PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 173-176 (1976)

In Vitro and *In Vivo* Activity of 2,4-diamino-6-[2-(3,4-dichlorophenyl)acetamido] quinazoline against *Cryptococcus neoformans*[1] (39168)

ALI R. HARIRI AND HOWARD W. LARSH[2]

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73069

Cryptococcus neoformans is an encapsulated yeast-like fungus that may cause primary pulmonary infection. It can spread to other parts of the body and has a special affinity for the central nervous system (CNS). Like most systemic mycoses, the infection is often secondary to other predisposing factors, such as Hodgkin's disease, leukemia, or other factors which repress the immune system (1–3).

Only a few antifungal agents have been useful in the treatment of mycotic diseases; among them, the most frequently used are amphotericin B (AmB) and 5-fluorocytosine (5-FC).

Amphotericin B was discovered by Gold *et al.* (4) in 1956 and characterized by Vandeputte *et al.* (5). With this discovery, a new era in treatment of systemic mycoses commenced. Amphotericin B is a polyene antibiotic that affects eukaryotic cells by binding sterols in the membranes (6, 7). Although treatment of systemic mycoses with AmB has been relatively successful, prolonged treatment results in toxicity to the host, especially renal damage (8).

In 1957 Duschinsky (9) synthesized 5-FC. Unlike some base analogs, including 5-fluorouracil, 5-FC lacks cytotoxic and antitumor activities in mammalian cells. Although this compound did not show bacteriostasis, it did possess selective antifungal activity against yeast-like fungi *in vitro* (10). It affects the nucleic acid synthesis by inhibiting the pyrimidine biosynthetic pathways. Utz *et al.* (11) have shown 5-FC to be effective in the treatment of cryptococcosis in man. However, many patients treated with 5-FC for cryptococcal meningitis relapsed after showing initial improvement. Many strains of yeast cells isolated from patients treated with 5-FC have proved resistant to the drug. Resistance may result from failure of the pyrimidine analog to penetrate the yeast (12).

Since there are so few antifungal antibiotics, the search for new potent, less toxic drugs for the treatment of systemic mycoses seemed desirable.

This investigation was primarily concerned with a new antifungal agent and its effect on *C. neoformans* at the cellular and molecular levels. In addition, its *in vivo* antifungal activity was determined against *C. neoformans*.

Materials and Methods. Organism. Stock culture of *C. neoformans*, strain 184, originally isolated from a human case by Dr. L. Friedman, Charity Hospital, New Orleans, La., was maintained on modified Sabouraud's dextrose agar. Transfers of the organism to fresh slants were made at 10-day intervals.

Medium. A modified Wickerham's medium (13) was employed in *in vitro* experiments. The medium consisted of Wickerham's concentration of trace elements and salts, 1% glucose, 0.5% vitamin free casamino acids (Difco), and thiamin at 0.2 μg/ml.

Sabouraud's dextrose agar medium, containing 20 units penicillin and 40 units streptomycin per ml, was used to determine viable colony forming units.

Antibiotic and chemicals. 2,4-diamino-6-[2-(3,4-dichlorophenyl) acetamido] quinazoline (QU) was supplied by the Department of Microbiology, Medical University of South Carolina. The chemicals, [^{3}H]uridine (46.2Ci/mmole) and [^{3}H]L-leucine (31.9 Ci/mmole) were purchased from New England Nuclear.

Experimental animal. All male, inbred

[1] Supported in part by USPHS Grant No. 5 T01 AI00123, National Institutes of Health.

[2] To whom all correspondence should be addressed.

BALB/cJ mice were used throughout the *in vivo* experiments.

Determination of the minimal inhibitory concentration (MIC). The tube dilution sensitivity method was utilized to determine the MIC of the drug.

C. neoformans cells were grown in 500-ml Erlenmeyer flasks containing 100 ml of the modified Wickerham's medium for 24 hr. Cells were harvested by centrifugation at 1200*g* for 10 min at 25°C. This was followed by two washings with the same medium. The cells were resuspended in physiological saline to a final concentration of 1×10^5 cells/ml. One ml of this cell suspension was dispensed into sterile tubes and different concentrations of the test antibiotic, in 1-ml volumes, were added to each tube. Modified Wickerham's medium was added to the tubes to a final volume of 10 ml. Tubes were placed at a 30° angle in a 37°C gyrotory shaker (120 rpm).

Viable particle counts were determined to establish the relative increase in population of the yeast. The MIC was defined as the lowest concentration of drug that completely inhibited multiplication of the fungus as determined by colony counts.

Effect on synthesis of macromolecules. Ribonucleic acid and protein synthesis were studied in *C. neoformans* by following the incorporation of appropriately labeled radioactive precursors into trichloroacetic acid (TCA) insoluble material. Cells were grown on defined medium for 6 hr and harvested in the manner previously described. Cell suspensions were adjusted to 5×10^5/ml. The experimental drug and radioactively labeled material were added. One-milliliter aliquots were withdrawn from the cultures after 4 hr and further reactions were arrested by addition of an equal volume of 10% cold TCA. The acid insoluble material was collected on 0.45 μm membrane filter, washed three times with 20 ml of 5% cold TCA, and dried. Radioactivity was measured by counting each filter in toluene base fluor with a Beckman L-S 100 liquid scintillation counter. Control groups of nonviable *C. neoformans* cells were processed in the same manner as were the living yeast. There was no evidence of uptake of the labeled precursors by the nonliving cells.

Animal experiments. A 24-hr culture of *C. neoformans*, grown in modified Sabouraud's dextrose broth medium, was harvested and washed twice with physiological saline. The cells were resuspended in physiological saline. Mice, weighing 18–22 g, were infected by injection into a lateral tail vein with 0.1 ml containing 5×10^6 viable yeast cells. Animals were housed in groups of 10 and given food and water *ad libitum* and observed daily. Therapy was begun 72 hr postinfection. Ten mice were included in each control and test group. Each mouse received 14 ip injections, or oral doses, of drug, one every 48 hr. Controls consisted of groups of uninfected mice treated (ip or oral) with the highest concentration of the drug (800 μg/kg/dose), uninfected mice treated (ip or oral) with physiological saline, and infected untreated mice. One added group was incorporated as a sham, operating control for ip experiments. After administering seven doses of the drug, each mouse was weighed and the kilogram dosage was adjusted for any weight gain. Dead mice, collected during daily routine observations, were autopsied and their organs (brains, blood, and spleen) cultured on modified Sabouraud's dextrose agar plates. On Day 31 postinfection, the surviving mice were sacrificed and organ cultures were made.

Results. In vitro studies. The data in Fig. 1 show the MIC value of QU for *C. neoformans*. The MIC values differed at each time interval and increased as the incubation time was prolonged.

QU at the concentrations under MIC ap-

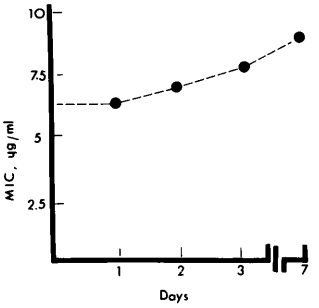

FIG. 1. Minimal inhibitory concentration of quinazoline derivative for *C. neoformans* at designated periods of incubation. Colony counts of the yeasts were performed after treatment with several concentrations of the drug.

peared to be effective in preventing the uptake of labeled precursors by the macromolecules of *C. neoformans* (Fig. 2). The inhibitory effect increased with higher concentrations of QU, inhibiting 40% of RNA and 50% of protein synthesis with use of the MIC level.

In vivo studies. The curing dose 50 (CD_{50}) was the dosage required to cure approximately 50% of the infected mice and was confirmed by the absence of *C. neoformans* yeast cells in their cultured organs.

In our experimental mouse model, using iv injections of *C. neoformans* yeast cells into the tail vein, results proved reproducible. In all cases, infected control animals, which received no drug treatment, died between 12 and 30 days postinfection.

A minimum of two different experiments were completed to determine the CD_{50}. Cumulative data from these studies are shown in Fig. 3.

There was 100% survival of animals treated with 800 μg QU/kg/dose, ip, and their organ cultures were negative, indicating the complete recovery from the disease. The CD_{50} was approximately 200 μg/kg/dose.

Discussion. Quinazoline derivatives have been used extensively against eukaryotic parasites, especially *Plasmodium* species

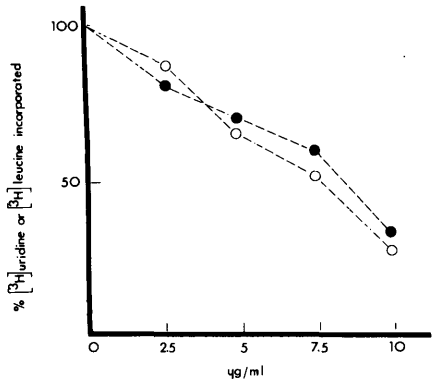

FIG. 2. Inhibition of RNA and protein synthesis by quinazoline derivative. [^{3}H]uridine (1 μCi/ml) (●), or [^{3}H]leucine (5 μCi/ml) (○) was added to an exponentially growing culture of *Cryptococcus neoformans*, 184, in the presence of different concentrations of the drug. Duplicate samples of 1 ml were taken after 4 hr. One hundred percent incorporation into TCA precipitable material for [^{3}H]uridine was 13.9×10^3 cpm/ml, and for [^{3}H]leucine was 9.6×10^3 cpm/ml.

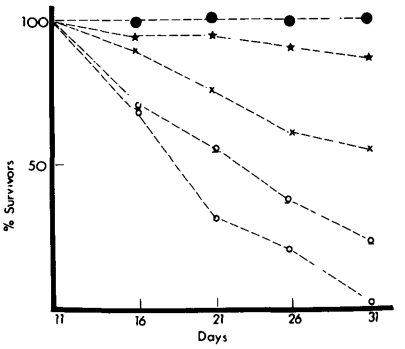

FIG. 3. Percentage of survivors of mice infected with *C. neoformans* and treated ip with different concentrations of QU over period of 31 days. Controls: (●) A-sham operating, B-uninfected treated with PBS, and C-uninfected treated with 800 mg/kg/dose, (○) infected untreated. Tests: infected and treated with (○) 100 μg/kg/dose, (×) 200 μg/kg/dose, (★) 400 μg/kg/dose, and (●) 800 μg/kg/dose.

(14, 15). Nevertheless, QU showed no activity against *Plasmodium* species. Subsequent *in vitro* screening against several yeasts revealed that this compound had antifungal properties. The MIC values were in the range of 6–8.25 μg/ml against *C. neoformans*, but the fungistatic dose varied slightly as time of incubation increased. The *in vitro* fungistatic or fungicidal properties of most antibiotics depend upon many factors, such as the nature of the organism, length of exposure to antibiotic, temperature being used, and dosage of the antibiotic. In our experiments, increasing the dose of QU above the MIC level eventually reached a fungicidal level.

At the molecular level, partial inhibition of cell RNA and protein synthesis was achieved using concentrations below MIC.

Results with *in vivo* mouse model indicated a statistically significant dose-related therapeutic effect of QU. It was encouraging to observe that no apparent deaths occurred due to drug toxicity at experimental dosages.

Experimentally infected mice did not respond to oral administration of QU as a therapeutic agent. This was probably due to lack of proper absorption or denaturation of the drug in the gastrointestinal tract. Intraperitoneal injections of infected animals with an adequate concentration of QU resulted in complete recovery from the disease.

We are currently investigating the synergistic effect of QU and AmB in combination. The mechanism of AmB action on model membranes (7) indicates that this antibiotic probably facilitates the passage of other drugs through the cellular membrane and increases their availability in the cytosol (16). Experimental evidence indicates that this phenomenon can be used to manipulate the permeability of the yeast cell. Lower, less toxic doses of AmB can be used in combination with other antifungal agents having a different mode of action than AmB (17). This has proved feasible in our studies using QU along with AmB (unpublished data).

Further evaluations and investigations are needed to provide sufficient information required to allow safe application of this agent against mycotic infections.

Summary. The minimum inhibitory concentration of quinazoline derivative was determined by the tube dilution method for *Cryptococcus neoformans*, strain 184.

The effect of this chemical agent on macromolecular metabolism indicated an inhibition of incorporations of labeled precursors into RNA and protein of *C. neoformans*.

A mouse model infection with *C. neoformans* was established. Following this, the animals were given ip or oral doses of different concentrations of the experimental drug. Infected mice responded to ip administration of the drug in that the percentage of surviving mice increased progressively with increasing drug dosage. The curing dose 50 (CD_{50}) was determined, based on the isolation of *C. neoformans* from organs of animals during or at the termination of the experiments.

1. Gendel, B. R., Endes, M., and Norman, S. L., Amer. J. Med. **9**, 343 (1949).
2. Zimmerman, L. E., Amer. J. Clin. Path. **25**, 46 (1955).
3. Hutter, R. V. P., and Collins, H. S., Lab. Invest. **11** (1962).
4. Gold, W., Stout, H. A., Pagano, J. F., and Donovick, R., Antibiot. Annu. 579 (1955–1956).
5. Vandeputte, J., Wachtel, J. L., and Stiller, E. T., Antibiot. Annu. 587 (1955–1956).
6. Kinsky, S. C., Antimicrob. Ag. Chemother. p. 387 (1963).
7. Kinsky, S. C., Ann. Rev. Pharmacol. **10**, 119 (1970).
8. Weissmann, C., Pras, M., and Hirschhorn, R., J. Clin. Invest. **45**, 1084 (1966).
9. Duschinsky, R., Pleven, E., and Heidelberger, C., J. Amer. Chem. Soc. **79**, 4559 (1957).
10. Grunberg, E., Titsworth, E., and Bennet, M., Antimicrob. Ag. Chemother. p. 566 (1963).
11. Utz, J. P., Tybes, B. S., Shadomy, H. J., Duma, R. J., Kannan, M. M., and Mason, K. N., Antimicrob. Ag. Chemother. p. 344 (1968).
12. Jund, R., and Lacroute, F., J. Bacteriol. **102**, 607 (1970).
13. Wickerham, L. J., J. Bacteriol. **52**, 293 (1946).
14. Thompson, P. F., Bayles, A., and Olszewski, B., Exp. Parasitol. **25**, 32 (1969).
15. Thompson, P. F., Bayles, A., and Olszewski, B., Amer. J. Trop. Med. Hyg. **19**, 12 (1970).
16. Medoff, G., Comfort, M., and Kobayashi, G. S., Proc. Soc. Exp. Biol. Med. **138**, 571 (1971).
17. Kwan, C. N., Medoff, G., Kobayashi, G. S., Schlessinger, D., and Raskas, H. J., Antimicrob. Ag. Chemother. **2**, 61 (1972).

Received September 2, 1975. P.S.E.B.M. 1976, Vol. 151.