# CHAPTER I
# INTRODUCTION

*Cryptococcus neoformans* is the etiologic agent of cryptococcosis in both immunocompetent and immunocompromised hosts, especially patients with AIDS, one of the most serious global fungal diseases and one of the most common pathogens isolated from the central nervous system in the world today. In Thailand, epidemiological data during September 1984 to May 2008 from Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health revealed 47,861 (14.41%) cases of cryptococcosis which is the second most common fungal opportunistic infection in AIDS patients after *Pneumocystis carinii* pneumonia (PCP) (Epidemiological Information Section Bureau of Epidemiology, 2008). The economic impact of *C. neoformans* on health care systems is overwhelming since there is presently no cure for AIDS and costly lifelong antifungal prophylaxis or maintenance therapy is required for these patients. Because of prolonged maintenance therapies with antifungal drugs, isolations of drug resistant strains have been increasing. The initial treatment in Thai AIDS patient for cryptococcal meningitis is amphotericin B for two weeks plus fluconazole for an additional 8 weeks. After completion of induction treatment, fluconazole 200 mg/day has been used for standard secondary prophylaxis until immune reconstitution occurs because of antiretroviral therapy (Mootsikapun *et al*., 2004, Tansuphaswadikul *et al*., 2006). Fluconazole is considered the treatment of choice for cryptococcal meningitis because it penetrates extensively into body tissues and fluids and is cleared primarily by renal excretion, with 80% of the administered dose excreted unchanged by the kidney. The long plasma elimination half-life permits once-daily dosing (Jura and Hillenbrand, 2006).

The MICs (Minimal Inhibitory Concentrations) were determined for the level of resistance of *C. neoformans* isolates according to the procedure of the microdilution method of antifungal susceptibility testing of the National Committee for Clinical Laboratory Standards (NCCLS) or E-test method (AB BIODISK, Solna,

Sweden). The MIC is determined by observing the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test. The antifungal susceptibility data were generated by NCCLS reference MIC methods (NCCLS M27-A2, 2002), indicating that *in vitro* resistance to commonly used antifungal agents, such as amphotericin B, fluconazole, itraconazole and ketoconazole, remains uncommon among *C. neoformans* and have been found fluconazole resistance strains that show MIC of fluconazole ≥ 64 μg/ml. For instance, fluconazole MICs for the incident surveillance *C. neoformans* isolate was ≥ 64 μg/ml for isolates from 6/253 (2.4%) detected between 1992-1994 and isolates from 2/269 (0.7%) detected between 1996-1998 in United State (Brandt *et al*., 2001). Similarly, Chandenier *et al*. (2004) demonstrated that the presence of 7/110 (6.3%) Cambodian isolates and 3/152 (5.8%) African isolates for which the fluconazole MICs ≥ 64 μg/ml were resistant to fluconazole (Chandenier *et al*., 2004). Sar *et al*. (2004) reported an increase in resistance to fluconazole from 2.5%-14% between 2001 and 2002 in a hospital in Cambodia (Sar *et al*., 2004). In Kenya, fluconazole resistance rate was 11.2% (9/80) from clinical isolates collected in 2003-2004 (Bii *et al*., 2007). In addition, fluconazole resistant isolates were found in 4/22 of *C. neoformans* var. *grubii* environmental isolates in Barcelona, Spain (Lopez *et al*., 2005).

Many studies revealed that high or rising MIC of fluconazole was associated with clinical relapse or untreated primary infection in pateints with AIDS-associated cryptococcal meningitis. The increase in the MICs of fluconazole has been described from sequential clinical isolates from AIDS patient with recurrent meningitis (Rodero *et al*., 2003). In addition, Friese *et al*. reported a cryptococcal meningitis case in which the emergence of a fluconazole-resistant strain (fluconazole MIC ≥ 64 μg/ml) was documented after three episodes of meningitis (Friese *et al*., 2001). Previous surveillance data from Thailand, *C. neoformans* isolates were inhibited by fluconazole at concentrations of ≤16 μg/ml (Poonwan *et al*., 1997). In addition, Manosuthi *et al* (2006) reported a retrospective cohort study to 98 *C. neoformans* isolates from HIV-infected patients diagnosed with cryptococcal meningitis who were admitted to

Bamrasnaradura Institute, Nonthaburi, Thailand between January 2003 and October 2004. Approximately 30% of *C. neoformans* isolates of patients who did or did not receive fluconazole therapy had MIC 16-32 μg/ml (Manosuthi *et al*., 2006). A study in 9 patients with untreated primary cryptococcosis in Thailand, fluconazole MICs of 7/22 isolates were ≥ 256 μg/ml by E-test (Sukroongreung *et al*., 2001).

Molecular mechanisms for fluconazole resistance in *C. neoformans* include increased efflux of fluconazole and mutation of *ERG11*, the gene encoding the target of fluconazole, lanosterol 14α-demethylase. This key enzyme in the ergosterol biosynthesis pathway catalyzes the oxidative removal of the 14α-methyl group from lanosterol. This drug binds to the heme in the active site of 14α-demethylase, competing with substrate binding. Mutations within the *ERG11* structural gene reduce fluconazole binding which are responsible for the amino acid substitution glycine 484 for serine (abbreviated as G484S) (Rodero *et al*., 2003). In addition to the mutations in *ERG11*, overexpression of the gene results in the production of high concentrations of target enzyme, creating the need for higher intracellular fluconazole concentrations to inhibit all of the enzyme present in the cell. It has been reported that fluconazole-resistant *C. albican*s isolates express *ERG11* mRNA at higher levels than matched susceptible isolates in the presence of the drug (Franz *et al*., 1998). Until now, there is no information about the presence of *ERG11* overexpression causes resistance to fluconazole in *C. neoformans*.

Another important mechanism of fluconazole resistance is reduced intracellular accumulation of the drug. Thereafter, it became evident that fluconazole is actively transported out of the cells in an energy-dependent manner and that an enhanced drug efflux is caused by the overexpression of genes encoding membrane transport proteins such as *AFR1*; antifungal resistance1 (Posteraro *et al*., 2003) *MDR1*; multidrug resistance1 (Thornewell *et al*., 1997). Both genes are classified in ATP-binding cassette (ABC) transporters, which use adenosine triphosphate (ATP) as the energy source. Each of the mechanisms of fluconazole resistant genes described above can cause reduced susceptibility of *C. neoformans* to fluconazole.

Although cryptococcosis is still an important public health problem in northern Thailand, there is no information on the *in vitro* antifungal susceptibility of *C. neoformans* isolates in this area. The results of this study will perform the antifungal susceptibility profiles and mechanism of fluconazole resistance in *C. neoformans* fluconazole resistant isolates from Chiang Mai. It may provides the information necessary to predict clinical outcome of treatment of this infection, a guide for selecting appropriate doses of these agents and to find suitable clinical management strategies for the treatment of *C. neoformans* infections in the future. The molecular techniques and the current understanding of resistance mechanisms may be sufficient to explain the resistance phenotype in *C. neoformans* fluconazole resistant isolates. Study on the expression of fluconazole resistant genes will be able to complement the *in vitro* antifungal susceptibility testing for detecting and monitoring the emergence of azole antifungal drug resistance. The identification of amino acid substitutions responsible for azole resistance may provide new insights into the way the drugs interact with target molecule. This knowledge may aid in the development of more active molecules of antifungal agents.