malignancy. Onkologie **2007**;30(4):185-91.

103. Park SH, Choi SM, Lee DG, et al. Intravenous itraconazole vs. amphotericin B deoxycholate for empirical antifungal therapy in patients with persistent neutropenic fever. Korean J Intern Med **2006**;21(3):165-72.

104. Gigliotti F, Shenep JL, Lott L, Thornton D. Induction of prostaglandin synthesis as the mechanism responsible for the chills and fever produced by infusing amphotericin B. J Infect Dis **1987**;156(5):784-9.

105. Le T, Wolbers M, Chi NH, Quang VM, Chinh NT, Lan HPN, Lam PS, Kozal KM, Shikuma CM, Day NJ, Farrar J. Epidemiology, seasonality and Predictors of Outcome in *Penicillium marneffei* Infection in Ho Chi Minh City, Viet Nam. Clin Infect Dis 2011 Arp 1; 52(7):945–952.

Appendix A: Study Flow Diagram



Appendix B: Trial Procedure Chart

Event	SCR	Baseline D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	W4 (+/- 3d)	W8 (+/- 3d)	W12 (+/- 3d)	W16 (+/- 3d)	W20 (+/- 3d)	W 24 (+/- 3d)
Informed Consent	x																				
Inclusion/ Exclusion Criteria	x																				
Medical History	x																				
Pregnancy Test *	x																				
Clinical Assessment**		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Medication Adherence Assessment																x	x	x	x	x	x
AEs Assessment		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
CXR¥		x																			
Blood (ml)																					
CBC£		1			1			1				1		1		1		1			
CD4£ α		1																			
Chemistry & Liver Function Test£		2			2			2				2		2		2		2			
Blood culture¥, ©		5		5		5		5		5		5		5							
Blood Fungal Colony Count ©, β		1	1	1	1	1	1	1		1		1		1							
Molecular & Serology		5		5		5		5						5		5	5	5			5
IRD testing							10									10	10	10	10	10	10
Maximum total blood volume (ml)		**15**	**1**	**11**	**4**	**11**	**11**	**14**	**0**	**6**	**0**	**9**	**0**	**14**	**0**	**18**	**15**	**18**	**10**	**10**	**15**
Urine (ml)																					
Urinary Antigen£		20		20		20		20		20		20		20		20	20	20			20
Skin lesion																					
Smear & Culture¥		x																			
Sputum																					
Zn smear ¥		x	x	x																	

*Pregnancy Test: for female with child-bearing potential only
** Clinical Assessments including vital signs, weight, physical exam
¥ Can be repeated at any time as clinically indicated.
£ Blood tests scheduled for D2-14 may be done within a +/- 1 day window period
© Stop taking these samples when two consecutive sample tests are negative
α To be done when subjects are suspected to have an IRD event
β Only done at HTD and NHTD during working hour

Appendix C: Pharmacokinetic Study Schedule

I. Intensive PK (30 patients ARV-naïve, 15 in each arm)

Day of treatment	**Day 1**			**Day 2**					**Day 8**								
Itraconazole arm	0h (pre-drug administration)	0.5h	2h	1h	3h	4h	12h		0h (pre-drug administration)	0.5h	1h	2h	3h	4h	6h	12h	
Amphotericin B arm	0h (pre-drug administration)	0.5h	2h	1h	3h	4h	12h	24h	0h (pre-drug administration)	0.5h	1h	2h	4h	6h	12h	16h	24h

II. Time for taking sample for Population PK (200 patients, 100 in each arm)

Day of treatment	**Day 1**	Day 1-4 (only 1 sample is collected each day during the following randomized time blocks)				Day 8 and 10 (only 1 sample is collected each day at the following randomized timeslot)		**Day 12**		**Wk 4, 8, 12,24**
Itraconazole arm	0h (pre-drug administration)	0-2h	2-4h	4-8h	8-12h	0h (pre-drug administration)	3h	0h (pre-drug administration)	3h	Before AM dose at follow-up visit
Amphotericin arm	0h (pre-drug administration)	0-3h	3-6h	6-12h	12-18h	0h (pre-drug administration)	6h (or right after infusion is completed)	0h (pre-drug administration)	6h (or right after infusion is completed)	Before AM dose at follow-up visit

Note: h refers to the number of hours after a patient takes itraconazole by mouth or after the infusion of amphotericin B is completed

Appendix D: Secondary Objective #9 - case control study to evaluate the exposure risk factors for penicilliosis

Purpose: to investigate the risk exposure and risk behaviors in equally susceptible individuals with HIV/AIDS and not the host susceptibility to penicilliosis.
Our hypothesis is that the reservoir of P. marneffei is in the environment, in decaying organic materials and in a combination of a type of soil, humidity, and a tropical flora that forms a symbiotic relationship with the fungus. Proximity to water and humidity provide a favorable environment for germination and transmission. Sharing needles is a risk for bloodborne transmission from person to person.

Background: Please refer to section 1.2 of the protocol.

Experimental Plan: (See flow chart next page)
This is a hospital-based case-control study that is built into the main trial. Cases (N=200) will be conveniently and randomly recruited from a pool of subjects who enter the trial with culture-confirmed penicilliosis at selected trial sites.

Controls (or disease reference group, N=400) will be randomly selected from a pool of patients with AIDS who come to the outpatient clinic for routine care or who are admitted in the hospital for acute care at our trial centers. Controls may have an active opportunistic infection, but penicilliosis should be ruled out. Controls will be recruited simultaneously (within <1 wk of cases) and will be individually matched 2:1 to cases. The following matching scheme is designed to ensure that controls are similar to cases in term of host characteristics: age by 5 years, sex, and susceptibility to penicilliosis (CD4 by 50 cells/µL or WHO disease categories).

After signing a separate informed consent form, 5 cc of blood and 20 cc of urine will be collected and stored at -70ºC for serological tests. All subjects will complete a one-to-one 20-30 minute interview by a standardized questionnaire with a study staff in a private room. Global positioning system (GPS) mapping technology will be used to characterize the geo-spatial distribution of cases and controls.

Data Analysis:
Univariate and multivariate logistic-regression models will be used to estimate the odds ratios and associated 95% confidence intervals of exposure variables and disease in pair-matched data. Assessment of presence of exposure, duration of exposure and recent/past exposure will be made for all exposure variables. Multivariate models will be created through stepwise elimination of variables of interest from univariate analysis while relevant variables will be retained. Additive and multiple interactions among exposure variables will be evaluated.

Case Control Study Flow Chart

Case Group (200 patients)

HIV-infected patients with microbiological confirmed diagnosis of penicilliosis who participated in the trial

Control Group (400 patients)

HIV-infected patients admitted to the same hospital or seen in the outpatient clinic for routine or acute care during the same time but do not have culture-confirmed penicilliosis, matched sex, age, CD4 or WHO disease staging.

Inclusion criteria for control patients:

- HIV-infected patients >18 years old
- Patients with fever and/or non-specific constitutional symptoms
- All patients with other opportunistic fungal infections: cryptococcosis, candidiasis, candidemia, histoplasmosis, PCP
- All patients with other opportunistic infections: tuberculosis, CMV…

Exclusion criteria for control patients:

- Healthy and asymptomatic HIV-infected subjects

Epidemiologic factors to be investigated in the survey:

- Home and work addresses
- Type/s of work and specific activities at work
- Specific activities most days of the week in the past 3, 6, 12 months
- Present/past exposure/contact with bamboo and/or bamboo rats
- Present/past exposure to healthy/ill domestic animals (dogs, cats, birds including chickens and ducks, reptiles, pigs, rabbits or rodents)
- Present/past exposure to healthy/ill farm or wild animals
- Types of plant/trees around home/work
- Live by or close contact with any body of water
- Eat exotic food including raw or rarely cooked food
- Current/past smoking of cigarettes/marijuana/opium/others
- Current/past intravenous drug use (heroin or others) and injection practices

Appendix E: Secondary Objective #10 - Molecular Epidemiology of Penicillium marneffei

Purpose: to investigate the molecular epidemiology of *P. marneffei* infection using a number of cutting-edge molecular technologies including highly discriminatory mutilocus microsatetlite typing (MLMT) and correlate the identified genotypes with clinical and geo-spatial epidemiology data

Background: Please refer to section 1.8 of the protocol.

Experimental Plan:
Pure sub-cultured isolates of *Penicillium marneffei* from subjects enrolled into this study will be stored with micro beads (called Microbank^{TM}) obtained from Pro-Lab Diagnostics in cryovials containing cryopreservative at -70ºC at OUCRU laboratories in Ho Chi Minh City and Ha Noi. Typing of P.marneffei isolates will be performed by various typing technologies, namely multilocus sequences typing (MLST), multilocus microsatellite typing (MLMT), and direct sequencing of the cell wall glycoprotein called Manoprotein-1 (MP-1) in collaborations with Dr. Brent Lasker from the US Centers for Disease Control and Prevention and Dr. Matthew Fisher from Imperial College London. Most of the typing works will be performed in Vietnam, with some samples shipped to collaborators labs overseas for confirmation/comparison of typing results. Please refer to the references for detailed molecular typing protocols [75, 76, and 77].

Parallel to typing isolates from clinical population, we will collect soil specimens and set up air sampling booths from different geographical areas in North and South Vietnam. Both standard culture and quantitative PCR assays will be used to detect presence of *Penicillium marneffei* from the environment, and direct sequencing of MP-1 protein will be used to type environmental isolates.

Data Analysis:
Typing data from human clinical populations and from environmental sources from different geographical areas in Vietnam will be integrated with clinical data to identify the genetic variations within populations of *Penicillium marneffei* in Vietnam. These data can then be shared among collaborating laboratories interested in typing and ecological/epidemiological studies of *Penicillium marneffei* through the endemic regions, allowing sophisticated temporal epidemiological surveillance analysis, and greater understanding of the evolution and adaptation of this important emerging opportunistic pathogen.

Appendix F: Secondary Objective #11 - Urinary Antigen of Penicillium marneffei for Diagnosis and Monitor of Treatment

Purpose: to prospectively evaluate an ELISA and a latex agglutination assay to detect *P. marneffei* urinary antigen for diagnostic accuracy and as a surrogate marker for microbiological and clinical outcomes of penicilliosis.

Background: Please refer to section 1.5.3 of the protocol. In summary, simple, rapid, robust dot blot ELISA and a latex agglutination assays for detection of *P. marneffei* antigenuria using a polyclonal hyperimmune IgG have been developed and prospectively tested in smaller scale studies (37 cases, 300 controls) with sensitivities and specificities in the upper 90% [53]. We plan to validate these tests in our large-scale case-control study (secondary objective #9, 200 cases, 400 controls) for diagnostic accuracy and for following/correlating *P. marneffei* antigenuria titers with fungal clearance and clinical response during the 3 months of antifungal therapy.

Experimental Plan:
Urine specimens from all patients participating in the trial will be collected at enrollment, 3 times a weeks for 2 weeks during acute hospitalization, week 4, 8, 12, and 24 (see appendix B – Trial Flow Chart). Simultaneously urine specimens will be collected from the control subjects but only at enrollment. Control subjects will be HIV-infected subjects with similar CD4 count or WHO disease staging but do not have culture evidence of penicilliosis. They ideally will be patients with a variety of other common opportunistic infections seen in Vietnam, including other fungal infections such as cryptococcosis, candidemia/candidiasis, PCP, undiagnosed histoplasmosis…Inclusion of other fungal infections will add to the reliability of the specificity.

Urine samples will be stored at −30°C and thawed only at the time of testing. Control *P. marneffei* antigen and purified rabbit anti- *P. marneffei* IgG will be obtained from our collaboration with Dr. Desakorn (Mahidol University, Thailand). *P. marneffei* IgG will be labeled with FITC conjugate, and ELISA and agglutination assays will be performed at OUCRU according to Desalorn et al [53]. All samples will be tested in duplicate, and each test was repeated three times.

Analysis Plan:
Data will be analyzed with the assistance of Dr. Marcel Wolbers, OUCRU biostatistician using R computer software. At each ELISA cutoff titer, the sensitivity and the specificity will be calculated. A receiver operating characteristic (ROC) curve is then constructed by plotting sensitivity against (1 − specificity) at each value. We will also evaluate baseline *P. marneffei* antigen titer as an independent predictors of disease outcome and evaluate the role of serial *P. marneffei* antigen titers in predicting treatment response.

Appendix G: Secondary Objective #12 - Cost effectiveness of itraconazole vs. amphotericin B for penicilliosis

Purpose: to conduct an economic evaluation to estimate the net cost of itraconazole versus amphotericin B therapy for penicilliosis

Background: As the cost differential between itraconazole and amphotericin B treatment is one of the reasons for undertaking the trial, it will be important to conduct a formal economic evaluation alongside the trial to ensure that all costs are accurately recorded, and to permit a cost-effectiveness analysis in the event that non-inferiority is not demonstrated (i.e. if itraconazole turns out to be cheaper but less effective). Hence, an economic evaluation will be conducted in collaboration with the Health Economics Research Centre, University of Oxford (PI: Prof. Alastair Gray).

Experimental and data analysis plan:
The objective of the analysis will be to estimate the net cost of itraconazole versus amphotericin B therapy, including medication costs, other treatments, hospital stays, and patient incurred costs, including loss of income for the patients and their care takers, out-of-pocket costs, and the need for transfer to tertiary centres. These information will be prospectively collected on each patient during the study and recorded in the health economic CRFs. Unit costs will be obtained from each trial centre and used to produce a net cost per patient in each arm of the study over the 2 week (primary) and 6 month (secondary) follow-up periods. In the event that non-inferiority is not demonstrated, the economic evaluation will assess cost-effectiveness as the ratio of the difference in cost to the difference in survival, expressed as life years gained. Although it is possible that itraconazole is better tolerated than amphotericin B, it is unlikely that these differences will be large enough to be detected in any form of simple disability adjustment or quality of life adjustment, and so it is not proposed that a cost per DALY averted or QALY gained is reported, or that information is collected prospectively on these metrics. Life years gained will be based on the primary outcome measure of survival at 2 weeks and also at 6 months. All estimates of costs, outcomes and cost-effectiveness will be reported with full recognition of uncertainty, including cost-effectiveness acceptability curve and sensitivity analyses around key parameters.

Appendix H: Secondary Objective #13 - Penicilliosis Immune Reconstitution Disease

Purpose: to study the incidence, clinical features, outcome, and outcome predictors of immune reconstitution disease (IRD) in penicilliosis

Background: HIV-associated IRD occurs in up to 30% of patients with opportunistic infections starting ART and is associated with higher morbidity and mortality, particularly in tuberculosis and cryptococcal meningitis. IRD has been reported but has not been systematically studied in penicilliosis. It is unknown whether penicilliosis IRD has worse clinical outcome. And as with other HIV-associated IRD, biomarkers to diagnose and to predict IRD in penicilliosis need further investigations.

Experimental Plan:
All trial participants with penicilliosis who are ART-naïve (estimated 80%) will be evaluated monthly for the development of IRD over a period of 6 month as they begin ART. 10ml of blood will be collected at enrollment to look for predictive biomarkers of IRD. For the patients who develop IRD during the first 6 months of ART, we will continue to follow the patients monthly during their routine clinic visit and will collect information about treatment and outcome of the IRD event. IRD events are defined based on the consensus criteria for general IRD according to the International Network for the study of HIV-associated IRIS. Biomarkers of immune dysfunction (levels and profile of cytokines/chemokines) that have been identified to predict and to differentiate IRD from other complications in other fungal opportunistic diseases such as cryptococcal meningitis will be studied. Other laboratory predictor variables that will be studied include: fungal clearance by quantitative culture and by serological assays, serum CRP, and D-dimer. Other AIDS-related or non-AIDS-related events that occur during the study follow up period will be classified and recorded.

Data Analysis:
Incidence, clinical features, management and outcome of penicilliosis IRD will be described. Clinical and laboratory variables will be compared between those with and without IRD in the study cohort. Multiple logistic regression analysis will be performed to identify independent predictors of IRD. Biomarkers that differentiate IRD from non-IRD events will be identified.

Appendix I: WHO clinical staging for HIV/AIDS

Clinical Stage 1
Asymptomatic Persistent generalised lymphadenopathy (PGL) Performance scale 1: asymptomatic, normal activity
Clinical Stage 2
Weight loss, <10% of body weight Minor mucocutaneous manifestations (seborrheic dermatitis, prurigo, fungal nail infections, recurrent oral ulcerations, angular cheilitis) Herpes zoster, within the last 5 years Recurrent upper respiratory tract infections (e.g. bacterial sinusitis) And/or performance scale 2: symptomatic, normal activity.
Clinical stage 3
Weight loss, >10% of body weight Unexplained chronic diarrhoea, > 1 month Unexplained prolonged fever (intermittent or constant), > 1 month Oral candidiasis (thrush) Oral hairy leukoplakia Pulmonary tuberculosis, within the past year. Severe bacterial infections (e.g. pneumonia, pyomyositis) And/or Performance scale 3: bed-ridden, < 50% of the day during the last month
Clinical stage 4
HIV wasting syndrome, as defined by CDC[1] Pneumocystis carinii pneumonia Toxoplasmosis of the brain Cryptosporidiosis with diarrhoea, >1 month Cryptococcosis, extra pulmonary Cytomegalovirus (CMV) disease of an organ other than liver, spleen or lymph nodes Herpes Simplex Virus (HSV) infection, mucocutaneous >1 month, or visceral any duration Progressive multifocal leukoencephalopathy (PML) Any disseminated endemic mycosis (e.g. histoplasmosis, coccidioidomycosis) Candidiasis of the oesophagus, trachea, bronchi or lungs Atypical mycobacteriosis, disseminated Non-typhoid Salmonella septicaemia Extra-pulmonary tuberculosis Lymphoma Kaposi's sarcoma (KS) HIV encephalopathy, as defined by CDC[2] And/or Performance scale 4: bed-ridden, > 50% of the day during the last month

(Note: Both definitive and presumptive diagnoses are acceptable)

[1] HIV wasting syndrome: weight loss of >10% of body weight, plus either unexplained chronic diarrhoea (>1 month), or chronic weakness and unexplained prolonged fever (>1 month).

[2] HIV encephalopathy: clinical finding of disabling cognitive and/or motor dysfunction interfering with activities of daily living, progressing over weeks to months, in the absence of a concurrent illness or condition other than HIV infection that could explain the findings.

Appendix J: Itraconazole Drug Interactions

Itraconazole and its major metabolite, hydroxyitraconazole, are inhibitors of CYP3A4. Therefore, the following drug interactions may occur (See Table 1 below and the following drug class subheadings that follow):

Itraconazole may decrease the elimination of drugs metabolized by CYP3A4, resulting in increased plasma concentrations of these drugs when they are administered with itraconazole. These elevated plasma concentrations may increase or prolong both therapeutic and adverse effects of these drugs. Inducers of CYP3A4 may decrease the plasma concentrations of itraconazole. Itraconazole may not be effective in patients concomitantly taking itraconazole and one of these drugs. Therefore, administration of these drugs with itraconazole is not recommended. Other inhibitors of CYP3A4 may increase the plasma concentrations of itraconazole. Patients who must take itraconazole concomitantly with one of these drugs should be monitored closely for signs or symptoms of increased or prolonged pharmacologic effects of itraconazole.

Table 1: Selected Drugs that are predicted to alter the plasma concentration of itraconazole or have their plasma concentration altered by itraconazole[1]

	Drug plasma concentration increased by itraconazole
Antiarrhythmics	digoxin, dofetilide[2], quinidine[2], disopyramide
Anticonvulsants	carbamazepine
Antimycobacterials	rifabutin
Antineoplastics	busulfan, docetaxel, vinca alkaloids
Antipsychotics	pimozide[2]
Benzodiazepines	alprazolam, diazepam, midazolam,[2,3] triazolam[2]
Calcium Channel Blockers	dihydropyridines, verapamil
Gastrointestinal Motility Agents	cisapride[2]
HMG CoA-Reductase Inhibitors	atorvastatin, cerivastatin, lovastatin,[2] simvastatin[2]
Immunosuppressants	cyclosporine, tacrolimus, sirolimus
Oral Hypoglycemics	Oral hypoglycemics

Drug plasma concentration increased by itraconazole	
Protease Inhibitors	indinavir, ritonavir, saquinavir
Other	levacetylmethadol (levomethadyl), ergot alkaloids, halofantrine, alfentanil, buspirone, methylprednisolone, budesonide, dexamethasone, trimetrexate, warfarin, cilostazol, eletriptan
Decrease plasma concentration of itraconazole	
Anticonvulsants	carbamazepine, phenobarbital, phenytoin
Antimycobacterials	isoniazid, rifabutin, rifampin
Gastric Acid Suppressors/Neutralizers	antacids, H_2-receptor antagonists, proton pump inhibitors
Non-nucleoside Reverse Transcriptase Inhibitors	nevirapine
Increase plasma concentration of itraconazole	
Macrolide Antibiotics	clarithromycin, erythromycin
Protease Inhibitors	indinavir, ritonavir

[1]This list is not all-inclusive.
[2]Contraindicated with itraconazole based on clinical and/or pharmacokinetics studies.
[3]For information on parenterally administered midazolam, see the Benzodiazepine paragraph below.

Antiarrhythmics: The class IA antiarrhythmic quinidine and class III antiarrhythmic dofetilide are known to prolong the QT interval. Co administration of quinidine or dofetilide with itraconazole may increase plasma concentrations of quinidine or dofetilide which could result in serious cardiovascular events. Therefore, concomitant administration of itraconazole and quinidine or dofetilide is contraindicated.

The class IA antiarrhythmic disopyramide has the potential to increase the QT interval at high plasma concentrations. Caution is advised when itraconazole and disopyramide are administered concomitantly.

Concomitant administration of digoxin and itraconazole has led to increased plasma concentrations of digoxin.

Anticonvulsants: Reduced plasma concentrations of itraconazole were reported when itraconazole was administered concomitantly with phenytoin. Carbamazepine, phenobarbital, and phenytoin are all inducers of CYP3A4. Although interactions with carbamazepine and phenobarbital have not been studied, concomitant administration of itraconazole and these drugs would be expected to result in decreased plasma concentrations of itraconazole. In addition, *in vivo* studies have demonstrated an increase in plasma carbamazepine concentrations in subjects concomitantly receiving ketoconazole. Although there are no data regarding the effect of itraconazole on carbamazepine metabolism, because of the similarities between ketoconazole and itraconazole, concomitant administration of itraconazole and carbamazepine may inhibit the metabolism of carbamazepine.

Antimycobacterials: Drug interaction studies have demonstrated that plasma concentrations of azole antifungal agents and their metabolites, including itraconazole and hydroxyitraconazole, were significantly decreased when these agents were given concomitantly with rifabutin or rifampin. *In vivo* data suggest that rifabutin is metabolized in part by CYP3A4. Itraconazole may inhibit the metabolism of rifabutin. Although no formal study data are available for isoniazid, similar effects should be anticipated. Therefore, the efficacy of itraconazole could be substantially reduced if given concomitantly with one of these agents. Co administration is not recommended.

Antineoplastics: Itraconazole may inhibit the metabolism of busulfan, docetaxel, and vinca alkaloids.

Antipsychotics: Pimozide is known to prolong the QT interval and is partially metabolized by CYP3A4. Co administration of pimozide with itraconazole could result in serious cardiovascular events. Therefore, concomitant administration of itraconazole and pimozide is contraindicated.

Benzodiazepines: Concomitant administration of itraconazole and alprazolam, diazepam, oral midazolam, or triazolam could lead to increased plasma concentrations of these benzodiazepines. Increased plasma concentrations could potentiate and prolong hypnotic and sedative effects. Concomitant administration of itraconazole and oral midazolam or triazolam is contraindicated. If midazolam is administered parenterally, special precaution and patient monitoring is required since the sedative effect may be prolonged.

Calcium Channel Blockers: Edema has been reported in patients concomitantly receiving itraconazole and dihydropyridine calcium channel blockers. Appropriate dosage adjustment may be necessary.

Calcium channel blockers can have a negative inotropic effect which may be additive to those of itraconazole; itraconazole can inhibit the metabolism of calcium channel blockers such as dihydropyridines (e.g., nifedipine and felodipine) and verapamil. Therefore, caution should be used when co-administering itraconazole and calcium channel blockers.

Gastric Acid Suppressors/Neutralizers: Reduced plasma concentrations of itraconazole were reported when itraconazole capsules were administered concomitantly with H2-receptor antagonists. Studies have shown that absorption of itraconazole is impaired when gastric acid production is decreased. Therefore, itraconazole should be administered with a cola beverage if the patient has achlorhydria or is taking H2-receptor antagonists or other gastric acid suppressors. Antacids should be administered at least 1 hour before or 2 hours after administration of itraconazole capsules. In a clinical study, when itraconazole capsules were administered with omeprazole (a proton pump inhibitor), the bioavailability of itraconazole was significantly reduced.

Gastrointestinal Motility Agents: Co administration of itraconazole with cisapride can elevate plasma cisapride concentrations which could result in serious cardiovascular events. Therefore, concomitant administration of itraconazole with cisapride is contraindicated.

HMG CoA-Reductase Inhibitors: Human pharmacokinetic data suggest that itraconazole inhibits the metabolism of atorvastatin, cerivastatin, lovastatin, and simvastatin, which may increase the risk of skeletal muscle toxicity, including rhabdomyolysis. Concomitant administration of itraconazole with HMG CoA-reductase inhibitors, such as lovastatin and simvastatin, is contraindicated.

Immunosuppressants: Concomitant administration of itraconazole and cyclosporine or tacrolimus has led to increased plasma concentrations of these immunosuppressants. Concomitant administration of itraconazole and sirolimus could increase plasma concentrations of sirolimus.

Macrolide Antibiotics: Erythromycin and clarithromycin are known inhibitors of CYP3A4 (See Table 1) and may increase plasma concentrations of itraconazole. In a small pharmacokinetic study involving HIV infected patients, clarithromycin was shown to increase plasma concentrations of itraconazole. Similarly, following administration of 1 gram of erythromycin ethyl succinate and 200 mg itraconazole as single doses, the mean Cmax and $AUC_{0-\infty}$ of itraconazole increased by 44% (90% CI: 119-175%) and 36% (90% CI: 108-171%), respectively.

Non-nucleoside Reverse Transcriptase Inhibitors: Nevirapine is an inducer of CYP3A4. *In vivo* studies have shown that nevirapine induces the metabolism of ketoconazole, significantly reducing the bioavailability of ketoconazole. Studies involving nevirapine and itraconazole have not been conducted. However, because of the similarities between ketoconazole and itraconazole, concomitant administration of itraconazole and nevirapine is not recommended.

In a clinical study, when 8 HIV-infected subjects were treated concomitantly with itraconazole capsules 100 mg twice daily and the nucleoside reverse transcriptase inhibitor zidovudine 8 ± 0.4 mg/kg/day, the pharmacokinetics of zidovudine were not affected. Other nucleoside reverse transcriptase inhibitors have not been studied.

Oral Hypoglycemic Agents: Severe hypoglycemia has been reported in patients concomitantly receiving azole antifungal agents and oral hypoglycemic agents. Blood glucose concentrations should be carefully monitored when itraconazole and oral hypoglycemic agents are coadministered.

Polyenes: Prior treatment with itraconazole, like other azoles, may reduce or inhibit the activity of polyenes such as amphotericin B. However, the clinical significance of this drug effect has not been clearly defined.

Protease Inhibitors: Concomitant administration of itraconazole and protease inhibitors metabolized by CYP3A4, such as indinavir, ritonavir, and saquinavir, may increase plasma concentrations of these protease inhibitors. In addition, concomitant administration of itraconazole and indinavir and ritonavir (but not saquinavir) may increase plasma concentrations of itraconazole. Caution is advised when itraconazole and protease inhibitors must be given concomitantly.

Other:

- Levacetylmethadol (levomethadyl) is known to prolong the QT interval and is metabolized by CYP3A4. Co-administration of levacetylmethadol with itraconazole could result in serious

cardiovascular events. Therefore, concomitant administration of itraconazole and levacetylmethadol is contraindicated.

- Elevated concentrations of ergot alkaloids can cause ergotism, ie. a risk for vasospasm potentially leading to cerebral ischemia and/or ischemia of the extremities. Concomitant administration of ergot alkaloids such as dihydroergotamine, ergometrine (ergonovine), ergotamine and methylergometrine (methylergonovine) with itraconazole is contraindicated.
- Halofantrine has the potential to prolong the QT interval at high plasma concentrations. Caution is advised when itraconazole and halofantrine are administered concomitantly.
- *In vitro* data suggest that alfentanil is metabolized by CYP3A4. Administration with itraconazole may increase plasma concentrations of alfentanil.
- Human pharmacokinetic data suggest that concomitant administration of itraconazole and buspirone results in significant increases in plasma concentrations of buspirone.
- Itraconazole may inhibit the metabolism of certain glucocorticosteroids such as budesonide, dexamethasone and methylprednisolone.
- *In vitro* data suggest that trimetrexate is extensively metabolized by CYP3A4. *In vitro* animal models have demonstrated that ketoconazole potently inhibits the metabolism of trimetrexate. Although there are no data regarding the effect of itraconazole on trimetrexate metabolism, because of the similarities between ketoconazole and itraconazole, concomitant administration of itraconazole and trimetrexate may inhibit the metabolism of trimetrexate.
- Itraconazole enhances the anticoagulant effect of coumarin-like drugs, such as warfarin.

Cilostazol and eletriptan are CYP3A4 metabolized drugs that should be used with caution when co-administered with itraconazole.

Section 2 – IVAP final protocol

A Randomized, Open-Label, Comparative Study of the Effectiveness of Itraconazole versus Amphotericin B in the Induction Treatment of Penicilliosis in HIV-Infected Adults

Itraconazole versus Amphotericin B for the Treatment of Penicilliosis

Lead Principal Investigator (Lead PI):

Nguyen Van Kinh, MD PhD
National Hospital for Tropical Diseases, Hanoi

Thuy Le, MD
Oxford University Clinical Research Unit, Viet Nam
University of Hawaii at Manoa, Honolulu, Hawaii USA

Principal Investigator (PI):

Ngo T Kim Cuc, MD
Hospital for Tropical Diseases, Ho Chi Minh City

Pham Thanh Thuy, MD
Bach Mai hospital, Ha Noi

Doan T Hong Hanh, MD
Vietnam-Sweden Uong Bi Hospital, Quang Ninh

Pham Hanh Phuc, MD
Viet Tiep General Hospital, Hai Phong

Co-Investigators:

Nguyen Tran Chinh, MD PhD
Hospital for Tropical Diseases, Ho Chi Minh City

Nguyen Huu Chi, MD
Hospital for Tropical Diseases, Ho Chi Minh City

Vo Minh Quang, MD
Hospital for Tropical Diseases, Ho Chi Minh City

Nguyen Tien Lam, MD
National Hospital for Tropical Diseases, Hanoi

Cao Thi Thanh Thuy, MD
National Hospital for Tropical Diseases, Hanoi

Hoang Dang Mich, Associate Professor, PhD, MD
Viet Tiep General Hospital, Hai Phong

Pham Si Lam, MD
Oxford Univerity Clinical Research Unit, Vietnam

Jeremy Day, MD
Oxford University Clinical Research Unit, Vietnam

Marcel Wolbers, PhD
Oxford University Clinical Research Unit, Vietnam

Heiman Wertheim, MD PhD
Oxford University Clinical Research Unit in Ha Noi

Mattias Larsson, MD
Oxford University Clinical Research Unit in Ha Noi

Alastair Gray, Prof
Health Economic Research Centre at Oxford University, Oxford, UK

Cecilia Shikuma, MD
Hawaii Center of AIDS, Honolulu, Hawaii, USA

Jeremy Farrar, MD PhD
Oxford University Clinical Research Unit, Viet Nam

Study statistician:
Marcel Wolbers, PhD.
Oxford University Clinical Research Unit, Viet Nam

Study Sponsor:
University of Oxford

Study Sites:
Hospital for Tropical Diseases, Ho Chi Minh City
190 Ben Ham Tu, District 5
Ho Chi Minh City, Viet Nam

National Hospital for Tropical Diseases, Hanoi
78 Giai Phong Street
Hanoi, Viet Nam

Bach Mai Hospital, Hanoi
78 Giai Phong Street
Hanoi, Viet Nam

Vietnam-Sweden Uong Bi Hospital, Quang Ninh

Thanh Son, Uong Bi, Quang Ninh, Viet Nam

Viet Tiep General Hospital
1 Nha Thuong, Le Chan, Hai Phong, Viet Nam

Study Funders:
The Department of International Development, UK
The Wellcome Trust, UK
The Medical Research Council, UK

Précis

Penicillium marneffei is an emerging endemic pathogenic fungus that can cause a fatal systemic mycosis in patients infected with human immunodeficiency virus (HIV) in areas of Southeast Asia. The mortality rate is close to 100% when diagnosis and treatment are delayed [1]. Since the HIV/AIDS pandemic arrived in Southeast Asia and since the first case of penicilliosis reported in Thailand in 1988, penicilliosis has become one of the most serious and common AIDS-defining illnesses in this region [2]. Increasingly immunocompromised patients in other parts of the world where the disease is not endemic are diagnosed with penicilliosis after traveling to Southeast Asia and the illness has been reported either in patients with recent or very remote travel histories to these areas [3-10].

Despite being one of the most common and fatal opportunistic infection in HIV-infected patients in Southeast Asia for nearly two decades, there has been a complete lack of clinical trials on the treatment of penicilliosis. Treatment choices therefore must be based upon data from case series and non-comparative studies. The most objective evidence came from a study by Supparatpinyo et al. who described treatment responses (defined by absence of fungal growth and resolution of clinical signs and symptoms) in a series of 80 HIV-infected patients with disseminated penicilliosis. Antifungal choices were at the discretion of clinicians without prior knowledge of antifungal susceptibility testing. Response rates were 77% for amphotericin B, 75% for itraconazole, and 36% for fluconazole [1]. A few years later the same group described a case series of 74 HIV-infected patients with penicilliosis treated with intravenous amphotericin B 0.6 mg/kg/day for 2 weeks followed by oral itraconazole 400 mg/day for 10 weeks [11]. The treatment response rate (defined by negative blood culture and resolution of fever and skin lesions at the end of 12 weeks therapy) was 97%. Remarkably there was only one death. Unfortunately this has not been the experience in Vietnam and elsewhere in Southeast Asia. The basis for choosing intravenous amphotericin B for initial therapy followed by oral itraconazole as maintenance therapy and the reported treatment success rate need to be subjected to clinical trials rather than be accepted currently as the “standard of care”.

Amphotericin B is an expensive drug for most patients at risk of penicilliosis. The need for intravenous access and side effect monitoring requires hospitalization, which adds to the cost burden of patients. By comparison, oral itraconazole is more tolerable and is readily available at a fraction of the price. Itraconazole has been shown to be at least as efficacious and is better tolerated compared to amphotericin B in the empirical treatment of febrile neutropenia [12]. Further, itraconazole (in various formulations) has been shown in case series to effectively treat other serious systemic fungal infections such as invasive aspergillosis, coccidioidomycosis, cryptococcosis, and blastomycosis [13-21]. For this reason physicians in Thailand, Burma, India, and Vietnam often use itraconazole alone in patients who either cannot afford amphotericin B therapy or are able to be treated as outpatient and anecdotally report comparable success rates compared to amphotericin B (personal communications: Nicolas White, MD., Former Director of Wellcome Trust Mahidol University Oxford in Thailand; Nguyen Huu Chi, MD., Director of HIV for inpatients at the Hospital for Tropical Diseases (HTD); and Vo Minh Quang, MD. Director of HTD’s outpatient HIV clinic). Indeed, Ranjana et al. recently reported a success rate of 97% using itraconazole alone at 400 mg/d for 3-4 weeks from India (n=50) [22].

The vast majority of patients with penicilliosis are able to take oral medication. The capsule formulation of itraconazole is the only formulation widely available in pharmacies across Asia. Itraconazole oral suspension was developed (co-formulated with cyclodextrin) to improve the

bioavailability of the capsule formulation, resulting in 30% increase in the area under the curve (AUC) [23]. This formulation however is not widely available and is associated with nausea due to cyclodextrin's osmotic effect, which may affect compliance and potentially be counter-productive in the goal to improve bioavailability [24].

We aim to conduct a randomized, open-label, comparative non-inferiority trial of the efficacy and safety of itraconazole versus amphotericin B for the acute-phase treatment of penicilliosis. If our hypothesis is correct, that itraconazole is at least as effective as amphotericin B, it becomes difficult to justify using amphotericin B in most areas of Southeast Asia where cost has a major role in the therapeutic decision process. However if our hypothesis is incorrect, that amphotericin B is found to be more effective than itraconazole, then there will be empirical evidence for Ministries of Health and policy makers across Asia to make amphotericin B more widely available and affordable. This study provides opportunities to investigate the microbiologic and pharmacokinetic basis for observed efficacies from the 2 antifungal regimens. The questions whether time to negative fungal blood culture and/or whether early fungicidal activities do correlate with treatment outcomes are relevant both to clinicians as well as clinical trial investigators studying fungal diseases. Population kinetic models for the 2 antifungal drugs will be constructed and pharmacokinetic variables such as peak/trough serum drug concentration, area under the curve in a drug concentration versus time analysis, and drug minimal inhibitory concentration (MIC) will be correlated with microbiological and treatment outcomes. These results will further implement treatment strategies for this infection.

1 Background

Introduction

Penicillium marneffei is an emerging endemic pathogenic fungus that can cause a fatal systemic mycosis in patients infected with human immunodeficiency virus (HIV) and advanced acquired immunodeficiency syndrome (AIDS) in areas of Southeast Asia. The mortality rate is close to 100% if left untreated or when diagnosis and treatment are delayed [1]. Since the first case of disseminated penicilliosis was reported in an HIV-positive patient in Thailand in 1988, penicilliosis has become the third most common AIDS-defining illness (after tuberculosis and cryptococcosis) in Northern Thailand [2]. Penicilliosis has been reported from Northeast India across Myanmar, Thailand, Cambodia, Viet Nam, Taiwan, Hong Kong, southern China to Malaysia and Indonesia [25]. Increasingly immunocompromised patients in other parts of the world where the disease is not endemic are diagnosed with penicilliosis after traveling to Southeast Asia, and the illness has been reported either in patients with recent or very remote travel histories to these areas [3-5].

Epidemiology

Penicillium marneffei was first isolated by Segretain from hepatic lesions of a captive bamboo rat (*Rhizomys sinensis*) used for experimental infections at the Pasteur Institute in Dalat, Vietnam in 1956. The bamboo rat died spontaneously from the reticuloendothelial mycosis [26]. The fungus was named *Penicillium marneffei* in honor of Hubert Marneffei, Director of the Pasteur Institute of Vietnam. Human penicilliosis was first described by Segretain himself after pricking his own finger with a needle filled with *P. marneffei* used to inoculate hamsters [27]. He developed a small nodule at the site of inoculation with maxillary lymphadenopathy. The infection was cured by 30 days of oral nystatin. Fourteen years later Di Salvo reported the first disseminated penicilliosis in 1973 in a US missionary with Hodgkin's disease who lived in South Carolina and had traveled through Southeast Asia [28]. The patient had recurrent hemoptysis and underwent pneumonectomy. Pathology showed granuloma with yeast-like cells on tissue sections, and *P. marneffei* grew on culture. The same year 5 more cases were reported from Bangkok, Thailand. The rarity of human penicilliosis changed when the HIV pandemic arrived in Southeast Asia. In 1988, cases of *P. marneffei* infection were first being observed in patients with advanced AIDS. *P. marneffei* has now become the third most common AIDS-defining illness (after tuberculosis and cryptococcosis) in Northern Thailand [2].

The only known natural hosts are bamboo rats (*Rhizomys* and *Cannomys* species) and humans [29-32]. *P. marneffei* can be isolated from the soil around bamboo rats' burrows, though only rarely from other environmental sources [33]. The exact route of acquisition in humans is unknown but it is thought unlikely to be from direct contact with the rodents and presumed to be via inhalation and, rarely, inoculation [34]. In Thailand human infection is seasonal – particularly coinciding with rainy seasons – and has been associated with soil exposure [34, 35]. There is no evidence of person-to-person spread. Infections have been described solely in those exposed in Asia except for one case in an HIV-infected African male with no such travel history [36]. It has become the third most common HIV-related opportunistic infection in Southeast Asia – accounting for 15% of all HIV-related illness in Northern Thailand [2], affecting 10% of the AIDS patients in Hong Kong [37], and is the second most common single pathogen isolated from blood cultures in the Hospital for Tropical Diseases (HTD), Ho Chi Minh City, Viet Nam after *Cryptococcus neoformans*. Patients with cellular immune deficiency are at risk for this

disease. Patients with advanced AIDS tend to develop disseminated disease at CD4 count <50 cells//µL. Despite more than a decade of research efforts, the natural reservoir and vehicle of transmission of *P. marneffei*, whether it is a zoonosis or a sapronosis, remains perplexing.

Microbiology

P. marneffei is the only known *Penicillium* species that exhibits temperature-dependent dimorphic feature. At 25^{o}C the fungus grows as mycelia (a mold) with septate hyphae that bear conidiophores and conidia (similar to *Aspergillus* spp), producing a deep wine red, water-soluble pigment that diffuses into the Sabouraud agar medium. This feature is similar to other 220 Penicillium species; of those 8 species are known to be pathogenic. At 37^{o}C on artificial medium or in human tissue, *P. marneffei* converts to yeast-like spherical that multiplies by binary fission and not budding. The fission yeast cells represent the parasitic form of *P. marneffei*. This form is seen in the intracellular infection of the macrophages. The mold to yeast transformation or phase transition, which is thermally regulated, is a diagnostic characteristic of *P. marneffei* and is thought to be the key factor in its' virulence.

Clinical Features

Patients with penicilliosis have various manifestations and degrees of severity. Common clinical presentations include fever, fatigue, weight loss, non productive cough, generalized lymphadenopathy, hepatosplenomegaly, and characteristic skin lesions [1, 11, 38]. CD4 count at presentation is generally less than 50/mm^{3}. Blood culture is positive in about 88% of patient while skin lesions are present in 85% of patients in one series [11]. Skin lesions tend to be papules with central necrosis, generally referred to as "molluscum-like" lesions on face, neck, oral mucosa, upper more than lower extremities and trunk. The skin lesions are very similar to those seen in disseminated cryptococcosis, and concomitant cryptococcosis (5% in one study in Thailand) and other opportunistic infections are not un-common in patients with penicilliosis. The most common laboratory abnormality is anemia. 76% of patients have hemoglobin level of 10 g/dl or less, but it was not possible to unequivocally attribute anemia to *P. marneffei* alone in patients with late stage HIV. Other reported manifestations include ulcerated oral mucosal lesions [39], consolidated pneumonia or pulmonary nodule [40], hepatic penicilliosis without any skin lesion [41], pericarditis, osteroarticular lesions of ribs, long bones, skull, lumbar vertebrae, scapula, and temporomandibular region [42, 43].

Laboratory Diagnosis

Laboratory diagnosis is currently based on direct microscopic identification of the fungus with confirmation by culture, though there has been increasing interest in the use of immunodiagnostics and molecular assays.

1.1.1 Microscopy & Culture

Microscopically *P. marneffei* can be seen as oval or round intracellular and extracellular yeasts in biopsies of cutaneous lesions, bone marrow, lymph node, liver and blood smear using Wright, Wright-Giemsa, or Gomori-Grocott methenamine (GMS) stains. More rarely, the infection has been diagnosed directly from sputum, pleural fluid, cerebro-spinal fluid, pericardium, stool, urine and fine needle aspirates of lymph nodes [2, 44, 45]. *P. marneffei* has characteristic central septate or cross-wall formation that is essentially diagnostic. The differential diagnosis of such

intracellular yeasts include histoplasmosis (which also has similar clinical presentations), cryptococcosis (which is associated with more neurological symptoms and less respiratory involvement, lymphadenopathy and hepatosplenomegaly), and *Candida glabrata* [46, 47].

Unlike many other endemic dimorphic fungi, *P. marneffei* grows readily in standard media and Sabouraud dextrose agar and can take up to 4-14 days. The classical culture characteristics of thermal dimorphism and the production of red pigment are easily demonstrated. Bone marrow, blood, and biopsies of skin lesions all have high culture yield (100%, 76%, and 90% respectively) [48].

1.1.2 Immunodiagnosis

Various methods have been developed assessing host antibody production (such as immunoblot, indirect fluorescent antibody test [IFAT], latex agglutination, and enzyme-linked immunosorbent assay [ELISA]); however they have so far been studied on only small numbers of patients or there have been issues with sensitivity and specificity [49-51]. There has been recent interest in detecting circulating galactomannan. The *Penicillium* galactomannan has considerable homology to that of *Aspergillus* and commercial assays for the detection of the latter have recently been investigated in *P. marneffei* infection. Sera from 11 of 15 culture confirmed penicilliosis cases were positive though 9% of HIV positive controls were apparent false positives [52].

1.1.3 Urinary Antigen Assay

An ELISA test for detection of *P. marneffei* antigen in urine has been developed and prospectively evaluated in 33 HIV-positive Thai patients with culture-confirmed *P. marneffei* and 248 patients with other diagnoses [53]. This ELISA detected *P. marneffei* antigen in the urine samples of all 33 (100%) patients with penicilliosis with a median titer of 1:20,480. *P. marneffei* was not detected in 94% of samples from healthy volunteer; however it was detected in 27% of 248 urine samples from inpatients with diagnoses other than penicilliosis (include cryptococcosis, melioidosis, and other bacteria septicemia). Sensitivity and specificity for this assay to detect penicilliosis at a cut off titer of 1:40 was 97% and 98% with the positive predictive value of 84.2% and negative predictive value of 99.7%.

The same polyclonal hyperimmune IgG was used to develop a simplified dot blot ELISA and a latex agglutination test for detecting *P. marneffei* antigenuria and prospectively evaluated in urine specimens from 37 patients with culture proven penicilliosis and 300 controls (52 healthy and 248 hospitalized patients without penicilliosis). The sensitivities for ELISA, dot blot ELISA, and agglutination test were 97.3%, 94.6%, and 100% respectively; specificities were 98%, 97.3%, and 99.3%, respectively. Of these 3 promising tests, the agglutination test seems to be the simplest, most rapid and robust and needs to be validated in larger prospective cohort studies for both diagnostic purpose and for use as a surrogate marker of treatment response and treatment relapse.

1.1.4 Molecular Diagnosis

Polymerase chain reaction (PCR) assays, detecting fungal DNA in blood samples, have been developed. High sensitivity and specificity have been reported. However the protocols remain labor (and equipment) intensive and they have yet to enter routine clinical practice [54].

Treatment

Disseminated penicilliosis has a high mortality if untreated. All 9 patients who were not treated died from disseminated disease in an early series [1]. In vitro *P. marneffei* is highly sensitive to itraconazole, ketoconazole, miconazole, voriconazole, terbinafine, and 5-flourocytosine - intermediately sensitive to amphotericin B but largely resistant to fluconazole [1, 55-58]. No clear data are presently available for the echinocandins, though they may work poorly against the pathogenic yeast phase [59].

1.1.5 Acute infection

There have been no comparative trials on the acute treatment of penicilliosis, and thus treatment choices must be based upon data from case series and in vitro data on antifungal sensitivities. An early case series of 80 consecutive HIV positive Thai patients with penicilliosis described responses to treatment with amphotericin B, itraconazole, or fluconazole. In addition, 30 isolates underwent antifungal sensitivity testing. The failure rates (defined as persistent fungemia, clinical deterioration, or lack of clinical improvement) were 22.8%, 25%, 63.6%, and 100% for amphotericin B, itraconazole, fluconazole, and no treatment respectively. Treatment choice was at the discretion of the attending physician without knowledge of the minimum inhibitory concentration (MIC) of antifungal drugs for the isolates. Consistent with the poorer response to fluconazole, there were consistently higher in vitro MICs for this drug (73% of isolates were classified as borderline susceptible or resistant). 41% of isolates tested for amphotericin B susceptibility were classified as only moderately sensitive or resistant, but despite this the *Penicillium*-attributable death rate was low (12.8%) in patients receiving amphotericin B. All isolates were sensitive to 5-fluorocytosine [1].

1.1.6 Amphotericin B therapy

A subsequent series described 74 HIV patients with disseminated penicilliosis treated with amphotericin B 0.6 mg/kg/day for 2 weeks followed by itraconazole 400 mg/day for 10 weeks [4]. All patients received cotrimoxazole as primary prophylaxis for *Pneumocystis jirovecii*. Remarkably there were no deaths in the study. The treatment success rate (defined by negative blood culture and resolution of fever and skin lesions at the end of 12 weeks therapy) was 97%. It is not clear from the report how this treatment strategy was chosen and the basis for the high success rate compared to early trials. Nevertheless, this treatment regimen has become the “standard of care”.

Unfortunately amphotericin B is a prohibitively expensive drug for most patients at risk of penicilliosis, and the requirement for hospitalization adds to the cost burden to patients. For this reason, physicians in Thailand, Burma, India, and Vietnam in practice use itraconazole alone in patients who either cannot afford amphotericin B therapy or who are clinically stable enough to be treated as outpatient and report comparable success rates compared to amphotericin B (personal communications: Nicolas White, MD. former Director of Wellcome Trust Mahidol University Oxford in Thailand, Nguyen Huu Chi, MD. Former director of HIV inpatient at Hospital for Tropical Diseases (HTD), and Vo Minh Quang, MD. Director of outpatient HIV clinic at HTD).

1.1.7 Itraconazole therapy

In a small case series of 10 HIV-infected Thai patients with penicilliosis who were treated with itraconazole 400 mg/day monotherapy for 2 months, two patients died while on therapy; the

other 8 achieved clinical improvement, but the mean duration to culture negative was unacceptably long at 57 days [60]. A more recent study from India described successful treatment with itraconazole 400 mg/day for 3-4 weeks with a remarkable success rate of 97% (N=40 patients) [22]. However if the number of loss to follow up (N=10) is stringently considered as failure, the success rate is reduced to 78%. Oral itraconazole has been shown to be at least as efficacous and have less side effects compared to amphotericin B in empirical treatment of febrile neutropenia [12]. Further, itraconazole has been shown in case series to effectively treat other serious systemic fungal infections such as invasive aspergillosis, coccidioidomycosis, cryptococcosis, and blastomycosis [13-21]. Unfortunately treatment response rates for different drugs cannot be compared across different studies that employ different study designs and study endpoints.

1.1.8 Secondary prophylaxis

Before the widespread introduction of highly active antiretroviral therapy (HAART) it was recognized that disease relapse rate after initial treatment success is as high as 57% with the median relapse time of 24 weeks [11]. A subsequent randomized, double-blind, placebo-controlled study of itraconazole secondary prophylaxis (200 mg once/day) was discontinued early as all relapses were within the placebo arm [61]. Long term maintenance therapy with itraconazole has since been adopted.

1.1.9 Discontinuation of secondary prophylaxis

Several reports have investigated the discontinuation of itraconazole secondary prophylaxis after immune reconstitution from HAART. However, all have been retrospective observational studies. There were no relapses after itraconazole discontinuation in 33 patients with a CD4 lymphocyte count >100/μL for >6 months who were followed for a median time of 18 months, nor in another study on those stabilized on HAART which unfortunately did not specify CD4 counts [62, 63]. One relapse was described in a series of 19 patients who discontinued prophylaxis at a median CD4 lymphocyte count of 95/μL (18 patients had a CD4 count <200/μL and ten <100/μL) equating to a relapse rate of 1.72/100 patient-years [64]. It therefore appears reasonable to discontinue secondary prophylaxis after significant immune restoration from antiretrovirals, though exact criteria need to be established in larger, prospective, randomized studies.

1.1.10 Primary prophylaxis

The potential for primary prophylaxis for fungal opportunistic infection in advanced HIV patients has been explored with a randomized placebo-controlled double-blinded study of itraconazole (200 mg/day) in those with CD4 lymphocyte counts <200/μL [65]. There was a significant decrease in the incidence of both cryptococcosis and penicilliosis in the intervention group (principally in those with CD4 count <100/μL); however there was no survival advantage to being on itraconazole (though the study was not powered for this end-point). This intervention has not been adopted in clinical practice.

Immune Responses

The mechanism of host-fungus interaction and host immune response to *P. marneffei* are not completely understood. Infection is presumably via inhalation of conidia from the environment;

although this has never been definitely shown. Phagocytic cells are likely the primary line of host defense against this fungus. *P. marneffei* conidia are able to recognize fibronectin and bind to laminin via a sialic acid-specific lectin [66]. This may play an important role in the attachment of conidia to bronchoalveolar epithelia before ingestion by host mononuclear phagocytes. Studies in mouse model have shown that *P. marneffei* can be cleared within 2 to 3 weeks in healthy hosts, whereas in nude mice or in T-cell-depleted mice, *P. marneffei* infection is fatal, demonstrating that T cells, and CD4+ T cells in particular, are necessary for clearing this fungal infection in mice [67]. Recently by use of an in vitro analysis of a sublethal *P. marneffei* infection in BALB/c mice, it was demonstrated that protective immunity follows a Th1 response, with high levels of interleukin-12, IFN-γ, and TNF-α being developed [68]. This finding is consistent with the general knowledge that a Th1 response plays a crucial role in host resistance to intracellular pathogens such as mycobacteria infections and infections with other fungi.

Circulating human monocytes have been shown to respond to *P. marneffei* conidia with an oxidative burst which was significantly enhanced by a macrophage colony-stimulating factor [69]. Human neutrophils are found to have antifungal activity against the yeast form of *P. marneffei* but not the conidia. This activity was mediated by exocytosis of the granular cytolytic molecules from neutrophils rather than by oxygen radical-dependent mechanisms [70].

Molecular Epidemiology

Modern molecular methods such as multilocus genotypes have provided opportunities to identify isolates of a similar or identical genetic background that are derived from a common infective population, to describe the hierarchical organization of population structure, to identify the reproductive mode and to provide information on the deeper phylogenetic and evolutionary history of the pathogen [71]. Until recently, molecular approaches to typing *P. marneffei* have relied on surveying the genome by using methods that randomly sample for genetic variation.

1.1.11 Restriction fragment length polymorphism

A group from Thailand has used HaeIII digests of genomic DNA to search for restriction fragment length polymorphisms (RFLPs) in order to differentiate *P. marneffei* isolates from Chiang Mai region [72]. The 22 human isolates in their study were classified into 2 DNA types (type I, 73%; type II, 27%). Another group study of 20 *P. marneffei* isolates from Taiwanese patients that used the same restriction digestion assayuncovered the same 2 HaeIII RFLP patterns that had been found in Thailand with the same frequencies. However, the use of randomly amplified polymorphic DNA (RAPD) assays yielded 8 different RAPD patterns, suggested that there was greater genetic diversity than had been uncovered by the RFLP assay [73].

1.1.12 Pulse-field gel electrophoresis

A separate study used pulsed-field gel electrophoresis of 69 *P. marneffei* isolates from several regions of Thailand using restriction enzyme NotI revealed 2 macro-restriction patterns (MPI and MPII) that could be grouped into 9 sub-types, yielding 54 genotypes in total [74]. Another assay using the tetranucleotide repeat primer $(GACA)_4$ and the phage M13 core sequence identified 4 genotypes that varied in frequency between northern and southern Thailand [55]. However there has been no correlation between the restriction patterns from various *P. marneffei* isolates and geographic regions or clinical phenotypes. The drawbacks of these

typing systems are the low discriminatory power due to small numbers of alleles, the reproducibility of RAPD and macrorestriction profiles between laboratories, and variation within alleles.

1.1.13 Mutilocus sequence typing (MLST)

Recently, sequence-specific assays of genetic variation in the *P. marneffei* genome have been developed to address the above drawbacks. These are the multilocus sequence typing (MLST) and mutilocus microsatellite typing (MLMT). MLST characterizes isolates by sequencing housekeeping genes (usually seven), and is becoming the technique of choice for bacterial species and *Candida albicans* [75]. The alleles present at each locus are combined into a mutilocus sequence type, which is deposited in a species-specific online database held at http://www.mlst.net/. However, the use of MLST is limited when it is unclear whether the species being typed (*P. marneffei* in this case) contain insufficient genetic variation in the house keeping loci to discriminate between isolates.

1.1.14 Multilocus microsatellite typing (MLMT)

MLMT was designed to circumvent the problem of low levels of genetic variation. It targets loci that contain di-, tri-, or tetranucleotide repeats. These repeats (or microsatellites) are more highly variable than housekeeping loci due to the accumulation of length polymorphisms as a consequence of slippage by DNA polymerase during genome replication [76, 77]. The alleles at each locus are scored by electrophoresing PCR-amplified loci through an automated sequencer, typing the length polymorphisms, and then combining the alleles from each locus into a mutilocus microsatellite types that can be used to query online databases held at http://www.mutilocus.net/. The resulting outputs from these queries can be used to analyze the population genetic structure of the organism or to test epidemiological hypotheses.

Fisher et al. [76] screened 1.7 Mb of *P. marneffei* genome sequence for microsatellite motifs, using all possible permutations of di-, tri-, and tetranucleotide motifs with a minimum repeat number of six. This research resulted in 30 dinucleotide, 14 trinucleotide, and 5 tetranucleotide repeats being discovered. However, a similar study on the same genome sequence by Lasker and Ran [78] uncovered only 3 microsatellites. It is unclear why there is such a discrepancy, although the software used in the later study excluded tri- and tetra nucleotide repeats. Of the 49 loci identified by Fisher et al [76], 24 were chosen and amplified as multiplex PCRs in four groups of six loci and used to type a panel of 29 clinical and bamboo rat isolates chosen from across the endemic range of *P. marneffei* [25, 31, 76]. Of the 24 loci, 23 were amplifiable and 21 were polymorphic with between 2 and 14 alleles present at each locus, comprising 19 unique microsatellites in total. Clustering of isolates based on the microsatellite genetic distance D_1 [79] showed that isolates occur within 2 geographically separated clades that account for 26% of the total observed genetic diversity [25]. The “eastern” clade contained isolates from mainland China, Hong Kong, Indonesia, and Vietnam, while the “western” clade contained isolates from Thailand and India, showing that *P. marneffei* has a geographic component to its population genetic structure. A study over a smaller geographical scale in Manipur, India showed that while the microsatellites of isolates were identical within bamboo plantations, they were dissimilar between bamboo plantations [31]. This finding suggests that the population genetic structure of *P. marneffei* may in fact be partitioned over local, as well as large, geographical scales, although further studies are necessary to confirm the generality of this finding.

These molecular methods, particularly the highly discriminatory MLMT techniques provide unique means to screen samples from human clinical populations, from bamboo rat populations, and environmental sources from different geographical areas and to identify the natural cycle of infection by *P. marneffei* in nature.

Pharmacokinetics - Pharmacodynamics of Itraconazole and Amphotericin B

1.1.15 Itraconazole spectrum of activity and mechanism of action

Itraconazole is a triazole compound that has in general broader spectrum of antifungal activity than other azole antifungals, from activity against mucocutaneous candidiasis, dermatomycosis, to deep mycoses including aspergillosis, candidiasis, cryptococcosis, histoplasmosis, and several endemic mycoses such as paracoccidioidomycosis, chromoblastomycosis, and penicilliosis. Itraconazole, like other azoles, has 3 nitrogen atoms in its azole ring which might improve tissue penetration, prolong half-life, and increase specificity for fungal enzymes [80]. The nitrogen atoms interact with the heme iron of the fungal cytochrome P450 3A (CYP3A), inhibiting the function of lanosine 14α-demethylase which converts lanosterol to ergosterol, the main sterol in the fungal cell membrane. This inhibits replication and promotes cell death, or in the case of yeast cells of *Candida albicans*, transformation into hypothetically invasive hyphae [81]. Itraconazole has little effect on mammalian cytochome P450 enzymes even at high concentrations or on the sterol and steroid pathways of the human pituitary-adrenal-testicular axis [82]. Resistance to azole antifungals rarely develops and appears to be a problem mainly with fluconazole in HIV-positive subjects [81, 82].

1.1.16 Pharmacokinetics of itraconazole

Plasma level of itraconazole can be measured either by high performance liquid chromatography (HPLC) or by bioassay. HPLC has a high specificity and sensitivity (2 ng/mL plasma) and has been used in most pharmacokinetic studies [83]. The absolute bioavailability of oral itraconazole is 55% (±15%). Oral itraconazole should be administered with food since the bioavailability is reduced by 40% when it is administered under fasting condition [84]. The bioavailability of itraconazole is reduced by 50% when administered with H_2 blocker [85]. Since the bioavailability of oral itraconazole is affected by gastric acidity, acid-reducing drugs (H2 blockers, proton pump inhibitors) should be administered at least 2 hours after administration of itraconazole. Itraconazole is highly lipophilic, is strongly protein binding (99.8%), and has a high tissue penetration. Body fluids such as cerebrospinal fluid (CSF), eye fluid and saliva contain low to non-detectable amounts of itraconazole, whereas in many organs and tissues the concentrations exceed the corresponding plasma levels by a factor of 1.5 to 20 [86].

Metabolism of itraconazole is extensive in the liver, and excretion of inactive metabolites occurs primarily in the urine and feces. Dosing of oral itraconazole does not need to be adjusted for renal insufficiency. A hepatic metabolite, hydroxyitraconazole, is bioactive and has activity similar to that of the parent compound [87]. Because of the high volume distribution of itraconazole, oral or intravenous loading doses are needed to reach protective level quickly especially when given for treatment of systemic mycosis. It is recommended that 600 mg/day in two divided doses for 3 days is used for oral loading dose, and 400 mg/day in 2 divided doses for 2 days is used for intravenous loading doses.

1.1.17 Itraconazole formulations

Oral itraconazole suspension and intravenous formulations have recently been developed to circumvent the variation in serum concentrations of itraconazole capsules. In general the oral suspension (with cyclodextrin) preparation is more readily absorbed than the tablets, resulting in roughly a 30% larger AUC than with the tablet preparation. Peak serum concentration at steady state, after the oral solution at a dose of 200 mg twice daily, ranged from 513 to 2,278 ng/L with a median concentration of 1,326 ng/L. In contrast, the peak serum concentration at steady state after administration of the capsule formulation at the same dose ranged from 297 to 1,609 ng/L with a median value of 741 ng/L [88]. Opposite to the tablet formulation, the absorption of the liquid suspension is enhanced when it is taken in a fasted state and has a more predictable absorption. Nausea is more common with the liquid formulation due to the osmotic effects of cyclodextrin. This may affect compliance and is potentially counter-productive in the goal to improve bioavailability.

The same vehicle (cyclodextrin) is used to solubilise the IV formulation as the oral solution. This vehicle is known to accumulate in patients with impaired renal function and therefore, use of the intravenous preparation is limited to patients with a creatinine clearance >30 mL/min and is usually reserved for patients with severe infections who are intolerant of amphotericin B. The intravenous formulation is no longer manufactured in the United States but is available in some other countries.

1.1.18 Pharmacodynamics of itraconazole

The concentration-effect relationship for any systemic antifungal agent remains a controversial issue. Historically the target plasma level for itraconazole has been estimated at 250 ng/mL (by HPLC) based on the in vitro IC_{90} (the concentration needed to achieve 90% reduction in replication) [89, 90]. Numerous itraconazole concentration-effect studies have been undertaken and each has demonstrated a link to drug efficacy [15, 17, 91]. A similar relationship for toxicity has not been identified. The pharmacodynamic efficacy investigations include both preclinical animal model and clinical trials using itraconazole both as prophylaxis to prevent the development of invasive fungal disease and as treatment of invasive fungal diseases. In a group of 21 patients with invasive aspergillosis, mean itraconazole concentration in responders was 6.5 mg/L and 4.2 mg/L in nonresponders (based on a microbiologic assay) [17]. A similar quantitative relationship was observed in a group of patients with nonmeningeal coccidioidomycosis. In this cohort of 39 patients, itraconazole concentrations measured by bioassay were 6.5 ± 4.2 mg/L in the 28 patients who had a clinical response and 4.0 ± 3.2 mg/L in 11 nonresponders [91]. In another study of 25 patients with HIV and cryptococcal meningitis, trough itraconazole concentrations exceeding 1 mg/L was observed in the group of patients with 100% response rate; whereas trough concentrations below 1 mg/L was observed in the group of patients with a 66% response rate [15].

In regards to investigations of itraconazole use as prophylaxis to prevent the development of invasive fungal disease, the relationship is similar to that observed in treatment studies; however, the concentrations associated with effective disease prevention is two to fourfold lower than that shown necessary for fungal disease treatment [92-94]

1.1.19 Clinical experiences with itraconazole for prophylaxis and treatment of invasive fungal diseases

In clinical trials, itraconazole oral solution (5 mg/kg/day) was more effective at preventing systemic fungal infection in patients with hematological malignancy than placebo, fluconazole suspension (100 mg/day), oral amphotericin B (2 g/kg/day) and was highly effective at preventing fungal infections in liver transplant recipients [13, 13, 95]. There were no unexpected AEs with the itraconazole oral solution in any of these trials. In a randomized clinical trial, intravenous itraconazole solution is at least as effective as intravenous amphotericin B in the empirical treatment of neutropenic patients with systemic fungal infections, and drug-related AEs are more frequent in patients treated with amphotericin B [12]. Itraconazole has been successfully used to treat a variety of invasive fungal infections including invasive aspergillosis, coccidioidomycosis, cryptococcosis, and blastomycosis in case series [13-21]. However, both the lack of direct systematic comparative studies and the reported variable bioavailability of the tablet formation of itraconazole have contributed to the slow coming of this drug.

1.1.20 Amphotericin B introduction

Amphotericin B is a polyene antibiotic first isolated in 1955 from *Streptomyces nodosus*. It is a broad antimycotic agent and a highly antiparasitic agent. After 5 decades of experiences and the births of newer antifungal drug classes, amphotericin B remains the agent of choice for many invasive fungal infections. Amphotericin B has a broad spectrum of action that includes most of the major fungal pathogens of man. This drug binds to the membrane sterols of fungal cells, causing impairment of their barrier function and loss of cell constituents. Metabolic disruption and cell death are consequent upon membrane alterations.

1.1.21 Amphotericin formulations

The most important drawback to the formulation of amphotericin B is that it is scarcely soluble in water. The reference conventional formulation Fungizone® which was a mixture with deoxycholate was developed for intravenous administration; unfortunately this formulation is nephrotoxic. Second generationamphotericin B formulations which depend on different lipid-carrier systems were developed in the 1990s to circumvent this side effect. These are Abelcet® (ABLC), AmBisome® (L-AmB) and Amphotec® (ABCD). Abelcet® is a formulation with 2 phospholipids in a 1:1 drug-to-lipid molar ratio, has a better therapeudic index and lower risk of renal disorders at a dosage of 1-5 mg/kg/day. Amphotec® is a formulation with cholesterol sulfate in equimolar concentrations, has similar antifungal efficacy as Fungizone® but less cytotoxic and hemolytic. AmBisome® formulation is integrated into small unilamellar liposomes and is superior to Fungizone® in bioavailability and side effects. Ostrosky-Zeichner et al have summarized 10 major controlled clinical studies and concluded that no study has ever shown a lipid new amphotericin B formulation to be less effective than Fungizone®, and some studies show strong evidence that the new formulations may be more effective and consistently less toxic than Fungizone®. In resource rich countries, these new formulations are used more commonly as their lower rate of side effects are usually considered to outweigh their high costs and to afford the use of higher doses [96].

1.1.22 Amphotericin B pharmacokinetics

Due to its low solubility amphotericin B gastrointestinal uptake of oral formulation is minimal, and IV infusion remains the route of choice. Amphotericin B is extensively bound to plasma proteins (~95%) by ß-lipoproteins, albumin, and α_1-acid glycoprotein [97]. Amphotericin B is highly amphipathic in nature (being both hydrophilic and hydrophobic). In water it forms a

mixture of water-soluble monomers and oligomers with insoluble aggregates [96]. Different aggregation states can be present in the same formulation, the proportions of each association form has been shown to depend on the interaction between amphotericin B and solvents such as amphotericin B concentrations [98], the medium in which the drug is dispersed [99], the action of surfactants and serum albumin [100, 101], or the temperature they have been exposed to. The various aggregation states of amphotericin B may interact with membrane sterol in different ways to induce changes in cell membrane, and may have different impacts on amphotericin efficacy and toxicity.

1.1.23 Comparison of amphotericin B and itraconazole in empirical treatment of invasive fungal infection

In an open, randomized, controlled, multicenter trial, powered for equivalence, involving 60 oncology centers in 10 countries evaluated 384 neutropenic patients with cancer who had persistent fever that did not respond to antibiotic therapy, itraconazole and amphotericin B have at least equivalent efficacy, and itraconazole is associated with significantly less toxicity than amphotericin B [12]. In another open, randomized controlled study evaluated 162 patients with underlying hematological malignancy and febrile neutropenia, significantly fewer itraconazole patients discontinued treatment due to any AE(22.2 vs. 56.8% AMB [amphotericin B]; $p < 0.0001$). The main reason for discontinuation was a rise in serum creatinine (1.2% itraconazole vs. 23.5% AMB). Intention-to-treat (ITT) analysis showed favorable efficacy for itraconazole: response and success rate were both significantly higher than for AMB (61.7 vs. 42% and 70.4 vs. 49.3%, both $p < 0.0001$). Treatment failure was markedly reduced in itraconazole patients (25.9 vs. 43.2%), largely due to the better tolerability [102]. Another study from Korea compared the efficacy and tolerability of the two drugs as an empirical antifungal agent in 96 patients with febrile neutropenia. The overall success rates were 47.9% for itraconazole and 43.8% for amphotericin B deoxycholate (% difference: 4.1% [95% confidence interval for the difference: -15.8 to 24]), which fulfilled the statistical criteria for the non-inferiority of itraconazole. The proportions of patients who survived for at least seven days after discontinuation of therapy or who were prematurely discontinued from the study were not significantly different between the two groups. The rates of breakthrough fungal infections and resolution of fever during neutropenia were similar in both groups. More patients who received amphotericin B deoxycholate developed nephrotoxicity, hypokalemia or infusion-related events than did those patients who received itraconazole (nephrotoxicity: 16.7% vs. 1.8%, hypokalemia: 66.7% vs. 24.6%, and infusion-related events: 41.7% vs. 3.5%, respectively) [103].

2 Study Objectives

Primary Objective

To compare the efficacy of Itraconazole and amphotericin B in the acute-phase treatment of penicilliosis as assessed by the absolute risk of death during the first 2 weeks of therapy.

Secondary Objectives

14. Determine overall survival until week 24

15. Determine time to treatment success (defined by absence of fungal growth in follow up culture, temperature <38ºC for 3 days, and complete resolution of skin lesions or lesions in the final stages of healing as judged by treating clinicians)
16. Determine relapse-free survival until week 24 of therapy (i.e., time to the first treatment relapse or death). Relapse is defined as recurrence of culture-confirmed penicilliosis after achieving treatment success at week 12
17. Determine time to culture sterilization
18. Determine the rate of early antifungicidal activities as assessed by the decrease in colony forming unit (CFU) count per mL of blood in serial blood samples
19. Determine safety and tolerability as assessed by Grade 3 and Grade 4 adverse events (AEs) and serious adverse events (SAEs)
20. Identify baseline clinical, microbiological, and/or laboratory predictors of outcome
21. Develop population pharmacokinetic (PK) models of amphotericin B and itraconazole in HIV-infected patients to characterize the absorption, distribution, and clearance, and identify the sources of variance in pharmacokinetic parameters. Correlate PK variables to fungal clearance, early antifungicidal activity, and treatment outcomes.
22. Study the epidemiology of *P. marneffei* infection, focusing on finding the natural reservoir and vehicle of transmission of *P. marneffei*. A simultaneous case-control study will be performed to identify exposure risk factor/s for the development of penicilliosis in age, sex, CD4 or WHO-disease-stage matched HIV-infected patients with and without penicilliosis. Detailed exposure histories related to living and working environment (proximity/exposure to any body of water, tropical plants/trees, soil, domestic/farm/wild animals, types of raw/rarely cooked foods consumed, injection drug use history/practices, type/seasonality of jobs, current/past specific activities most days) will be investigated. Global positioning system (GPS) mapping technology will be used to characterize the geo-spatial distribution of cases and controls (Appendix D)
23. Investigate the molecular epidemiology of *P. marneffei* infection using a number of cutting-edge molecular technologies including highly discriminatory mutilocus microsatetlite typing (MLMT) and correlate the identified genotypes with clinical and geo-spatial epidemiology data (appendix E)
24. Evaluate an ELISA assay to detect *P. marneffei* urinary antigen for diagnostic accuracy of penicilliosis and as a surrogate marker for microbiological and clinical outcomes (Appendix F)
25. Determine the cost effectiveness of treating the acute-phase of penicilliosis with itraconazole versus amphotericin B (Appendix G)
26. Characterize the incidence, clinical features and outcome of patients who develop penicillium associated immune reconstitution disease (IRD) (Appendix H)

3 Study Plans

Study Designs and Overview

This study is a randomized, open-label, comparative, multi-center trial with the following treatment groups:

Group 1: intravenous amphotericin B 0.7 mg/kg/day x 2 wks
Group 2: oral itraconazole 400 mg/day x 2 weeks (including 600 mg/day x first 3 days for loading)

After the 2-week acute-phase therapy, all patients will continue on to the maintenance-phase therapy with oral itraconazole 400 mg/day x 10 weeks, followed by the suppressive-phase therapy with itraconazole 200 mg/day until CD4 count rises above 100 for 6 months on antiretroviral therapy (ART) for HIV.

Randomization will be 1:1 and stratified for the study site. Patients will be followed until 6 months post randomization.

Study Size

Planned enrollment of 440 subjects total

Study Duration

Study enrollment will anticipate to begin in 2012 and to end when 440 subjects are enrolled and have been followed for at least 6 months. This is anticipated to occur over 4 years.

Study Population

3.1.1 Screening Criteria

1. HIV positive

AND

2. Age ≥18 year

AND

3. Clinicians suspect penicilliosis illness in a patient with typical umbilicated skin lesions or a combination of the following features without skin lesions: fever, malaise, enlarged lymph nodes, hepatomegaly and/or splenomegaly, cough and/or respiratory complaints, gastrointestinal complaints, anemia, thrombocytopenia, elevated AST, and ALT.

3.1.2 Screening Procedure

Subjects that meet the above criteria will be invited to participate in the study. If the subjects agree to participate and sign the informed consent form, they will undergo the following screening procedures.

- Blood samples for blood culture and routine hematology, chemistry, and liver function tests
- Skin lesion scrapping for direct microscopy and culture as deemed appropriate by treating clinicians
- Urine sample to rule out pregnancy in females
- Peripheral lymph node aspiration if lymph node size is >1cm
- Bone marrow aspiration only if deemed appropriate by attending physicians
- HIV testing (in accordance with Vietnam MOH guidelines on diagnosis and treatment of HIV/AIDS dated Aug 2009) if not already done

During the screening period (while awaiting culture results), subjects will be treated as clinically indicated, by best medical practice. If empiric antifungal is deemed appropriate, patients will be randomized to receive either amphotericin B or itraconazole. If culture does not subsequently confirm the diagnosis of penicilliosis, subjects will be withdrawn from the trial. After signing the informed consent form, subjects that had culture confirmed penicilliosis at an outside hospital will not require a repeat culture part of the screening step and be directly evaluated with the other inclusion and exclusion criteria.

After screening results are available, eligibility for this treatment protocol will be assessed by the following inclusion and exclusion criteria:

3.1.3 Inclusion Criteria

1. HIV positive

AND

2. Age ≥18 year

AND

3. Syndrome consistent with penicilliosis (primary or relapse) PLUS culture-confirmed diagnosis of penicilliosis (from blood, skin lesion scrapping, lymph node or bone marrow biopsy).

3.1.4 Exclusion Criteria (any of the following):

1. Age <18
2. Pregnancy or urine β-hCG positive
3. History of allergy or severe reaction to either itraconazole or amphotericin B
4. Central nervous system involvement (assessed clinically and by evidence of inflammation and/or infection in the CSF)
5. Use of the following prohibited drugs: phenytoin, barbiturates, carbamazepine, rifampin, HMG-CoA reductase inhibitors, cisapride, terfenadine, midazolam, dihydropyridine Ca channel blocker, cyclosporine, cyclophosphamide, tacrolimus, digoxin, quinidine, ergot derivatives, pimozide, coumadin, or investigational drugs.
6. Baseline AST or ALT > 400U/L
7. Absolute neutrophil count < 500 cells/μL
8. Creatinine clearance of <30 by Cockcroft-Gault formula or on hemodialysis
9. Concurrent diagnosis of cryptococcal meningitis or active tuberculosis (as amphotericin B is the treatment of choice for cryptococcal meningitis, and tuberculosis treatment with INH and Rifampin is contraindicated when used with itraconazole)
10. Current treatment with an antifungal drug for confirmed or suspected penicilliosis for >48 hours

The reasons why patients who meet the screening criteria but are later excluded from the study will be recorded in a separate patient log.

Estimating creatinine clearance (mL/min)
Cockcroft and Gault equation:
CrCl = (140 - age) x weight(Kg) / (Cr(mg%) x 72) for males (x 0.85 for females)

(If unit for Cr is mmol/L, convert to mg% by Cr x 0.01)

Nornal range: Male = 90-140 ml/minute, Female = 85-135 ml/minute

Randomization

Randomization will be 1:1 and stratification by study site. Each site will have a separate randomization list to ensure the 1:1 ratio of the treatment arms at each site. In addition, to

ensure that the 1:1 ratio can be approximately obtained at any time during the study, the randomization list at each site is further divided into even block sizes of 4-10 patients, and within each randomization block, treatment allocation is maintained at 1:1.
A computer-generated randomization list will be produced by a study pharmacist with no clinical involvement in the trial. This list will then be incorporated into a web based program. This program can be assessed 24 hours/day with secured log in by study personnel from each centre. When a patient is enrolled to the study, an authorized study staff will enter patient details (patient ID, year of birth and patient initials) into the system to obtain the treatment allocation for that patient based on the randomization list. All transactions on the web server will be intermediately logged, unchangeable and auditable.

Criteria for Evaluation

3.1.5 Primary Endpoint

Absolute risk of death during the first 2 weeks after randomization

3.1.6 Secondary Endpoints

3.1.6.1Clinical endpoints

- Overall survival until week 24
- Time to treatment success (defined by absence of fungal growth in follow up culture, temperature <38ºC for 3 days, and complete resolution of lesions or lesions in the final stage of healing as judged by treating clinicians)
- Relapse-free survival until week 24 of therapy (i.e., time from treatment success to the first treatment relapse or death). (Relapse is defined as recurrence of culture-confirmed penicilliosis after achieving treatment success at week 12)
- Deaths from penicilliosis until week 24 (causes of death will be determined by investigators)
- Time to change of therapy from assigned study therapy
- Total number of patients with Grade 3 and Grade 4 AEs and SAEs, and the cumulative incidence of Grade 3 and Grade 4 AEs and SAEs, associated with cessation of randomly assigned therapy between treatment arms
- Antifungal medication adherence
- Incidence of Immune Reconstitution Diseases

3.1.6.2Microbiological endpoints

- Time to blood culture sterilization
- Rate of early fungicidal activity as determined by serial blood samplings during therapy and measured by the decrease in log colony forming units per mL of blood (CFUs/mL)
- Frequency and patterns of itraconazole and amphotericin B resistance emergence

3.1.6.3Pharmacological endpoints

- Antifungal concentration time curves
- Maximum antifungal concentrations/MIC, area under the curve (AUC) of antifungals/MIC over time

Statistical Considerations

3.1.7 Analysis of the primary endpoint and overall survival

This is a non-inferiority trial with a non-inferiority margin of Δ=10%; i.e., the aim is to prove that the absolute risks of death during the first 2 weeks of treatment in the two treatment arms differ by less than 10% (at worst) in favour of amphotericin B. Two-week mortality estimates will be based on the Kaplan-Meier method. Patients lost to follow-up before the week 2 assessments will be treated as censored. Based on these estimates and corresponding standard errors (calculated according to Greenwood's formula), a two-sided 95% confidence interval (CI) for the difference in the absolute risks of death will be calculated. If the CI excludes differences of 10% or more in favour of the amphotericin B arm, the primary objective of the trial will be met.

In addition, we will assess the joint effect of treatment assignment and the baseline covariates age, sex, injection drug use, ART naïve/experienced, and presence of fungemia on the primary endpoint. This adjusted analysis will be based on logistic regression. As we expect only few patients lost to follow-up during the first 2 weeks, these patients will be removed from the adjusted analysis.

In a second step, we will analyze overall survival, i.e., time to death during the entire follow-up period of 24 weeks. Overall survival will be summarized by Kaplan-Meier curves and the 2 arms will be compared with a Cox proportional hazards regression model with treatment as the only covariate. In addition, an adjusted analysis will be performed using the Cox model and the same baseline covariates as listed above.

Potential heterogeneity of the treatment effect will be explored in the following pre-defined subgroups:

- Injection drug use (yes vs no)
- ART status (naive vs experienced)
- Presence of fungemia (yes vs no)
- Baseline CD4 count

3.1.8 Analysis of secondary endpoints

Time to treatment success**:** The cumulative proportion of patients achieving treatment success over time will be summarized with the cumulative incidence function, which takes the competing risk of prior death into account. Comparison between the two arms will be based on the Fine and Gray model with treatment as the only covariate. An adjusted analysis including the same covariates as for the analysis of overall survival described above will also be conducted.

Relapse-free survival until week 24 of therapy (i.e., time from treatment success to the first treatment relapse or death): Relapse free survival in both arms will be summarized using Kaplan-Meier curves.

Other time-to-event endpoints**:** Will be summarized using Kaplan-Meier curves (in case they include death in the endpoint) or cumulative incidence functions (otherwise, to take into account the competing risk of death). In addition, they will be modeled with (cause-specific) Cox

proportional hazards models including the same covariates as for the analysis of overall survival.

Adverse events: Frequency tables and listings of Grade 3 and 4 AEs, SAEs, and AEs leading to discontinuation of the randomized treatment will be produced. The overall frequency of each of these types of adverse events will be compared between the 2 arms with Fisher's exact test.

3.1.9 Analysis of populations

The primary analysis will be based on the full analysis population including all randomized patients following an intention-to-treat principle, but excluding subjects without microbiological confirmed penicilliosis. As the analysis of non-inferiority trials on the full analysis set is not necessarily conservative, the analysis of the primary endpoint will be repeated on the per-protocol population. This population excludes patients if they meet any exclusion criteria while in the study, are not treated according to the randomized treatment arm, or lost to follow-up before day 14.

3.1.10 Sample size calculation

The inpatient mortality rate of patients with HIV-associated penicilliosis at HTD in 2009 was 10% [5]. Not all of these patients received antifungal treatment before death. On the other hand, this rate did not include out-of-hospital deaths. In considering these opposing factors we estimate that the mortality in both treatment arms will be approximately 10% with a plausible range of 5-15%.

The primary aim of this trial is to demonstrate non-inferiority of itraconazole compared to amphotericin B treatment with respect to overall mortality at the end of 2 week induction therapy. The sample size calculation is based on an assumed mortality rate of 15% in both arms, a non-inferiority margin of 10% and a one-sided significance level of 2.5%. Based on these assumption, a total sample size of 400 patients will guarantee a power of 80% to show non-inferiority or, equivalently, that the two-sided 95% confidence interval for the difference in mortality between the two arm excludes an excess mortality of 10% or more in favour of amphotericin B therapy. We expect that the combined proportion of losses to follow-up and major protocol violations will be no more than 10%. To account for this, a total of **440 patients** (220 per treatment arm) will be randomized in this trial.

3.1.11 Justification of the non-inferiority margin

Given that without proper treatment, penicilliosis has almost a 100% mortality rate, a non-inferiority margin of 10% and a "cure" rate of at least 85% for patients receiving amphotericin B would allow us to prove that itraconazole retains at least 88% of the benefits of amphotericin B over placebo. A non-inferiority margin of 10% may seem large given that the primary outcome is mortality. We nevertheless regard it as acceptable due to the following reasons:

First, it should be highlighted that the 10% excess mortality for itraconazole refers to a worst-case scenario, i.e. the degree of inferiority that we aim to exclude with 95% confidence. Our actual best guess of the true mortality difference based on the trial data, i.e. the observed

difference, will be much less than 10%. For example, if the observed mortality risk for patients with amphotericin B is 15%, the observed mortality risk for patients with itraconazole must be <18% in order to guarantee that the 95% confidence interval excludes mortality differences of 10% or more.

Second, our sample size calculation is based on a conservative assumption regarding mortality. If the true mortality in both arms is equal but lower than 15%, e.g. 5% or 10%, we will have 80% power to exclude excess mortalities of >6% or >8%, respectively, in the itraconazole arm.

Third, in case itraconazole is substantially inferior to amphotericin B treatment, the trial will also have sufficient power to detect this: If the true mortality in the itraconazole arm is 15% and the true mortality in the amphotericin B arm is 6.5% or less, we will have >80% power that the 95% confidence interval excludes 0, i.e. to confirm a difference between the two arms.

Fourth, the possibility of some excess mortality in the itraconazole arm should be balanced with the unavailability of amphotericin B (particularly in provincial/district hospitals), prohibitive costs, the more complex administration, and the less favourable safety profile..

Finally, practicability and feasibility of the trial must be considered [20]. A non-inferiority margin of 7.5% appears to provide little gain but would lead to a sample size of 792 (80% increase), whereas a margin of 5% would result in a prohibitively large sample size of 1,320 (300% increase).

Subject and Study Modification or Discontinuation

3.1.12 Subject withdrawal/discontinuation

Participants, or their surrogates if the patient is otherwise unable to make informed decisions, can terminate study participation at any point they wish to. If a patient is withdrawn prior to completion of the study, the reason for this decision will be recorded in the case report forms (CRFs). The remaining follow-up evaluation will be conducted if patient consent is obtained.

4 Study Treatment

Overview

This protocol will compare the two current treatment strategies for acute penicilliosis: itraconazole versus amphotericin B followed by itraconazole therapy. There is no placebo arm (i.e. no arm without active drug administered).

Eligible patients will be randomized to receive either:

C. Itraconazole: 400 mg/day in two divided doses for 12 weeks (including 600 mg/day in two divided doses x 3 days for loading).
D. Amphotericin B: 0.7 mg/kg/day IV x 2 weeks, followed by itraconazole 400 mg/day po for 10 weeks.

Products

4.1.1 Itraconazole

Itraconazole capsules (Itranstad) purchased by the trial pharmacist from licensed suppliers in Viet Nam and provided to the study participants free of charge throughout the whole 12 weeks duration of the treatment. Study participants will be transferred to the National HIV/AIDS Treatment Program which provides free opportunistic infection treatment and anti-retroviral therapy (ART) as soon as possible.

4.1.2 Amphotericin B

Amphotericin B intravenous formulation purchased by the trial pharmacist and provided to the study participants free of charge for the 2 week duration of the treatment.

Storage and Handling

The itraconazole capsules will be kept at room temperature (approximately 25ºC or 77ºF). Amphotericin B intravenous formulation will be kept under refrigeration (2-8ºC or 36-46ºF) and not allow to freeze. All medication storage and administration will be regulated through the central pharmacy departments at each study site to ensure good quality and control of medication handling.

Study Drug Dosing

4.1.3 Itraconazole dosing

Because of the high volume distribution of itraconazole, oral loading dose is needed to reach protective level quickly for treatment of systemic mycosis. Note that itraconazole capsules are to be taken only with food and/or an acidic drink (likely cola drink) as its absorption is dependent on gastric pH. Any gastric acid reducing drugs (H2 blocker, proton pump inhibitor) are not allowed, and concomitant therapies with these drugs are exclusion criteria for the trial. If an acid blocking agent needs to be given, H2 blocker is recommended to be used 6 hours before or after administration of oral itraconazole.

Itraconazole oral loading dose: 3 capsules 100 mg po bid (or 600 mg/day) for 3 days, followed by the standard treatment dose of 2 capsules 100 mg po bid (or 400 mg/day) for a total of 12 weeks.

4.1.4 Amphotericin B dosing

Amphotericin B 0.7 mg/kg/day IV x 2 weeks, followed by itraconazole 2 capsules 100 mg po bid (or 400 mg/day) for 10 weeks. A loading dose of itraconazole is not necessary for subjects already on amphotericin B.

Product Administration

The initial dose of both components of the study drug should be given as soon as possible after enrollment and randomization. These can be administered with food or a snack whenever possible. Itraconazole needs to be taken with food or an acidic drink (likely cola drink).

Post Dose Emesis

If emesis occurs within 60 minutes after oral study drug administration, and is thought to be of sufficient volume to evacuate the study drug from the stomach (i.e., 5 cc vomitus probably would not remove the study drug from the stomach), a repeat dose of the study drug should be administered. The maximum number of repeat doses is two (after initial dose) per dosing interval. If all three doses are vomited, this will be recorded and participants will continue with the next scheduled dose. If a patient vomits all given doses within a 24 hour period or if a patient is judged by the treating clinician to be intolerant of oral medication, a nasal gastric tube placement is indicated. Patients who cannot tolerate nasal gastric tube placement will be considered intolerant to treatment, recorded as such and may be switched to appropriate treatment at the discretion of the treating physician.

Concomitant and Prohibited Medications

4.1.5 Prohibited medications

Concomitant administration with cisapride, dofetilide, ergot derivatives, levomethadyl, lovastatin, midazolam, pimozide, quinidine, simvastatin, or triazolam is prohibited during administration of study drug. Rare cases of serious cardiovascular AEs (including death), ventricular tachycardia, and torsade de pointes have been observed due to increased cisapride, pimozide, quinidine, dofetilide or levomethadyl concentrations induced by itraconazole. Concurrent use of these drugs is contraindicated.

4.1.6 Category C drugs with amphotericin B where monitor therapy is recommended

Amphotericin B may enhance the nephrotoxic effect of aminoglycosides and cyclosporine. Corticosteroids (systemic) may enhance the hypokalemic effect of amphotericin B.

4.1.7 Category B drugs with amphotericin B where no action is needed

Amphotericin B may enhance the adverse/toxic effect of Cardiac Glycosides such as Digoxin and neuromuscular-blocking effect of Neuromuscular-Blocking Agents such as Atracurium; Cisatracurium; Doxacurium [Off Market]; Metocurine Iodide; Mivacurium [Off Market]; Pancuronium; Rocuronium; Succinylcholine; Vecuronium.

4.1.8 Anti-pyretic

If an anti-pyretic is needed, acetaminophen / paracetamol is recommended.

4.1.9 Anti-emesis

As itraconazole can cause nausea and vomiting, an anti-emetic may be used for intractable symptoms. The drugs listed in Section 8.1.1 may be considered.

5 Study Procedure

See Appendix B for graphical representations of study assessments and frequency.

Hospitalization

After signing the informed consent form, all enrolled patients will be admitted to the hospital at the participating study site and will remain hospitalized through the first 2 weeks of therapy. Patients who choose to self-discharge before the end of the initial two week treatment will continue to be followed through out-patients visits or at home.

Initial Evaluation

5.1.1 History and physical examination on day 1

Including (but not limited to):

- Presence of symptoms
  - Fever
  - Weight loss
  - Enlarging lymph nodes
  - Fatigue/anorexia
  - Cough and/or shortness of breath
  - Nausea and/or vomiting
  - Skin and/or mucosal lesions
- Development of symptoms listed above
- Epidemiologic factors (only for patients participating in the case control study)
  - Home and work addresses
  - Type/s of work and specific activities at work
  - Specific exposure to soil, location and type of soil
  - Travel history
  - Exposure/contact with bamboo and/or bamboo rats
  - Animals in household (dogs, cats, birds including chickens and ducks, reptiles, pigs, rabbits or other rodents)
  - Animals in surrounding area (yard, farm etc)
  - Illness in animals noted above
  - Live by or close contact with any body of water
  - Exotic food including raw or rarely cooked food
  - Exposure to ill persons with similar symptoms
- Previous peniciliosis history
- HIV history
  - Injection drug use
  - Antiretroviral history and drugs
  - Latest CD4 count if known

- Allergies
- Physical Exam
  - Vital signs and weight
  - Detailed physical examination
- Clinical Data

5.1.2 Admission clinical laboratory tests

At the time of enrollment, the following routine laboratory tests will be performed:

- CBC
- Blood chemistries
- Urine pregnancy test for women at child-bearing age
- Blood culture
- Skin scraping for microscopy and culture
- Lymphnode aspiration for microcopy and culture if >1cm
- Bone marrow aspiration for microcopy and culture as deemed appropriate by the treating clinician
- Sputum microscopy (Zn stain)
- Liver function test: AST, ALT, bilirubin, LDH
- HIV testing (in accordance with Vietnam MOH guidelines on diagnosis and treatment of HIV/AIDS) if not already done

5.1.3 Admission research laboratory tests

At the time of enrollment, the following research laboratory tests will be performed:

5.1.3.1Blood draw for fungal colony count

1 mL of blood will be collected prior to the start of antifungal therapy.

5.1.3.2 Blood draw for routine culture

5 mL of blood will be collected at screening for routine culture (which will also culture *P. marneffei*).

5.1.3.3 Blood draw for PK-PD analyses

2 mL of heparinized blood will be collected prior to the start of antifungal therapy for all patients enrolled at Hospital for Tropical Diseases and National Hospital for Tropical Diseases.

5.1.3.4 Archived whole blood for molecular and serology research

5 mL of blood prior to the start of antifungal will be collected, processed and archived at -70ºC for serological and molecular research purposes as per protocol.

5.1.3.5 Urinary P. marneffei antigen test

20 mL of urine will be collected prior to the start of antifungal and stored at -20ºC.

5.1.3.6 Admission chest X-ray

A chest X-ray will be performed at enrollment.

Interval Assessments

5.1.4 Interval history and physical exam

The following will be performed according to the schedule in Appendix B

- Presence, worsening or improvement of admission symptoms
- Vital signs and weight
- Physical examination
- Signs and symptoms of AEs

5.1.5 Interval clinical laboratory tests

The following will be performed according to the schedule in Appendix B

- CBC with differential
- Blood chemistries including LFTs
- Sputum microscopy (Zn stain) in day 2 and 3

5.1.6 Interval research laboratory tests

5.1.6.1Blood draw for fungal colony count

1 mL of blood will be collected daily during the first week and every other day during 2nd week until *P. marneffei* yeast cells are no longer seen for 2 consecutive days.

5.1.6.2Blood draw for PK-PD analyses

Patients enrolled at Hospital for Tropical Diseases and National Hospital for Tropical Diseases will participate in a population pharmacokinetic (PK) study. In addition, 30 enrolled patients at the Hospital for Tropical Diseases will participate in the intensive PK analysis. The test schedule for these two groups is shown in the table in Appendix C. For the intensive PK study, we will invite enrolled subjects to participate in this sub-study from the 1st day of enrollment on a continuous basis, and this substudy will close when 30 subjects are enrolled. 2 mL of heparinized blood will be collected 15-17 times over 3 days (day 1, 2 and 8) following set time points as outlined in Appendix C. For the population PK study, 2ml of heparinized blood will be collected during randomized time blocks on day 1, 2, 3, 4, 8, 10 and 12 of hospitalization as outlined in Appendix C, when possible at times of routine hospital care in order to minimize blood sampling. After 2 weeks of hospitalization, PK samples will be collected at outpatient follow-up times at month one, three, and six into therapy. It is crucial that exact time of antifungal medication administration and subsequent blood collection times are recorded in the Case Report Forms.

5.1.6.3 Archived whole blood for molecular and serology research

5 mL of blood will be collected, processed and archived at -70ºC for serological and molecular research purposes as per protocol in week 12.

5.1.6.4 IRD blood tests

10mL of blood will be collected at day 6 and at week 16 to study the incidence and characteristics of *P. marneffei* associated immune reconstitution disease

Left over whole blood or serum from routine clinical or research laboratory tests will be stored at -70ºC in case there is not enough blood for a particular test, loss of specimen, etc. The schedule of assessments and collection of research samples will not change without Ethical Committee notification and approval.

5.1.6.5 Follow-up chest X-ray

A follow-up X-ray would be standard care for persons with pulmonary lesions found at enrollment.

Interval assessments can be done outside of the hospital if required by the patient.

The Pharmacokinetic samples may be sent to Manchester University, UK for analysis. Other research samples may be sent to OUCRU collaborating labs in UK, US, Singapore and Thai Lan for analysis.

Other Samples

If any of the following samples are obtained for clinical indications (or in the course of usual care), a small portion of these samples should be stored at -70ºC for later analyses detailed below.

5.1.7 Bronchial alveolar lavage

5 ml of fluid obtained from the bronchial alveolar lavage should be saved for further analyses.

5.1.8 Cerebral spinal fluid (CSF)

1 ml of fluid obtained from the lumber puncture should be saved for future studies.

5.1.9 Pleural fluid

5 ml of fluid obtained from the thoracentesis should be saved for future studies.

6 Clinical Response Assessments

All enrolled patients will be seen daily both by treating clinicians and study investigators. Daily vital signs and physical exams will be performed. Measures such as temperature, weight, progression or resolution of skin or mucosal lesions, lymphadenopathy, hepatosplenomegaly, fatigue, cough and/or shortness of breath, nausea, vomiting, abdominal pain, diarrhea etc will be recorded daily.

Clinical response is defined as resolution of fever (temperature <38ºC for 3 consecutive days) and resolution of skin lesions (either completely gone or in the final stage of healing) due to penicilliosis at the end week 12. This response will also be evaluated earlier during therapy at week 2, week 4, and later at week 24.

7 Clinical Failure or Relapse Assessments

7.1 Clinical failure assessments and management

Subjects that meet the following criteria after 7 days of therapy will be classified as a clinical failure:

- Persistent fungal blood culture, OR
- Persistent worsening of fever and/or skin lesions due to penicilliosis, AND
- The treating clinician judge the patient to be failing current therapy

Subjects who meet the clinical failure criteria at day 7 may be switched to other medications at the discretion of the treating clinician according to the best medical practice. The treating clinician will also make the decision regarding need for continued hospitalization beyond the first 2 weeks of therapy.

7.2 Treatment relapse assessments and management

Subjects who meet developed cultural-confirmed penicilliosis after achieving treatment success at 12 weeks (see section 6 above) will be classified as a treatment relapse.

8 Risks

Risk of Amphotericin B

8.1.1 Infusion-related reactions

Infusion-related reactions, particularly nausea and vomiting, are common with amphotericin B administration, usually occurring between 15 minutes to 3 hours following the initiation of the dose. Nausea and vomiting may require the use of a phenothiazine such as promethazine (usual adult dose - 12.5 to 25 mg every 4 to 6 hours via deep IM only) or prochlorperazine (usual adult dose - 10 mg IM or IV or 25 mg PR every 4 to 6 hours).

Phlebitis is a complication that primarily occurs in patients receiving infusions via a small peripheral vein. The addition of hydrocortisone (usual adult dose - 25 mg) or heparin (usual final concentration — 500 to 1000 U/L) to the infusion may lessen infusion-related thrombophlebitis, but are not routinely recommended.

Other ways to minimize amphotericin B-induced thrombophlebitis include:

- Infusion of the drug using a central line or a large peripheral vein via a catheter
- Use of alternating infusion sites
- Avoidance of final amphotericin B infusion concentrations exceeding 0.1 mg/mL
- Avoidance of infusion times of less than four hours

Drug-induced fever, chills, and headache can also be seen. These symptoms can be minimized or prevented by premedication with paracetamol (usual adult dose - 500 to 1000 mg PO every 4 hours) and/or diphenhydramine (usual adult dose — 25 to 50 mg PO or IV). Nonsteroidal anti-inflammatory agents may also be useful in this setting. In a double-blind, placebo-controlled trial, ibuprofen administered 30 minutes prior to amphotericin B deoxycholate reduced the rate of occurrence of chills from 87 percent to 49 percent [104].

8.1.2 Nephrotoxicity

Amphotericin B administration may result in nephrotoxicity. With amphotericin B deoxycholate, a reversible and often transient decline in glomerular filtration rate (GFR) has been described in 5 to 80 percent of patients. The net effect is an elevation (above baseline) in the plasma creatinine concentration. Although more severe renal failure due to amphotericin B alone is uncommon, the risks of such reactions increase with diuretic-induced volume depletion or the concurrent administration of another nephrotoxin such as an aminoglycoside, cyclosporine, or foscarnet.

Volume expansion with intravenous sodium chloride (a practice commonly known as "sodium loading") may ameliorate the decline in GFR; 500 mL of 0.9 percent sodium chloride is typically given prior to the amphotericin B infusion.

8.1.3 Electrolyte abnormalities

Hypokalemia, hypomagnesemia, and hyperchloremic acidosis are reflections of an increase in distal tubular membrane permeability. Many patients require potassium and/or magnesium supplementation during therapy. Correction of hypokalemia may be difficult in patients with persistent hypomagnesemia.

8.1.4 Other reactions

A reversible, normochromic, normocytic anemia occurs in most patients receiving amphotericin B, but the onset may be delayed for as long as 10 weeks after the initiation of therapy. Transfusions are infrequently required.

Severe allergic reactions (including anaphylaxis) are extremely rare but have been reported.

8.1.5 Patient monitoring

Patients receiving amphotericin B intravenously will be monitored clinically for infusion-related reactions following each administration. Measurements of renal function will be performed 3 times in the first week and 2 times in the second week. If the plasma creatinine concentration exceeds 2.5 mg/dL (265 µM /L), amphotericin B will be permanently discontinued, and the subject switched to itraconazole, and these patients will not be analyzed per protocol.

Serum electrolytes (particularly potassium and magnesium) will be assessed at baseline and 3 times in the first week and 2 times in the second week. Complete blood counts will be measured 3 times in the first week and 2 times in the second week of therapy.

Risk of Itraconazole capsule formulation

8.1.6 Hepatotoxicity

Itraconazole has been associated with rare cases of serious hepatotoxicity, including liver failure and death. Some of these cases had neither pre-existing liver disease nor a serious underlying medical condition. If clinical signs or symptoms develop that are consistent with liver disease, treatment will be discontinued and liver function testing performed and monitored. The risks and benefits of itraconazole use will be reassessed.

8.1.7 Other adverse events

Most common: dyspepsia, abdominal pain, nausea, vomiting, constipation, diarrhoea, headache, and dizziness.
Rarely: increase liver enzyme values, some cases of hepatitis and cholestatic jaundice, especially in those treated for more than one month. There have been rare cases of liver failure and death. Heart failure and pulmonary oedema and serious cardiovascular events including arrhythmias and sudden death have been attributed to drug interactions in patients receiving itraconazole. Alopecia, oedema, and hypokalaemia with prolonged use, menstrual disorders, and peripheral neuropathy have been reported in a few patients.

Others: allergic reaction such as pruritus, rash, urticaria, and angioedema; the Stevens-Johnson syndrome.

8.2.3 Post-marketing experience

Worldwide post-marketing experiences with the use of itraconazole include adverse events of gastrointestinal origin, such as dyspepsia, nausea, vomiting, diarrhea, abdominal pain and constipation. Other reported AEs include peripheral edema, congestive heart failure and pulmonary edema, headache, dizziness, peripheral neuropathy, menstrual disorders, reversible increases in hepatic enzymes, hepatitis, liver failure, hypokalemia, hypertriglyceridemia, alopecia, allergic reactions (such as pruritus, rash, urticaria, angioedema, anaphylaxis), Stevens-Johnson syndrome, anaphylactic, anaphylactoid and allergic reactions, photosensitivity and neutropenia. There is limited information on the use of itraconazole during pregnancy. Cases of congenital abnormalities including skeletal, genitourinary tract, cardiovascular and ophthalmic malformations as well as chromosomal and multiple malformations have been reported during post-marketing experience. A causal relationship with itraconazole has not been established.

8.1.8 Patient monitoring

Patients receiving itraconazole will be monitored clinically for evidence of hepatic dysfunction. Liver function tests will be performed 3 times in the first week and 2 times in the second week. If the transaminitis (AST/ALT) exceeds 10 times the upper limit of normal or other laboratory evidence of grade IV hepatic dysfunction while on itraconazole, itraconazole will be discontinued, and the subject switched to amphotericin B, and these patients will not be analyzed per protocol.

Risk of Phlebotomy and of Intravenous Catheter Placement

The primary risks of phlebotomy include local discomfort, occasional bleeding or bruising of the skin at the site of needle puncture, and rarely hematoma, infection or fainting. At the time of enrollment and during study visits, each subject will be asked about participation in other research studies to ensure that blood draws do not exceed the following for all research protocols combined: 450 mL over any 6-week period for adults.

Only subjects who are assigned to the amphotericin B group will have a midline peripheral catheter placed in the arm for amphotericin B infusion. The risks for a peripheral catheter placement are similar to the risks of phlebotomy above, plus a possibility of vein inflammation. These risks are minimized by performance of only experienced medical persons. The study

doctors must examine subjects with a catheter every day to look for signs of infection and inflammation and will replace the catheter immediately upon such a concern.

Risk of Diagnosis

The risk associated with the diagnosis of penicilliosis is that the infection is an AIDS- defining illness, thus potentially exposing patients underlying HIV status that will potentially cause social isolation and stigmatism. All information about patients will be kept confidential and will not be shared outside the clinical and research team.

9 Benefit(s)

Benefits of Treatment

The benefit of treatment for penicilliosis is clear as penicilliosis is fatal if not diagnosed and treated. The relative benefit of treatment with itraconazole versus amphotericin B is entirely unknown. It has been shown in case series that treatment with either amphotericin B followed by itraconazole strategy or itraconazole alone strategy are both quite effective. Treatment medications (amphotericin B and/or itraconazole) will be provided by the study for the entire duration of the 3 month treatment, which represents a significant relief of financial burden for patients whose access to this treatment may have otherwise been limited.

Benefit of Diagnosis

The benefit of knowing the diagnosis of penicilliosis is also clear as penicilliosis is fatal if not diagnosed and treated. For the majority of infectious diseases in general, early diagnosis often leads to better treatment outcomes.

10 Alternatives

The alternative to participation in this study is routine standard care by the doctors in the hospital. For confirmed penicilliosis, patients will generally receive an antifungal (amphotericin B or itraconazole) based largely on ability to pay the costs and perhaps disease severity. Patients will have to pay for the cost of drugs and the care for the entire treatment duration. Follow up might not be as stringent compared to patients who participate in this study.

11 Data Management

Source documents will be generated during the study by the site study staff at participating institutions. Source documents include all original recordings of observations or notations of clinical activities, and all reports and records necessary for the evaluation and reconstruction of the clinical trial. Source documents include, but are not limited to, the subject's medical records, research case record forms (paper or electronic), laboratory reports, ECG tracings, x-rays, radiologist's reports, subject's diaries and questionnaires, biopsy reports, ultrasound photographs, progress notes, pharmacy records, and any other similar reports or records of procedures performed during the subject's participation in the study.

Access to applicable source documents will need to be made available for study purposes. The site investigators are responsible for maintaining any source documentation related to the study. Source documentation should support the data collected on the CRF when the CRF is not the original site of recording**,** and must be signed and dated by the person recording and/or

reviewing the data. Source documentation must be available for review or audit by the sponsor or designee and any applicable national authorities.

Case Report Forms (CRFs) will be used as a data collection tool. The study team will transfer the information from the source documents onto the CRFs. CRFs may be used as source documents if they are the primary data collection tool for specified data as documented in written standard operating procedures. The site Investigators are responsible for maintaining accurate, complete and up-to-date records and for tracking receipt of CRFs for each participant. These forms are to be completed on an ongoing basis during the course of the study by authorized individuals. All subject CRFs will be reviewed by the designated staff and signed as required.

The CRFs and instructions will be distributed to the site(s) by the Principle Investigator. Data entries on paper CRFs must be completed legibly with pen. Corrections must be made by striking through the incorrect entry with a single line (taking care not to obliterate or render the original entry illegible) and entering the correct information adjacent to the incorrect entry. Corrections to paper CRFs must be initialed and dated by the person making the correction. All CRFs should be reviewed by the designated study staff and signed as required with written or electronic signature, as appropriate.

Selected study members will be trained by a Data Manager on how to enter all clinical data as source information, from the CRFs and from laboratory source documents into a internet-based computerized data entry system called CliRes. This is a single computerized data entry that occurs simultaneously as clinical/research data are being collected during the trial as soon as possible after the information is generated. Source documents and electronic data will be verified according to the Trial Monitoring Plan.

12 Monitoring

Study Monitoring

The trial will be conducted in compliance with this protocol, Medical Research Council Guidelines of Good Clinical Practice, International Conference on Harmonization (ICH) Guidelines for Good Clinical Practice (GCP) and all applicable regulatory requirement(s).

As per ICH-GCP 5.18 clinical protocols are required to be adequately monitored by the study sponsor. Monitors will visit the clinical research site to monitor all aspects of the study in accordance with the appropriate regulations and the approved protocol. The objectives of a monitoring visit will be: 1) to verify the existence of signed informed consent documents for each monitored subject; 2) to verify the prompt and accurate recording of all monitored data points, and prompt reporting of all unexpected SAEs; 3) to compare abstracted information with individual subjects' records and source documents (subjects' charts, case report forms, laboratory analyses and test results, physicians' progress notes, nurses' notes, and any other relevant original subject information); and 4) to ensure protection of study subjects, investigators' compliance with the protocol, and completeness and accuracy of study records. The monitors also will inspect the clinical site regulatory files to ensure that regulatory requirements and applicable guidelines are being followed. During the monitoring visits, the investigator (and/or designee) and other study personnel will be available to discuss the study progress and monitoring visit.

Data and Safety Monitoring Plan

An independent data monitoring and ethical committee (DMEC) will oversee the trial. Unexpected serious adverse events will be reported to the DMEC and to the responsible Ethical Committees within ten working days of occurrence.

The DMEC will perform interim analyses after recruitment of 100 patients or after 20 deaths, whichever comes first. The review will include review of summary tables of grade 3 and 4 adverse events, serious adverse events and an analysis of mortality.
Based on these data, the committee will make one of the following recommendations:

- Continue the trial without modification
- Continue the trial with modification
- Discontinue the trial due to safety or other concerns

The DMEC may also suggest discontinuation if the trial results indicate "beyond reasonable doubt" that one of the allocated strategies is better than the other in primary outcome. The Haybittle-Peto boundary, requiring $p<0.001$ at interim analysis to consider stopping for efficacy, should be used as a guidance. However, the DMEC recommendation should not be based purely on statistical tables but also requires clinical judgment.

As the dissemination of preliminary summary data could influence the further conduct of the trial and introduce bias, access to interim data and results will be confidential and strictly limited to the involved statistician and the monitoring board and results (except for the recommendation) will not be communicated to the outside and/or clinical investigators involved in the trial.

Further reviews will be at the discretion of the DMEC or the request of the Trial Steering Committee. All DMEC reports, replies or decisions will be sent to the Trial Steering Committee and the responsible Research Ethical Committees.

13 Definition and Assessment of Adverse Events

Definition of Adverse Events

An adverse event (AE) is any undesirable event that occurs to a study participant during the course of the study whether or not that event is considered related to the study drug. An AE can, therefore, be any unfavorable and unintended sign (including an abnormal laboratory finding, for example), symptom, or disease temporally associated with the study drug, whether or not considered related to the study drug.
Examples include:

- An increase in severity or frequency of a pre-existing abnormality or disorder (events that are marked by a change from the participant's baseline/entry status)
- All reactions from sensitivity or toxicity to study drug
- Injuries or accidents (e.g., for a fall secondary to dizziness, record "dizziness" as the event and include the information about the fall in the comment/narrative section and information about the injury secondary to the fall as part of the "outcome")
- New clinically significant abnormalities in clinical laboratory values, physiological testing or physical examination.

Stable chronic conditions, such as arthritis, which are present prior to clinical trial entry and do not worsen are not considered AEs and will be documented in the subject's clinical chart as medical history.

Clinical or laboratory events are considered adverse events only if they occur after the first dose of study treatment and before the patient completes trial participation. (See below for reporting of adverse events.)

Definition of Serious Adverse Events

An AE is considered to be "serious" if it results in one of the following outcomes

- Death,
- Life-threatening event (the subject was at immediate risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe),
- Inpatient hospitalization or prolongation of existing hospitalization
- Persistent or significant disability/incapacity (a substantial disruption of a person's ability to conduct normal life functions),
- Congenital anomaly/birth defect
- Important medical event that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the other outcomes listed in the definition above.

An AE needs to meet only one of the above criteria to be considered serious.

Definition of Unexpected Serious Adverse Events

Untoward medical events which fit one or more criteria of SAE above and which are not considered a part of normal clinical progression of disease or expected drug reaction or any event which becomes of concern to the investigators or study doctors during the course of the trial may be reported as a USAE.

Assessment of Adverse Events

All adverse events that occur after the initiation of trial itraconazole or amphotericin B therapy will be graded according to the scale below.

- **Mild:** (Grade 1): Transient or mild symptoms; no limitation in activity; no intervention required. The AE does not interfere with the participant's normal functioning level.

- **Moderate** (Grade 2): Symptom results in mild to moderate limitation in activity; no or minimal intervention required. The AE produces some impairment of functioning, but it is not hazardous to health.

- **Severe** (Grade 3): Symptom results in significant limitation in activity; medical intervention may be required. The AE produces significant impairment of functioning or incapacitation.

- **Life-threatening** (Grade 4): Extreme limitation in activity, significant assistance required; significant medical intervention or therapy required; hospitalization.

[Note: "Life-threatening" as a severity grade is not necessarily the same as "life-threatening" as a "serious" criterion. The former is a "potential" threat to life and the latter is an "immediate" threat to life.]

A laboratory abnormality is an adverse event if it is associated with an intervention. Intervention includes, but is not limited to, discontinuation of treatment, dose reduction/delay, or concomitant treatment. In addition, any medically important laboratory abnormality may be reported as an adverse event at the discretion of the investigator. This would include a laboratory result for which there is no intervention but the abnormal value suggests a disease or organ toxicity. Laboratory events will be graded according to the following criteria:

- Events resulting in severe symptoms, condition or intervention will be classified as Grade 3.
- Events which are deemed to be life-threatening will be classified as Grade 4.

If clinical sequelae are associated with a laboratory abnormality, the diagnosis or medical condition should be reported as the adverse event (e.g., renal failure, hematuria) not the laboratory abnormality (e.g., elevated creatinine, urine RBC increase).

14 Adverse Event Reporting

Since there is extensive experience with both amphotericin B and itraconazole in clinical practice, the fact that evaluation of safety is not a primary objective in this trial, and the fact that both drugs and the dosages used in the protocol are approved by Vietnam Ministry of Health for treatment of penicilliosis, only unexpected Serious Adverse Events (SAEs) which occur at any time during the trial will be reported to the DMEC and Ethical Committees within ten working days of occurrence.

Grade 3 adverse events, grade 4 adverse events and serious adverse events which occur between initial dose of study medication and up to 6 months after initial dose will be recorded in the case report form. These events will be entered into the study database and provided to the DMEC upon safety review as required. Grade 1 and grade 2 adverse events will not be recorded. Events which are not unexpected serious adverse events will not be recorded after 6 months of study participation.

15 Human Subject Protections

Ethical Approval

This protocol, patient information sheet, informed consent document, relevant supporting information will be submitted to the designated Ethical Committee (EC) and must be approved before the study is initiated.

Any amendments must also be approved by the designated EC prior to implementing changes in the study.

The investigators are responsible for keeping the designated EC appraised of the progress of the study as deemed appropriate, but in any case at least once a year.

Compliance with Good Clinical Practice

This study will be conducted in compliance with the conditions stipulated by the Ethical Committee of the Viet Nam Ministry of Health and the Oxford Tropical Research Ethics Committee, Medical Research Council Guidelines of Good Clinical Practice and International Conference on Harmonisation, Good Clinical Practice (ICH/GCP) Guidelines. In addition, all local regulatory requirements will be adhered to, in particular those which afford greater protection to the safety of the trial participants.

Informed Consent

The informed consent for this study will be translated into Vietnamese and must be signed by the study participant or legal representative before participation in the study, including any screening procedures. A copy of the signed consent must be provided to the study participant. Signed consents must remain in each study participants study file, and be available for verification by study monitors at any time.

In the case of illiterate subjects, the consent will be read in Vietnamese to the subjects in the presence of a literate witness who will sign to confirm the accurate reading of the form.

If the subject is too ill to consent, the next of kin may consent for the subject. Once the subject is able, the subject will be consented for continuation in the study.

Separate informed consent forms will be signed for participation in the intensive pharmacokinetic portion of the study. Study sites participating in the case control portion of the study will have appropriate information included in the informed consent form. Participants in the control arm of the case control study will have a separate consent specific only to procedures in that portion of the study.

Rationale for Research Subject Selection

15.1.1 Inclusion of adults male and female age ≥18 years

The study will only include adult patients from both sexes and age ≥18 years as the study sites only treat adult HIV-infected patients. Although in Vietnam a patient is considered an adult at the physiologic age of ≥15 years, the actual number HIV-infected patients who is at WHO stage IV disease from age 15 to 18 is likely to be too low to justify their inclusion in this protocol.

15.1.2 Justification of Exclusions

The exclusion criteria are primarily to increase subject safety. The exclusion of pregnant women is to minimize any potential thread to the fetus (with itraconazole) and to prevent significant variation in interpretation of PK-PD data. Children <15 years of age are excluded as there are not enough pediatric HIV-infected patients and therefore not enough of those patients with penicilliosis to feasibly set up a study site in a pediatric hospital. Penicilliosis patients with CNS signs/symptoms might have *P. marneffei* CNS infection and should not receive itraconazole as this drug does not penetrate the CNS very well. Patients with transaminases >10 times upper limit of normal should not be on itraconazole. Patients with absolute neutrophil count <500 cells/µL should not be on amphotericin B. Patients with cryptococcal meningitis need to be treated with the current standard of care which is IV amphotericin B. Patients with

active TB or being treated for TB with rifampicin should not be on itraconazole because of drug-drug interactions.

Record Retention

The investigator is responsible for retaining all essential documents listed in the ICH Good Clinical Practice Guidelines. All essential documentation for all study subjects are to be maintained by the investigators in a secure storage facility for 15 years according to the requirements of the Viet Nam Ministry of Health. All stored records are to be kept confidential. It is the investigator's responsibility to retain copies of source documents

Storage of Samples

Approximately 15 ml of blood and cultured strains of *P.marneffei* will be stored in the hospital freezer at -70ºC only for the secondary analyses specified in the protocol. In the future, other investigators may wish to study these samples and/or data. In that case, EC approval must be sought prior to any sharing of samples and/or data. Any clinical information shared about the sample would similarly require prior EC approval.

Access to stored samples will be limited using a locked room under the control of Oxford University Clinical Research Unit. Samples and data will be stored using codes (not subjects' names) assigned by the investigators. Only investigators will have access to the samples and data. At the end of the study, samples will continue to be stored indefinitely in the hospital freezer at -70ºC.

Subjects may decide at any point not to have their samples stored. In this case, the principal investigator will destroy all known remaining samples and report what was done to the subject.

Anonymity and Confidentiality

The information obtained during the conduct of this clinical study is confidential. The results of the research study may be published, but patient names or identities will not be revealed. Records will remain confidential. To maintain confidentiality, the principal investigators at each site will keep records in locked cabinets and the results of tests will be coded to prevent association with the subject's names.

Compensation

Monetary reimbursement will be provided in accordance with OUCRU policy for lost time and travel fees incurred to study participants.

The study will cover the costs of the 2 week hospitalization and all related research tests. The study will not cover long term care for disability after hospitalization resulting from the complications of the illness. Reasonable transportation cost for the follow-up visits will also be covered

References

1. Supparatpinyo K, Nelson KE, Merz WG, et al. Response to antifungal therapy by human immunodeficiency virus-infected patients with disseminated penicillium marneffei infections and in vitro susceptibilities of isolates from clinical specimens. Antimicrob Agents Chemother **1993**;37(11):2407-11.

2. Supparatpinyo K, Khamwan C, Baosoung V, Nelson KE, Sirisanthana T. Disseminated penicillium marneffei infection in southeast asia. Lancet **1994**;344(8915):110-3.

3. Antinori S, Gianelli E, Bonaccorso C, et al. Disseminated penicillium marneffei infection in an HIV-positive italian patient and a review of cases reported outside endemic regions. J Travel Med **2006**;13(3):181-8.

4. Filiotou A, Velegraki A, Giannaris M, et al. First case of penicillium marneffei fungemia in greece and strain susceptibility to five licensed systemic antifungal agents and posaconazole. Am J Med Sci **2006**;332(1):43-5.

5. Cristofaro P, Mileno MD. Penicillium marneffei infection in HIV-infected travelers. Aids Alert **2006**;21(12):140-2.

6. Depraetere K, Colebunders R, Ieven M, et al. Two imported cases of penicillium marneffei infection in belgium. Acta Clin Belg **1998**;53(4):255-8.

7. Jones PD, See J. Penicillium marneffei infection in patients infected with human immunodeficiency virus: Late presentation in an area of nonendemicity. Clin Infect Dis **1992**;15(4):744.

8. Julander I, Petrini B. Penicillium marneffei infection in a swedish HIV-infected immunodeficient narcotic addict. Scand J Infect Dis **1997**;29(3):320-2.

9. Nguyen K, Taylor S, Wanger A, Ali A, Rapini RP. A case of penicillium marneffei in a US hospital. J Am Acad Dermatol **2006**;54(4):730-2.

10. Sobottka I, Albrecht H, Mack D, et al. Systemic penicillium marneffei infection in a german AIDS patient. Eur J Clin Microbiol Infect Dis **1996**;15(3):256-9.

11. Sirisanthana T, Supparatpinyo K, Perriens J, Nelson KE. Amphotericin B and itraconazole for treatment of disseminated penicillium marneffei infection in human immunodeficiency virus-infected patients. Clin Infect Dis **1998**;26(5):1107-10.

12. Boogaerts M, Winston DJ, Bow EJ, et al. Intravenous and oral itraconazole versus intravenous amphotericin B deoxycholate as empirical antifungal therapy for persistent fever in neutropenic patients with cancer who are receiving broad-spectrum antibacterial therapy. A randomized, controlled trial. Ann Intern Med **2001**;135(6):412-22.

13. Boogaerts M, Maertens J. Clinical experience with itraconazole in systemic fungal infections. Drugs **2001**;61 Suppl 1:39-47.

14. Caillot D, Bassaris H, McGeer A, et al. Intravenous itraconazole followed by oral itraconazole in the treatment of invasive pulmonary aspergillosis in patients with hematologic malignancies, chronic granulomatous disease, or AIDS. Clin Infect Dis **2001**;33(8):e83-90.

15. Denning DW, Tucker RM, Hanson LH, Hamilton JR, Stevens DA. Itraconazole therapy for cryptococcal meningitis and cryptococcosis. Arch Intern Med **1989**;149(10):2301-8.

16. Denning DW, Tucker RM, Hanson LH, Stevens DA. Itraconazole in opportunistic mycoses: Cryptococcosis and aspergillosis. J Am Acad Dermatol **1990**;23(3 Pt 2):602-7.

17. Denning DW, Tucker RM, Hanson LH, Stevens DA. Treatment of invasive aspergillosis with itraconazole. Am J Med **1989**;86(6 Pt 2):791-800.

18. Diaz M, Puente R, de Hoyos LA, Cruz S. Itraconazole in the treatment of coccidioidomycosis. Chest **1991**;100(3):682-4.

19. Dismukes WE, Bradsher RW,Jr, Cloud GC, et al. Itraconazole therapy for blastomycosis and histoplasmosis. NIAID mycoses study group. Am J Med **1992**;93(5):489-97.

20. Graybill JR, Stevens DA, Galgiani JN, Dismukes WE, Cloud GA. Itraconazole treatment of coccidioidomycosis. NAIAD mycoses study group. Am J Med **1990**;89(3):282-90.

21. Tucker RM, Denning DW, Dupont B, Stevens DA. Itraconazole therapy for chronic coccidioidal meningitis. Ann Intern Med **1990**;112(2):108-12.

22. Ranjana KH, Priyokumar K, Singh TJ, et al. Disseminated penicillium marneffei infection among HIV-infected patients in manipur state, india. J Infect **2002**;45(4):268-71.

23. Barone JA, Moskovitz BL, Guarnieri J, et al. Enhanced bioavailability of itraconazole in hydroxypropyl-beta-cyclodextrin solution versus capsules in healthy volunteers. Antimicrob Agents Chemother **1998**;42(7):1862-5.

24. Saag M. Itraconazole oral solution: Pharmacokinetics and absorption. AIDS Patient Care STDS **1997**;11 Suppl 1:S16-7.

25. Fisher MC, Aanensen D, de Hoog S, Vanittanakom N. Multilocus microsatellite typing system for penicillium marneffei reveals spatially structured populations. J Clin Microbiol **2004**;42(11):5065-9.

26. Capponi M, Sureau P, Segretain G. Penicillose de rhizomys sinensis. Bull Soc Pathol Exot **1956**;49:418-21.

27. SEGRETAIN G. Penicillium marneffei n.sp., agent of a mycosis of the reticuloendothelial system. Mycopathologia **1959**;11:327-53.

28. DiSalvo AF, Fickling AM, Ajello L. Infection caused by penicillium marneffei: Description of first natural infection in man. Am J Clin Pathol **1973**;60(2):259-63.

29. Ajello L, Padhye AA, Sukroongreung S, Nilakul CH, Tantimavanic S. Occurrence of penicillium marneffei infections among wild bamboo rats in thailand. Mycopathologia **1995**;131(1):1-8.

30. Chariyalertsak S, Vanittanakom P, Nelson KE, Sirisanthana T, Vanittanakom N. Rhizomys sumatrensis and cannomys badius, new natural animal hosts of penicillium marneffei. J Med Vet Mycol **1996**;34(2):105-10.

31. Gugnani H, Fisher MC, Paliwal-Johsi A, Vanittanakom N, Singh I, Yadav PS. Role of cannomys badius as a natural animal host of penicillium marneffei in india. J Clin Microbiol **2004**;42(11):5070-5.

32. Deng ZL, Yun M, Ajello L. Human penicilliosis marneffei and its relation to the bamboo rat (rhizomys pruinosus). J Med Vet Mycol **1986**;24(5):383-9.

33. Gugnani HC, Paliwal-Joshi A, Rahman H, et al. Occurrence of pathogenic fungi in soil of burrows of rats and of other sites in bamboo plantations in india and nepal. Mycoses **2007**;50(6):507-11.

34. Chariyalertsak S, Sirisanthana T, Supparatpinyo K, Praparattanapan J, Nelson KE. Case-control study of risk factors for penicillium marneffei infection in human immunodeficiency virus-infected patients in northern thailand. Clin Infect Dis **1997**;24(6):1080-6.

35. Chariyalertsak S, Sirisanthana T, Supparatpinyo K, Nelson KE. Seasonal variation of disseminated penicillium marneffei infections in northern thailand: A clue to the reservoir? J Infect Dis **1996**;173(6):1490-3.

36. Lo Y, Tintelnot K, Lippert U, Hoppe T. Disseminated penicillium marneffei infection in an african AIDS patient. Trans R Soc Trop Med Hyg **2000**;94(2):187.

37. Wong KH, Lee SS. Comparing the first and second hundred AIDS cases in hong kong. Singapore Med J **1998**;39(6):236-40.

38. Wong SS, Wong KH, Hui WT, et al. Differences in clinical and laboratory diagnostic characteristics of penicilliosis marneffei in human immunodeficiency virus (HIV)- and non-HIV-infected patients. J Clin Microbiol **2001**;39(12):4535-40.

39. Chiewchanvit S, Mahanupab P, Hirunsri P, Vanittanakom N. Cutaneous manifestations of disseminated penicillium marneffei mycosis in five HIV-infected patients. Mycoses **1991**;34(5-6):245-9.

40. Cheng NC, Wong WW, Fung CP, Liu CY. Unusual pulmonary manifestations of disseminated penicillium marneffei infection in three AIDS patients. Med Mycol **1998**;36(6):429-32.

41. Kantipong P, Panich V, Pongsurachet V, Watt G. Hepatic penicilliosis in patients without skin lesions. Clin Infect Dis **1998**;26(5):1215-7.

42. Jayanetra P, Nitiyanant P, Ajello L, et al. Penicilliosis marneffei in thailand: Report of five human cases. Am J Trop Med Hyg **1984**;33(4):637-44.

43. Louthrenoo W, Thamprasert K, Sirisanthana T. Osteoarticular penicilliosis marneffei. A report of eight cases and review of the literature. Br J Rheumatol **1994**;33(12):1145-50.

44. Chaiwun B, Khunamornpong S, Sirivanichai C, et al. Lymphadenopathy due to penicillium marneffei infection: Diagnosis by fine needle aspiration cytology. Mod Pathol **2002**;15(9):939-43.

45. Chan YH, Wong KM, Lee KC, et al. Pneumonia and mesenteric lymphadenopathy caused by disseminated penicillium marneffei infection in a cadaveric renal transplant recipient. Transpl Infect Dis **2004**;6(1):28-32.

46. Sun HY, Chen MY, Hsiao CF, Hsieh SM, Hung CC, Chang SC. Endemic fungal infections caused by cryptococcus neoformans and penicillium marneffei in patients infected with human immunodeficiency virus and treated with highly active anti-retroviral therapy. Clin Microbiol Infect **2006**;12(4):381-8.

47. Mootsikapun P, Srikulbutr S. Histoplasmosis and penicilliosis: Comparison of clinical features, laboratory findings and outcome. Int J Infect Dis **2006**;10(1):66-71.

48. Supparatpinyo K, Chiewchanvit S, Hirunsri P, Uthammachai C, Nelson KE, Sirisanthana T. Penicillium marneffei infection in patients infected with human immunodeficiency virus. Clin Infect Dis **1992**;14(4):871-4.

49. Chaiyaroj SC, Chawengkirttikul R, Sirisinha S, Watkins P, Srinoulprasert Y. Antigen detection assay for identification of penicillium marneffei infection. J Clin Microbiol **2003**;41(1):432-4.

50. Panichakul T, Chawengkirttikul R, Chaiyaroj SC, Sirisinha S. Development of a monoclonal antibody-based enzyme-linked immunosorbent assay for the diagnosis of penicillium marneffei infection. Am J Trop Med Hyg **2002**;67(4):443-7.

51. Vanittanakom N, Mekaprateep M, Sittisombut N, et al. Western immunoblot analysis of protein antigens of penicillium marneffei. J Med Vet Mycol **1997**;35(2):123-31.

52. Huang YT, Hung CC, Hsueh PR. Aspergillus galactomannan antigenemia in penicilliosis marneffei. AIDS **2007**;21(14):1990-1.

53. Desakorn V, Simpson AJ, Wuthiekanun V, et al. Development and evaluation of rapid urinary antigen detection tests for diagnosis of penicilliosis marneffei. J Clin Microbiol **2002**;40(9):3179-83.

54. Vanittanakom N, Vanittanakom P, Hay RJ. Rapid identification of penicillium marneffei by PCR-based detection of specific sequences on the rRNA gene. J Clin Microbiol **2002**;40(5):1739-42.

55. Imwidthaya P, Thipsuvan K, Chaiprasert A, Danchaivijitra S, Sutthent R, Jearanaisilavong J. Penicillium marneffei: Types and drug susceptibility. Mycopathologia **2001**;149(3):109-15.

56. Radford SA, Johnson EM, Warnock DW. In vitro studies of activity of voriconazole (UK-109,496), a new triazole antifungal agent, against emerging and less-common mold pathogens. Antimicrob Agents Chemother **1997**;41(4):841-3.

57. Ryder NS. Activity of terbinafine against serious fungal pathogens. Mycoses **1999**;42 Suppl 2:115-9.

58. Sar B, Boy S, Keo C, et al. In vitro antifungal-drug susceptibilities of mycelial and yeast forms of penicillium marneffei isolates in cambodia. J Clin Microbiol **2006**;44(11):4208-10.

59. Nakai T, Uno J, Ikeda F, Tawara S, Nishimura K, Miyaji M. In vitro antifungal activity of micafungin (FK463) against dimorphic fungi: Comparison of yeast-like and mycelial forms. Antimicrob Agents Chemother **2003**;47(4):1376-81.

60. Supparatpinyo K, Chiewchanvit S, Hirunsri P, et al. An efficacy study of itraconazole in the treatment of penicillium marneffei infection. J Med Assoc Thai **1992**;75(12):688-91.

61. Supparatpinyo K, Perriens J, Nelson KE, Sirisanthana T. A controlled trial of itraconazole to prevent relapse of penicillium marneffei infection in patients infected with the human immunodeficiency virus. N Engl J Med **1998**;339(24):1739-43.

62. Chaiwarith R, Charoenyos N, Sirisanthana T, Supparatpinyo K. Discontinuation of secondary prophylaxis against penicilliosis marneffei in AIDS patients after HAART. AIDS **2007**;21(3):365-7.

63. Hung CC, Chen MY, Hsieh SM, Sheng WH, Hsiao CF, Chang SC. Discontinuation of secondary prophylaxis for penicilliosis marneffei in AIDS patients responding to highly active antiretroviral therapy. AIDS **2002**;16(4):672-3.

64. Sun HY, Chen MY, Hsiao CF, Hsieh SM, Hung CC, Chang SC. Endemic fungal infections caused by cryptococcus neoformans and penicillium marneffei in patients infected with human immunodeficiency virus and treated with highly active anti-retroviral therapy. Clin Microbiol Infect **2006**;12(4):381-8.

65. Chariyalertsak S, Supparatpinyo K, Sirisanthana T, Nelson KE. A controlled trial of itraconazole as primary prophylaxis for systemic fungal infections in patients with advanced human immunodeficiency virus infection in thailand. Clin Infect Dis **2002**;34(2):277-84.

66. Hamilton AJ, Jeavons L, Youngchim S, Vanittanakom N. Recognition of fibronectin by penicillium marneffei conidia via a sialic acid-dependent process and its relationship to the interaction between conidia and laminin. Infect Immun **1999**;67(10):5200-5.

67. Kudeken N, Kawakami K, Kusano N, Saito A. Cell-mediated immunity in host resistance against infection caused by penicillium marneffei. J Med Vet Mycol **1996**;34(6):371-8.

68. Sisto F, Miluzio A, Leopardi O, Mirra M, Boelaert JR, Taramelli D. Differential cytokine pattern in the spleens and livers of BALB/c mice infected with penicillium marneffei: Protective role of gamma interferon. Infect Immun **2003**;71(1):465-73.

69. Roilides E, Lyman CA, Sein T, Petraitiene R, Walsh TJ. Macrophage colony-stimulating factor enhances phagocytosis and oxidative burst of mononuclear phagocytes against penicillium marneffei conidia. FEMS Immunol Med Microbiol **2003**;36(1-2):19-26.

70. Kudeken N, Kawakami K, Saito A. Mechanisms of the in vitro fungicidal effects of human neutrophils against penicillium marneffei induced by granulocyte-macrophage colony-stimulating factor (GM-CSF). Clin Exp Immunol **2000**;119(3):472-8.

71. Taylor JW, Geiser DM, Burt A, Koufopanou V. The evolutionary biology and population genetics underlying fungal strain typing. Clin Microbiol Rev **1999**;12(1):126-46.

72. Vanittanakom N, Cooper CR,Jr, Chariyalertsak S, Youngchim S, Nelson KE, Sirisanthana T. Restriction endonuclease analysis of penicillium marneffei. J Clin Microbiol **1996**;34(7):1834-6.

73. Hsueh PR, Teng LJ, Hung CC, et al. Molecular evidence for strain dissemination of penicillium marneffei: An emerging pathogen in taiwan. J Infect Dis **2000**;181(5):1706-12.

74. Trewatcharegon S, Sirisinha S, Romsai A, Eampokalap B, Teanpaisan R, Chaiyaroj SC. Molecular typing of penicillium marneffei isolates from thailand by NotI macrorestriction and pulsed-field gel electrophoresis. J Clin Microbiol **2001**;39(12):4544-8.

75. Maiden MC, Bygraves JA, Feil E, et al. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A **1998**;95(6):3140-5.

76. Fisher MC, DE Hoog S, Akom NV. A highly discriminatory multilocus microsatellite typing (MLMT) system for penicillium marneffei. Mol Ecol Notes **2004**;4(3):515-8.

77. Fisher MC, Koenig G, White TJ, Taylor JW. A test for concordance between the multilocus genealogies of genes and microsatellites in the pathogenic fungus coccidioides immitis. Mol Biol Evol **2000**;17(8):1164-74.

78. Lasker BA. Nucleotide sequence-based analysis for determining the molecular epidemiology of penicillium marneffei. J Clin Microbiol **2006**;44(9):3145-53.

79. Goldstein DB, Ruiz Linares A, Cavalli-Sforza LL, Feldman MW. An evaluation of genetic distances for use with microsatellite loci. Genetics **1995**;139(1):463-71.

80. Zuckerman JM, Tunkel AR. Itraconazole: A new triazole antifungal agent. Infect Control Hosp Epidemiol **1994**;15(6):397-410.

81. Haria M, Bryson HM, Goa KL. Itraconazole. A reappraisal of its pharmacological properties and therapeutic use in the management of superficial fungal infections. Drugs **1996**;51(4):585-620.

82. Van Cauteren H, Heykants J, De Coster R, Cauwenbergh G. Itraconazole: Pharmacologic studies in animals and humans. Rev Infect Dis **1987**;9 Suppl 1:S43-6.

83. Woestenborghs R, Lorreyne W, Heykants J. Determination of itraconazole in plasma and animal tissues by high-performance liquid chromatography. J Chromatogr **1987**;413:332-7.

84. Heykants J, Van Peer A, Van de Velde V, et al. The clinical pharmacokinetics of itraconazole: An overview. Mycoses **1989**;32 Suppl 1:67-87.

85. Lange D, Pavao JH, Wu J, Klausner M. Effect of a cola beverage on the bioavailability of itraconazole in the presence of H2 blockers. J Clin Pharmacol **1997**;37(6):535-40.

86. De Beule K. Itraconazole: Pharmacology, clinical experience and future development. Int J Antimicrob Agents **1996**;6(3):175-81.

87. Odds FC, Bossche HV. Antifungal activity of itraconazole compared with hydroxy-itraconazole in vitro. J Antimicrob Chemother **2000**;45(3):371-3.

88. Cartledge JD, Midgely J, Gazzard BG. Itraconazole solution: Higher serum drug concentrations and better clinical response rates than the capsule formulation in acquired immunodeficiency syndrome patients with candidosis. J Clin Pathol **1997**;50(6):477-80.

89. Van Cutsem J. In-vitro and in-vivo activity of itraconazole. Med Klin (Munich) **1991**;86 Suppl 1:5-8.

90. Van Cutsem J. In vitro antifungal spectrum of itraconazole and treatment of systemic mycoses with old and new antimycotic agents. Chemotherapy **1992**;38 Suppl 1:3-11.

91. Tucker RM, Denning DW, Arathoon EG, Rinaldi MG, Stevens DA. Itraconazole therapy for nonmeningeal coccidioidomycosis: Clinical and laboratory observations. J Am Acad Dermatol **1990**;23(3 Pt 2):593-601.

92. Boogaerts MA, Verhoef GE, Zachee P, Demuynck H, Verbist L, De Beule K. Antifungal prophylaxis with itraconazole in prolonged neutropenia: Correlation with plasma levels. Mycoses **1989**;32 Suppl 1:103-8.

93. Glasmacher A, Hahn C, Leutner C, et al. Breakthrough invasive fungal infections in neutropenic patients after prophylaxis with itraconazole. Mycoses **1999**;42(7-8):443-51.

94. Tricot G, Joosten E, Boogaerts MA, Vande Pitte J, Cauwenbergh G. Ketoconazole vs. itraconazole for antifungal prophylaxis in patients with severe granulocytopenia: Preliminary results of two nonrandomized studies. Rev Infect Dis **1987**;9 Suppl 1:S94-9.

95. Prentice AG, Glasmacher A, Djulbegovic B. In meta-analysis itraconazole is superior to fluconazole for prophylaxis of systemic fungal infection in the treatment of haematological malignancy. Br J Haematol **2006**;132(5):656,8; author reply 658-9.

96. Torrado JJ, Espada R, Ballesteros MP, Torrado-Santiago S. Amphotericin B formulations and drug targeting. J Pharm Sci **2008**;97(7):2405-25.

97. Bennett J. Antimicrobial agents: Antifungal agents. In: Hardman J, Limbird L, eds. The Goodman & Gilman's pharmacological basis of therapeudics. New York: McGraw-Hill, **1995**:1175-790.

98. Kajtar M, Vikmon M, Morlin E, Szejtli J. Aggregation of amphotericin B in the presence of gamma-cyclodextrin. Biopolymers **1989**;28(9):1585-96.

99. Mazerski J, Grzybowska J, Borowski E. Influence of net charge on the aggregation and solubility behaviour of amphotericin B and its derivatives in aqueous media. Eur Biophys J **1990**;18(3):159-64.

100. Tancrede P, Barwicz J, Jutras S, Gruda I. The effect of surfactants on the aggregation state of amphotericin B. Biochim Biophys Acta **1990**;1030(2):289-95.

101. Aramwit P, Yu BG, Lavasanifar A, Samuel J, Kwon GS. The effect of serum albumin on the aggregation state and toxicity of amphotericin B. J Pharm Sci **2000**;89(12):1589-93.

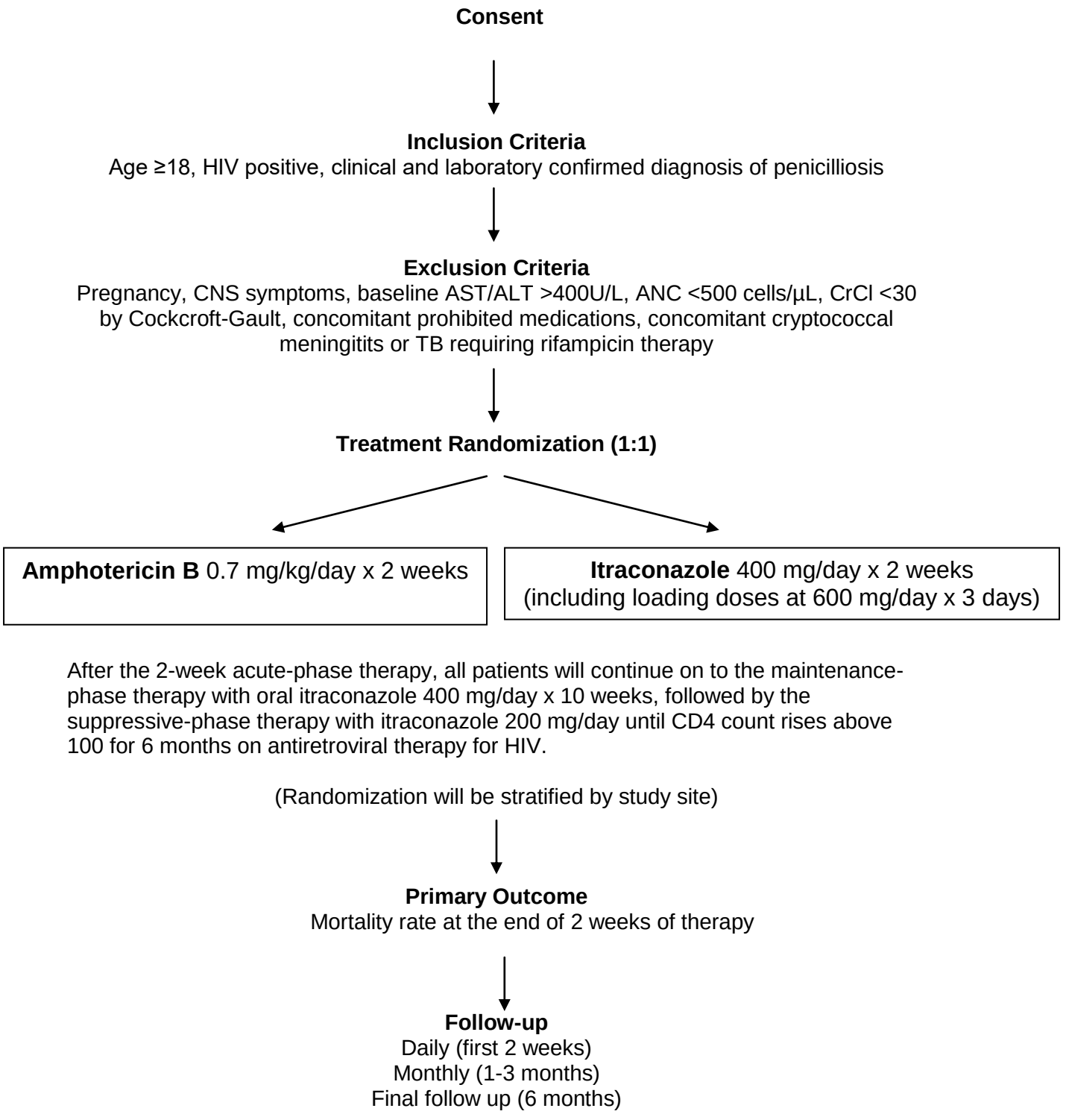
102. Schuler U, Bammer S, Aulitzky WE, et al. Safety and efficacy of itraconazole compared to amphotericin B as empirical antifungal therapy for neutropenic fever in patients with haematological malignancy. Onkologie **2007**;30(4):185-91.

103. Park SH, Choi SM, Lee DG, et al. Intravenous itraconazole vs. amphotericin B deoxycholate for empirical antifungal therapy in patients with persistent neutropenic fever. Korean J Intern Med **2006**;21(3):165-72.

104. Gigliotti F, Shenep JL, Lott L, Thornton D. Induction of prostaglandin synthesis as the mechanism responsible for the chills and fever produced by infusing amphotericin B. J Infect Dis **1987**;156(5):784-9.

105. Le T, Wolbers M, Chi NH, Quang VM, Chinh NT, Lan HPN, Lam PS, Kozal KM, Shikuma CM, Day NJ, Farrar J. Epidemiology, seasonality and Predictors of Outcome in *Penicillium marneffei* Infection in Ho Chi Minh City, Viet Nam. Clin Infect Dis 2011 Arp 1; 52(7):945–952.

Appendix A: Study Flow Diagram



Appendix B: Trial Procedure Chart

Event	SCR	Baseline D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	W4 (+/- 3d)	W8 (+/- 3d)	W12 (+/- 3d)	W16 (+/- 3d)	W20 (+/- 3d)	W 24 (+/- 3d)
Informed Consent	x																				
Inclusion/ Exclusion Criteria	x																				
Medical History	x																				
Pregnancy Test *	x																				
Clinical Assessment**		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Medication Adherence Assessment																x	x	x	x	x	x
AEs Assessment		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
CXR¥		x																			
Blood (ml)																					
CBC£		1			1			1				1		1		1		1			
CD4 α		1																			
Chemistry & Liver Function Test£		2			2			2				2		2		2		2			
Blood culture¥, ©		5																			
Blood Fungal Colony Count ©, β		1	1	1	1	1	1	1		1		1		1							
Molecular & Serology		5																5			
IRD testingλ							10												10		
Maximum total blood volume (ml)		**15**	**1**	**1**	**4**	**1**	**11**	**4**	**0**	**1**	**0**	**4**	**0**	**4**	**0**	**3**	**0**	**8**	**10**	**0**	**0**
Urine (ml)																					
Urinary Antigen£		20																			
Skin lesion																					
Smear & Culture¥		x																			
Sputum																					
Zn smear ¥		x	x	x																	

*Pregnancy Test: for female with child-bearing potential only

** Clinical Assessments including vital signs, weight, physical exam

¥ Can be repeated at any time as clinically indicated.

£ Blood tests scheduled for D2-14 may be done within a +/- 1 day window period

© Stop taking these samples when two consecutive sample tests are negative

α To be done during 2 week in-patients, if necessary and when subjects are suspected to have an IRD event

β Only done at HTD and NHTD during working hour

λ Besides the above schedule, one more IRIS sample can be taken when the patient is suspected to have IRD

Appendix C: Pharmacokinetic Study Schedule

III. Intensive PK (30 patients ARV-naïve, 15 in each arm)

Day of treatment	**Day 1**			**Day 2**					**Day 8**								
Itraconazole arm	0h (pre-drug administration)	0.5h	2h	1h	3h	4h	12h		0h (pre-drug administration)	0.5h	1h	2h	3h	4h	6h	12h	
Amphotericin B arm	0h (pre-drug administration)	0.5h	2h	1h	3h	4h	12h	24h	0h (pre-drug administration)	0.5h	1h	2h	4h	6h	12h	16h	24h

IV. Time for taking sample for Population PK (200 patients, 100 in each arm)

Day of treatment	**Day 1**	Day 1-4 (only 1 sample is collected each day during the following randomized time blocks)				Day 8 and 10 (only 1 sample is collected each day at the following randomized timeslot)		**Day 12**		**Wk 4, 8, 12,24**
Itraconazole arm	0h (pre-drug administration)	0-2h	2-4h	4-8h	8-12h	0h (pre-drug administration)	3h	0h (pre-drug administration)	3h	Before AM dose at follow-up visit
Amphotericin arm	0h (pre-drug administration)	0-3h	3-6h	6-12h	12-18h	0h (pre-drug administration)	6h (or right after infusion is completed)	0h (pre-drug administration)	6h (or right after infusion is completed)	Before AM dose at follow-up visit

Note: h refers to the number of hours after a patient takes itraconazole by mouth or after the infusion of amphotericin B is completed

Appendix D: Secondary Objective #9 - case control study to evaluate the exposure risk factors for penicilliosis

Purpose: to investigate the risk exposure and risk behaviors in equally susceptible individuals with HIV/AIDS and not the host susceptibility to penicilliosis.
Our hypothesis is that the reservoir of P. marneffei is in the environment, in decaying organic materials and in a combination of a type of soil, humidity, and a tropical flora that forms a symbiotic relationship with the fungus. Proximity to water and humidity provide a favorable environment for germination and transmission. Sharing needles is a risk for bloodborne transmission from person to person.

Background: Please refer to section 1.2 of the protocol.

Experimental Plan: (See flow chart next page)
This is a hospital-based case-control study that is built into the main trial. Cases (N=200) will be conveniently and randomly recruited from a pool of subjects who enter the trial with culture-confirmed penicilliosis at selected trial sites.

Controls (or disease reference group, N=400) will be randomly selected from a pool of patients with AIDS who come to the outpatient clinic for routine care or who are admitted in the hospital for acute care at our trial centers. Controls may have an active opportunistic infection, but penicilliosis should be ruled out. Controls will be recruited simultaneously (within <1 wk of cases) and will be individually matched 2:1 to cases. The following matching scheme is designed to ensure that controls are similar to cases in term of host characteristics: age by 5 years, sex, and susceptibility to penicilliosis (CD4 by 50 cells/µL or WHO disease categories).

After signing a separate informed consent form, 5 cc of blood and 20 cc of urine will be collected and stored at -70ºC for serological tests. All subjects will complete a one-to-one 20-30 minute interview by a standardized questionnaire with a study staff in a private room. Global positioning system (GPS) mapping technology will be used to characterize the geo-spatial distribution of cases and controls.

Data Analysis:
Univariate and multivariate logistic-regression models will be used to estimate the odds ratios and associated 95% confidence intervals of exposure variables and disease in pair-matched data. Assessment of presence of exposure, duration of exposure and recent/past exposure will be made for all exposure variables. Multivariate models will be created through stepwise elimination of variables of interest from univariate analysis while relevant variables will be retained. Additive and multiple interactions among exposure variables will be evaluated.

Case Control Study Flow Chart

Case Group (200 patients)

HIV-infected patients with microbiological confirmed diagnosis of penicilliosis who participated in the trial

Control Group (400 patients)

HIV-infected patients admitted to the same hospital or seen in the outpatient clinic for routine or acute care during the same time but do not have culture-confirmed penicilliosis, matched sex, age, CD4 or WHO disease staging.

Inclusion criteria for control patients:

- HIV-infected patients >18 years old
- Patients with fever and/or non-specific constitutional symptoms
- All patients with other opportunistic fungal infections: cryptococcosis, candidiasis, candidemia, histoplasmosis, PCP
- All patients with other opportunistic infections: tuberculosis, CMV…

Exclusion criteria for control patients:

- Healthy and asymptomatic HIV-infected subjects

Epidemiologic factors to be investigated in the survey:

- Home and work addresses
- Type/s of work and specific activities at work
- Specific activities most days of the week in the past 3, 6, 12 months
- Present/past exposure/contact with bamboo and/or bamboo rats
- Present/past exposure to healthy/ill domestic animals (dogs, cats, birds including chickens and ducks, reptiles, pigs, rabbits or rodents)
- Present/past exposure to healthy/ill farm or wild animals
- Types of plant/trees around home/work
- Live by or close contact with any body of water
- Eat exotic food including raw or rarely cooked food
- Current/past smoking of cigarettes/marijuana/opium/others
- Current/past intravenous drug use (heroin or others) and injection practices

Appendix E: Secondary Objective #10 - Molecular Epidemiology of Penicillium marneffei

Purpose: to investigate the molecular epidemiology of *P. marneffei* infection using a number of cutting-edge molecular technologies including highly discriminatory mutilocus microsatetlite typing (MLMT) and correlate the identified genotypes with clinical and geo-spatial epidemiology data

Background: Please refer to section 1.8 of the protocol.

Experimental Plan:
Pure sub-cultured isolates of *Penicillium marneffei* from subjects enrolled into this study will be stored with micro beads (called Microbank^{TM}) obtained from Pro-Lab Diagnostics in cryovials containing cryopreservative at -70ºC at OUCRU laboratories in Ho Chi Minh City and Ha Noi. Typing of P.marneffei isolates will be performed by various typing technologies, namely multilocus sequences typing (MLST), multilocus microsatellite typing (MLMT), and direct sequencing of the cell wall glycoprotein called Manoprotein-1 (MP-1) in collaborations with Dr. Brent Lasker from the US Centers for Disease Control and Prevention and Dr. Matthew Fisher from Imperial College London. Most of the typing works will be performed in Vietnam, with some samples shipped to collaborators labs overseas for confirmation/comparison of typing results. Please refer to the references for detailed molecular typing protocols [75, 76, and 77].

Parallel to typing isolates from clinical population, we will collect soil specimens and set up air sampling booths from different geographical areas in North and South Vietnam. Both standard culture and quantitative PCR assays will be used to detect presence of *Penicillium marneffei* from the environment, and direct sequencing of MP-1 protein will be used to type environmental isolates.

Data Analysis:
Typing data from human clinical populations and from environmental sources from different geographical areas in Vietnam will be integrated with clinical data to identify the genetic variations within populations of *Penicillium marneffei* in Vietnam. These data can then be shared among collaborating laboratories interested in typing and ecological/epidemiological studies of *Penicillium marneffei* through the endemic regions, allowing sophisticated temporal epidemiological surveillance analysis, and greater understanding of the evolution and adaptation of this important emerging opportunistic pathogen.

Appendix F: Secondary Objective #11 - Urinary Antigen of Penicillium marneffei for Diagnosis and Monitor of Treatment

Purpose: to prospectively evaluate an ELISA and a latex agglutination assay to detect *P. marneffei* urinary antigen for diagnostic accuracy and as a surrogate marker for microbiological and clinical outcomes of penicilliosis.

Background: Please refer to section 1.5.3 of the protocol. In summary, simple, rapid, robust dot blot ELISA and a latex agglutination assays for detection of *P. marneffei* antigenuria using a polyclonal hyperimmune IgG have been developed and prospectively tested in smaller scale studies (37 cases, 300 controls) with sensitivities and specificities in the upper 90% [53]. We plan to validate these tests in our large-scale case-control study (secondary objective #9, 200 cases, 400 controls) for diagnostic accuracy and for following/correlating *P. marneffei* antigenuria titers with fungal clearance and clinical response during the 3 months of antifungal therapy.

Experimental Plan:
Urine specimens from all patients participating in the trial will be collected at enrollment, 3 times a weeks for 2 weeks during acute hospitalization, week 4, 8, 12, and 24 (see appendix B – Trial Flow Chart). Simultaneously urine specimens will be collected from the control subjects but only at enrollment. Control subjects will be HIV-infected subjects with similar CD4 count or WHO disease staging but do not have culture evidence of penicilliosis. They ideally will be patients with a variety of other common opportunistic infections seen in Vietnam, including other fungal infections such as cryptococcosis, candidemia/candidiasis, PCP, undiagnosed histoplasmosis…Inclusion of other fungal infections will add to the reliability of the specificity.

Urine samples will be stored at −30°C and thawed only at the time of testing. Control *P. marneffei* antigen and purified rabbit anti- *P. marneffei* IgG will be obtained from our collaboration with Dr. Desakorn (Mahidol University, Thailand). *P. marneffei* IgG will be labeled with FITC conjugate, and ELISA and agglutination assays will be performed at OUCRU according to Desalorn et al [53]. All samples will be tested in duplicate, and each test was repeated three times.

Analysis Plan:
Data will be analyzed with the assistance of Dr. Marcel Wolbers, OUCRU biostatistician using R computer software. At each ELISA cutoff titer, the sensitivity and the specificity will be calculated. A receiver operating characteristic (ROC) curve is then constructed by plotting sensitivity against (1 − specificity) at each value. We will also evaluate baseline *P. marneffei* antigen titer as an independent predictors of disease outcome and evaluate the role of serial *P. marneffei* antigen titers in predicting treatment response.

Appendix G: Secondary Objective #12 - Cost effectiveness of itraconazole vs. amphotericin B for penicilliosis

Purpose: to conduct an economic evaluation to estimate the net cost of itraconazole versus amphotericin B therapy for penicilliosis

Background: As the cost differential between itraconazole and amphotericin B treatment is one of the reasons for undertaking the trial, it will be important to conduct a formal economic evaluation alongside the trial to ensure that all costs are accurately recorded, and to permit a cost-effectiveness analysis in the event that non-inferiority is not demonstrated (i.e. if itraconazole turns out to be cheaper but less effective). Hence, an economic evaluation will be conducted in collaboration with the Health Economics Research Centre, University of Oxford (PI: Prof. Alastair Gray).

Experimental and data analysis plan:
The objective of the analysis will be to estimate the net cost of itraconazole versus amphotericin B therapy, including medication costs, other treatments, hospital stays, and patient incurred costs, including loss of income for the patients and their care takers, out-of-pocket costs, and the need for transfer to tertiary centres. These information will be prospectively collected on each patient during the study and recorded in the health economic CRFs. Unit costs will be obtained from each trial centre and used to produce a net cost per patient in each arm of the study over the 2 week (primary) and 6 month (secondary) follow-up periods. In the event that non-inferiority is not demonstrated, the economic evaluation will assess cost-effectiveness as the ratio of the difference in cost to the difference in survival, expressed as life years gained. Although it is possible that itraconazole is better tolerated than amphotericin B, it is unlikely that these differences will be large enough to be detected in any form of simple disability adjustment or quality of life adjustment, and so it is not proposed that a cost per DALY averted or QALY gained is reported, or that information is collected prospectively on these metrics. Life years gained will be based on the primary outcome measure of survival at 2 weeks and also at 6 months. All estimates of costs, outcomes and cost-effectiveness will be reported with full recognition of uncertainty, including cost-effectiveness acceptability curve and sensitivity analyses around key parameters.

Appendix H: Secondary Objective #13 - Penicilliosis Immune Reconstitution Disease

Purpose: to study the incidence, clinical features, outcome, and outcome predictors of immune reconstitution disease (IRD) in penicilliosis

Background: HIV-associated IRD occurs in up to 30% of patients with opportunistic infections starting ART and is associated with higher morbidity and mortality, particularly in tuberculosis and cryptococcal meningitis. IRD has been reported but has not been systematically studied in penicilliosis. It is unknown whether penicilliosis IRD has worse clinical outcome. And as with other HIV-associated IRD, biomarkers to diagnose and to predict IRD in penicilliosis need further investigations.

Experimental Plan:
All trial participants with penicilliosis who are ART-naïve (estimated 80%) will be evaluated monthly for the development of IRD over a period of 6 month as they begin ART. 10ml of blood will be collected at enrollment to look for predictive biomarkers of IRD. For the patients who develop IRD during the first 6 months of ART, we will continue to follow the patients monthly during their routine clinic visit and will collect information about treatment and outcome of the IRD event. IRD events are defined based on the consensus criteria for general IRD according to the International Network for the study of HIV-associated IRIS. Biomarkers of immune dysfunction (levels and profile of cytokines/chemokines) that have been identified to predict and to differentiate IRD from other complications in other fungal opportunistic diseases such as cryptococcal meningitis will be studied. Other laboratory predictor variables that will be studied include: fungal clearance by quantitative culture and by serological assays, serum CRP, and D-dimer. Other AIDS-related or non-AIDS-related events that occur during the study follow up period will be classified and recorded.

Data Analysis:
Incidence, clinical features, management and outcome of penicilliosis IRD will be described. Clinical and laboratory variables will be compared between those with and without IRD in the study cohort. Multiple logistic regression analysis will be performed to identify independent predictors of IRD. Biomarkers that differentiate IRD from non-IRD events will be identified.

Appendix I: WHO clinical staging for HIV/AIDS

Clinical Stage 1
Asymptomatic Persistent generalised lymphadenopathy (PGL) Performance scale 1: asymptomatic, normal activity
Clinical Stage 2
Weight loss, <10% of body weight Minor mucocutaneous manifestations (seborrheic dermatitis, prurigo, fungal nail infections, recurrent oral ulcerations, angular cheilitis) Herpes zoster, within the last 5 years Recurrent upper respiratory tract infections (e.g. bacterial sinusitis) And/or performance scale 2: symptomatic, normal activity.
Clinical stage 3
Weight loss, >10% of body weight Unexplained chronic diarrhoea, > 1 month Unexplained prolonged fever (intermittent or constant), > 1 month Oral candidiasis (thrush) Oral hairy leukoplakia Pulmonary tuberculosis, within the past year. Severe bacterial infections (e.g. pneumonia, pyomyositis) And/or Performance scale 3: bed-ridden, < 50% of the day during the last month
Clinical stage 4
HIV wasting syndrome, as defined by CDC[1] Pneumocystis carinii pneumonia Toxoplasmosis of the brain Cryptosporidiosis with diarrhoea, >1 month Cryptococcosis, extra pulmonary Cytomegalovirus (CMV) disease of an organ other than liver, spleen or lymph nodes Herpes Simplex Virus (HSV) infection, mucocutaneous >1 month, or visceral any duration Progressive multifocal leukoencephalopathy (PML) Any disseminated endemic mycosis (e.g. histoplasmosis, coccidioidomycosis) Candidiasis of the oesophagus, trachea, bronchi or lungs Atypical mycobacteriosis, disseminated Non-typhoid Salmonella septicaemia Extra-pulmonary tuberculosis Lymphoma Kaposi's sarcoma (KS) HIV encephalopathy, as defined by CDC[2] And/or Performance scale 4: bed-ridden, > 50% of the day during the last month

(Note: Both definitive and presumptive diagnoses are acceptable)

[1] HIV wasting syndrome: weight loss of >10% of body weight, plus either unexplained chronic diarrhoea (>1 month), or chronic weakness and unexplained prolonged fever (>1 month).

[2] HIV encephalopathy: clinical finding of disabling cognitive and/or motor dysfunction interfering with activities of daily living, progressing over weeks to months, in the absence of a concurrent illness or condition other than HIV infection that could explain the findings.

Appendix J: Itraconazole Drug Interactions

Itraconazole and its major metabolite, hydroxyitraconazole, are inhibitors of CYP3A4. Therefore, the following drug interactions may occur (See Table 1 below and the following drug class subheadings that follow):

Itraconazole may decrease the elimination of drugs metabolized by CYP3A4, resulting in increased plasma concentrations of these drugs when they are administered with itraconazole. These elevated plasma concentrations may increase or prolong both therapeutic and adverse effects of these drugs. Inducers of CYP3A4 may decrease the plasma concentrations of itraconazole. Itraconazole may not be effective in patients concomitantly taking itraconazole and one of these drugs. Therefore, administration of these drugs with itraconazole is not recommended. Other inhibitors of CYP3A4 may increase the plasma concentrations of itraconazole. Patients who must take itraconazole concomitantly with one of these drugs should be monitored closely for signs or symptoms of increased or prolonged pharmacologic effects of itraconazole.

Table 1: Selected Drugs that are predicted to alter the plasma concentration of itraconazole or have their plasma concentration altered by itraconazole[1]

Drug plasma concentration increased by itraconazole	
Antiarrhythmics	digoxin, dofetilide[2], quinidine[2], disopyramide
Anticonvulsants	carbamazepine
Antimycobacterials	rifabutin
Antineoplastics	busulfan, docetaxel, vinca alkaloids
Antipsychotics	pimozide[2]
Benzodiazepines	alprazolam, diazepam, midazolam,[2,3] triazolam[2]
Calcium Channel Blockers	dihydropyridines, verapamil
Gastrointestinal Motility Agents	cisapride[2]
HMG CoA-Reductase Inhibitors	atorvastatin, cerivastatin, lovastatin,[2] simvastatin[2]
Immunosuppressants	cyclosporine, tacrolimus, sirolimus
Oral Hypoglycemics	Oral hypoglycemics

Drug plasma concentration increased by itraconazole	
Protease Inhibitors	indinavir, ritonavir, saquinavir
Other	levacetylmethadol (levomethadyl), ergot alkaloids, halofantrine, alfentanil, buspirone, methylprednisolone, budesonide, dexamethasone, trimetrexate, warfarin, cilostazol, eletriptan
Decrease plasma concentration of itraconazole	
Anticonvulsants	carbamazepine, phenobarbital, phenytoin
Antimycobacterials	isoniazid, rifabutin, rifampin
Gastric Acid Suppressors/Neutralizers	antacids, H_2-receptor antagonists, proton pump inhibitors
Non-nucleoside Reverse Transcriptase Inhibitors	nevirapine
Increase plasma concentration of itraconazole	
Macrolide Antibiotics	clarithromycin, erythromycin
Protease Inhibitors	indinavir, ritonavir

[1]This list is not all-inclusive.
[2]Contraindicated with itraconazole based on clinical and/or pharmacokinetics studies.
[3]For information on parenterally administered midazolam, see the Benzodiazepine paragraph below.

Antiarrhythmics: The class IA antiarrhythmic quinidine and class III antiarrhythmic dofetilide are known to prolong the QT interval. Co administration of quinidine or dofetilide with itraconazole may increase plasma concentrations of quinidine or dofetilide which could result in serious cardiovascular events. Therefore, concomitant administration of itraconazole and quinidine or dofetilide is contraindicated.

The class IA antiarrhythmic disopyramide has the potential to increase the QT interval at high plasma concentrations. Caution is advised when itraconazole and disopyramide are administered concomitantly.

Concomitant administration of digoxin and itraconazole has led to increased plasma concentrations of digoxin.

Anticonvulsants: Reduced plasma concentrations of itraconazole were reported when itraconazole was administered concomitantly with phenytoin. Carbamazepine, phenobarbital, and phenytoin are all inducers of CYP3A4. Although interactions with carbamazepine and phenobarbital have not been studied, concomitant administration of itraconazole and these drugs would be expected to result in decreased plasma concentrations of itraconazole. In addition, *in vivo* studies have demonstrated an increase in plasma carbamazepine concentrations in subjects concomitantly receiving ketoconazole. Although there are no data regarding the effect of itraconazole on carbamazepine metabolism, because of the similarities between ketoconazole and itraconazole, concomitant administration of itraconazole and carbamazepine may inhibit the metabolism of carbamazepine.

Antimycobacterials: Drug interaction studies have demonstrated that plasma concentrations of azole antifungal agents and their metabolites, including itraconazole and hydroxyitraconazole, were significantly decreased when these agents were given concomitantly with rifabutin or rifampin. *In vivo* data suggest that rifabutin is metabolized in part by CYP3A4. Itraconazole may inhibit the metabolism of rifabutin. Although no formal study data are available for isoniazid, similar effects should be anticipated. Therefore, the efficacy of itraconazole could be substantially reduced if given concomitantly with one of these agents. Co administration is not recommended.

Antineoplastics: Itraconazole may inhibit the metabolism of busulfan, docetaxel, and vinca alkaloids.

Antipsychotics: Pimozide is known to prolong the QT interval and is partially metabolized by CYP3A4. Co administration of pimozide with itraconazole could result in serious cardiovascular events. Therefore, concomitant administration of itraconazole and pimozide is contraindicated.

Benzodiazepines: Concomitant administration of itraconazole and alprazolam, diazepam, oral midazolam, or triazolam could lead to increased plasma concentrations of these benzodiazepines. Increased plasma concentrations could potentiate and prolong hypnotic and sedative effects. Concomitant administration of itraconazole and oral midazolam or triazolam is contraindicated. If midazolam is administered parenterally, special precaution and patient monitoring is required since the sedative effect may be prolonged.

Calcium Channel Blockers: Edema has been reported in patients concomitantly receiving itraconazole and dihydropyridine calcium channel blockers. Appropriate dosage adjustment may be necessary.

Calcium channel blockers can have a negative inotropic effect which may be additive to those of itraconazole; itraconazole can inhibit the metabolism of calcium channel blockers such as dihydropyridines (e.g., nifedipine and felodipine) and verapamil. Therefore, caution should be used when co-administering itraconazole and calcium channel blockers.

Gastric Acid Suppressors/Neutralizers: Reduced plasma concentrations of itraconazole were reported when itraconazole capsules were administered concomitantly with H2-receptor antagonists. Studies have shown that absorption of itraconazole is impaired when gastric acid production is decreased. Therefore, itraconazole should be administered with a cola beverage if the patient has achlorhydria or is taking H2-receptor antagonists or other gastric acid suppressors. Antacids should be administered at least 1 hour before or 2 hours after administration of itraconazole capsules. In a clinical study, when itraconazole capsules were administered with omeprazole (a proton pump inhibitor), the bioavailability of itraconazole was significantly reduced.

Gastrointestinal Motility Agents: Co administration of itraconazole with cisapride can elevate plasma cisapride concentrations which could result in serious cardiovascular events. Therefore, concomitant administration of itraconazole with cisapride is contraindicated.

HMG CoA-Reductase Inhibitors: Human pharmacokinetic data suggest that itraconazole inhibits the metabolism of atorvastatin, cerivastatin, lovastatin, and simvastatin, which may increase the risk of skeletal muscle toxicity, including rhabdomyolysis. Concomitant administration of itraconazole with HMG CoA-reductase inhibitors, such as lovastatin and simvastatin, is contraindicated.

Immunosuppressants: Concomitant administration of itraconazole and cyclosporine or tacrolimus has led to increased plasma concentrations of these immunosuppressants. Concomitant administration of itraconazole and sirolimus could increase plasma concentrations of sirolimus.

Macrolide Antibiotics: Erythromycin and clarithromycin are known inhibitors of CYP3A4 (See Table 1) and may increase plasma concentrations of itraconazole. In a small pharmacokinetic study involving HIV infected patients, clarithromycin was shown to increase plasma concentrations of itraconazole. Similarly, following administration of 1 gram of erythromycin ethyl succinate and 200 mg itraconazole as single doses, the mean Cmax and $AUC_{0-\infty}$ of itraconazole increased by 44% (90% CI: 119-175%) and 36% (90% CI: 108-171%), respectively.

Non-nucleoside Reverse Transcriptase Inhibitors: Nevirapine is an inducer of CYP3A4. *In vivo* studies have shown that nevirapine induces the metabolism of ketoconazole, significantly reducing the bioavailability of ketoconazole. Studies involving nevirapine and itraconazole have not been conducted. However, because of the similarities between ketoconazole and itraconazole, concomitant administration of itraconazole and nevirapine is not recommended.

In a clinical study, when 8 HIV-infected subjects were treated concomitantly with itraconazole capsules 100 mg twice daily and the nucleoside reverse transcriptase inhibitor zidovudine 8 ± 0.4 mg/kg/day, the pharmacokinetics of zidovudine were not affected. Other nucleoside reverse transcriptase inhibitors have not been studied.

Oral Hypoglycemic Agents: Severe hypoglycemia has been reported in patients concomitantly receiving azole antifungal agents and oral hypoglycemic agents. Blood glucose concentrations should be carefully monitored when itraconazole and oral hypoglycemic agents are coadministered.

Polyenes: Prior treatment with itraconazole, like other azoles, may reduce or inhibit the activity of polyenes such as amphotericin B. However, the clinical significance of this drug effect has not been clearly defined.

Protease Inhibitors: Concomitant administration of itraconazole and protease inhibitors metabolized by CYP3A4, such as indinavir, ritonavir, and saquinavir, may increase plasma concentrations of these protease inhibitors. In addition, concomitant administration of itraconazole and indinavir and ritonavir (but not saquinavir) may increase plasma concentrations of itraconazole. Caution is advised when itraconazole and protease inhibitors must be given concomitantly.

Other:

- Levacetylmethadol (levomethadyl) is known to prolong the QT interval and is metabolized by CYP3A4. Co-administration of levacetylmethadol with itraconazole could result in serious

cardiovascular events. Therefore, concomitant administration of itraconazole and levacetylmethadol is contraindicated.

- Elevated concentrations of ergot alkaloids can cause ergotism, ie. a risk for vasospasm potentially leading to cerebral ischemia and/or ischemia of the extremities. Concomitant administration of ergot alkaloids such as dihydroergotamine, ergometrine (ergonovine), ergotamine and methylergometrine (methylergonovine) with itraconazole is contraindicated.
- Halofantrine has the potential to prolong the QT interval at high plasma concentrations. Caution is advised when itraconazole and halofantrine are administered concomitantly.
- *In vitro* data suggest that alfentanil is metabolized by CYP3A4. Administration with itraconazole may increase plasma concentrations of alfentanil.
- Human pharmacokinetic data suggest that concomitant administration of itraconazole and buspirone results in significant increases in plasma concentrations of buspirone.
- Itraconazole may inhibit the metabolism of certain glucocorticosteroids such as budesonide, dexamethasone and methylprednisolone.
- *In vitro* data suggest that trimetrexate is extensively metabolized by CYP3A4. *In vitro* animal models have demonstrated that ketoconazole potently inhibits the metabolism of trimetrexate. Although there are no data regarding the effect of itraconazole on trimetrexate metabolism, because of the similarities between ketoconazole and itraconazole, concomitant administration of itraconazole and trimetrexate may inhibit the metabolism of trimetrexate.
- Itraconazole enhances the anticoagulant effect of coumarin-like drugs, such as warfarin.

Cilostazol and eletriptan are CYP3A4 metabolized drugs that should be used with caution when co-administered with itraconazole.

Section 3 – Summary of changes

Study: **A Randomized, Open-Label, Comparative Study of the Effectiveness of Itraconazole versus Amphotericin B in the Induction Treatment of Penicilliosis in HIV-Infected Persons**

Previous approved version:

- Protocol version **5.1** dated 26.07.2012,

Amended version:

- Protocol version **5.2** dated 12.10.2012, approved by Vietnam Ministry of Health on 25 Feb 2013

Below are the changes of final protocol compared to original approved versions:

1. Modification of an exclusion criteria: from excluding patients with pretreatment "AST and ALT >5 times the upper limit of normal (ULN)" to excluding patients with pretreatment AST and ALT "**> 400 U/L**". The reason is because elevated AST/ALT levels are common laboratory manifestations of penicilliosis. In our retrospective cohort of 513 patients[1], 35% of patients had AST/ALT levels >5 times ULN and 13% with AST/ALT levels >10 times ULN. If we exclude patients with AST/ALT >5 times ULN, we would have to exclude nearly 50% of patients with penicilliosis, and the study would loose its generalizability. Amongst 143 patients who died, only 2 died of liver related diseases. Therefore, the modified exclusion criteria (excluding patients with AST/ALT >400U/L) would allow a more representative study population. To maximize the safety of patients, if AST/ALT rose >600U/L during itraconazole therapy, itraconazole will be changed to amphotericin B.

2. Based on preliminary research findings at OUCRU lab, the frequency of sample collection (blood culture, molecular, serology, and IRIS lab tests) have been reduced as they do not provide more information (Please see updated Appendix B and updated wordings in Section 3 and Section 5 accordingly).

Reference:
1. Le T, Wolbers M, Chi NH, Quang VM, Chinh NT, Lan HPN, Lam PS, Kozal KM, Shikuma CM, Day NJ, Farrar J. Epidemiology, Seasonality and Outcome Predictors of *Penicillium marneffei* Infection in Ho Chi Minh City, Viet Nam. Clin Infect Dis 2011 Arp 1; 52(7):945–952.

Statistical analysis plan for the 11CN study (ISRCTN97524945) "A Randomized, Open-Label, Comparative Study of the Effectiveness of Itraconazole versus Amphotericin B in the Induction Treatment of Penicilliosis in HIV-Infected Adults"

Authors: Marcel Wolbers, Nhan Ho Thi, Thuy Le
Final version: v1.00, 13July2016 (finalized before release of the randomization list)
Current version: v1.01, 03August2016 (identical to the final version but with additional post-hoc exploratory analyses which were specified after release of the randomization list added to the end of this document)

Purpose

This document details the planned analyses and endpoint derivations for the ISRCTN97524945 trial as outlined in the published study protocol. It focuses on the analysis for the main clinical trial publication and does not include analysis for any subsidiary studies.

Statistical software

Data derivations will be performed with the statistical software SAS v9.4 (SAS Insitute, Cary, North Carolina, US). Statistical analyses will be performed with the statistical software R using the current R version at the time of the final analysis (R Foundation for Statistical Computing, Vienna, Austria).

Analysis populations

Intention-to treat and modified intention to treat population

The primary analysis population for all analysis is the full analysis population containing all randomized patients except for those without microbiologically confirmed penicilliosis. Participants not receiving any study treatment will still be included in the intention to treat population (ITT). All participants will be analyzed according to their randomized arm.

As several study participants did not receive study treatment, the analysis of the primary endpoint and overall survival will be repeated in the population of all subjects in the ITT who received at least one dose of the randomized study treatment (modified ITT).

Per-protocol population

The analysis of non-inferiority trials in the intention-to-treat population is not necessarily conservative. Therefore, the analysis of the primary endpoint and survival will also be repeated in the per-protocol population. The per-protocol population will exclude the following patients: patients without microbiological confirmed penicilliosis, major protocol violators, subjects who did not receive a single dose of the randomized study drug, patients who received <7 days of randomized treatment due to reasons other than adverse events (including death), and patients lost to follow-up before day 14. Analyses in the per protocol population will also be according to the randomized treatment arm.

Derivation rules for the definition of study populations:

- No microbiological confirmation of penicilliosis: INPOC.Disontinue=1
- Major protocol violators are defined as subjects who meet at least one of the following exclusion criteria:
  - Concurrent diagnosis/treatment of cryptococcal meningitis (using amphotericin B) or active tuberculosis (using rifampicin): SCR.MeningTB=1
  - On amphotericin or itraconazole (dose 400mg/day) for >48 hours prior to enrolment: SCR.ItraAmpUse48=1
  - AST/ALT >400 U/L: SCR.AstAlt10=1
  - Absolute neutrophil count <500 cells/µL: SCR.WBC500=1
  - Creatinine Clearance (Cockgraft-Gault formula) <30 ml/min: SCR.CreatinineCG=1
  - Use of contraindicated drugs: SCR.DrugUse=1
- No study treatment received: Study treatment is recorded in the DRUG dataset (DRUG.drugname=1 (amphotericin B) or 2 (itraconazole)/3 (itraconazole with modified dose for patients with TB)) and patients not receiving study treatment are those without a single record in that dataset corresponding to the randomized treatment arm (TREAT.Arm; 1= amphotericin B, 2= itraconazole).
- <7 days of randomized treatment due to reasons other than adverse events (including death):
  All inpatient administrations of the randomized treatment are recorded in the DRUG dataset. The total duration of inpatient study drug treatment is defined as the sum of the durations (datepart(DRUG.datestop)-datepart(DRUG.datestart)+1) of all drug records matching the randomized treatment group (DRUG.drugname=1 for TREAT.Arm=1, DRUG.drugname=2 or 3 for TREAT.Arm=2).
  The drug will be considered to have been stopped for reasons other than adverse events if the patient's last drug record has a stopping reason 'Other' (DRUG.ReasonStop=3).
  Notes:
  - For DRUG.drugname= 3, the stop date was not systematically recorded. If it is missing, it will be imputed with the date of hospital discharge (INPOC.dischargedate).
  - For some subjects, itraconazole treatment beyond hospital discharge was recorded in the DRUG dataset. To calculate inpatient administration only, the drug stop date will be replaced by the hospital discharge date in this case.
- Lost to follow up before day 14: Patient is not recorded as having died and the last date the patient is known to be alive (see derivation for overall survival below for details) is <14 days after the baseline date.

Treatment of missing data

As the expected amount of missing data is minimal in this trial, all analyses will be based on complete case analyses and the number of missing data points / excluded observations will be clearly identified from all outputs.

Baseline characteristics

Baseline characteristics will be summarized as median (upper and lower quartile) for continuous data and frequency (percentage) for categorical data. The amount of missing data for each baseline characteristic will also be displayed. Formal comparisons of baseline characteristics between study arms are discouraged by most

statisticians (see e.g. Senn SS (2008): Statistical Issues in Drug Development, 2nd Edition, Wiley [p. 98f]) but are mandated by some journals. To satisfy all potential publishers, we will calculate p-values (based on the Wilcoxon rank sum test and Fisher's exact test for continuous and categorical data, respectively) but will only report them if mandated by the journal.
The baseline date is defined as the date of the first dose of study drug (first DRUG.datestart where DRUG.drugname is 1 (AmB) or 2 or 3 (Itra)). For subjects who did not receive any study drug, the date of enrolment recorded on the demographics page (DEMO.dateenrol) will be used instead.

The following baseline characteristics will be summarized by treatment arm [with derivation rules in brackets]:
Baseline – Demographic, history, investigations, and examination day 1

- Recruitment site (first two digits of patient identifier: 03="HTD", 20="NHTD", 21="Bach Mai", 26="Uong Bi", 27="Viet Tiep")
- Age [year(baseline date)-DEMO.yearbirth]
- Sex [DEMO.sex]
- Weight [TREAT.weight]
- IVDU [HIST.IVDU]
- On ARV [HIST.arvtreat]
- Duration of ARV [baseline date-HIST.datearv+1; missing day in datearv will be imputed as 1, missing month and day as 01July]
- History of previous TM infection [HIST.prepm]
- Illness history and examination (HIST form): duration of illness and symptoms (questions 7-16)
- Temperature (EXEN.temp) and respiratory rate (EXEN.resprate)
- Hepatosplenomegaly ["Yes" if INVEST.Hepa=1 or INVEST.Splen=1]
- Chest X-ray result [INVEST.CXR]
    - Baseline - Laboratory
    - All laboratory parameters recorded on the laboratory form (LABODD) form at baseline (latest value before or at the baseline date):
    - For WBC and PLT, two reporting units are recorded but they are equivalent, i.e. no conversion is required. For Hb, measurements in [g/L] will be converted to [g/dL] by dividing the measurement by 10.
    - For baseline CD4 cell count, any value recorded on the LABODD form is acceptable. If CD4 is missing, it will be imputed with the CD4 cell count from the history form (HIST.cd4) which was collected <3 months prior to enrolment (if available).
    - Skin culture positive for TM [INVEST.SkinCult=1 and INVEST.SkinCultSpec=1]
    - Blood culture positive for TM [INVEST.BloodCult=1 and INVEST.BloodCultSpec=1]
    - Baseline fungal load: original scale [CFU/ml blood] and log10-transformed [log10 CFU/ml blood] value.
    - Fungal loads are recorded in the BLFUNCT table and the fungal load can be calculated as Fungalx10(FungalPow).
    - If a fungal load at the baseline date is not available, it will be imputed by the value from the day before or after baseline (if available).

Primary endpoint – absolute risk of death during the first 2 weeks after randomization

Derivation

Derivation of overall survival:
Time to death: [date of death or censoring]-[date of baseline]+1
Event indicator: =1 if patient died, =0 otherwise

[Date of death]:
Deaths are recorded in the INPOC form (inpatient outcome, INPOC.ideath=1 with death date INPOC.DateDeath) and the OUPOC form (outpatient outcome, OUPOC.ideath=1 with death date OUPOC.DateDeath).

[Date of censoring]: Last date where the patient was known to be alive.
This is defined as the last date amongst the following dates for participants who did not die: enrolment date (DEMO.DateEnrol), daily inpatient or follow-up examination days (EXDAILY.dateassess, INVFU.dateassess), any in-patient drug start or stop date (DRUG.DateStart or DRUG.DateStop), hospital discharge or study discontinuation date (INPOC.DischargeDate or INPOC.DateDiscontinue), dates were blood was taken for lab assessments (LABODD.dateassess or INVADDFU.dateassess), adverse event start or stop dates (AE.StartDate or AE.StopDate), or the last outpatient visit date (OUPOC.DateLastVisit).

Derivation of overall survival during the first two weeks after randomization:
This is defined in the same way as overall survival. Subjects who were followed up for >15 days or those who died after study day 15 will be treated as censored observations on day 15.

Planned analyses for the primary endpoint

Primary analysis
This is a non-inferiority trial with a non-inferiority margin of Δ=10%; i.e., the aim is to prove that the absolute risks of death during the first 2 weeks of treatment in the two treatment arms differ by less than 10% (at worst) in favour of amphotericin B. The absolute risk of death by two weeks will be estimated with Kaplan-Meier method. Based on these estimates and corresponding asymptotic standard errors (calculated according to Greenwood's formula), a two-sided Wald-type 95% confidence interval (CI) for the difference in the absolute risks of death will be calculated. If the CI excludes differences of 10% or more in favour of the amphotericin B arm, the primary objective of the trial will be met.
The estimated risk difference and its standard error will also be used to generate p-values for the following Wald-type tests:

- Test for non-inferiority: One-sided test (at the 2.5% significance level) of the null hypothesis
  "H_0: absolute risk difference Δ≥10% in favor of amphotericin B" versus the alternative
  "H_1: absolute risk difference Δ<10% in favor of amphotericin B".
- Test for a difference between the drugs (i.e. superiority of either drug): Two-sided test (at the 5% significance level) of the null hypothesis
  "H_0: absolute risk difference Δ=0" versus the alternative
  "H_1: absolute risk difference Δ≠0".

The above analysis based on asymptotic Wald-type tests with standard errors from Greenwood's formula is the pre-defined analysis but it does not guarantee exact type I error and confidence interval coverage. As an additional exploratory analysis, we will also calculate the above confidence intervals and tests using the recently developed melded BPCP confidence intervals and tests (Fay MP et al (2015). "Combining One-Sample

Confidence Procedures for Inference in the Two-Sample Case." *Biometrics* 71, 146–156.) which have been implemented in the R function bpcp:: bpcp2samp.

Subgroup analyses

The following subgroups are pre-defined:

- *Modified intention-to-treat population*
- Per-protocol population
- Injection drug use [HIST.IVDU, 1="Yes", 2="No"]
- Presence of fungemia at baseline ["Positive for TM" if INVEST.BloodCult=1 ("positive") and INVEST.BloodCultSpec=1 ("TM"), "Negative for TM" otherwise if blood culture result is non-missing]
- *Baseline fungal load* ["0 CFU/ml" vs. "1-1000 CFU/ml" vs. ">1000 CFU/ml"]
- *Baseline dyspnea requiring oxygen* [HIST.ShortBreath =1]
- *Baseline oral ulcers* [HIST.ThroatUlcers =1]
- ARV status at baseline [HIST.ARVtreat, 2="ARV naive", 1="ARV experienced"]
- Baseline CD4 cell count ["<50cells/mm^3" vs. "≥50cells/mm^3"]

Note: Subgroups in *italic* were not pre-defined in the protocol but are added as pre-defined subgroup analysis in this analysis plan.

In each of these subgroups, the primary endpoint will be analyzed as described above for the ITT population. Heterogeneity of the absolute risk difference across subgroup levels will be tested with an overall Wald-type test.

Logistic regression analyses

The joint effect of treatment assignment and the baseline covariates injection drug use, presence of fungemia, dyspnea requiring oxygen, throat ulcers, and ARV status (naïve/experienced) on the subject's survival status at 2 weeks will be assessed using logistic regression. (Note: Age and sex (covariates specified in the protocol) were replaced by baseline dyspnea requiring oxygen and throat ulcers during the writing of the analysis plan as they are more clinically relevant.) As we expect only few patients lost to follow-up during the first 2 weeks, these patients will be removed from the adjusted analysis.

In a supplementary analysis, we will also examine the adjusted effect of baseline fungal count (categorized as "0 CFU/ml" vs. "1-1000 CFU/ml" vs. ">1000 CFU/ml") instead of fungemia.

Secondary endpoint – Survival until 24 weeks after randomization

Derivation

The endpoint will be derived in the same way as the primary endpoint (see above). Deaths documented on the database but occurring after the pre-defined follow-up period of 24 weeks will be listed separately but for the main analysis of this endpoints subjects with follow-up for >24 weeks or documented deaths after study day 169 will be treated as censored on study day 169.

Analysis

Overall survival in the two arms will be visualized using Kaplan-Meier curves. Absolute risks (1 minus the survival function) rather than survival functions will be displayed over time as the overall mortality in this trial is not very high.

The two arms will be formally compared with a Cox proportional hazards regression model with treatment allocation as the only covariate. The analysis will be performed in the ITT population and the same subgroups as for the primary endpoint. Potential heterogeneity of the treatment effect across levels of sub-grouping variables will be tested using likelihood ratio tests for an interaction term between treatment and the grouping variable. An adjusted multivariable Cox regression model will also be fitted using the same covariates as for the logistic regression model of the primary endpoint.

As the proportional hazards assumption may not be satisfied and absolute risk differences may be more interpretable than hazard ratios, we will also compare the absolute 24-week risk of death between the two treatment arms in the ITT population and pre-defined subgroups using the same methods as described for the primary endpoint.

Secondary endpoint – Time to treatment success (taking into account death without prior treatment success)

Derivation

Treatment success is defined by absence of fungal growth in follow up culture, temperature <38°C for 3 days, and complete resolution of lesions or lesions in the final stage of healing as judged by the treating clinicians. The endpoint is recorded on the in-patient form (INPOC.DaysToFinish).

Patients who died without prior documented treatment success will be considered as experiencing the competing endpoint "death without prior treatment success". Otherwise, they will be treated as censored at the last date they were known to be alive.

Analysis

The overall probability of treatment success will be estimated with the cumulative incidence function, which takes the competing risk of prior death into account. The main comparisons between the two arms will be based on a comparison of the cumulative incidence functions using a Fine-Gray competing risks regression model with treatment as the only covariate. Of note, the protocol pre-defined a comparison of rates using a cause-specific Cox proportional hazards regression model as the main analysis but a comparison of cumulative incidence functions appears to be more clinically relevant and hence constitutes the main analysis according to this analysis plan superceding the protocol. Finally, an adjusted analysis based on the Fine-Gray model will include the same covariates as for the analysis of the primary endpoint.

Secondary endpoint – Time to TM paradoxical IRIS, TM relapse, or death

Derivation

[Time to event]: Time from randomization to the earliest of a TM paradoxical IRIS event (AE.startdate of an adverse event with AE.pt="T.m paradoxical IRIS"), a TM relapse (AE.startdate of an adverse event with AE.pt="T.m relapse"), death, or censoring.

[Event type]:

0, if patient is censored (i.e. experienced none of the above events)

1, if first event is TM paradoxical IRIS

2, if first event is TM relapse

3, if first event is death (without prior relapse or IRIS)

Note: The protocol defined TM IRIS and TM relapse as separate endpoints but as they are difficult to distinguish clinically, we decided to analyze them jointly instead.

Analysis

The combined endpoint will be analyzed using a Cox proportional hazards regression model with treatment allocation as the only covariate. The absolute risk of TM IRIS and relapse by 24 weeks will be estimated with the cumulative incidence function (accounting for competing event types). The treatment effect measure of interest is the absolute risk differences for TM IRIS or relapse, respectively, with corresponding Wald-type 95% confidence intervals and tests.

Secondary endpoint – deaths from penicilliosis

Derivation

[Time to event]: As for the endpoint survival until 24 weeks.

[Event type]:
0, if patient is censored (i.e. did not die)
1, if the death cause is considered to be related to TM according to the independent serious adverse event review committee (i.e. if the patient has an adverse event with AE.saeresindeath="RELATED TO PM")
2, if the death is considered to be related to other causes than TM according to the independent review committee (i.e. if the patient has an adverse event with AE.saeresindeath="RELATED TO OTHER")
3, if the death cause could not be assessed by the review committee as there was insufficient information (i.e. if the patient died but the assessment by the committee is missing)
Note: The protocol says that the cause of death will be determined by the investigator but this has been superceded by a more objective independent review committee.

Analysis
The endpoint will be analyzed using competing risks methodology in the same way as for the time to treatment success except that a multivariable analysis will not be conducted.

Secondary endpoint – rate of early fungicidal activity (EFA) during the first 14 days after randomization

Derivation
Longitudinal quantitative fungal count measurements are recorded in the BLFUNCT dataset. The analysis population includes all subjects with a measurable (non-zero) fungal count at baseline and at least two fungal count measurements until study day 15 (including the baseline count). The analysis will be based on log10-transformed quantitative fungal count measurements and zero measurements will be assigned a log10-count of 0.
For each subject, their EFA is defined as the slope of a linear regression of the subject's longitudinal log10-fungal count measurements until study day 15 versus time. Only fungal counts until the first 'real' zero measurement will be included where a 'real' zero is defined as a zero measurement that is not immediately followed by a non-zero measurement. In addition, the first 'real' zero fungal count will only be included in the regression analysis if it is associated with a steeper rate of decline than an analysis that omits it. This ad-hoc adjustment has been frequently performed in analyses of fungal counts in cryptococcal meningitis and avoids that the rate of decline is underestimated because of the detection limit of 5 CFU/ml (i.e. true values below 5 CFU/ml are recorded as zeros).

Analysis
EFA will be compared between the two treatment arms based on a linear regression model with treatment as the main covariate and adjustment for the baseline log10-fungal count. In addition, spaghetti plots of the fungal trajectories of participants over time will be displayed by treatment arm.

Secondary endpoint – Clinical adverse events and new laboratory adverse abnormalities

Derivation
Adverse events (AE) are all events recorded on the adverse event form. Only grade 3 and 4 adverse events were collected in this study.

New laboratory abnormalities are defined as any worsening of a lab value to grade 3 or 4 (including changes from grade 3 to 4) compared to the subject's previous lab value. In addition, to be conservative, if a subject's baseline lab value was missing, the first post-baseline lab value was also considered a new lab abnormality if it was of grade 3 or 4. A grading table for laboratory abnormalities is provided in the Appendix.

Planned analysis

The number of patients with grade 3&4 adverse events, serious adverse events, and specific adverse events, respectively, will be summarized and compared between the two treatment arms using Fisher's exact test. The total number of grade 3&4 adverse events and serious adverse events per patient will additionally be compared between the two groups using a quasi-Poisson regression model with treatment as the only covariate.

Summaries tables by treatment arm will be performed for the following events:

- Summary of all grade 3&4 adverse events by system organ class
- Summary of all grade 3&4 adverse event by system organ class and preferred term
- Summary of grade 3&4 adverse events leading to changes in randomized treatment
- Summary of serious adverse events
- Summary of serious adverse events assessed by the committee and considered 'definitely', 'probably', or 'possibly' related to study drug
- Summary of serious adverse events with outcome death
- Summary of serious adverse events with outcome death and the death cause considered to be TM related
- Summary of laboratory abnormalities present at baseline
- Summary of new laboratory abnormalities

Secondary endpoint – Antifungal medication adherence (and other drug-administration related outcomes)

Antifungal drug intake was directly observed while the patient was hospitalized. Hence, there was no need to collect adherence information. However, the following outcomes will be summarized by treatment arm:

- Number of days that participants received the randomized study drug as an inpatient
- Did the patient receive amphotericin B?
- Time from baseline to hospital discharge

Adherence to maintenance itraconazole as an outpatient was collected via patient-interviews at each monthly follow-up visit as the proportion of mandated itraconazole doses since the last visit that were not taken (INVFU.MedAdherence). For each treatment arm, the proportion of missed doses (categorized as 0%, >0% to 20%, >20%-100%) will be summarized with the total number of visits as the denominator. In addition, adherence in patients with TM relapse will be described.

Pre-defined secondary endpoint that are not part of this analysis plan

- Time to change in therapy from assigned study treatment will not be explicitly analyzed. However, the analyses outlined above include a summary of all adverse events leading to changes in randomized treatment.
- Time to blood culture sterilization will not be analyzed as this endpoint was not collected.
- Frequency and patterns of itraconazole and amphotericin resistance, pharmacological and health economics endpoints will be analyzed and reported separately.

Post-hoc exploratory analyses specified after release of the randomization list

Primary endpoint: Logistic regression analysis

An alternative parameterization of the baseline fungal count was considered post-hoc which modeled it using two covariates: an indicator (0/1) variable whether the subject was sterile at baseline or not and a continuous variable of log10-fungal count (centered at a log10-count of 100 CFU/ml and set to 0 for sterile subjects).

Secondary endpoint – Survival until 24 weeks after randomization

As there was some evidence of non-proportional hazards, separate hazard ratios for the time periods 1-4, 5-8, 9-16, and 17-24 weeks were calculated post-hoc and added to the forest plot.

Secondary endpoint – Rate of early fungicidal activity (EFA) during the first 14 days after randomization

The following additional analyses regarding the association between longitudinal fungal counts and overall survival until week 24 were performed post-hoc in subjects with a positive fungal count at baseline:

- Joint modeling of the longitudinal fungal counts and 6 month survival. Specifically, the following model was implemented using R function jointModelBayes in package JMBayes version 0.7-9:
    - Longitudinal sub-model: Mixed effects model of longitudinal log10-fungal counts during the first two weeks with a fixed intercept, a fixed treatment group specific slope, and random intercepts and slopes. Values below the detection limit of 5 CFU/ml were modeled as left-censored at log10(4.5) CFU/ml.
    - Survival sub-model: Treatment arm as the only fixed covariate and shared random effects with the longitudinal sub-model (param="shared-RE").
- Assessment of the predictive value of EFA during the first 14 days, EFA during the first week, and sterility after 1 week for subsequent survival. This was examined using Cox regression models for overall survival until week 24 adjusted for log10-baseline fungal count and (optionally) treatment arm including only subjects who were still alive after 14 or 7 days, respectively.

Appendix: Grading of laboratory adverse events (NIH Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events 2009)

	Grade 3	**Grade 4**
Haematological		
Haemoglobin	6.5 –7.4g/dl	<6.5 g/dl
White cell count	1.0 - 1.49 K/μl or g/L	<1.0 K/μl or g/L
Neutrophils	NEU % xWBC=NEU K/μl :0.5 – 0.74 K/μl	NEU % xWBC=NEU K/μl <0.5 K/μl
Platelets	25 - 49 K/μl or g/L	<25 K/μl or g/L
Biochemical		
Potassium (low)	2.0 – 2.4 mmol/l	<2.0 mmol/l
Potassium (high)	6.6 – 7.0 mmol/l	>7.0 mmol/l
Magnesium	0.30 – 0.44 mmol/l	< 0.30 mmol/l
Creatinine	1.9 – 3.5X ULN	>3.5X ULN
Bilirubin	2.6 – 5X ULN	>5X ULN
AST	5.1 – 10X ULN	>10X ULN
ALT	5.1 – 10X ULN	>10X ULN

ULN for creatinine is 100 μmol/L for females and 120 μmol/L for males.
ULN for bilirubin is 17 μmol/L for females and males.
ULN for AST is 37 U/L for females and 40 U/L for males.
ULN for ALT is 33 U/L for females and 40 U/L for males.