Development of the Novel Triazole Antifungal, Isavuconazole

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Abstract

The involvement of fungi in life-threatening infections has been increasing over the last few decades, which can cause death in the terminal stage of patients that have been immunocompromised by a malignant tumor, or from contracting hematopoietic system diseases, or after receiving strong antitumor treatments. To discern trends in visceral mycoses, I have epidemiologically analyzed the data on visceral mycoses that were reported in the *Annual of the Pathological Autopsy Cases in Japan* from 1969 to 2009 by the Japanese Society of Pathology. In the annual total number of pathological autopsy cases, the frequency of visceral mycoses increased noticeably with the incidence of candidiasis and aspergillosis increasing the most. Before 1989, visceral mycoses were predominantly caused by *Candida*, followed by *Aspergillus*. Although the rate of candidiasis decreased by degrees from 1990, the rate of aspergillosis increased up to, and then surpassed, that of candidiasis. Despite advances in antifungal therapy, fungal infections which have fatality rates of 50% to 100% remain a major cause of morbidity and mortality in immunocompromised patient. This unmet medical need led me to develop a potent antifungal agent, isavuconazole (=RO0094815) which is a inhibitor of the lanosterol 14 α-demethylase (CYP51) that catalyzes the formation of ergosterol from lanosterol. Isavuconazole showed a potent inhibitory activity against fungal pathogens in both *in vitro* and *in vivo* models. However, most triazole antifungal agents, including isavuconazole, showed only limited activity against zygomycetes spp., *Fusarium* spp., *Pseudallescheria* spp., *Sporothrix* spp., and *Scedosporium* ssp. This limitation is a major drawback of azoles.

The following text in the abstract has been omitted pending regulatory approval of Isavuconazole.

Abbreviations

AIDS	Aquired immune deficiency syndrome
ALL	Acute lymphatic leukemia
AmB	Amphotericin B
AML	Acute myeloid leukemia
ATCC	American Type Culture Collection
ATL	Adult T cell leukemia
BMT	Bone marrow transplantation
BSI	Bloodstream infection
CLL	Chronic lymphatic leukemia
CLSI	Clinical Laboratory Standards Institute
CML	Chronic myeloid leukemia
CYP51	CYP51 Cytochrome P450 sterol C14α-demethylase (=lanosterol 14α-demethylase)
DIC	Disseminated intravascular coagulation
ERG11	= CYP51
FLC	Fluconazole
ICD-9	the International Classification of Diseases 9
ICD-10	the International Classification of Diseases 10
ITC	Itraconazole
MDS	Myelodysplastic syndrome
MIC	Minimum inhibitory concentration
MoL	Monocytic leukemia
NCBI	National Center for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
OD	Optical density
PK	Pharmacokinetics
RO0094815	Isavuconazole
VRC	Voriconazole

General introduction

Fungi are non-photosynthetic eukaryotic organisms with well defined nuclei and characteristic intracellular membrane-bound organelles such as endoplasmic reticulum, golgi apparatus, lysosome, and mitochondria, etc. Most fungi species are heterotrophic or saprophytic and are responsible for the breakdown of organic material in environments. Fungi comprise a very large and diverse group of microorganisms that chiefly exist as mycelia, the threadlike branching hyphae that form the fluffy structure usually seen on moldy food, and fungi in mycelial form are known as molds. The shape of these tube-like structures is maintained by apical growth and the presence of a robust cell wall composed of several kinds of polysaccharides. Although most fungi exist with mycelial growth, many species have a life-cycle stage as unicellular or yeast form. Growth of these cells results from budding at a specific site or at the site of separation of daughter cells from mother cells. Mushrooms are an example of Basidiomycotina fungi that has fruiting bodies.

In a recent classification, both Fungi and Metazoa (animals) belong to Opisthokonta, one of the 6 major eukaryotic supergroups (104), whereas land plants, which belong to the Archaeplastida supergroup, are only distantly related to Opisthokonta. Although the distantly-related fungi and land plants both possess cell wall, animals, the relative of fungi do not. The cell wall of fungi contains β-D-glucan, chitin, chitosan, and mannan, while the major components of the cell wall of land plants are cellulose, hemicellulose, and pectin. The cell membrane of all eukaryotic organisms is generally about 5 nm thick and contains phospholipids, sterols, and proteins that together form a lipid bilayer structure. Membrane sterols of fungi consist cheifly of

ergosterol and its derivatives, but those of animal cells consist of cholesterol. This is the most important difference between animal and fungal cells.

We often suffer from infection by pathogenic fungi or, in immunocompromised settings, by opportunistic fungi. About 200 species of fungi are known to be pathogenic to humans and animals, and some fungi also cause plant diseases. The most notable mycosis (fungal infection) agents of human visceral infection belong to three fungal groups, ascomycetes, basidiomycetes and zygomycetes. *Candida* and *Aspergillus* are major genus of the ascomycetes, *Cryptococcus* is in basidiomycetes, and the *Mucor* and *Rhizopus* species are zygomycetes. In the past few decades, interest in human mycoses has surged as a result of a significant increase in the relative importance of fungal infections in the western world. Factors predisposing humans to fungal infections are: congenital immune defects, diabetes, human immunodeficiency virus (HIV) infection, and malignant diseases. The incidence of fungi in life-threatening infections has been increasing, and severe visceral mycosis in particular is a cause of death in the terminal stage of immunocompromised patients who have suffered from a malignant tumor or a hematopoietic system disease, or have received strong antitumor treatment. Although some patients tend to be living longer due to recent advanced medical treatments, such as organ transplantation or hematopoietic stem cell transplantations with immunosuppressant treatments or radiation therapy and chemotherapy in cancer and leukemia settings, those patients still have a high risk of suffering from fungal infections because of their immunocompromised state.

Unfortunately, there have been very few reports of quantitative analyses on visceral mycoses and, even though mycoses are known to have increased, the trends of infections, their causative agents, underlying diseases, and gender of patients were not

noted in detail. Such epidemiological data is fundamental information and is a precious resource for deciding promptly what measures to take; therefore, an analysis of epidemiological trends of mycoses in the clinical setting in Japan would be indispensable.

An important strategy in preventing high-risk patients from fungal infections is treatment with antibiotics and antifungals prophylactically or empirically but, although many effective antibiotics and antibacterial agents are on the market, there are few effective antifungals. The only antifungal drugs available for systemic use in Japan are amphotericin B (AmB) (polyene macrolide analog) as a plasma membrane-disrupting agent; fluconazole (FLC), itraconazole (ITC) and voriconazole (VRC) (which are all triazole analogs) as inhibitors of ergosterol (a component of plasma membrane) synthesis; terbinafine (an allylamine analog) as an inhibitor of squalene epoxidase; and fluorocytosine as an inhibitor of DNA synthesis. Using these drugs to treat mycoses in humans ordinarily encounters problems of resistance and toxicity. Therefore, it is highly desirable to make a better antifungal agent with a broad antifungal spectrum that includes strains resistant to the antifungals already on the market, and that has good PK, high selectivity, and less toxicity to humans.

In part I, to outline the trend in systemic mycoses in Japan, I epidemiologically analyzed the data on visceral mycoses reported in the *Annual of the Pathological Autopsy Cases in Japan* over the past 40 years. From this analysis, I realized that total visceral mycoses have been increasing, and that aspergillosis in particular had increased remarkably. Furthermore, I recognized the major underlying diseases that pose the highest risk for patients who have been exposed to some predisposing factors. From these results, unmet medical needs for developing new antifungals were suggested.

In part II, I have compared the antifungal activity of isavuconazole, a lanosterol 14α-demethylase (CYP51) inhibitor that was screened from hundreds of synthesized triazole derivatives, against 140 reference fungal strains and 165 clinically isolated yeasts in Japan and compared the results with those of other azoles and AmB. I showed the inhibitory activity of isavuconazole and discussed the species that are intrinsically resistant to triazole antifungals in a framework of fungal phylogeny and evolution.

At present, this potent antifungal drug that was developed as a result of this research is now in clinical phase III studies in the US. Some studies have been completed and the latest result of one study has met the primary endpoint.

Part I

Epidemiology of Visceral Mycoses: Analysis of Data in *Annual of the Pathological Autopsy Cases in Japan*

Abstract

The data on visceral mycoses that had been reported in the *Annual of the Pathological Autopsy Cases in Japan* from 1969 to 2009 by the Japanese Society of Pathology were analyzed epidemiologically. The frequency of visceral mycoses among the annual total number of pathological autopsy cases increased noticeably from 1.60% in 1969 to a peak of 4.66% in 1990. Of them, the incidences of candidiasis and aspergillosis increased the most. After 1990, however, the frequency of visceral mycoses decreased gradually. Until 1989, the predominant causative agent was *Candida*, followed in order by *Aspergillus* and *Cryptococcus*. Although the rate of candidiasis decreased by degrees from 1990, the rate of aspergillosis increased up to and then surpassed that of candidiasis in 1991. After 1994, the rate of mycoses increased again and the frequency of aspergillosis also rose. In the 10 years between 1999 and 2009, the frequency of visceral mycoses varied between 4.01 and 4.70% with a peak in 2009. The major disease underlying the visceral mycoses was leukemia, followed by solid cancers and then by other blood and hematopoietic system diseases. Severe mycotic infection increased over the reported 25-year period from 6.6% of the total visceral mycosis cases in 1969 to 71% in 1994. But during the decade after 1994, severe infections decreased by degrees to between 40% and 50%, as did *Candida* infections. The reasons for this decrease of candidiasis combined with increase of aspergillosis and of severe mycotic infection in general might be that (i) both non-severe and severe *Candida* infections were excluded from the case totals when it became possible to control them by antifungal drugs such as FLC and ITC, but (ii) the available antifungal drugs were not efficacious against severe infections, such as pulmonary aspergillosis or central nervous system infections by zygomycetes species, and (iii) the number of patients living longer

in an immunocompromised state had increased because of developments in chemotherapy and progress in medical care. These epidemiological findings strongly suggested that novel effective antifungals are necessary to treat severe fungal infections, such as aspergillosis and drug-resistant candidiasis.

Introduction

Recently many reports have described an increase of systemic fungal infections, such as aspergillosis (13, 23), zygomycosis (17), fusariosis (98), and candidiasis due to non-*albicans Candida* species (15). It seems possible to control cutaneous or superficial candidiasis by using effective azoles; however, azole-resistant *C. albicans* strains and pathogenic non-*albicans Candida* species have been emerging (8, 15, 100). Furthermore, severe systemic aspergillosis has been increasing in bone marrow transplant patients (7) and in those with other immunocompromised conditions (6).

Over the past 40 years, medical mycologists and pathologists have published several papers on the trends of mycoses (23, 27, 67, 87, 92, 93). Groll et al. reported the trends of invasive fungal infections from autopsy findings at the university hospital of Frankfurt, Germany (22, 23), and Kappe et al. presented analyzed data for invasive aspergillosis cases culled from autopsy records in Heidelberg, Germany (68). In Japan, several studies analyzing data on mycoses from autopsies were reported previously, but reports on recent trends are few. A study by Miyake and Okudaira covered the 13 years between 1948 and 1961 (87), and a study by Hotchi et al. covered the 10 years between 1966 and 1975 (27). Okudaira et al. then reported the analyzed data from 1972 to 1981 (93), and now this report covers the periods after that. As I know wanted to know the impact of FLC and ITC launching, I concentrated my study on the period after 1989.

To discern current trends in visceral mycoses, I have epidemiologically analyzed the data on visceral mycoses that had been reported in the *Annual of the Pathological Autopsy Cases in Japan*, from 1969 to 2009 (31-66). A total numbers of mycosis cases was increasing until 1990, whereas the number of candidiasis cases stopped increasing and began to decrease after 1989 (75, 77), chiefly because of the

effects of a newly developed antifungal drug, FLC. Aspergillosis cases, on the other hand, were not decreasing but came to have the highest rate of mycosis among the total autopsies. In this report, I review the recent trends and also analyze the effect of antifungal agents introduced in the clinical settings.

Materials and Methods

Diagnostic criteria

The criteria for the pathological diagnosis of each class of mycosis described in the *Annual of the Pathological Autopsy Cases in Japan* are not defined definitively by the Japanese Society of Pathology. Basically, the description of each case is the responsibility of the reporting pathologist and depends on his or her ability to make a diagnostic determination. Most of the autopsies included both gross and histopathological examinations, but it cannot be expected that all pathologists were equally rigorous in their examination, and some differences may have been derived from their individual experiences of mycoses. Concerning fungemia or candidemia, reporting pathologists might have been given some clinical information on fungemia from the patient's medical records. Although I could not exclude any ambiguities on these diagnostic data, I used the original descriptions to define the class of mycosis for each case.

Definitions

Mycoses were defined as infections caused by eumycotic organisms such as *Candida*, *Aspergillus*, *Cryptococcus*, Zygomycete, and other fungal species. Infections caused by filamentous bacteria such as Actinomycetes (*Actinomadura*, *Nocardia*, and *Streptomyces* spp.) and pneumocystis pneumonia caused by *Pneumocystis jirovecii* were excluded from the criteria for mycoses. Superficial infections such as dermatophytoses were excluded from the category of visceral mycoses. The term "complicated infection" means a mixed infection with two or more species of fungi, as when cultures from specimens might be identified as containing more than two kinds of

fungi.

Mycotic infections from the autopsy records were defined as severe if they were (i) the direct cause of death; (ii) severe pulmonary infection involving both lobes of the lung; (iii) severe visceral infections of two or more organ systems, including those involving the central nervous system; (iv) multiorgan systemic infection of three or more organ systems; or (v) fungemia.

Data collection

The data for annual total deaths and the number of certified deaths from mycoses were taken from *Vital Statistics of Japan,* edited from 1969 to 1996 by the Minister's Secretariat, Ministry of Health and Welfare (105). And further years of data from 1997 to 2009 were collected from the database edited by the National Statistics Center, the Ministry of Internal Affairs and Communication (108).

Data on visceral mycoses occurring in Japan from 1969 to 2009 were collected in the *Annual of the Pathological Autopsy Cases in Japan*, which was published from 1970 to 2010 by the Japanese Society of Pathology (31-66). Those data were extracted and compiled to make a database for analysis; however, data from the years 1982 to 1988 other than 1985 were not used in this study because they were not available. Cases of stillborn babies were excluded from each annual total number of autopsy cases. The data were compiled into a database by using Filemaker Pro version 5.0, supplied by Filemaker, Inc., and used to look up causative agents of infection, age, sex, underlying diseases, and infected organs of patients.

Results

In recent years, the annual total number of deaths in Japan has been about 1,000,000, and of these, about 1.5 to 4% of bodies are examined by pathological autopsy annually. FIG. 1 shows that among the total deaths, the frequency of mycotic infection (that is, of candidiasis, aspergillosis, or other fungal infections) as the certified direct cause of death had increased noticeably from 1979 to 2009. Before 1994, diseases were classified using the International Classification of Diseases 9 (ICD-9) as the standard diagnostic tool for epidemiology, which did not provide a list of causative agents in detail. The list of fungal causative agents in ICD-10 includes not only *Candida* but also *Aspergillus* and other fungi. After 1994, *Aspergillus* infections have suddenly and dramatically increased.

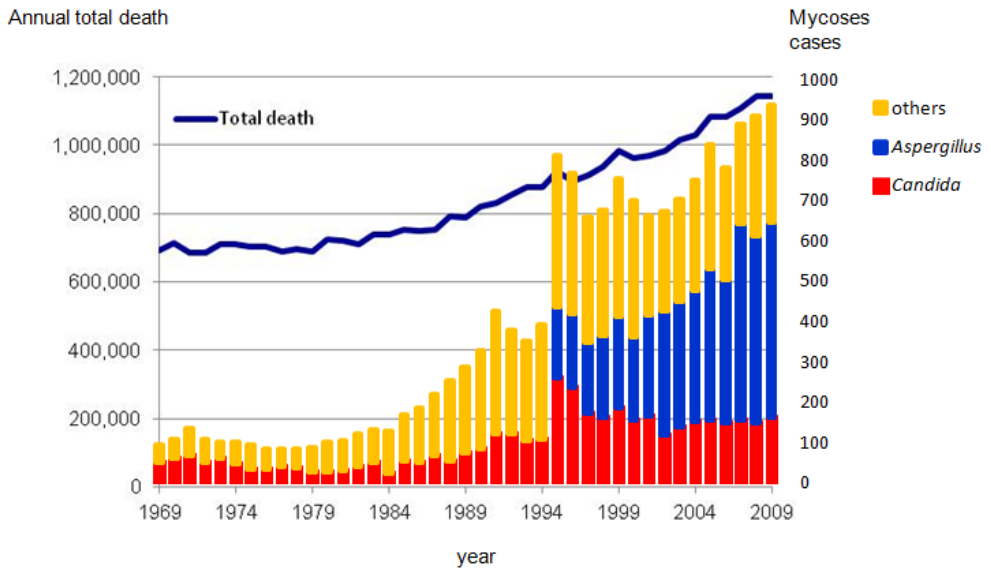


FIG.1 Annual trends of total death and of deaths certified as resulting from mycoses in Japan. Data were extracted from Vital Statistics of Japan, edited by the National Statistics Center, the Ministry of Internal affairs and Communication for the years 1969 through 2009.
—, number of annual total deaths; candidiasis cases; aspergillosis cases; other mycoses cases.

The occurrence of mycoses among total autopsy cases from 1969 to 2009 in

Japan, excluding data from 2006 and 2008, is shown in TABLE 1.

TABLE 1. Change in rates of mycoses among total autopsy cases and of causative agents of mycoses from 1969 to 2009 in Japan

year	Total no. of autopsies	Total no. of mycoses	% of mycoses among total autopsies	% of caes among total mycoses (% of cases among total autopsy)											
				Candida		*Aspergillus*		*Cryptococcus*		Zygomycetes		Others		Unknown [a]		Complicated [b]	
1969	24,715	396	1.60	25.8	(0.41)	24.5	(0.39)	8.8	(0.14)	0.8	(0.01)	0.3	(0.00)	37.6	(0.60)	2.3	(0.04)
1970	23,599	407	1.72	27.0	(0.47)	21.1	(0.36)	9.6	(0.17)	4.7	(0.08)	0.5	(0.01)	34.6	(0.60)	2.5	(0.04)
1971	23,245	433	1.86	28.9	(0.54)	24.7	(0.46)	9.9	(0.18)	3.7	(0.07)	0.2	(0.00)	28.9	(0.54)	3.7	(0.07)
1972	22,769	379	1.66	38.8	(0.65)	31.7	(0.53)	14.0	(0.23)	2.9	(0.05)	0.0	(0.00)	7.7	(0.13)	5.0	(0.08)
1973	23,274	466	2.00	37.8	(0.76)	23.4	(0.47)	12.7	(0.25)	1.3	(0.03)	0.0	(0.00)	21.7	(0.43)	3.2	(0.06)
1974	23,111	531	2.30	35.4	(0.81)	22.6	(0.52)	8.5	(0.19)	1.9	(0.04)	0.2	(0.00)	27.9	(0.64)	3.6	(0.08)
1975	23,048	620	2.69	34.5	(0.93)	17.3	(0.46)	8.1	(0.22)	2.9	(0.08)	0.0	(0.00)	34.5	(0.93)	2.7	(0.07)
1976	24,093	621	2.58	43.0	(1.11)	21.4	(0.55)	9.5	(0.24)	2.3	(0.06)	1.1	(0.03)	18.4	(0.47)	4.3	(0.11)
1977	25,897	664	2.56	37.5	(0.96)	22.6	(0.58)	6.9	(0.18)	3.5	(0.09)	0.2	(0.00)	25.3	(0.65)	4.1	(0.10)
1978	30,742	813	2.64	43.1	(1.14)	23.2	(0.61)	8.2	(0.22)	3.3	(0.09)	1.8	(0.05)	16.9	(0.45)	3.4	(0.09)
1979	32,844	861	2.62	35.4	(0.93)	21.7	(0.57)	9.1	(0.24)	3.0	(0.08)	0.0	(0.00)	25.9	(0.68)	4.9	(0.13)
1980	35,943	970	2.70	49.2	(1.33)	20.7	(0.56)	7.3	(0.20)	3.2	(0.09)	0.0	(0.00)	15.1	(0.41)	4.5	(0.12)
1981	38,841	1,096	2.82	42.0	(1.18)	26.0	(0.73)	7.4	(0.21)	3.8	(0.11)	0.1	(0.00)	15.0	(0.42)	5.7	(0.16)
1985	39,333	1,558	3.96	41.6	(1.65)	30.7	(1.22)	6.5	(0.26)	3.6	(0.14)	0.8	(0.03)	11.0	(0.44)	5.7	(0.23)
1989	37,557	1,672	4.45	42.3	(1.89)	30.1	(1.34)	5.4	(0.24)	3.6	(0.16)	0.2	(0.01)	13.0	(0.58)	5.1	(0.23)
1990	37,399	1,743	4.66	33.6	(1.57)	33.2	(1.55)	4.8	(0.22)	3.5	(0.16)	0.1	(0.00)	20.7	(0.96)	4.1	(0.19)
1991	35,618	1,350	3.79	33.7	(1.28)	34.7	(1.32)	4.7	(0.18)	3.6	(0.13)	0.0	(0.00)	19.2	(0.73)	4.1	(0.16)
1992	33,201	1,177	3.55	36.3	(1.29)	37.3	(1.32)	5.4	(0.19)	4.2	(0.15)	0.0	(0.00)	12.3	(0.44)	4.4	(0.16)
1993	31,207	1,136	3.64	37.2	(1.36)	36.5	(1.33)	4.5	(0.16)	3.4	(0.12)	0.3	(0.01)	14.5	(0.53)	3.5	(0.13)
1994	27,827	882	3.17	35.5	(1.12)	40.7	(1.29)	6.1	(0.19)	3.2	(0.10)	0.1	(0.00)	10.4	(0.33)	4.0	(0.13)
1995	27,926	1,022	3.66	31.0	(1.14)	39.8	(1.46)	6.8	(0.25)	3.7	(0.14)	0.2	(0.01)	15.1	(0.55)	3.3	(0.12)
1996	27,058	1,060	3.92	32.1	(1.26)	41.2	(1.62)	5.0	(0.20)	3.8	(0.15)	0.2	(0.01)	14.2	(0.56)	3.5	(0.14)
1997	26,681	1,143	4.28	34.7	(1.49)	40.9	(1.75)	3.8	(0.16)	3.6	(0.15)	0.2	(0.01)	13.5	(0.58)	3.2	(0.14)
1998	25,961	1,135	4.37	30.3	(1.33)	43.2	(1.89)	4.5	(0.20)	2.8	(0.12)	0.2	(0.01)	15.2	(0.67)	3.8	(0.17)
1999	27,194	1,243	4.57	29.0	(1.32)	43.5	(1.99)	4.5	(0.21)	2.3	(0.11)	0.0	(0.00)	16.7	(0.76)	3.9	(0.18)
2000	26,159	1,051	4.02	30.4	(1.22)	50.7	(2.04)	7.0	(0.28)	3.4	(0.14)	0.0	(0.00)	4.4	(0.18)	4.0	(0.16)
2001	25,459	1,165	4.58	27.4	(1.25)	46.0	(2.10)	5.3	(0.24)	3.5	(0.16)	0.3	(0.01)	14.1	(0.65)	3.4	(0.16)
2002	24,010	976	4.06	27.2	(1.10)	49.1	(2.00)	4.5	(0.18)	2.6	(0.10)	0.2	(0.01)	11.2	(0.45)	5.3	(0.22)
2003	20,948	841	4.01	32.7	(1.31)	45.1	(1.81)	6.2	(0.25)	3.2	(0.13)	0.0	(0.00)	9.2	(0.37)	3.7	(0.15)
2004	19,930	843	4.23	29.5	(1.25)	44.1	(1.87)	6.6	(0.28)	3.9	(0.17)	0.4	(0.02)	12.1	(0.51)	3.3	(0.14)
2005	18,924	872	4.61	28.3	(1.31)	43.3	(2.00)	5.5	(0.25)	4.5	(0.21)	0.2	(0.01)	14.0	(0.64)	4.1	(0.19)
2007	16,384	720	4.39	27.6	(1.21)	45.0	(1.98)	6.1	(0.27)	5.0	(0.22)	0.6	(0.02)	12.6	(0.56)	3.1	(0.13)
2009	13,787	648	4.70	27.9	(1.31)	44.6	(2.10)	5.4	(0.25)	3.9	(0.18)	0.3	(0.01)	14.4	(0.67)	3.5	(0.17)

[a] An unidentified hungus was observed in the infected organ.
[b] Mixed infection with two or more than two kinds of fungi in the infected organ.

The frequency of visceral mycoses in the annual total number of autopsy cases increased significantly from 1.60% in 1969 to a peak of 4.66% in 1990. After 1990, however, this frequency decreased gradually, from 3.79% in 1991 to 3.17% in 1994. After 1994, the mycoses rate increased again and during the 10 years between 1999 and 2009, the frequency of visceral mycoses fluctuated between 4.01 and 4.70%, with a peak in 2009. Candidiasis also increased from 0.41% in 1969 to a peak of 1.89% in 1989 and then decreased to around 1.3% by degrees after 1991. In contrast, the aspergillosis rate rose from 0.39% in 1969 to a peak of 2.1% in 2001 and has maintained a constant level of about 1.9% from 1999. During this period, the rate of zygomycosis increased slowly (ranging from 0.01% to 0.22%), whereas that of

cryptococcosis was not remarkably changed (ranging from 0.14% to 0.28%).

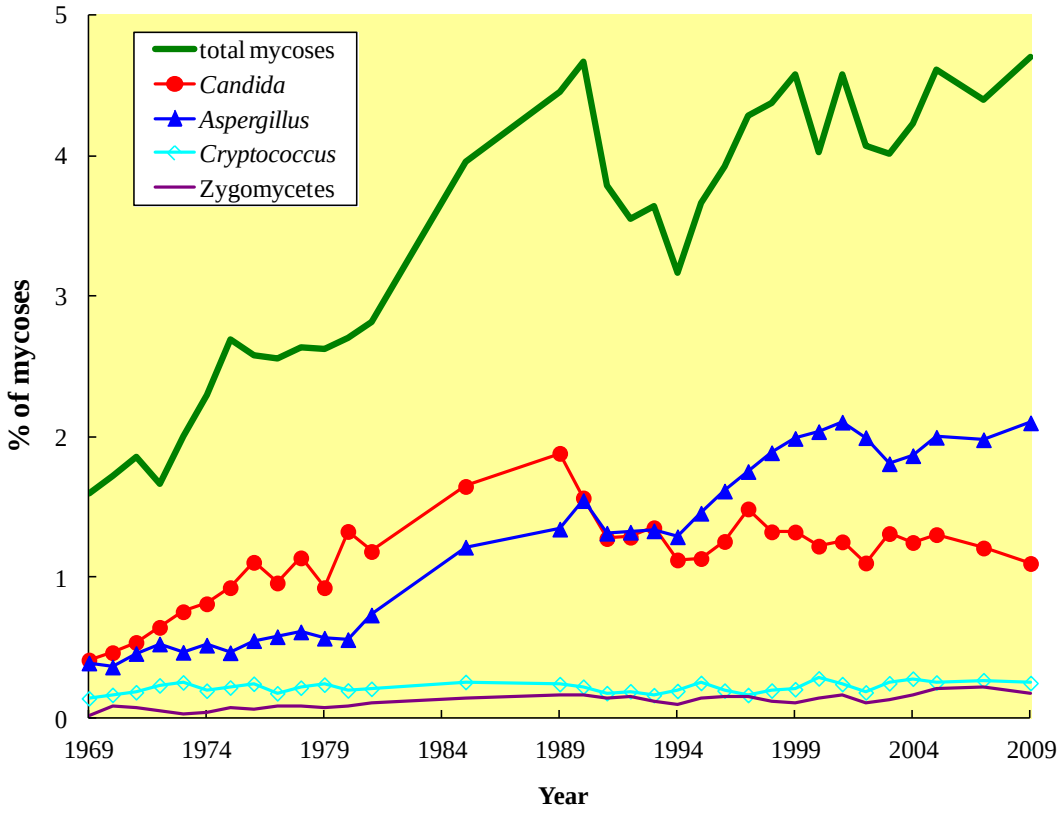


FIG. 2 Annual trends of total visceral mycoses reported in autopsy cases and the major causative agents.

Until 1989, the predominant causative agent was *Candida*, followed by *Aspergillus* and then *Cryptococcus*. Although the rate of candidiasis decreased by degrees from 1990, the rate of aspergillosis increased, and surpassed that of candidiasis in 1991. Since 1994, the rate of aspergillosis has increased rapidly, and matches an increase reported by the National Statistics Center in mycoses as the certified direct cause of death (FIG. 2). When the annual figures for causative agents of visceral mycoses in 1989, 1999, and 2009 were compared, the predominant causative agent has changed from *Candida* to *Aspergillus* over the two decades (FIG. 3).

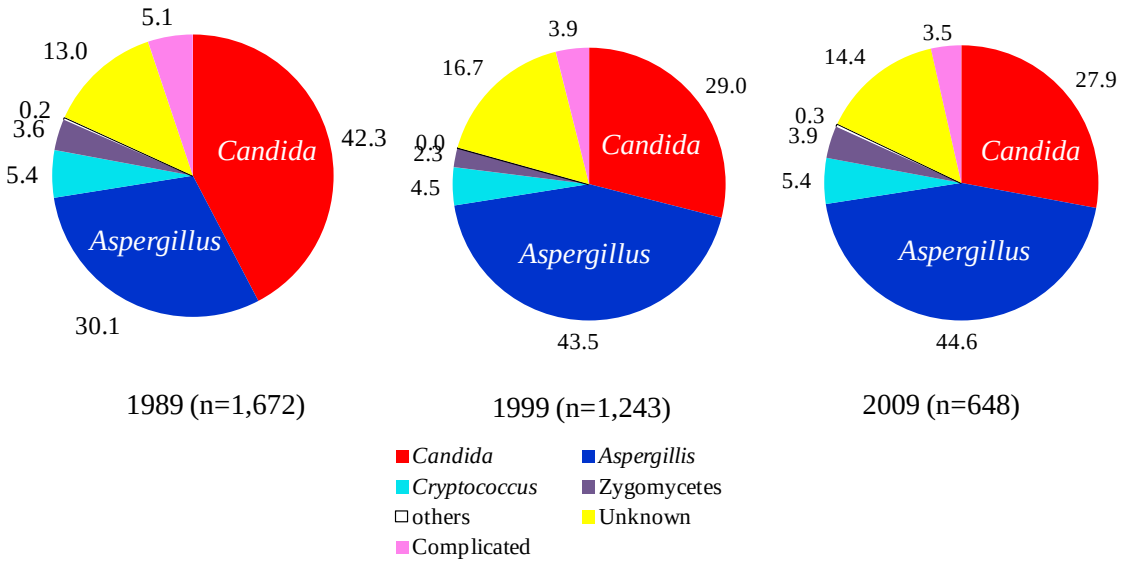


FIG. 3 A comparison of causative agents for visceral mycoses in autopsy cases.

Each agent is reported as a percentage of annual total mycoses during two decades.

TABLE 2 lists the organ distribution of these causative agents. TABLE 2a shows the frequency between 1989 and 1994 and reveals that 37.2% of candidiasis was observed in the digestive ducts (mouth and tongue 2.2%, esophagus 15.9%, stomach 11.1%, and intestine 8.0%), 34.7% was observed in the lung and bronchial system, and 23.3% in the kidney. The frequency of heart infections by *Candida* was also high at 13.4%, and high rates of systemic candidiasis (16.7%) and candidemia (13.7%) were also observed. For aspergillosis, the most commonly infected organ system comprised the lung and bronchia (83.9%); other organs were not involved at a high rate. *Cryptococcus* also infected the lungs and bronchia most frequently (64.0%), followed by the brain and meninx (21.5%). Zygomycosis was also observed most commonly in the lung and bronchia (69.4%), followed by the liver (9.6%) and kidney (9.1%). Ten year later, substantial changes in these distribution trends were seen only in candidiasis

(TABLE 2b). In particular, the rate of *Candida* infections other than candidemia decreased by a range of between a quarter and a half. Changes in the organ distribution of other causative agents were not remarkable.

TABLE 2

a) Distribution of causative agents of mycoses by organ; data from 1989 to 1994

Infection type or organ	% of infections caused by :			
	Candida (N=2,172)	*Aspergillus* (N=1,967)	*Cryptococcus* (N=289)	Zygomycetes (N=209)
Total	100.0	100.0	100.0	100.0
Systemic	16.7	8.6	13.5	9.6
Fungemia	13.7	4.3	4.8	5.3
Brain + meninx	4.1	3.3	21.5	5.3
Mouth + tongue	2.2	0.3	0.0	0.0
Esophagus	15.9	1.3	0.7	0.5
Stomach	11.1	2.9	0.3	7.2
Intestine	8.0	2.6	0.0	4.8
Liver	8.1	3.6	3.5	9.6
Larynx + pharynx	1.2	0.3	0.0	0.0
Lung + bronchia	34.7	83.9	64.0	69.4
Heart	13.4	7.4	3.8	7.7
Kidney	23.3	7.3	9.7	9.1
Bladder	3.5	0.5	0.0	0.0
Thyroid	3.5	3.5	2.4	3.3
Spleen	3.7	2.2	6.2	6.2

b) Distribution of causative agents of mycoses by organ; data from 2000 to 2005

Infection type or organ	% of infections caused by :			
	Candida (N=1,675)	*Aspergillus* (N=2,677)	*Cryptococcus* (N=336)	Zygomycetes (N=201)
Total	100.0	100.0	100.0	100.0
Systemic	8.2	5.9	12.2	17.9
Fungemia	13.5	3.3	4.8	8.5
Brain + meninx	2.0	1.9	19.3	7.0
Mouth + tongue	2.9	0.2	0.3	0.0
Esophagus	17.7	0.6	0.9	1.0
Stomach	8.7	1.8	0.9	4.5
Intestine	5.5	1.2	1.5	6.5
Liver	3.7	1.3	6.5	7.0
Larynx + pharynx	0.8	0.1	1.5	0.0
Lung + bronchia	25.6	74.9	55.4	49.3
Heart	7.9	5.0	1.8	9.5
Kidney	10.4	3.4	6.3	7.0
Bladder	2.4	0.1	0.0	1.0
Thyroid	1.1	1.2	3.0	1.5
Spleen	1.6	0.6	4.5	2.0

TABLE 3. Organ distribution data categorized by underlying diseases

Disease type	Diagnosis	a) Mycoses in 4 years total of two categories between 1989-1993: pts total[a]	mycosis	%	Diagnosis	b) Mycoses in 6 years total of two categories between 2000-2009: pts total[l]	mycosis	%
Solid cancer	Lung	15,340	369	2.4	Lung	13,724	368	2.7
	Stomach	12,719	227	1.8	Stomach	10,004	148	1.5
	Liver	13,985	167	1.2	Liver	10,307	164	1.6
	Pancreas	5,046	100	2.0	Pancreas	4,463	105	2.4
	Colon	7,140	82	1.1	Colon	6,934	84	1.2
	Esophagus	3,124	58	1.9	Esophagus	2,846	66	2.3
	Gall bladder	3,478	67	1.9	Gall bladder	2,101	32	1.5
	Breast	2,274	37	1.6	Breast	1,634	34	2.1
	Uterus	2,068	40	1.9	Uterus	1,388	25	1.8
	Prostate	3,278	29	0.9	Prostate	5,279	35	0.7
	Ovary	1,248	32	2.6	Ovary	945	40	4.2
	Kidney	2,110	38	1.8	Kidney	2,158	22	1.0
	Bladder	1,590	41	2.6	Bladder	1,610	29	1.8
	Brain	1,824	37	2.0	Brain	1,205	27	2.2
	Thyroid	3,005	15	0.5	Thyroid	2,695	4	0.1
	Pharynx	752	20	2.7	Pharynx	641	13	2.0
	total	*78,981*	*1,359*	*1.7*	*total*	*67,934*	*1,196*	*1.8*
Blood and hematopoietic system diseases	AML[b]	1,753	620	35.4	AML	1,883	590	31.3
	CML[c]	424	185	43.6	CML	454	127	28.0
	ALL[d]	745	319	42.8	ALL	533	264	49.5
	CLL[e]	119	17	14.3	CLL	126	18	14.3
	MoL[f]	278	49	17.6	MoL	135	30	22.2
	MDS[g]	473	105	22.2	MDS	974	192	19.7
	ATL[h]	535	116	21.7	ATL	540	121	22.4
	other leukemia[i]	1,740	95	5.5	other leukemia	899	101	8.3
	(Leukemia and MDS)	*6,067*	*1,506*	*24.8*	*(Leukemia and MDS)*	*5,544*	*1,443*	*26.0*
	Malignant lymphoma	4,649	487	10.5	Malignant lymphoma	5,782	505	8.7
	Multiple myeloma	1,834	170	9.3	Multiple myeloma	1,577	90	5.7
	Aplastic anemia	388	103	26.5	Aplastic anemia	1,613	95	5.9
	DIC[j]	1,761	18	1.0	DIC	5,549	54	1.0
	Purpura[k]	130	12	9.2	Purprua	226	23	10.2
	Immune dis.	52	10	19.2	Immune dis.	483	44	9.1
	AIDS	109	25	22.9	AIDS	208	61	29.3
	(Total for other hematopoietic system diseases)	*8,923*	*825*	*9.2*	*(Total for other hematopoietic system diseases)*	*15,438*	*872*	*5.6*
	Total	14,990	2,331	15.6	Total	20,982	2,315	11.0

[a]Data were taken from autopsy cases of 1989, 1990, 1991, and 1993.
[b]AML, acute myeloid leukemia; [c]CML, chronic myeloid leukemia; [d]ALL, acute lymphatic leukemia; [e]CLL, chronic lymphatic leukemia; [f]MoL, monocystic leukemia; [g]MDS, myeloldysplastic syndrome, [h]ATL, adult T-cell leukemia; [i]other leukemia: includes other leukemia and non-specified leukemia; [j]DIC, disseminated intravascular coagulation syndrome; [k]Purpura, idiopathic thrombocytopenic purpura or thrombotic thrombocytopenic purpura. [l]Data were taken from 2000, 2002-2004, 2007, and 2009.

In a more detailed analysis of mycoses in the underlying diseases, I compared the rate of mycoses in autopsy cases of solid cancer patients with that of patients with

blood and hematopoietic system diseases to understand which patients were at greater risk of dying from mycoses after contracting an underlying disease. Between 1989 and 1993, in autopsied patients with leukemia or myelodysplastic syndrome (MDS), 24.8% had visceral mycoses, whereas in patients with hematopoietic system diseases other than leukemia the figure was 9.2%, and in patients with solid cancers was 1.7%. Therefore, the combined frequency rate of 15.6% for the blood and hematopoietic system diseases, including leukemia, malignant lymphoma, aplastic anemia, multiple myeloma, etc., was 9.1 times the rate of 1.7% for solid cancers. If the comparison is narrowed down to autopsied leukemia and MDS patients, the frequency of mycosis was more than 14.6 times higher than in solid-cancer patients (24.8 and 1.7%, respectively). A breakdown of the frequency of mycosis within each of these major underlying diseases over the same period shows that visceral mycoses were observed in solid cancers more frequently in pharyngeal cancer (2.7%), ovarian cancer (2.6%), bladder cancer (2.6%), and lung cancer (2.4%), and were observed in blood and hematopoietic system diseases, more frequently in chronic myeloid leukemia (CML) (43.6%), acute lymphatic leukemia (ALL) (42.8%) and acute myeloid leukemia (AML) (35.4%) (TABLE 3a). In the data from 2000 to 2009, almost the same tendency can be observed (TABLE 3b). Visceral mycoses in autopsied leukemia and MDS patients accounted for 26.0%, followed by patients with other blood and hematopoietic system diseases other than leukemia at 5.6%, and by patients with solid cancers at 1.8%. The combined frequency rate for blood and hematopoietic system diseases of 11.0% is 6.5 times higher than that for solid cancers of 1.8%. In this period, mycoses were observed within the autopsied solid cancer patients most frequently in those with ovarian cancer (4.2%), lung cancer (2.7%), pancreatic cancer (2.4%), and esophageal cancer (2.3%), and within

patients with blood and hematopoietic system diseases most frequently in those with ALL (49.5%), AML (31.3%), AIDS (29.3%), and CML (28.0%).

In both periods, the number of patients with solid cancer that were autopsied (in each 78,981 and 67,934) in much greater than those with leukemia and MDS (in each 6,067 and 5,544), but an examination of the rate of mycoses in the two underlying diseases shows that mycoses may occur with a much higher frequency in patients with leukemia or MDS than in those with solid cancers (TABLE 3a, b).

TABLE 4. Causative agents of mycoses in the major underlying diseases compared for the periods 1989 to 1994 and 2000 to 2005

1989-1994	Leukemia and MDS		Soild cancers		Lymphoma		Myeloma		Organ transplantation	
	n	%	n	%	n	%	n	%	n	%
Candida	397	26.4	713	48.0	178	36.6	64	37.6	3	30.0
Aspergillus	564	37.5	377	25.4	151	31.0	50	29.4	2	20.0
Cryptococcus	25	1.7	90	6.1	19	3.9	13	7.6	2	20.0
Zygomycetes	101	6.7	23	1.5	17	3.5	12	7.1	0	0.0
Other	5	0.3	2	0.1	1	0.2	0	0.0	0	0.0
Unknown	296	19.7	246	16.6	102	20.9	23	13.5	2	20.0
Complicated	118	7.8	34	2.3	19	3.9	8	4.7	1	10.0
Total	1,506	100.0	1,485	100.0	487	100.0	170	100.0	10	100.0

2000-2005	Leukemia and MDS		Soild cancers		Lymphoma		Myeloma		Organ transplantation	
	n	%	n	%	n	%	n	%	n	%
Candida	217	18.2	450	41.6	156	28.8	44	22.6	68	17.9
Aspergillus	634	53.1	402	37.2	259	47.9	108	55.4	211	55.5
Cryptococcus	23	1.9	74	6.8	18	3.3	13	6.7	6	1.6
Zygomycetes	106	8.9	10	0.9	21	3.9	4	2.1	19	5.0
Other	4	0.3	0	0.0	1	0.2	0	0.0	2	0.5
Unknown	144	12.1	127	11.7	58	10.7	17	8.7	56	14.7
Complicated	66	5.5	18	1.7	28	5.2	9	4.6	18	4.7
Total	1,194	100.0	1,081	100.0	541	100.0	195	100.0	380	100.0

When the causative mycotic agents for the four major underlying diseases,

(leukemia including MDS, solid cancer, lymphoma, and myeloma) and solid-organ transplantation cases were compared (TABLE 4), aspergillosis was the most predominant causative agent in leukemia and MDS, between 1989 and 1994, followed in order by candidiasis, zygomycosis, and cryptococcosis; however, for other three underlying diseases, candidiasis was the most frequent disease, followed by aspergillosis and cryptococcosis. In contrast, from 2000 to 2005, the predominant causative agent in these four major underlying diseases and in organ transplantation was aspergillosis followed by candidiasis. Mycoses in organ transplanted patients were reported in only 10 cases in the earlier period, but featured in 380 cases in the later period. Most systemic or multiorgan mycotic infections caused patients to die within a short period, responded poorly to currently available antifungal agents, or required a long period of treatment.

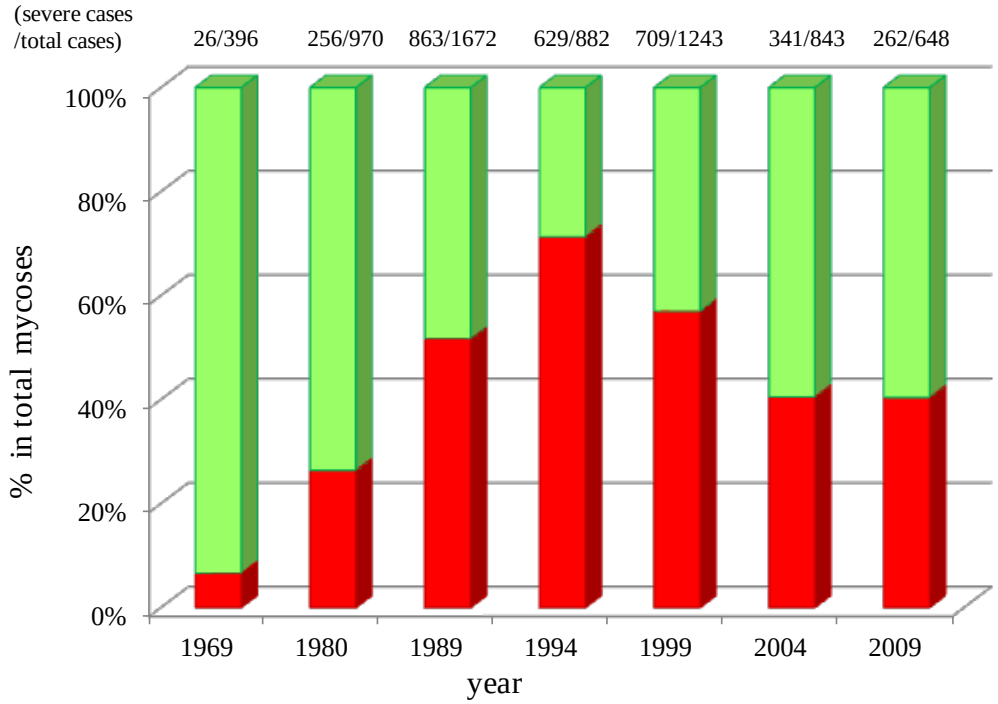
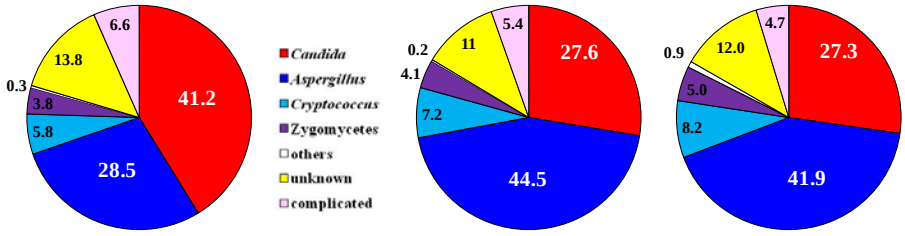


FIG. 4 Increase in the proportion of severe infections within total mycoses.
■ severe infection; ■ non-severe infection.

The frequency of severe mycotic infections has also increased dramatically over the 25-year study period, from 6.6% of the total visceral mycosis cases in 1969 to 71.3% in 1994 (FIG. 4). After 1994, however, the rate of severe infection decreased to around 40% over the next 15 years. When the proportions of causative agents for the severe mycoses were compared for the years 1989 and 1994, candidiasis (41.2%) was found to be the most frequent mycosis, followed by aspergillosis (28.5%), in 1989, but aspergillosis (44.5%) surpassed candidiasis (27.6%) in 1994 (FIG. 5). Furthermore, this ratio in 1994 was maintained in 2004.



(n=863) 1989 (n=629) 1994 (n=341) 2004

FIG. 5. Comparison of the proportion of causative agents for severe mycoses in 1989, in 1994 and in 2004.

Each agent is reported as a percentage of total mycoses. Severe candidiasis cases decreased but aspergillosis cases increased in 1994, a situation that was maintained in 2004.

Analysis of the frequency of severe infections for each causative agent showed that 93% of zygomycosis, 83% of cryptococcosis, 78% of aspergillosis, and 56% of candidiasis cases were found among the severe cases in 1994. The rate for each agent had been almost exactly 50% in 1989 (data not shown).

TABLE 5. Frequencies and rates of the visceral mycoses reported by age [a]

1993+1994

Age	Total no. of autopsy cases	Total mycoses		*Candida*		*Aspergillus*		*Cryptococcus*		Zygomycetes		others		unknown		complicated	
		cases	%	cases	%	cases	%	cases	%	cases	%	cases	%	cases	%	cases	%
Neonatal babies	1,491	23	1.54	18	1.21	0	0.00	0	0.00	0	0.00	0	0.00	5	0.34	0	0.00
0-9	1,324	40	3.02	17	1.28	12	0.91	0	0.00	2	0.15	0	0.00	7	0.53	2	0.15
10-19	532	52	9.77	14	2.63	25	4.70	0	0.00	4	0.75	0	0.00	6	1.13	3	0.56
20-29	836	84	10.05	36	4.31	34	4.07	2	0.24	5	0.60	0	0.00	6	0.72	1	0.12
30-39	1,203	84	6.98	29	2.41	31	2.58	5	0.42	5	0.42	0	0.00	11	0.91	3	0.25
40-49	3,942	156	3.96	39	0.99	75	1.90	10	0.25	5	0.13	0	0.00	20	0.51	7	0.18
50-59	8,169	312	3.82	105	1.29	120	1.47	14	0.17	10	0.12	0	0.00	47	0.58	14	0.17
60-69	16,385	544	3.32	192	1.17	209	1.28	27	0.16	18	0.11	2	0.01	75	0.46	22	0.13
70-79	15,561	537	3.45	194	1.25	220	1.41	30	0.19	11	0.07	1	0.01	62	0.40	19	0.12
80-	9,567	186	1.94	92	0.96	48	0.50	17	0.18	7	0.07	1	0.01	18	0.19	4	0.04
Total	59,010	2,018	3.42	736	1.25	774	1.31	105	0.18	67	0.11	4	0.01	257	0.44	75	0.13

2003+2004

Age	Total no. of autopsy cases	Total mycoses		*Candida*		*Aspergillus*		*Cryptococcus*		Zygomycetes		others		unknown		complicated	
		cases	%	cases	%	cases	%	cases	%	cases	%	cases	%	cases	%	cases	%
Neonatal babies	602	5	0.83	3	0.50	0	0.00	0	0.00	0	0.00	0	0.00	2	0.33	0	0.00
0-9	624	20	3.21	13	2.08	3	0.48	0	0.00	1	0.16	0	0.00	3	0.48	0	0.00
10-19	217	13	5.99	5	2.30	3	1.38	0	0.00	2	0.92	0	0.00	2	0.92	1	0.46
20-29	407	25	6.14	5	1.23	17	4.18	0	0.00	0	0.00	0	0.00	2	0.49	1	0.25
30-39	882	33	3.74	12	1.36	13	1.47	1	0.11	1	0.11	0	0.00	6	0.68	0	0.00
40-49	1,729	84	4.86	20	1.16	40	2.31	5	0.29	3	0.17	0	0.00	10	0.58	6	0.35
50-59	5,163	264	5.11	74	1.43	127	2.46	10	0.19	12	0.23	1	0.02	28	0.54	12	0.23
60-69	9,350	398	4.26	124	1.33	177	1.89	21	0.22	18	0.19	1	0.01	40	0.43	17	0.18
70-79	13,304	564	4.24	177	1.33	250	1.88	51	0.38	17	0.13	1	0.01	49	0.37	19	0.14
80-	8,336	276	3.31	91	1.09	119	1.43	20	0.24	6	0.07	0	0.00	37	0.44	3	0.04
Total	40,614	1,682	4.14	524	1.29	749	1.84	108	0.27	60	0.15	3	0.01	179	0.44	59	0.15

[a] The highest frequency of mycoses was observed for individuals in their 20s, whereas the highest incidence was in those in their 60s and 70s in each period. Neonatal babies showed a tendency to suffer from candidiasis due to an endogenous pathogen. These data were taken from the data of 1993, 1994, 2003, and 2004.

In comparison of the mycoses rate over 2 years from 1993 to 1994 and that from 2003 to 2004, the highest frequency of mycoses by age was observed in patients in their 20s, whereas the highest incidence was observed in those in their 60s and 70s. Observing the figures over the decade, there was no difference between the period 1993 to 1994 and 2003 to 2004. Particularly the neonatal babies showed a tendency to suffer from candidiasis, which might be caused by endogenous pathogens (TABLE 5).

There was no remarkable difference between genders in the frequency of visceral mycoses in total autopsies as determined from the 1993 and 1994 compiled data. There was little difference in candidiasis between males and females (1.21% and 1.31%, respectively); cryptococcosis was found at a higher frequency in females than in males (0.29% and 0.13%, respectively) in total autopsy cases, whereas aspergillosis was found

slightly more frequently in males (1.39%) than in females (1.18%). The frequency of mycoses in both sexes relative to total autopsies was almost the same (3.4%). Almost the same trend was observed in the data compiled in 2003 and 2004 (TABLE 6).

TABLE 6. The ratio between the sexes of mycoses in total autopsy cases

a. 1993+1994

	mycoses cases		ratio of pathogen (%)		rate in total autopsies (%)	
	M[a]	F[a]	M	F	M	F
Candida	453	282	35.4	37.8	1.21	1.31
Aspergillus	520	254	40.7	34.0	1.39	1.18
Cryptococcus	43	62	3.4	8.3	0.11	0.29
Zygomycetes	42	25	3.3	3.3	0.11	0.12
others	13	1	1.0	0.1	0.03	0.00
unknown	162	95	12.7	12.7	0.43	0.44
complicated	46	28	3.6	3.7	0.12	0.13
total	1,279	747	100.0	100.0	3.41	3.47

b. 2003+2004

	mycoses cases		ratio of pathogen (%)		rate in total autopsies (%)	
	M	F	M	F	M	F
Candida	327	197	29.5	34.1	1.23	1.38
Aspergillus	516	235	46.6	40.7	1.94	1.65
Cryptococcus	54	54	4.9	9.4	0.20	0.38
Zygomycetes	44	16	4.0	2.8	0.17	0.11
others	2	1	0.2	0.2	0.01	0.01
unknown	129	50	11.7	8.7	0.49	0.35
complicated	35	24	3.2	4.2	0.13	0.17
total	1,107 0	577	100.0	100.0	4.16	4.05

[a] M: male, F: female. There was no remarkable difference between the gender in the data of 1993 and 1994, and of 2003 and 2004

Discussion

Candidiasis is the most common mycotic disease in Japan, requiring the treatment of 74,000 patients (ca. 6,000 males and ca. 68,000 females) who received medical treatment in hospitals or clinics in 1993 (106). The most frequently presented symptoms were of *Candida* vaginitis in women in their 20s and 30s; however, very few of the visceral-candidiasis patients succumbed to the disease. FIG. 1 shows the frequency of mycoses that were certified as a direct cause of death. Total deaths increased from 700,000 in 1969 to 1,150,000 in 2009, and mycotic infections also increased from 93 cases in 1969 to 939 cases in 2009. This total may well be inaccurate because, even if a patient suffers from a mycosis and dies from that mycosis in the presence of an underlying disease, the difficulty in making a diagnosis may mean that the direct cause of death is certified as being the underlying disease. From 1995, when ICD-10 started to be used to ascribe causative agents, *Aspergillus* came to be a major causative agent directly responsible for death. From then until 2009, and despite the change in categorizing the causative agents, total mycoses cases have been increasing and the involvement of aspergillosis has been increasing explosively.

This epidemiological and etiological study used the retrospective autopsy data that was compiled by the Japanese Society of Pathology gathered from university hospitals, public hospitals, and large private hospitals all over Japan. Visceral mycoses continued to increase up to 1990, but from 1991 they started to decrease, with candidiasis cases in particular tending to decrease after 1989. In contrast, the increase in aspergillosis cases up to 1990 remained constant at 1.3% of total autopsy cases from 1990 to 1994. Groll et al. also reported almost the same trend for the increase of aspergillosis in their pathological autopsy data obtained in Frankfurt, Germany (22, 23).

The major reason for this turnaround in the data is thought to be the introduction of FLC in Japan. FLC treatment has likely decreased the cases of both severe and non-severe *Candida* infections; however, its activity against aspergillosis would be limited (2, 10, 74). Kujath and Lerch reported their clinical experience in treating *Aspergillus* infections of multiple soft-tissue injuries, which did not improve when treated with FLC (300 mg daily for 16 days) (74). Anaissie et al. also reported that one patient with pneumonia due to *Aspergillus glaucus* responded only partially to FLC at 2,000 mg/day and that three patients did not respond to high-dose FLC treatment (more than 800 mg/day) (2). Thus, they concluded that the activity of FLC was limited in severe infections by *Aspergillus* species and other molds.

In Japan, the introduction of FLC in the middle of 1989 was a kind of turning point against candidiasis (18, 29, 75) but not against other severe mycoses such as invasive aspergillosis. Although ITC was introduced in 1993, the effect of ITC on clinical outcomes is not clear within this surveyed period. When the causative agents for 1989 and 1994 were compared, we could see that the predominant causative agent for severe mycotic infection had shifted from *Candida* to *Aspergillus*, and the proportion of severe infections among the total mycoses had increased from 6.6% in 1969 to 71.3% in 1994 (FIG. 4, 5). I surmise that the reasons for this decrease of candidiasis combined with an increase of aspergillosis or of other severe mycotic infections might be that (i) antifungal-responsive infections were excluded from the case totals, because they had become controllable by antifungal drugs such as FLC (launched in the middle of 1989 in Japan); (ii) empirical therapy was commonly given to prevent immunocompromised patients from acquiring primary mycoses; (iii) available antifungal drugs were partially efficacious for severe infections; (iv) diagnostic techniques for both candidiasis and

aspergillosis were inadequate and not yet fully developed until the end of 1994 (19, 72, 110, 119); or (v) the number of patients living longer in an immunocompromised state increased as a result of developments in chemotherapy, solid organ transplantation, and bone marrow transplantation (BMT).

Until 1994, kidney transplantation was not uncommon, even in Japan, but liver transplantation was still rare and BMT was so rare that we could not find the report of mycoses after BMT in the reports of pathological autopsy cases up to 1994. Recently, the number of solid organ transplantation cases is increasing, and BMT is a common procedure in Japan. Similarly, although many AIDS patients have been diagnosed in the United States and European countries, AIDS patients were rare in Japan until 1994 (15, 21, 23). In fact, more recent data shows that more than 20% of patients suffering from AIDS have been reported to have mycotic infection as a complication (TABLE 3). Even now, cases of severe visceral mycoses are essentially uncontrollable, and especially *Aspergillus* infections were still increasing in 2009 (7, 14, 23, 117). Therefore, there is an urgent unmet medical need for drugs that are highly potent against visceral mycoses and have efficacy against aspergillosis, azole-resistant *Candida* strains, or non-*albicans* *Candida* species, as well as other mycoses.

With respect to the affected-organ distribution data (TABLE 2), the rate of candidemia was higher than that of fungemia from *Aspergillus*, *Cryptococcus*, or zygomycetes. The increasing use of indwelling intravenous catheters might be one major infection route for fungemia, and *Candida* as an endogenous pathogen infects more easily than other pathogens. According to the report of the nosocomial bloodstream infections (BSIs) in the US hospitals, of 24,179 patients with BSIs from 1995 to 2002, 9.0% was caused by *Candida* (118). *Candida* BSI was the fourth most

common in all BSIs and followed the coagulase negative *Staphylococcus*, *Staphylococcus aureus*, and enterococci. However, BSIs from other fungal species was not reported. Although the causative agents affected organs differently, the lung and bronchial system were most frequently involved, regardless of the pathogen species. This suggests that the lung and bronchia are at the highest risk of being exposed to not only exogenous pathogens, such as *Aspergillus*, *Cryptococcus*, or zygomycetes, but also to *Candida* species. Although *Candida* species are normally found commensally in the digestive tract, it is possible for them to be a major causative agent of systemic infection in immunocompromised patients and of topical infections in healthy individuals. Further, *Candida* is known to be involved in nosocomial transmission (5), which might explain why the lungs and bronchia are found to be the organs predominantly infected by *Candida*. To support this, *Candida* infections were also observed more commonly than those of *Aspergillus*, *Cryptococcus*, and zygomycetes species in the esophagus and stomach.

The data from autopsy surveys include patients of antemortem-diagnosed cases as well as those recognized by postmortem necropsy. The former cases include those not completely cured at the time that death occurred from the underlying disease or the mycosis. In Japan, only about 3% of bodies are subjected to pathological autopsy. I assume that even if we were able to analyze all patients who died from disease, the frequency of mycotic infection would probably not change much statistically. However, if I could count all mycosis patients regardless of their good or bad convalescence, the rate of mycoses would probably be greatly different from the result that we arrived at in this study. This is because most of the data on mycoses obtained at autopsy must be biased toward those patients who had died from malignant diseases. Almost all systemic

fungal infections, especially those caused by *Aspergillus* or zygomycetes, in immunocompromised patients are refractory to all antifungal agents currently available, even though effective drugs do exist for less severe or locally limited infections. I would expect, however, that many such patients could be cured of or prevented from contracting systemic mycoses with aggressive effective treatments. Despite the limitations inherent in individual autopsy data in Japan, they are still worthy of use to gain much epidemiological and etiological information. Nevertheless, further information could be derived by using newer data, as they become available, and with other means of analysis.

Part II

In vitro Activity of Isavuconazole against 140 Reference Fungal Strains and 165 Clinically Isolated Yeasts from Japan

Abstract

In vitro susceptibilities of 140 laboratory reference strains of fungi, including type strains, and 165 clinical yeast isolates from Japan towards a CYP51 inhibitor, isavuconazole, compared with fluconazole (FLC), itraconazole (ITC), voriconazole (VLC) and amphotericin B (AmB) were measured. Broth microdilution methods based on Clinical and Laboratory Standards Institute (CLSI) methods were used for yeasts, and RPMI-MOPS medium semi-solidified with 0.2% low-melting-point agarose, based on CLSI guidelines was used for molds. The range of isavuconazole minimum inhibitory concentrations (MICs) was 0.0004–0.21 mg/L for *Candida albicans*, 0.0036–0.4 mg/L for *Candida glabrata*, 0.023–0.058 mg/L for *Candida krusei*, 0.0026–0.032 mg/L for *Cryptococcus neoformans*, 0.1–0.39 mg/L for *Aspergillus fumigatus* and 0.2–0.39 mg/L for *Aspergillus terreus*. Isavuconazole was as active as ITC against the dimorphic true pathogenic fungi, with a range of MICs from <0.0004 mg/L to 0.0063 mg/L for *Blastomyces dermatitidis* and *Histoplasma capsulatum*. It was also active against uncommon dematiaceous fungi such as *Exophiala* spp. and *Phialophora* spp. as well as against dermatophytic species. Isavuconazole showed very good *in vitro* antifungal activity with a broad spectrum, including against FLC-resistant *Candida* spp., *Aspergillus* spp. and uncommon opportunistic fungal species. No cross-resistance was found to isavuconazole amongst FLC-resistant strains. Against *Fusarium, Pseudallescheria, Scedosporium*, and zygomycetes species, isavuconazole showed a limited or partial activity, and these fungi species are probably intrinsically resistant to azoles.

The following text in the abstract has been omitted pending regulatory approval of Isavuconazole.

Introduction

Despite advances in antifungal therapy, fungal infections remain a major cause of morbidity and mortality in immunocompromised patients, with fatality rates of 50–100% in such patients (85). *Candida* and *Aspergillus* are the major opportunistic pathogens and are the predominant causative agents of mortality in patients with hematological malignancies (76). *Candida albicans* is the leading cause of neonatal fungal sepsis, but non-*albicans Candida* species have become more frequent causative organisms (82).

AmB has long been regarded as the gold standard for the treatment of invasive and disseminated fungal infections in critically ill patients. However, the drug has serious safety concerns associated with renal toxicity, hypokalemia, and hypomagnesemia (111), and recent case reports documented clinical failure with this agent against infections caused by non-*albicans Candida* such as *C. rugosa* (9), *C. lusitaniae* (84) and *C. glabrata* (73). Azoles are potent compounds; however, some of the drugs on the market have problems such as a narrow spectrum and resistance to FLC, or specific issues, such as the variability in the bioavailability of posaconazole (112) and the drug-drug interactions of ITC (11). The echinocandins have emerged as useful therapeutic options for invasive candidiasis but case reports describing reduced *in vitro* activity and clinical failure of caspofungin against *C. albicans*, *C. glabrata*, and *C. krusei* have appeared (25, 26, 71, 86). There exists an eminent need for more potent and safer antifungal compounds available for both parenteral and oral administration. Therefore, the target enzyme of the ergosterol synthesis pathway has been chosen to make a novel antifungal agent. The enzyme of CYP51 (=ERG11) is a lanosterol C14 α-demethylase that converts lanosterol into ergosterol, and plays an important role in

ergosterol synthesis. Azole compounds are well-known validated inhibitors of CYP51. RO0094815 was screened from hundreds of triazole derivatives synthesized, and showed a potent inhibitory activity against fungal pathogens *in vitro* and *in vivo* models (121). RO0094815 was named isavuconazole (81). As the isavuconazole is poorly soluble in water, that compound was specially designed as a water-soluble prodrug derivative as isavuconazo-lium (-nium) for both oral and intravenous administration available (91).

I show here that isavuconazole has a broad spectrum of action *in vitro*. The results of the comparison of the antifungal activity data of four azoles and AmB against reference strains suggest that using broth microdilution methods and spectrophotometry to turbidimetrically measure growth are very helpful when performing *in vitro* antifungal susceptibility tests.

At present, this potent antifungal drug that was developed as a result of this research is now in clinical phase III studies in the US. Some studies have been completed and the latest result of one study has met the primary endpoint.

Materials and Methods

Antifungal drugs

Isavuconazole and VRC were synthesized at the Nippon Roche Research Center (now Kamakura Research Laboratories, Chugai Pharmaceutical Co., Kanagawa, Japan), and ITC and FLC were prepared from the commercial products Itrizole® (Janssen Pharmaceutical K.K., Tokyo, Japan) and Diflucan® (Pfizer Japan Inc., Tokyo, Japan). AmB was purchased from Sigma Chemical Co. (St Louis, MO). All drugs were dissolved at a concentration of 20 mg/mL with dimethyl sulfoxide (DMSO) and were stored at temperatures below −20ºC.

Fungal strains

In this study, 140 laboratory reference strains of fungi and 165 yeast clinical isolates from Japan were examined. Strains purchased from the American Type Culture Collection (ATCC, Manassas, VA) included the Clinical and Laboratory Standards Institute (CLSI) quality control (QC) strains *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 as well as reference strains *C. albicans* ATCC 90028, *C. albicans* ATCC 24433, *C. parapsilosis* ATCC 90018, and *C. tropicalis* ATCC 750. Strains designated NCPF were purchased from the National Collection of Pathogenic Fungi (Bristol, UK). Strains purchased from the Institute for Fermentation Osaka were designated IFO and are deposited in the NITE Biological Resource Center (Tokyo, Japan). Strains purchased from the Institute of Applied Microbiology (University of Tokyo, Japan) were designated IAM and are deposited in the RIKEN BioResource Center (Saitama, Japan). Other clinical isolates were from clinical specimens of sputum, blood, stool, urine, and vaginal discharge collected in Japan between 1991 and 1998.

***In vitro* antifungal susceptibility**

Fungal strains used in this report were maintained as frozen stock cultures at −80ºC. Subcultures for most yeasts were made on Sabouraud dextrose agar or YPD agar (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar); potato dextrose agar was used for common molds and MYSA (1% malt extract, 0.1% yeast extract, 0.1% soytone, 1% dextrose and 2% agar) was used for uncommon molds such as zygomycete species and dematiaceous fungi. Czapek yeast extract agar supplemented with 20% sucrose (69) was used for collecting conidia effectively from *Aspergillus* spp. and *Penicillium* spp.

For dimorphic true pathogenic fungi such as *Histoplasma capsulatum* and *Blastomyces dermatitidis* (*Ajellomyces dermatitidis*), ATCC Culture Medium 906 Pine and Drouhet's *Histoplasma* yeast-phase medium were used for the subculture. The inocula of yeast-phase conidia were prepared as suspension of $1–3\times10^6$ conidia/mL with saline. The inocula of filamentous fungi (molds) were prepared as conidial suspensions of $1–3\times10^6$ conidia/mL with BSG80 comprised of 0.85% NaCl, 0.03% KH_2PO_4, 0.06% Na_2HPO_4, 0.1% Tween 80 and 0.01% gelatin (Difco Laboratories, Detroit, MI). For antifungal susceptibility testing, MICs were determined using both a spectrophotometer and a reading mirror by the modified broth microdilution methods outlined by the CLSI (88, 89). Modifications were as follows: first, the drugs were diluted in DMSO from 100% to 10% with sterile distilled water instead of the medium to avoid precipitation of hydrophobic drugs owing to the salts in the medium; second, the RPMI-MOPS medium, semi-solidified by adding 0.2% low-melting-point agarose (LMPA) (Gibco BRL, Grand Island, NY) was used for the filamentous fungi (molds) to avoid precipitation of conidia and to reduce aerobic growth on the medium surface; and third, 96-well flat-bottomed microtitre plates were used, to facilitate the use of spectrophotometry to measure the

optical density (OD) of molds. Before the susceptibility test, QC strains of yeasts were used to confirm that there were no differences between the MICs determined using a semi-solid medium and an ordinary liquid medium. Brain–heart infusion medium (Difco) supplemented with 0.1% cysteine and 1.0% dextrose, solidified with 0.2% LMPA, was used for the examinations of the dimorphic true pathogenic fungi.

Drugs serially diluted two-fold with 100% DMSO (from 20 mg/mL to 0.04 mg/L) were diluted ten-fold with sterile distilled water (see above) in microtitre plates with a U-shaped well (Falcon, Lincoln Park, NJ). Aliquots (20 μL) of the drug solutions were transferred to flat-bottomed 96-well microtitre plates (Falcon) and 80 μL of conidia-free medium and 100 μL of 2× inoculum suspension were added. The inoculum sizes for common yeast strains were from 1 to 3×10^3 conidia/mL, and for the molds and dimorphic fungi strains were from 1 to 3×10^4 conidia/mL at the final concentration. The final concentration of drug ranged from 0.0004 mg/L to 200 (or 100) mg/L. Before incubation, well-mixed media in microtitre plates for the molds were refrigerated for 5 min to solidify the medium and were then incubated at 35ºC for a suitable period until the OD of the control wells was >0.15 for each species. The incubation period was typically 1–2 days for fast-growing species and 6–11 days for slow-growing species such as *Malassezia*, dermatophytes, dematiaceous fungi, and dimorphic true pathogenic fungi. The MIC endpoint of AmB was defined as the lowest concentration that prevented any discernible growth (score 0) for yeasts and molds. Before measuring the yeasts, plates containing liquid media were agitated by a plate agitator. The MIC endpoint of azoles was defined as the calculated concentration of drug that produced a 50% reduction of turbidity (score 2) compared with that of the drug-free control. For molds, the MICs of ITC, VRC, and isavuconazole were determined from the lowest

drug concentration that prevented any discernible growth (score 0), and that of FLC was determined by the calculated concentration of drug that produced 80% reduction of turbidity (score 1) by spectrophotometry at an OD of 600 nm, with the exception of the dermatophytes. For the dermatophyte species, the MICs of all azoles tested were determined only from the calculated concentration of drug that produced an 80% reduction of turbidity (score 1) measured by spectrophotometry. The MICs of azoles against *Fusarium*, *Pseudallescheria*, *Scedosporium* and zygomycete strains were determined using the two endpoints (score 0, 100% growth inhibition; and score 1, 80% growth inhibition) and that of FLC was determined using one endpoint (score 1). For calculation of the geometric mean, a MIC of ≤0.0004 mg/L was considered to be 0.0004 mg/L and, similarly, a MIC of ≥100 mg/L or ≥200 mg/L was 100 mg/L or 200 mg/L, respectively.

The interpretive breakpoints of azoles have been defined (20, 24, 89) as follows: for FLC, strains with a MIC ≤8 mg/L are considered susceptible and those with a MIC ≥64 mg/L are considered resistant; and for ITC, strains with a MIC ≤0.125 mg/L are considered susceptible and those with a MIC ≥1 mg/L are considered resistant. For VRC and isavuconazole, those with a MIC ≤1 mg/L are considered susceptible and those with a MIC ≥4 mg/L as resistant (96).

The sequence alignment and phylogenetic analysis in the Materials and Methods have been omitted pending regulatory approval of Isavuconazole.

TABLE 7

In vitro activity [MIC endpoint [a] (mg/L)] of isavuconazole and comparator agents against 48 laboratory yeast reference strains by spectrophotometric determination.

Species	*N*	Days [b]	FLC			ITC			VRC			Isavuconazole			AmB		
			Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
Candida albicans	8	1	0.02-20	0.14	20	0.0005-0.091	0.0022	0.091	0.0007-0.29	0.0027	0.29	0.0004-0.21	0.0022	0.21	0.05-0.2	0.05	0.2
GM for *C. albicans* (mg/L)			0.27			0.004			0.006			0.004			0.08		
Candida glabrata	9	1-2	0.5-67	3.0	35	0.0025-0.29	0.024	0.29	0.009-0.81	0.046	0.62	0.0036-0.40	0.02	0.27	0.1-0.2	0.1	0.2
GM for *C. glabrata* (mg/L)			3.7			0.03			0.06			0.03			0.13		
Candida tropicalis	4	1	0.16-0.36	0.24	0.36	0.004-0.006	0.004	0.1	0.011-0.04	0.012	0.04	0.0049-0.02	0.009	0.02	0.1-0.39	0.2	0.39
GM for *C. tropicalis* (mg/L)			0.25			0.005			0.016			0.01			0.23		
Candida krusei	3	1	11-25	12	25	0.0094-0.022	0.017	0.022	0.099-0.12	0.06	0.12	0.023-0.058	0.058	0.058	0.39-0.39	0.39	0.39
GM for *C. krusei* (mg/L)			15			0.02			0.09			0.04			0.39		
Candida parapsilosis	3	2	0.26-1.5	0.65	1.5	0.0011-0.0099	0.0081	0.0099	0.0019-0.024	0.017	0.024	0.0007-0.013	0.011	0.013	0.1-0.2	0.1	0.2
GM for *C. parapsilosis* (mg/L)			0.63			0.0045			0.01			0.0046			0.13		
Candida guilliermondii	2	2	0.7-2.2			0.0093-0.027			0.029-0.037			0.021-0.061			0.1-0.39		
Candida kefyr IFO0586	1	2	0.17			0.004			0.0018			0.0006			0.05		
Candida lusitaniae	2	1	0.074-0.16			0.0011-0.0024			0.0021-0.0042			0.0020-0.0052			0.05-0.1		
GM for all *Candida* spp. (mg/L)	32		0.9			0.008			0.018			0.011			0.13		
Cryptococcus neoformans	8	2	0.36-2.6	1.3	2.6	0.0013-0.011	0.0059	0.11	0.005-0.034	0.015	0.034	0.0026-0.032	0.012	0.032	0.025-0.1	0.05	0.1
GM for *C. neoformans* (mg/L)			1.2			0.005			0.015			0.012			0.05		
Malassezia furfur	3	6	1.6-9.9	2.7	9.9	0.053-0.078	0.073	0.078	0.011-0.063	0.023	0.063	0.18-0.81	0.18	0.81	6.3-12.5	6.25	12.5
GM for *M. furfur* (mg/L)			3.5			0.07			0.03			0.33			7.9		
Malassezia pachydermatis	2	7-9	1.8-2.7			<0.0004			0.013-0.02			0.0021-0.0027			0.1		
GM for all *Malassezia* spp. (mg/L)	5		2.9			0.009			0.021			0.046			1.4		
Trichosporon beigelii	2	3	0.024-0.19			0.0029-0.013			0.0017-0.01			0.005			0.1-0.39		
Trichosporon asahi IFO10844	1	3	5.4			0.051			0.034			0.31			0.39		
GM for all *Trichosporon* spp. (mg/L)	3		0.29			0.012			0.008			0.020			0.25		

MIC, minimum inhibitory concentration; $MIC_{50/90}$, MIC for 50% and 90% of the organisms, respectively; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; AmB, amphotericin B; GM, geometric mean.

[a] MIC endpoint: score 2 (50% inhibitory concentration by spectrophotometry) for FLC, ITC, VRC and isavuconazole; and score 0 (optically clear, i.e. no discernable growth) for AmB.

[b] Number of incubation days for MIC measurement.

TABLE 8

In vitro activity [MIC endpoint [a] (mg/L)] of isavuconazole and comparator agents against 57 laboratory mould reference strains by spectrophotometric determination.

Species	*N*	Days [b]	FLC			ITC			VRC			Isavuconazole			AmB		
			Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
Aspergillus fumigatus	12	2	45->200	190	>200	0.025-0.1	0.05	0.1	0.1-0.2	0.2	0.2	0.1-0.39	0.2	0.39	0.2-0.39	0.39	0.39
GM for *A. fumigatus* (mg/L)			144			0.047			0.16			0.22			0.34		
Aspergillus terreus	3	2	>200	>200	>200	0.05-0.1	0.05	0.1	0.39	0.39	0.39	0.2-0.39	0.2	0.39	0.39	0.39	0.39
GM for *A. terreus* (mg/L)			>200			0.06			0.39			0.25			0.39		
Aspergillus flavus IAM13835	1	2	200			0.1			0.39			0.78			0.39		
Aspergillus niger ATCC9642	1	2	200			0.2			1.56			1.56			n.d.		
Aspergillus oryzae IAM13881	1	2	>200			0.39			0.78			0.39			n.d.		
GM for all *Aspergillus* spp. (mg/L)	18		161			0.063			0.25			0.28			0.35		
Trichophyton mentagrophytes [c]	3	7	3.6-22	12	22	0.0004-0.011	0.011	0.011	0.02-0.024	0.021	0.024	0.003-0.038	0.028	0.038	0.39	0.39	0.39
GM for *T. mentagrophytes* (mg/L)			11			0.0036			0.022			0.015			0.39		
Trichophyton tonsurans [c]	3	7	2.2-8.3	3.8	8.3	<0.0004-0.051	0.0014	0.0051	0.011-0.047	0.041	0.047	0.003-0.049	0.017	0.049	0.025-0.2	0.2	0.2
GM for *T. tonsurans* (mg/L)			4.1			0.0014			0.028			0.011			0.1		
Trichophyton rubrum [c]	2	7	0.39-3.1			0.0016-0.0056			0.011			0.011-0.012			0.2		
Epidermophyton floccosum [c]	1	7	1.1			0.0014			0.0074			0.023			0.05		
Microsporum canis [c]	1	6	77			0.047			0.03			0.025			0.39		
Microsporum gypseum [c] IFO8231	1	7	12			0.007			0.023			0.015			0.1		
GM for all dermatophyte spp. (mg/L)	11		4.7			0.0033			0.021			0.015			0.17		
Alternaria alternata	1	6	180			0.39			1.56			3.1			0.39		
Alternaria mali IFO8984	1	6	23			0.39			0.78			1.56			0.1		
Aureobasidium pullulans	1	7	7.8			0.013			0.025			0.05			0.1		
Cladosporium trichoides	1	6	25			0.013			0.78			0.39			0.39		
Cladosporium carrionii	1	6	2.9			<0.0004			0.013			0.013			0.78		
Cladosporium herbarum IFO4458	1	3	48			0.2			1.56			6.25			0.39		
Exophiala dermatitidis	2	7	22-26			0.05-0.1			0.2-0.39			0.39-1.56			0.2-0.39		
Exophiala jeanselmei	2	6-9	18-20			0.0063-0.05			0.2-0.78			0.2-3.1			0.2-0.39		
Exophiala spinifera	1	7	36			0.013			0.39			0.78			1.56		
Exophiala moniliae	1	7	25			0.0031			0.025			0.05			0.2		
Fonsecaea pedrosoi	1	6	19			0.025			0.2			0.39			3.1		
Fonsecaea compacta	1	9	33			0.1			0.2			0.39			3.1		
Paecilomyces variotii	1	6	>200			0.0031			0.78			3.1			0.05		
Penicillium marneffei	1	6	2.7			0.0031			0.025			0.05			0.39		
Penicillium oxalicum	1	3	>200			0.2			0.78			0.39			0.2		
Phialophora verrucosa	1	6	11			0.1			0.2			0.2			1.56		
Phialophora parasitica	1	6	170			>200			0.39			0.78			0.78		
Sporothrix schenckii	3	7	>200	>200	>200	>200	>200	>200	12.5-50	25	50	12.5-25	25	25	0.78-3.1	0.78	3.1
Blastomyces dermatitidis	3	5-11	0.06-0.71	0.11	0.71	<0.0004-0.0008	<0.0004	0.0008	0.0016-0.0063	0.0063	0.0063	<0.0004-0.0008	<0.0004	0.0008	0.025-0.2	0.05	0.2
Histoplasma capsulatum	3	5-7	0.55-1.2	0.77	1.2	<0.0004	<0.0004	<0.0004	0.0063-0.025	0.0063	0.025	0.0031-0.0063	0.0063	0.0063	0.013-0.05	0.05	0.05
GM for all dimorphic, true pathogens (mg/L)	6		0.37			0.0004			0.0063			0.0016			0.045		

MIC, minimum inhibitory concentration; $MIC_{50/90}$, MIC for 50% and 90% of the organisms, respectively; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; AmB, amphotericin B; GM, geometric mean; N/T, not tested.

[a] MIC endpoint: score 2 (50% inhibitory concentration by spectrophotometry) for FLC, ITC, VRC and isavuconazole; and score 0 (optically clear, i.e. no discernable growth) for AmB.

[b] Number of incubation days for MIC measurement.

[C] MIC endpoint of dermatophytes species: score 1 for all azoles and score 0 for AmB.

TABLE 9

In vitro activity [MIC endpoint [a] (mg/L)] of isavuconazole and comparator agents against *Fusarium*, *Scedosporium* and zygomycetes.

Species	*N*	Days [b]	FLC	ITC		VRC		Isavuconazole		AmB
			GM score 1(range)	GM score 1 (range)	GM score 0 (range)	GM score 1(range)	GM score 0 (range)	GM score 1(range)	GM score 0 (range)	GM score 0 (range)
Fusarium solani	6	2	>200 (>200)	117 (59->200)	>200 (>200)	2.1 (1.2-2.9)	8.8 (3.1-25)	13 (5.4-18)	71 (25->200)	0.68 (0.39-1.56)
Fusarium moniliforme	2	2	(>200)	(0.69-27)	(>200)	(0.45-0.5)	(3.1-6.25)	(1.2-2.8)	(3.1-6.25)	(1.56-3.1)
Fusarium oxysporum IFM41530	1	2	(>200)	(>200)	(>200)	(1.3)	(6.25)	(2.3)	(12.5)	(1.56)
All *Fusarium* spp.	9		>200	55	>200	1.4	7.3	6.8	32	0.9
Pseudallescheria boydii (Sedosporium apiospermum)	3	2-6	15 (7.8-27)	0.26 (0.16-0.6)	5.0 (0.39->200)	0.10 (0.052-0.19)	0.39 (0.2-0.78)	0.29 (0.12-0.77)	2.5 (1.56-3.1)	40 (1.56->200)
Sedosporium prolificans	3	2	>200 (>200)	>200 (>200)	>200 (>200)	6.6 (5.5-8.7)	50 (50)	6.9 (5.5-10)	>200 (>200)	>200 (200->200)
All *Sedosporium* spp.	6		100	7.2	32	0.8	4.4	1.4	22	63
Absidia corymbifera	4	1-2	>200 (>200)	0.056 (0.021-0.086)	0.14 (0.1-0.2)	8.4 (11-28)	30 (12.5-50)	1.2 (0.2-2.1)	1.9 (0.78-3.1)	0.025 (0.025)
Absidia hyalopsora IFO8084	1	2	(>100)	(0.048)	(0.1)	(2.7)	(12.5)	(0.18)	(0.78)	N/T
Cunninghamella bertholletiae	2	2	(>100)	(0.15-0.33)	(0.2-0.78)	(10-11)	(12.5-25)	(5.9)	(>100)	N/T
Mucor circinelloides IFO4554	1	1	(>200)	(0.2)	(0.39)	(5.5)	(12.5)	(1.9)	(3.1)	(0.05)
Mucor rouxianus IFO5773	1	2	(>200)	(>200)	(>200)	(>200)	(>200)	(>200)	(>200)	(0.05)
Mucor ramosissimus ATCC28933	1	3	(>100)	(0.11)	(0.2)	(13)	(50)	(2.5)	(6.25)	N/T
Rhizomucor pusillus	3	1-2	153 (90->200)	0.04 (0.012-0.33)	0.2 (0.1-0.78)	4.1 (2.7-9.2)	21 (12.5-25)	0.68 (0.24-4.3)	3.1 (1.56-6.25)	0.04 (0.025-0.05)
Rhizopus oryzae	4	1-2	>200 (>200)	0.33 (0.083-2.2)	1.56 (0.39-100)	6.2 (2.7-12)	21 (6.25-50)	0.92 (0.33-2.9)	3.1 (1.56-12.5)	0.07 (0.05-0.1)
Rhizopus microsporus	3	2	>100 (>100)	0.27 (0.19-0.33)	0.62 (0.39-0.78)	5.7 (5.3-6.0)	12.5 (12.5)	0.64 (0.59-0.69)	2.0 (1.56-3.13)	N/T
All zygomycete species	20		>100	0.18	0.52	8.4	22	1.2	4.4	0.05

MIC, minimum inhibitory concentration; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; AmB, amphotericin B; GM, geometric mean; N/T, not tested.

[a] MIC endpoint: score 1(80% inhibitory concentration by spectrophotometry) for FLC; score 1/score 0 (optically clear, i.e. no discernable growth) for ITC, VRC, isavuconazole; and score 0 for AmB.

[b] Number of incubation days for MIC measurement.

TABLE 10

In vitro activity [MIC endpoint [a] (mg/L)] of isavuconazole and comparator agents against 165 clinical isolates from Japan

Species	*N*	**FLC**			**ITC**			**VRC**			**Isavuconazole**			**AmB**		
		GM (range)	MIC_{50}	MIC_{90}	GM (range)	MIC_{50}	MIC_{90}	GM (range)	MIC_{50}	MIC_{90}	GM (range)	MIC_{50}	MIC_{90}	GM (range)	MIC_{50}	MIC_{90}
Candida albicans	33	0.054 (0.018-0.17)	0.045	0.15	0.0009 (<0.0004-0.0029)	0.001	0.0022	0.0010 (<0.0004-0.0032)	0.001	0.0025	0.0007 (<0.0004-0.0026)	0.0007	0.0013	0.032 (0.0031-0.39)	0.05	0.05
Candida glabrata	25	0.97 (0.093-63)	0.64	3.4	0.0056 (0.0013-0.19)	0.005	0.019	0.010 (0.0016-1.1)	0.0076	0.043	0.0055 (0.0011-0.64)	0.0039	0.02	0.057 (0.0063-0.10)	0.05	0.1
Candida tropicalis	24	0.18 (0.04-0.91)	0.20	0.48	0.0027 (<0.0004-0.012)	0.004	0.0095	0.0082 (<0.0004-0.049)	0.011	0.017	0.0030 (<0.0004-0.0094)	0.0046	0.008	0.069 (0.025-0.2)	0.05	0.2
Candida guilliermondii	15	1.54 (0.65-2.4)	1.5	2.4	0.044 (0.0083-0.15)	0.042	0.14	0.025 (0.0056-0.062)	0.031	0.046	0.042 (0.0069-0.18)	0.051	0.11	0.057 (0.025-0.39)	0.05	0.39
Candida krusei	10	6.2 (3.7-9.4)	5.5	8.8	0.0042 (0.0025-0.008)	0.0041	0.0079	0.019 (0.012-0.04)	0.016	0.03	0.006 (0.032-0.015)	0.0041	0.011	0.13 (0.1-0.2)	0.1	0.2
Candida parapsilosis	17	0.22 (0.077-1.1)	0.23	0.61	0.0019 (0.00042-0.0088)	0.0019	0.0059	0.0026 (0.00054-0.018)	0.0035	0.015	0.0013 (<0.0004-0.017)	0.0015	0.011	0.085 (0.05-0.2)	0.1	0.2
Candida lusitaniae	17	0.069 (0.016-0.16)	0.076	0.15	0.0008 (<0.0004-0.0034)	0.0006	0.0021	0.0009 (<0.0004-0.0023)	0.0012	0.0021	0.0008 (<0.0004-0.0025)	0.00059	0.0024	0.016 (0.0063-0.025)	0.013	0.025
Candida lypolitica	8	6.2 (1.4-11)	7.0	11	0.005 (<0.0004-0.07)	0.0056	0.07	0.048 (0.032-0.074)	0.049	0.074	0.011 (0.032-0.074)	0.049	0.074	0.24 (0.1-0.39)	0.2	0.39
Trichosporon beigelii	2	0.35 (0.3-0.42)			0.014 (0.0095-0.02)			0.004 (0.0028-0.0055)			0.009 (0.0031-0.026)			0.14 (0.1-0.2)		
Cryptococcus neoformans	14	1.5 (0.42-2.6)	1.7	2.3	0.0027 (<0.0004-0.0052)	0.0033	0.005	0.0074 (0.0021-0.014)	0.0077	0.012	0.0039 (0.0009-0.011)	0.0053	0.0086	0.012 (0.0063-0.025)	0.013	0.025

MIC, minimum inhibitory concentration; $MIC_{50/90}$, MIC for 50% and 90% of the organisms, respectively; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; AmB, amphotericin B; GM, geometric mean.

[a] MIC endpoint: score 2 (50% inhibitory concentration by spectrophotometry) for FLC, ITC, VRC and isavuconazole; and score 0 (optically clear, i.e. no discernable growth) for AmB.

Results

Isavuconazole was tested against 140 laboratory strains of fungi and 165 contemporary clinical yeast isolates from Japan. TABLE 7 shows the activity against 48 yeasts, including representative strains of *C. albicans*, non-*albicans Candida* spp., and *Cryptococcus neoformans*. The activity of isavuconazole was more potent *in vitro* than that of FLC or AmB against all species and was, for the most part, comparable with that of ITC and VRC. The respective geometric means of the MICs (mg/L) for isavuconazole, FLC, ITC, VRC and AmB for each species were as follows: 0.004, 0.27, 0.004, 0.006, and 0.08 for *C. albicans*; 0.03, 3.7, 0.03, 0.06, and 0.13 for *C. glabrata*; 0.04, 15, 0.02, 0.09, and 0.39 for *C. krusei*; 0.01, 0.25, 0.005, 0.016, and 0.23 for *C. tropicalis*; 0.012, 1.2, 0.005, 0.015, and 0.05 for *C. neoformans*; and 0.33, 3.5, 0.07, 0.03, and 7.9 for *Malassezia furfur*. For *Candida* spp., isavuconazole showed lower *in vitro* activity than ITC and greater activity than VRC, but all these new triazoles showed more potent activity than FLC. For *C. neoformans*, the new triazoles showed similar activity. *M. furfur* showed resistance to AmB, whereas VRC was the most potent azole, followed by ITC and isavuconazole. As shown in TABLE 8, isavuconazole was also active against *Aspergillus* spp. and dermatophyte species. The respective geometric means of the MICs (mg/L) for isavuconazole, FLC, ITC, VRC and AmB were as follows: all *Aspergillus* spp., 0.28, 161, 0.063, 0.25, and 0.35 (*Aspergillus fumigatus* 0.22, 144, 0.047, 0.16, and 0.34); and dermatophyte species, 0.015, 4.7, 0.0033, 0.021, and 0.17. *Trichophyton mentagrophytes*, *Microsporum canis*, and *Microsporum gypseum* showed rather high MIC values for FLC. However, the MICs of the newer triazoles were lower. Isavuconazole was also active against uncommon opportunistic fungi and dimorphic pathogens. Against *Sporothrix schenckii* all four triazoles showed

low activity, but AmB showed improved *in vitro* activity. Furthermore, isavuconazole was more potent *in vitro* than FLC or AmB and was as active as ITC against dimorphic pathogens, *B. dermatitidis* (*A. dermatitidis*) and *H. capsulatum*, with MIC values <0.01 mg/L.

The activity of isavuconazole against *Fusarium*, *Scedosporium*, and zygomycete species is shown in TABLE 9. For these species, the MIC endpoints of score 0 (100% growth inhibition) and score 1 (80% growth inhibition) were determined according to the criteria of Guinea et al. (24). Against *Fusarium* spp. and *Scedosporium prolificans*, ITC was less active; however, isavuconazole showed limited activity against *Fusarium moniliforme* and *Fusarium oxysporum* with higher activity than ITC. VRC had stronger activity than isavuconazole against *Fusarium solani*. Isavuconazole, ITC, and VRC showed activity against *Pseudallescheria boydii* (*Scedosporium apiospermum*). Using MICs of the azoles against all of the zygomycete species tested, the respective geometric means of MICs (score 1/score 0) (mg/L) for isavuconazole, ITC, and VRC were determined to be 1.2/4.4, 0.18/0.52, and 8.4/22, respectively. The MIC values of isavuconazole were lower than those of VRC, but ITC presented the lowest values *in vitro*. *Mucor rouxianus* was not susceptible to any of the four triazoles but was susceptible to AmB and showed MICs (score 1/score 0) (mg/L) for isavuconazole, ITC, VRC, and AmB of >200/>200, >200/>200, >200/>200 and 0.5, respectively. The susceptibility of 165 clinically isolated yeasts in Japan to the four azoles and AmB was also examined. Isavuconazole was more potent *in vitro* compared with FLC in all clinical isolates of *Candida* spp., *Trichosporon beigelii*, and *C. neoformans* examined (TABLE 10). Comparing the activities of the azoles by MIC90 (MIC for 90% of the organisms) against clinical isolates, isavuconazole showed no

cross-resistance to FLC and showed potency comparable with ITC and VRC. Isavuconazole was the most potent against *C. albicans* and *C. tropicalis*; VRC was the most potent against *Candida guilliermondii* and *T. beigelii*; and ITC was the most potent against *C. krusei*, *C. parapsilosis*, and *C. neoformans*. As the sensitivity for VCZ and isavuconazole was defined in the '*In vitro* antifungal susceptibility' part of Materials and Methods, a MIC value of ≤1 mg/L was considered susceptible and that with a MIC ≥4 mg/L as resistant. A partially sensitive was defined between 1 and 4 mg/L, in this report. Isavuconazole-insensitive or -partially sensitive species were regarded as isavuconazole 'resistant' species.

The text on the mechanism of resistance and FIGURES in the Results have been omitted pending regulatory approval of Isavuconazole.

Discussion

Modified CLSI M27-A2 and CLSI M38-A procedures (88, 89) were applied in this study. MICs were measured by OD measurement as well as visual assessment. In the antimicrobial susceptibility tests, the test agent used was prepared in the Japanese style of two-fold serial dilutions from 100 mg/L, though the dilution style of the USA and Europe (a descending dilution from 128 mg/L or 64 mg/L) has recently become more common. As MIC values were to be calculated from OD values using spectrophotometry instead of visual endpoints, any concentration of drug can be used for the first well. Therefore, the MIC values found with our procedure might be slightly different when using the original CLSI method. The incubation period was set to attain a minimum OD of 0.15 in control wells. Under this condition for the yeast susceptibility test, the MIC endpoint used (measured spectrophotometrically) was the lowest concentration giving 50% growth inhibition (score 2). The QC strains provided MICs in almost the same range as, or a little lower than, the values for azoles suggested in CLSI M27-A2 (89). My data with investigated drugs were in the same range of MIC values as those reported by Illnait-Zaragozi et al. (30) for *C. neoformans*. Regarding the yeasts, the azole-resistant strain *C. albicans* NCPF 3303 showed MICs for FLC, ITC, VRC, isavuconazole and AmB of 20, 0.091, 0.29, 0.21, and 0.1 mg/L, respectively (28). According to CLSI interpretive guidelines (89), this strain was susceptible-dose dependent (S-DD; 16–32 mg/L) to FLC and was susceptible to all other azoles. All three *C. krusei* strains, including the QC strain, were S-DD (12–25 mg/L) to FLC but susceptible to other antifungals (ITC 0.0094–0.017 mg/L, VRC 0.0099–0.12 mg/L, isavuconazole 0.023–0.058 mg/L, and AmB 0.39 mg/L). Semi-solid medium was used to study mold and dimorphic pathogenic fungi to avoid conidial precipitation and

growth on the medium surface. The 80% inhibitory concentration values were determined by spectrophotometry and thus completely excluded experimenter subjectivity. Since it is difficult to read the OD of wells covered with growing aerobic mycelia, we concluded that semi-solidified media were preferable to determine the MICs of the molds. The semi-solid medium provided homogeneously scattered conidia growth and more consistent values of OD measured by a spectrophotometer. With the *Aspergillus* spp., no resistant strain to the new triazoles was found and all MICs for isavuconazole were <2 mg/L. MIC values against *A. fumigatus* (TABLE 8) were similar to those reported using either the CLSI method or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method (95, 99). My results were a little lower than the values reported for the CLSI method, possibly owing to the different growth properties of the assay conditions. In our method using a semi-solid medium in a flat-bottomed well, the conidia remained in suspension. In the CLSI method using a liquid medium in a U-shaped well, the conidia precipitate and form a pellet at the bottom of the well. Therefore, with the CLSI method, judgment of the endpoint and determination of score 0 at higher concentrations may be easier. The strains of *Aspergillus* spp. tested here were not contemporary clinical isolates. Therefore, these strains might not have been exposed to azoles. As a wild-type strain is not expected to have acquired resistance to a particular agent such as an azole (95), the *Aspergillus* strains tested can be regarded as wild-type strains. Amino acid substitutions in CYP51A at glycine 54 (G54), at G448, and at methionine 220 (M220), and amino acid substitution at position 98 of leucine for histidine (L98H) together with tandem repeat, as well as high-level expression of a multidrug resistance efflux pump have been reported as reasons for azole resistance (96, 99). Understanding the reason for the

development of resistance is very important in the development of novel drugs. *Aspergillus* species are generally susceptible to newer triazoles; however, intrinsic and acquired resistance have been documented (109). Pfaller et al. (95) reported that new triazole resistance amongst *A. fumigatus* is uncommon but increasing. Verweij et al. (109) reviewed acquired azole-resistant *A. fumigatus* isolated in clinical settings throughout Europe and the USA and they considered azole-resistant *Aspergillus* might be more common. Against uncommon fungal species, isavuconazole showed a broad antifungal spectrum that included dematiaceous species such as *Exophiala*, *Phialophora*, and *Fonsecaea* spp., but not *S. schenckii*. The four newer azoles tested were not active against *S. schenckii* (TABLE 8). Here I demonstrate that isavuconazole showed very good *in vitro* antifungal activity overall against a broad range of fungi, including FLC-resistant *Candida* spp., *Aspergillus* spp. and uncommon opportunistic fungal species, but showed limited antifungal activity against zygomycete species. With *Fusarium* spp., *P. boydii* (*S. apiospermum*), *S. prolificans*, and zygomycete species, the antifungal activities of isavuconazole and ITC was consistent with reports that isavuconazole is more active than VRC against zygomycete species but has limited activity against *Fusarium* spp. (FIG. 8) (24, 94). I compared the *in vitro* activity of isavuconazole with that of VRC using the waterfall plot charts, and each drug concentration in plasma after daily dosing to human; 2.6 µg/mL with isavuconazole (101) and 1.8 µg/mL with VCR (80, 97). It is important for each drug concentration in plasma to be greater than the MIC of fungi. This result suggests that isavuconazole is more effective than VRC against zygomycetes infections in human.

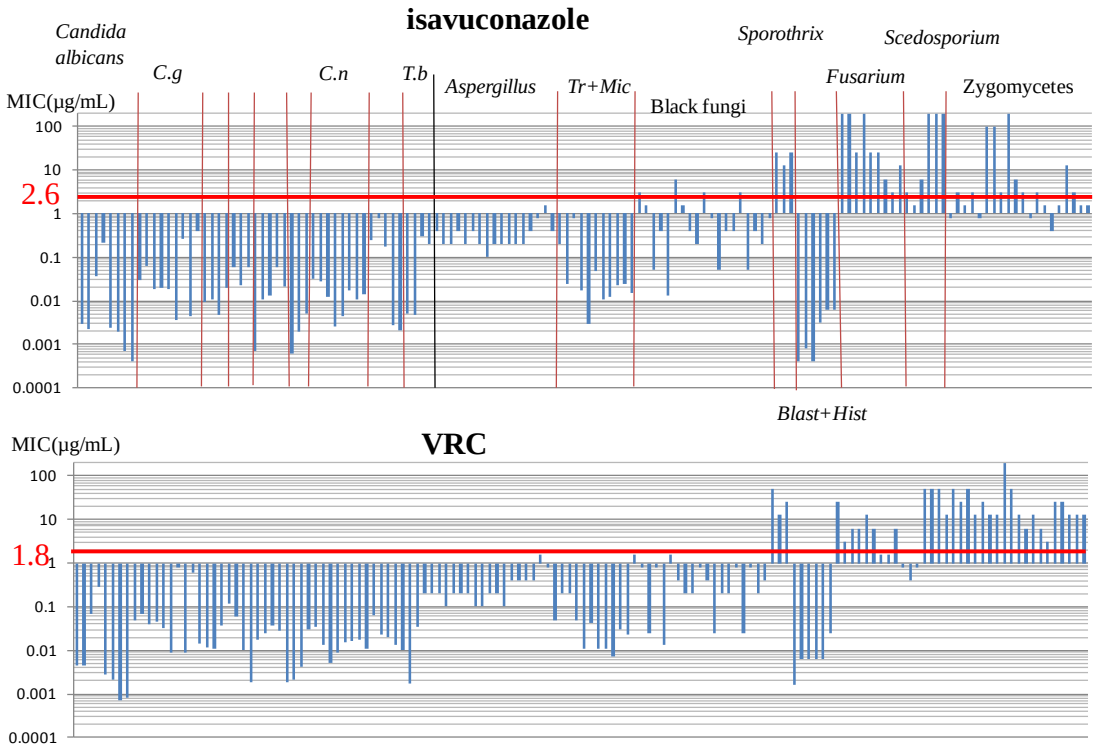


FIG. 8 *In vitro* activity of isavuconazole compared with that of voriconazole in waterfall plot.

Antifungal activity was drawn in the waterfall plot and — represents drug concentration in plasma after administration with daily dose. *C.g*, *Candida glabrata*; *C.n*, *Cryptococcus neoformans*; *T.b*, *Trichosporon beigelii*; *Tr+Mic*, *Trichophyton* and *Microsporum* spp.; *Blast+Hist*, *Blastomyces* and *Histoplasma* spp.

Similar findings were reported by Guinea et al. (24). Seifert et al. (103) and Curfs-Breuker et al. (12) have reported good *in vitro* activity of isavuconazole against yeasts including *Candida* isolates, and Warn et al. (114) reported *in vitro* activity of isavuconazole against *Aspergillus* spp. The efficacy of isavuconazole has been demonstrated in murine models of disseminated *Candida* infection (121) and infection by *Aspergillus flavus* (115) and *A. fumigates* (113). The activity of isavuconazole against refractory zygomycetes is of interest in light of the reported low *in vitro* efficacy of VRC (107) and the occurrence of breakthrough zygomycosis during therapy with the latter agent (83). The ergosterol synthesis pathway is a validated target of azole antifungal agents, and FLC is still an effective agent against sensitive candidiasis.

In clinical settings, ITC and VRC are used for infections due to *Aspergillus* species. VRC is used to treat most frequently because of its efficacy against *Candida* species and *Aspergillus* species, even though VRC shows a limited activity against *Scedosporium*, *Fusarium* species and zygomycete species. This issue of limited or partial activity of azole antifungals prompted me to research the reason why an intrinsic resistance is observed in those insensitive species. In this report, although MIC values were determined using two endpoints (score 1 and score 0) in partially sensitive or resistant strains (TABLE 9), only clinical study results can clarify which endpoint will be more relevant to evaluate clinical efficacy.

The text on the mechanism of resistance and FIGURES in the Discussion have been omitted pending regulatory approval of Isavuconazole.

Isavuconazole showed good activity against clinical isolates from Japan. I previously reported *in vitro* susceptibility testing against Japanese clinical isolated yeasts. FLC-resistant *C. albicans* was not isolated but FLC-resistant *C. glabrata* was isolated from two patients who had been treated with FLC over a long period, and these isolates were not cross-resistant to isavuconazole *in vitro*. *Candida krusei* and *C. lipolytica* had intermediate susceptibility to FLC and were susceptible to all new triazoles. Therefore, it is important to diagnose the causative agent from patient when determining which antifungal should be used to treat mycoses.

The high water solubility of isavuconazolium (the prodrug of isavuconazole) (90) allows for either parenteral application or oral administration. The results of phase I

studies (101, 102) indicate potential for a convenient treatment with both intravenous infusion and oral administration for patients during hospitalization and oral administration for the outpatient setting. Furthermore, infections due to emerging uncommon fungi (3, 24) and intrinsic FLC-resistant or AmB-resistant species have been increasing in recent decades. Isavuconazole will be one of the key players against invasive fungal infection by such unfavorable pathogens. There is also the possibility of use as a prophylactic, especially against *Candida* urinary tract infections, catheter-related candidemia and *Aspergillus* infections in immunocompromised patients who are neutropenic after receiving immunosuppressant medications, anticancer chemotherapies, or hematopoietic cell transplantation (16, 122). These results confirmed the antifungal spectrum of isavuconazole, showing for the first time that it has activity against clinical isolates of Japanese origin as well as against isolates from North America and Europe.

General Discussion

In epidemiological studies over the past 40 years, visceral mycoses have been increasing; in particular, life-threatening severe mycoses are still more than 40% of the total visceral mycoses in autopsy cases. During this period, the launch of FLC in 1989 showed a strong impact to clinical settings for treating *Candida* infection. Superficial infections or esophageal and vaginal infections by *Candida albicans* was treated very frequently, and many patients benefitted from the advantages of this antifungal; however, FLC-resistant *Candida* appeared immediately. From the result of an analysis of causative agents in autopsy cases, the decrease of candidiasis after 1989 was noticeable. However, aspergillosis has been increasing and continued to do so. Knoke et al. also reported the results of epidemiological study of mycoses in autopsy cases and insisted on the importance of pathological autopsy for diagnostic and therapeutic strategy in the management of fungal diseases (70). Their review of the autopsy rate in the western world concluded that the increment of *Aspergillus* infections are increasing and *Candida* infections are decreasing, just as I reported for Japan (120).

I mentioned that azole-resistant fungal infections are increasing, and severe infections remain at 40% of all mycoses in autopsied patients. The major cause of FLC resistance is the induction of the ATP binding cassette (ABC) transporter in *Candida* species, which limits the membrane permeability of FLC into fungal cell plasma. The mechanism of FLC resistance in *Aspergillus* seems to be a low affinity to CYP51 of *Aspergillus*, which means that FLC does not show any efficacy for aspergillosis. Furthermore, amino acid substitutions of CYP51 confer azole resistance by changing the binding affinity of azole antifungal drugs. As I mentioned before, the amino acid

substitution in CYP51A of *Aspergillus* at glycine 54 (G54), at G448, and at methionine 220 (M220), and amino acid substitution at position 98 of leucine for histidine (L98H) together with tandem repeat, have been reported as reasons for azole resistance (96, 99).

The text concerning about membrane sterols in the General Discussion has been omitted pending regulatory approval of Isavuconazole.

The novel triazole antifungal drug isavuconazole showed very potent antifungal activity against various medically important fungi such as *Candida* species, *Aspergillus* species, and *Cryptococcus neoformans*. Following the research described in this thesis, developing this antifungal drug was continued by Basilea Pharmaceurtica International AG, a spin-off from F. Hoffmann La-Roche. The end product, isavuconazolium salt, is a good water-soluble prodrug that can be used for both intravenous injection and oral administration.

This thesis has described in detail a small portion of the data used in the lengthy process of developing a drug. The results of my data analysis revealed that many unmet medical needs existed and helped our team to select what was the best target at that time. After the best candidate from hundreds of compounds has been chosen as the clinical candidate, its activity or efficacy needed to be confirmed in both non-clinical and clinical settings. The many non-clinical experiments that were a consequence of the data analysis described here were helpful when introducing the new drug to doctors and encouraging them to take part in the clinical trials. Additionally, the experiments were helpful for preparing the documents for isavuconazole as an Investigational New Drug (IND). Currently, isavuconazole is under Phase III studies,

one of which is for invasive aspergillosis (SECURE) and which has met the primary endpoint. The knowledge that invasive aspergillosis is a life-threatening disease primarily afflicting immunocompromised patients and that a new antifungal agent was required to treat these critically ill patients. It is gratifying to note that isavuconazole will be on the market in the very near future.

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