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IN VITRO AND *IN VIVO* STUDY OF THE NEW ANTIFUNGAL AGENTS

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IN VITRO AND _IN VIVO_ STUDY OF THE NEW ANTIFUNGAL AGENTS

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

FIGURE PAGE

IN VITRO AND *IN VIVO* STUDY OF THE NEW ANTIFUNGAL AGENTS

CHAPTER I

INTRODUCTION

Cryptococcus neoformans is a pathogenic fungus which may cause a primary pulmonary infection. Disseminating to other parts of the body, *C. neoformans* has shown a special affinity for the central nervous system (CNS).

Cryptococcus neoformans history is unique among all the fungi pathogenic to man. It was discovered almost simultaneously to exist as a highly virulent agent of human disease and as a saprophyte in nature. In 1894, Busse (7) reported a case involving skin and bone lesions in a human caused by a yeast-like fungus. During the same year, Sanfelice (34) recovered a yeast-like organism from peaches that proved to be pathogenic for laboratory animals and which he named *Saccharomyces neoformans*. Later Weis (52) reported isolates from peaches and milk were microscopically and culturally identical to those recovered from human cases.

The first human case of cryptococcosis of the nervous system was diagnosed post mortem by Verse (49)

Shortly afterward, Stoddard (44) reported two additional human cases and named the organism Torula histolitica. He also described the pathological differences between cryptococcosis and North American blastomycosis. In 1935, Benham (2,3) systematically investigated 22 isolates of Cryptococcus and named the species hominis. Finally, in 1952 Lodder (29) in his taxonomic study classified this encapsulated yeast as Cryptococcus neoformans.

Saprophytic varieties of cryptococcus species were found to occur on the skin and in the intestinal tract, in over half of the normal subjects studied by Benham (4) and Ravits (33). Man and animals frequently acquire this fungus by inhalation of soil and pigeon materials. Ajello (1) suggests that although heavily exposed, man may frequently have undetected asymptomatic infections. Resistance to the disease may be explained on the basis of one's ability to resist primary infection. Except under unusual circumstances, such resistance may result from development of a specific immunological response to the agent. Therefore, it appears more likely that natural resistance must be altered before actual infection occurs (11). Gendel (12) and Littman (27) reported that disseminated cryptococcosis developed more frequently in persons with low host resistance or in those individuals having reticuloendothelial diseases such as leukemia, malignant lymphoma, or Hodgkin's disease. In addition, Hutter (19) and Zimmerman (55) have

REPORTED THAT SYSTEMIC CRYPTOCOCCOSIS FREQUENTLY DEVELOPS IN PATIENTS WHO RECEIVE EXTENSIVE STEROIDS AND BROAD SPECTRUM ANTIBIOTICS TREATMENT. DISSEMINATION OFTEN OCCUR FROM FOCI IN THE LUNG VIA THE BLOOD STREAM TO ALL ORGANS INCLUDING THE CNS.

THE FIRST TRULY ANTIFUNGAL ANTIBIOTIC UTILIZED IN THE TREATMENT OF CRYPTOCOCCAL MENINGITIS WAS CYCLOHEXIMIDE (ACTIDIONE), WHICH WAS PRODUCED BY *STREPTOMYCES GRISEUS*. WAKSMAN (50) AND WHIFFEN (54) HAVE SHOWN THAT THIS ANTIBIOTIC POSSESSED HIGH *IN VITRO* FUNGICIDAL ACTION AGAINST *C. NEOFORMANS* AND OTHER YEASTS. HOWEVER, LITTMAN (28) PROVED THIS ANTIBIOTIC WAS INEFFECTIVE CLINICALLY.

LATER FISHER (10) USED DIAMIDINE (PROPAMIDINE) AGAINST *C. NEOFORMANS* IN A CONCENTRATION OF 620 µg/ml WHICH WAS FUNGICIDAL AND 80 µg/ml WHICH WAS FUNGISTATIC TO 2 STRAINS. *IN VITRO* STUDIES BY SOLOTOROVSKY (41) REVEALED PROPAMIDINE AND HYDROXYSTILBAMIDINE TO BE THE MOST ACTIVE DIAMIDINES AGAINST *C. NEOFORMANS*.

IN 1956, GOLD (14) MADE THE SIGNIFICANT DISCOVERY OF THE POLYENE ANTIBIOTIC, AMPHOTERICIN B, PRODUCED BY *STREPTOMYCES NODOSUS* WHICH WAS ISOLATED FROM A VENEZUELAN SOIL SAMPLE. AMPHOTERICIN A WAS DISTINGUISHED FROM AMPHOTERICIN B BY VANDEPUTTE (48). THESE FINDINGS MARKED THE BEGINNING OF A NEW ERA IN THE TREATMENT OF SYSTEMIC MYCOSES, PARTICULARLY CRYPTOCOCCAL MENINGITIS. *IN VITRO* AND *IN VIVO* EFFECTS OF AMPHOTERICIN B WERE EVALUATED BY STEINBERG

(42,43). These studies proved that the drug was effective *in vitro* against 9 strains of deep and 8 strains of superficial fungi. In experimental coccidioidomycosis and moniliasis in mice, intraperitoneal administration of amphotericin B produced protective and therapeutic effects with dosages ranging between 1 to 2.5 mg/kg. Oral administration of amphotericin B proved effective only against coccidioidomycosis in experimental mice. No toxic effects were revealed in the mice by either route of administration. Lampen (25,26) showed that the primary site of drug action was on the membranous structure of the cell. His studies of cytoplasmic membranes from yeast cells treated with polyene and isolated by differential centifugation revealed a close relationship between the content of sterols and polyene found in the lipid fraction of the membranes. Gottlieb (16) demonstrated that the antifungal activity of polyenes was reversed by the presence of various sterols in the medium. Weissman (53) also found that amphotericin B caused significant damage to lysosomes derived from kidney. This phenomenon may partially explain the renal toxicity frequently associated with amphotericin B.

Treatment of systemic mycoses with amphotericin B has been relatively successful. In the review by Hildick-Smith (18) 72% of the 66 reported cases showed a favorable response to this antibiotic. Nevertheless, there remains a need for more active and less toxic drugs in the treatment

OF CRYPTOCOCCOSIS AND OTHER HUMAN SYSTEMIC FUNGAL INFECTIONS.

THE AGENT 5-FLUOROCYTOSINE (5-FC) WAS FIRST SYNTHESIZED BY DUSCHINSKY (8) AND UNLIKE OTHER COMPOUNDS OF THE SERIES INCLUDING 5-FLUOROURACIL (5-FU), IT 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 (17). IT EFFECTS THE NUCLEIC ACID SYNTHESIS BY INHIBITING THE PYRIMIDINE BIOSYNTHETIC PATHWAY. SHADOMY (37,38,39,40) AND UTZ (47) HAVE SHOWN THAT 5-FC WAS EFFECTIVE IN THE TREATMENT OF CRYPTOCOCCOSIS IN MAN. HOWEVER, MANY PATIENTS TREATED WITH 5-FC FOR CRYPTOCOCCAL M 'INGITIS RELAPSED AFTER SHOWING INITIAL IMPROVEMENT. RESISTANCE TO 5-FC HAS DEVELOPED IN MANY STRAINS OF YEAST CELLS CULTURED FROM PATIENTS TREATED WITH 5-FC. RESISTANCE MAY RESULT FROM FAILURE OF THE PYRIMIDINE ANALOG TO PENETRATE THE YEAST (21).

ONLY A FEW ANTIFUNGAL AGENTS HAVE SHOWN EFFECTS AGAINST VARIOUS MYCOTIC INFECTIONS. THEREFORE, THE SEARCH FOR NEW POTENT, LESS TOXIC DRUGS FOR THE TREATMENT OF SYSTEMIC MYCOSES SEEMED DESIRABLE.

THIS INVESTIGATION WAS PRIMARILY CONCERNED WITH NEW ANTIFUNGAL AGENTS AND THEIR EFFECTS ON C. NEOFORMANS, INDIVIDUALLY AND IN COMBINATION WITH AMPHOTERICIN B. SPECIFIC EFFECTS WERE STUDIED AT THE CELLULAR AND MOLECULAR LEVELS AND IN ADDITION THEIR ANTIFUNGAL ACTIVITIES WERE DETERMINED IN VIVO AGAINST C. NEOFORMANS.

CHAPTER II

MATERIALS AND METHODS

Organism:

Stock cultures of *C. neoformans*, strain 184, originally isolated from a human case by Dr. Lorraine Friedman, Charity Hospital, New Orleans, Louisiana, were maintained on modified Sabouraud's dextrose agar and defined agar slants at room temperature. Transfers of the organism to fresh slants were made at ten day intervals.

Medium:

The defined medium used in our experiments consisted of: 0.01 mg boric acid, 0.01 mg copper sulfate, 0.1 mg potassium iodine, 0.05 mg ferric chloride, 0.07 mg zinc sulfate, 0.1 mg manganous sulfate, 0.01 mg sodium molybdate, 1 gram ammonium sulfate, 0.15 gram potassium phosphate dibasic, 0.1 gram potassium phosphate monobasic, 0.5 gram magnesium chloride, 0.1 gram sodium chloride, 0.1 gram calcium chloride, 5 grams casamino acids, 10 grams glucose, 200 µg thiamine hydrochloride in 1000 ml of distilled water. Solutions of buffered salts, casamino acids, trace salts and glucose were autoclaved separately and mixed after cooling.

THIAMINE SOLUTIONS WERE STERILIZED BY FILTERATION AND ADDED TO ENTIRE MEDIUM.

SABOURAUD'S DEXTROSE AGAR MEDIUM CONTAINING 20 UNITS PENICILLIN AND 40 UNITS STREPTOMYCIN PER ml WAS USED TO DETERMINE VIABLE COLONY FORMING UNITS.

EXPERIMENTAL ANIMAL:

AN INBRED STRAIN OF MALE MICE, THE BALB/cj (ORIGINAL STOCK OBTAINED FROM JACKSON LABORATORY, BAR HARBOR, MAINE, AND MAINTAINED THROUGH 110 GENERATIONS IN OUR LABORATORIES) WERE USED THROUGHOUT THE IN VIVO EXPERIMENTS.

ANTIBIOTICS AND CHEMICALS:

AMPHOTERICIN B (Amb) WAS PURCHASED FROM E. R. SQUIBB AND SONS; NALIDIXIC ACID (NA) WAS DONATED BY STERLING-WINTHROP RESEARCH INS., RENSSELAER, NEW YORK 12144; MICONAZOLE NITRATE (Mn) (1-{2-(2,4-DICHLOROPHENYL)-2-[(2,4-DICHLOROPHENYL) METHOXY]ETHYL} IMIDAZOLE MONONITRATE, WAS SUPPLIED BY JANSSEN PHARMACEUTICA, 2340 BEERSE, BELGIUM; AND QUINAZOLINE DERIVATIVE (Qu) (2,4-DIAMINO-6-[2-(3,4-DICHLOROPHENYL) ACDTAMIDO] QUINAZOLINE WAS OBTAINED FROM THE DEPARTMENT OF MICROBIOLOGY AND COLLEGE OF PHARMACY AT THE MEDICAL UNIVERSITY OF SOUTH CAROLINA, CHARLESTON, SOUTH CAROLINA 29401.

[^{3}H] URIDINE (SPECIFIC ACTIVITY: 46.2 Ci/mmole), [^{3}H] THYMIDINE (SPECIFIC ACTIVITY: 6.7 Ci/mmole) AND [^{3}H] L-LEUCINE (SPECIFIC ACTIVITY: 31.9 Ci/mmole) WAS PURCHASED

FROM NEW ENGLAND NUCLEAR, 575 ALBANY STREET, BOSTON, MASSACHUSETTS 02118.

DETERMINATION OF THE MINIMAL INHIBITORY CONCENTRATION (MIC):

THE TUBE DILUTION SENSITIVITY METHOD WAS UTILIZED TO DETERMINE THE MIC OF THE DRUGS.

C. NEOFORMANS CELLS WERE GROWN IN 500 ml ERLENMEYER FLASKS CONTAINING 100 ml OF THE DEFINED MEDIUM FOR 24 HOURS. CELLS WERE HARVESTED BY CENTRIFUGATION (1200 x G FOR 10 MINUTES AT 25 C IN 50 ml PLASTIC CENTRIFUGE TUBES IN INTERNATIONAL CENTRIFUGE MODEL HN). THIS WAS FOLLOWED BY TWO WASHINGS WITH THE SAME MEDIUM AND THE SUPERNATANTS WERE DISCARDED. THE CELLS WERE RESUSPENDED IN DEFINED MEDIUM AND ADJUSTED TO A CONCENTRATION OF 1×10^5 CELLS PER ml DETERMINED BY HEMOCYTOMETER COUNTS AND CONFIRMED BY COLONY COUNTS. ONE ml OF THIS CELL SUSPENSION WAS DISPENSED TO STERILE, COTTON PLUGGED TUBES (SIZE 16 x 150 BELLCO GLASS INC.). VARIOUS CONCENTRATIONS OF THE TEST ANTIBIOTICS IN ONE ml VOLUMES WERE ADDED TO EACH TUBE. DEFINED MEDIUM WAS ADDED TO THE TUBES TO THE FINAL VOLUME OF TEN ml. TUBES WERE PLACED AT A 30° ANGLE IN A GYROTORY SHAKER AT 120 RPM AT 37 C.

TURBIDIMETRIC MEASUREMENTS (600 nm, BAUSCH LOMB SPECTRONIC 20) ALONG WITH VIABLE PARTICLE COUNTS WERE DETERMINED AT TIMED INTERVALS TO ESTABLISH THE RELATIVE INCREASE IN POPULATION OF THE YEAST. THE MIC WAS DEFINED AS THE LOWEST CONCENTRATION OF EACH DRUG THAT COMPLETELY INHIBITED

THE FUNGUS AS DETERMINED BY COLONY COUNTS.

SYNERGISM STUDIES:

CELLS WERE GROWN, WASHED AND RESUSPENDED AS PREVIOUSLY DESCRIBED. A CONCENTRATION OF 0.1 µg/ml Amb WHICH WAS 1/4 OF MIC, WAS USED ALONE OR IN COMBINATION WITH DIFFERENT CONCENTRATIONS OF ONE OF THE EXPERIMENTAL DRUGS (Qu, Mn, AND NA). DRUGS WERE ADDED SIMULTANEOUSLY TO EACH TUBE IN ONE ml PORTIONS BEGINNING BELOW THEIR RESPECTIVE MIC's. ADDITIONAL TESTS WERE PERFORMED APPLYING DIFFERENT CONCENTRATIONS OF THE THREE DRUGS IN THE ABSENCE OF Amb. THE DEFINED MEDIUM WAS ADDED TO ALL TUBES TO A FINAL VOLUME OF 10 ml AFTER WHICH THE TUBES WERE PLACED ON THE SHAKER.

ANTIFUNGAL DRUG SYNERGY WAS DEFINED AS A DECREASE OF A HUNDRED FOLDS OR MORE IN THE COLONY COUNTS WHEN DRUGS WERE USED IN COMBINATION WITH Amb AS COMPARED TO COLONY COUNTS WHEN A SINGLE DRUG WAS USED (32).

EFFECT ON SYNTHESIS OF MACROMOLECULES:

NUCLEIC 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 SIX HOURS AND HARVESTED IN THE MANNER PREVIOUSLY DESCRIBED. CELL SUSPENSIONS WERE ADJUSTED TO OPTICAL DENSITY OF 0.15, APPROXIMATELY 5×10^5 CELLS PER ml. THE EXPERIMENTAL DRUG AND RADIOACTIVELY LABELED MATERIAL WERE ADDED AND

ONE ml ALIQUOTS WERE WITHDRAWN FROM THE CULTURES AT VARIOUS TIME INTERVALS. FURTHER REACTIONS WERE ARRESTED BY THE ADDITION OF AN EQUAL VOLUME OF 10% COLD TCA AND THE ACID INSOLUBLE MATERIALS WERE COLLECTED ON 0.45 μm MEMBRANE FILTERS (MILLIPORE). PRECIPITATES WERE WASHED THREE TIMES WITH 20 ml OF 5% COLD TCA, DRIED AND DISSOLVED IN 10 ml SOLUTION CONTAINING TWO PARTS TOLUENE, ONE PART TRITON X 100 AND 0.8% 2,4-DIPHENYLOXAZOLE (PPO). COUNTS WERE MADE WITH THE BECKMAN L-S 100 LIQUID SCINTILLATION COUNTER.

SYNERGISTIC EFFECTS ON SYNTHESIS OF MACROMOLECULES:

THE SAME PROCEDURES AS DESCRIBED PREVIOUSLY FOR SINGLE ANTIBIOTIC WERE USED; HOWEVER, BOTH ANTIBIOTICS WERE ADDED SIMULTANEOUSLY. THE CONCENTRATION OF 0.1 μg/ml Amb WAS USED IN COMBINATION WITH EACH ONE OF THE OTHER DRUGS (Qu, Mn, AND NA). A WIDE RANGE OF CONCENTRATIONS OF THE OTHER DRUGS, STARTING WELL UNDER EACH MIC'S, WERE INVESTIGATED.

MICE EXPERIMENTS:

A 24 HOUR CULTURE OF C. NEOFORMANS GROWN IN MODIFIED SABOURAUD'S DEXTROSE BROTH MEDIUM WAS HARVESTED BY CENTRIFUGATION (1200 x G FOR 10 MINUTES AT 25 C IN 50 ml PLASTIC CENTRIFUGE TUBES) FOLLOWED BY TWO WASHINGS WITH PHYSIOLOGICAL SALINE. THE SUPERNATANTS WERE DISCARDED AND THE CELLS WERE RESUSPENDED IN PHYSIOLOGICAL SALINE. MICE WEIGHING 18-22 GRAMS WERE INFECTED BY INJECTION OF 0.1 ml CONTAINING

5×10^6 VIABLE YEAST CELLS, INTO A LATERAL TAIL VEIN. THEY WERE HOUSED IN GROUPS OF TEN, GIVEN FOOD AND WATER AD LIBITUM, AND OBSERVED DAILY. THERAPY WAS BEGUN 72 HOURS POST INFECTION. TEN MICE WERE INCLUDED IN EACH CONTROL AND TEST GROUP. EACH MOUSE RECEIVED 14 INTRAPERITONEAL INJECTIONS OR ORAL DOSAGES OF DRUG INCORPORATED IN THE DAILY DIET, ONE EVERY 48 HOURS. CONTROLS CONSISTED OF GROUPS OF UNINFECTED MICE TREATED WITH HIGH CONCENTRATION (S) OF DRUG, UNINFECTED MICE TREATED WITH IP INJECTIONS OF PHYSIOLOGICAL SALINE, AND INFECTED UNTREATED MICE. ONE ADDED GROUP WAS INCORPORATED AS A SHAM OPERATING CONTROL FOR IP EXPERIMENTS. AFTER ADMINISTRATING 7 DOSAGES OF DRUGS, EACH MOUSE WAS WEIGHED AND DOSAGE PER kg WAS ADJUSTED. DURING DAILY OBSERVATION, DEAD MICE WERE AUTOPSIED AND THEIR ORGANS (BRAIN, BLOOD, AND SPLEEN) CULTURED ON MODIFIED SABOURAUD'S DEXTROSE AGAR PLATES WITH PENICILLIN AND STREPTOMYCIN. ON DAY 31 POST INFECTION, THE SURVIVING MICE WERE SACRIFICED AND ORGANS WERE CULTURED.

CHAPTER III

RESULTS

The data in Figure 1 show the MIC values of the experimental drugs (Amb, Qu, Mn, and NA) for C. neoformans. The MIC values of Qu and NA differed at each time interval and increased as the incubation time was prolonged; whereas, these values were relatively constant for Amb and Mn.

The synergism, resulting from different concentrations of Qu, Mn, or NA in combination with Amb, is illustrated in Figures 2-4. The concentration of Amb employed in each of the synergy studies was 1/4 of minimum inhibitory concentration for C. neoformans. At this concentration, Amb has no inhibitory effect on growth of the yeast-like organism. Several concentrations of Qu, Mn, and NA, starting well below the respective MIC values were used. Synergy was demonstrated with the combined use of Qu or NA and Amb by a decrease of 100 fold or more in the colony counts, as compared to using either drug alone. However, synergism was not observed using various concentrations of Mn with Amb.

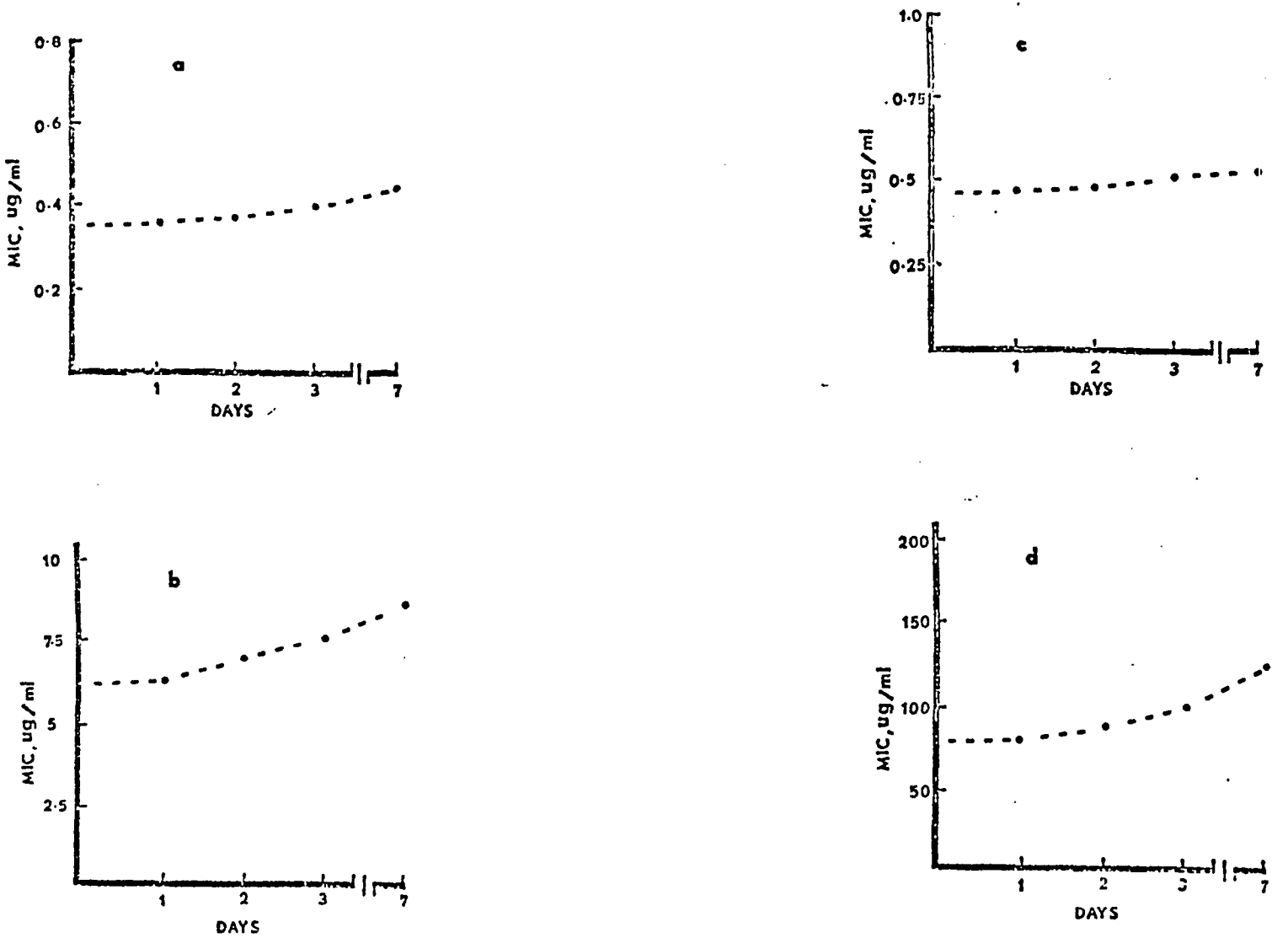
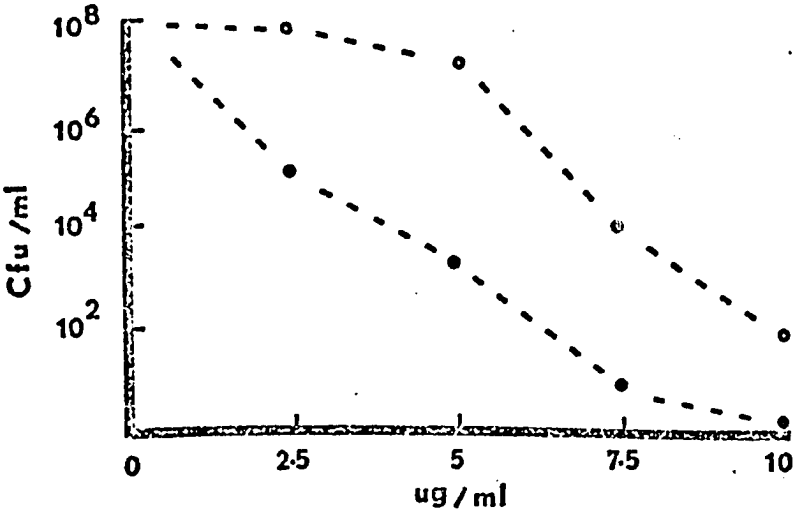


FIGURE 1. MINIMAL INHIBITORY CONCENTRATIONS OF AMPHOTERICIN B (A), QUINAZOLINE DERIVATIVE (B), MICONAZOLE NITRATE (C), AND NALIDIXIC ACID (D) FOR C. NEOFORMANS AT DESIGNATED PERIODS OF INCUBATION. COLONY COUNTS OF THE YEASTS WERE PERFORMED AFTER TREATMENT WITH SEVERAL CONCENTRATIONS OF EACH CHEMICAL COMPOUND.

FIGURE 2. DOSE RESPONSE OF THE VIABILITY OF C. NEOFORMANS TO DIFFERENT CONCENTRATIONS OF Qu ALONE (o--o) AND IN COMBINATION WITH 0.1 µg/ml Amb (•--•). COLONY COUNTS WERE DETERMINED AFTER 3 DAYS OF INCUBATION.

FIGURE 3. DOSE RESPONSE OF THE VIABILITY OF C. NEOFORMANS TO DIFFERENT CONCENTRATIONS OF Mn ALONE (o--o) AND IN COMBINATION WITH 0.1 µg/ml Amb (•--•). INCUBATIONS WERE 3 DAYS. COLONY COUNTS WERE DETERMINED AT THE END OF THIRD DAY.

FIGURE 4. DOSE RESPONSE OF THE VIABILITY OF C. NEOFORMANS TO DIFFERENT CONCENTRATIONS OF NA ALONE (o--o) AND IN COMBINATION WITH 0.1 µg/ml Amb (•--•). VIABILITY STUDIES WERE PERFORMED AFTER 3 DAYS OF INCUBATION.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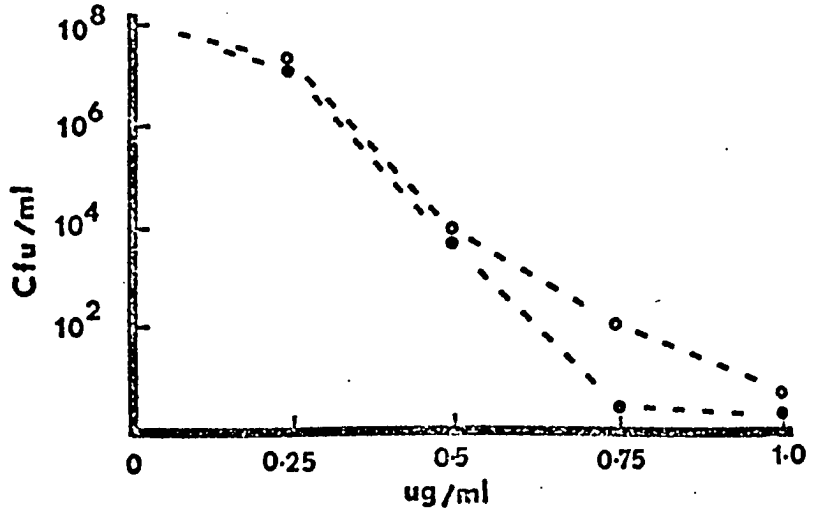
10^6
10^4
10^2
Cfu/ml
0
2·5
5
7·5
10
ug/ml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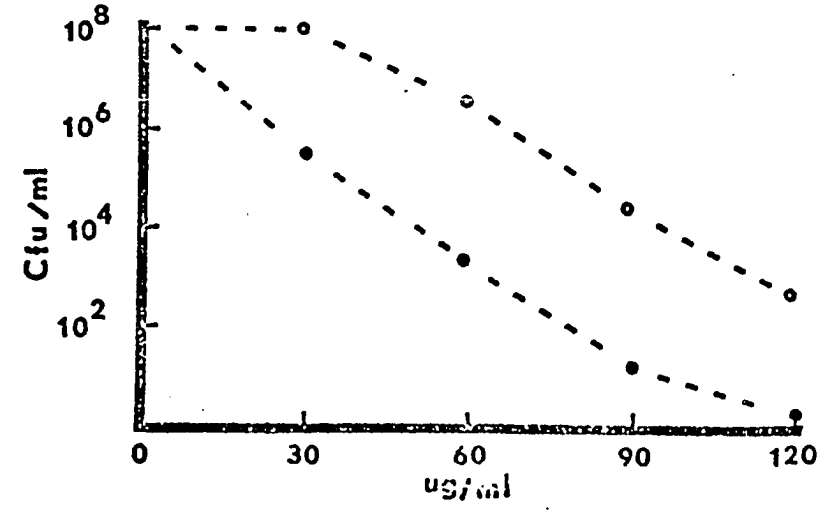
10^6
10^4
10^2
Cfu/ml
0
0·25
0·5
0·75
1·0
ug/ml


10^8
10^6
10^4
10^2
Cfu/ml
0
30
60
90
120
ug/ml

Amphotericin B, at concentrations under the MIC value appeared to be ineffective in preventing the uptake of labeled precursors by the macromolecules of C. neoformans (Figure 5). However, Qu and NA inhibited labeled uridine incorporation into RNA and labeled leucine incorporation into protein in C. neoformans. In addition, NA inhibited thymidine incorporation into the DNA of the yeast cells (Figures 6,7). The presence of 0.1 µg/ml Amb, well below the concentrations which depress macromolecular biosynthesis, potentiated the effectiveness of Qu up to 50% and NA up to 35%. In contrast, the data in Figure 8 indicate that macromolecular synthesis was not inhibited by Mn alone or in combination with 0.1 µg/ml Amb.

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 model system, using IV injections of C. neoformans yeast cells into the tail vein of mice, results were reproducible. In all cases, control mice which received no drug treatment died between 12 and 30 days post infection. Surviving control groups included, uninfected mice receiving the highest drug dosages reported, mice uninfected and treated with physiological saline and the sham operating uninfected mice.

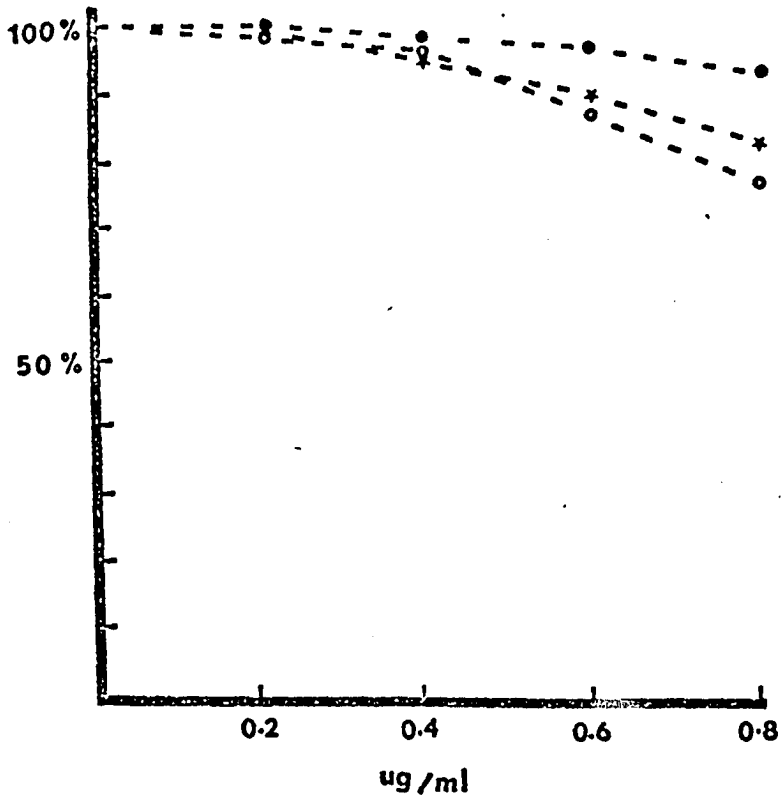


FIGURE 5. DOSE RESPONSE TO Amb OF [^{3}H] THYMIDINE (●--●), [^{3}H] URIDINE (o--o), AND [^{3}H] LEUCINE (*--*) INCORPORATION INTO TCA PRECIPITABLE FRACTION OF C. NEOFORMANS. THE YEASTS WERE GROWN IN [^{3}H] THYMIDINE (30 µci/ml), [^{3}H] URIDINE (1 µci/ml) AND [^{3}H] LEUCINE (5 µci/ml) AT INDICATED CONCENTRATIONS OF Amb FOR 4 HOURS. ONE HUNDRED PERCENT VALUE FOR [^{3}H] THYMIDINE WERE 4.4×10^3 COUNTS PER MIN PER ml, FOR [^{3}H] URIDINE WERE 16.2×10^3 cpm/ml, AND FOR [^{3}H] LEUCINE WERE 7.4×10^3 cpm/ml.

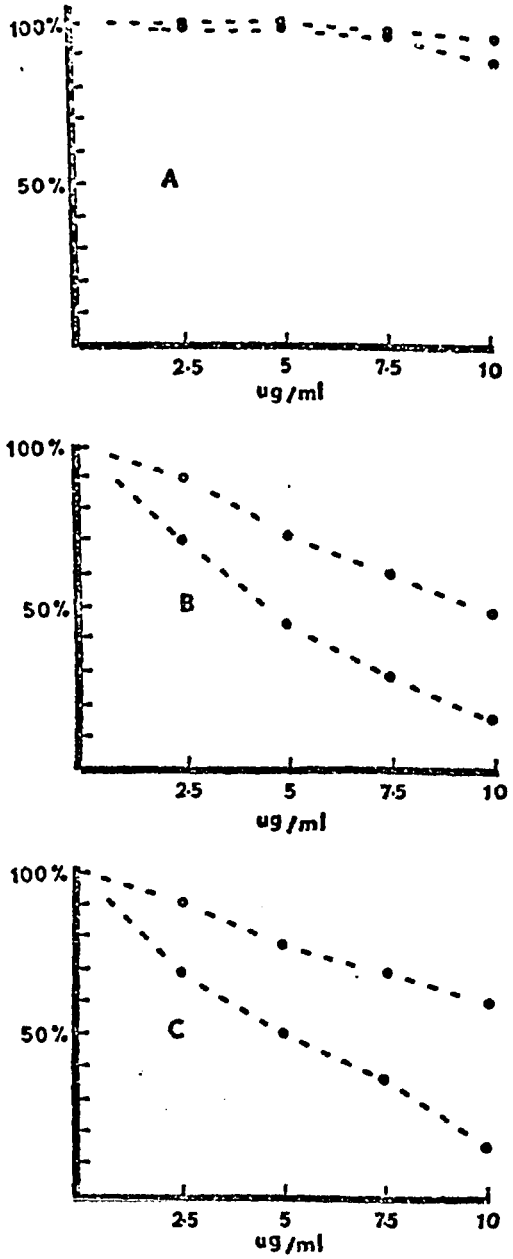


FIGURE 6. DOSE RESPONSE TO Qu OF [^{3}H] THYMIDINE (30 µci/ml) (A), [^{3}H] URIDINE (1 µci/ml) (B), AND [^{3}H] LEUCINE (5 µci/ml) (C) INCORPORATION AFTER 4 HOURS OF INCUBATION OF C. NEOFORMANS IN THE PRESENCE (●--●) AND ABSENCE (o--o) OF 0.1 µg/ml Amb. ONE HUNDRED PERCENT VALUE FOR [^{3}H] THYMIDINE INCORPORATION INTO TCA PRECIPITABLE MATERIALS WAS 3.1 x 10^3 cpm/ml, FOR [^{3}H] URIDINE WAS 13.9 x 10^3 cpm/ml AND FOR [^{3}H] LEUCINE WAS 6.6 x 10^3 cpm/ml.

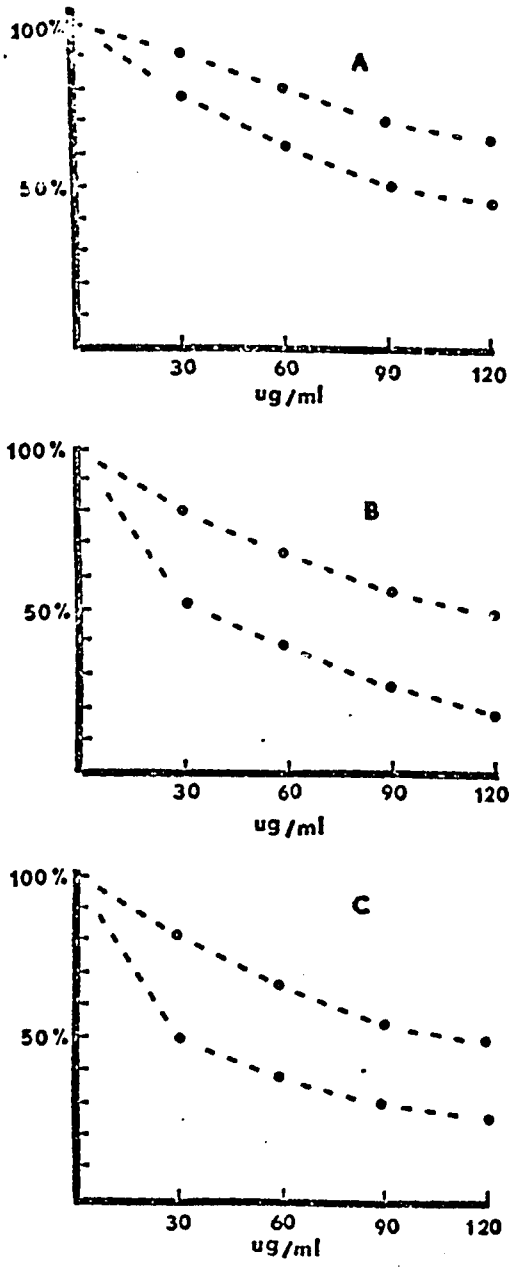


FIGURE 7. DOSE RESPONSE TO NA OF [^{3}H] THYMIDINE (30 µci/ml) (A), [^{3}H] URIDINE (1 µci/ml) (B) AND [^{3}H] LEUCINE (5 µci/ml) (C) INCORPORATION AFTER 4 HOURS OF INCUBATION OF C. NEOFORMANS IN THE PRESENCE (●--●) AND ABSENCE (o--o) OF 0.1 µg/ml Amb. ONE HUNDRED PERCENT VALUE FOR [^{3}H] THYMIDINE INCORPORATION INTO TCA PRECIPITABLE MATERIALS WAS 3.9×10^3 cpm/ml, FOR ^{3}H URIDINE WAS 16×10^3 cpm/ml AND FOR [^{3}H] LEUCINE WAS 7.5×10^3 cpm/ml.

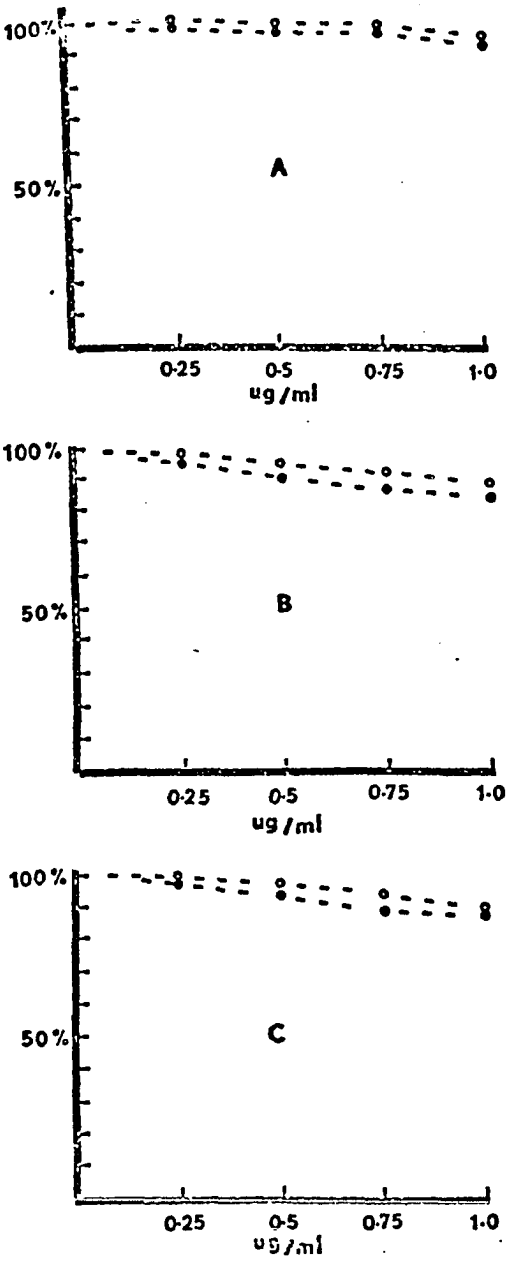


FIGURE 8. DOSE RESPONSE TO Mn OF [^{3}H] THYMIDINE (30 µci/ml) (A), [^{3}H] URIDINE (1 µci/ml) (B) AND [^{3}H] LEUCINE (5 µci/ml) (C) INCORPORATION AFTER 4 HOURS OF INCUBATION OF C. NEOFORMANS IN THE PRESENCE (●--●) AND ABSENCE (o--o) OF 0.1 µg/ml Amb. ONE HUNDRED PERCENT VALUE FOR [^{3}H] THYMIDINE INCORPORATION INTO TCA PRECIPITABLE MATERIALS WAS 3.3×10^3 cpm/ml, FOR [^{3}H] URIDINE WAS 17.3×10^3 cpm/ml AND FOR [^{3}H] LEUCINE WAS 8.1×10^3 cpm/ml.

To establish the CD-50 for Amb, Qu, Mn, and NA, a minimum of two different experiments for each drug were performed and accumulated data are shown in Figures 9-13. These data indicate that the percentage of surviving mice increased progressively with increasing drug dosage.

The dose response to Amb therapy is presented in Figure 9. Mortality was prevented completely and cultures of various organs (blood, spleen, and brain) were negative after a complete course of treatment with 1.5 mg/kg/dose. With the use of 1.0 mg/kg/dose, although all mice survived, C. neoformans was isolated from the spleen of two and the brain of one mouse. The CD-50 was 0.5 mg/kg/dose.

The survival rates of infected mice, treated with oral or IP Qu at different concentrations, are presented in Figures 10 and 11. After terminating therapy with at least an 800 µg/kg/dose IP, all mice survived and the cultures were negative for C. neoformans. The CD-50 of Qu for IP therapy was approximately 200 µg/kg/dose. Animals failed to respond to oral treatment in that 95% died due to C. neoformans infection.

The results obtained from oral administrations of various concentrations of Mn to mice infected with C. neoformans are shown in Figure 12. Data indicate total survival of the mice when one mg/kg/dose was used. The CD-50 for Mn was approximately 500 µg/kg/dose.

Oral treatment of infected mice with a 400 µg/kg/dose

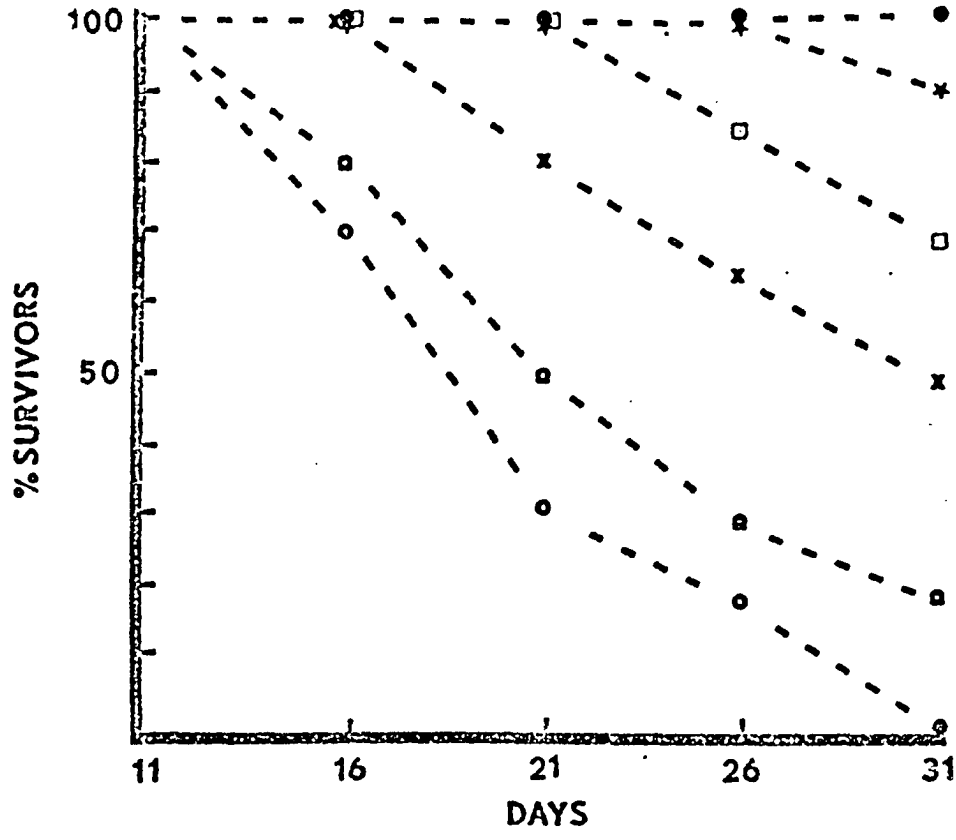


FIGURE 9. PERCENTAGE OF SURVIVORS OF MICE INFECTED WITH C. NEOFORMANS AND TREATED IP WITH DIFFERENT CONCENTRATIONS OF Amb OVER 31 DAYS TESTING PERIOD. CONTROLS: (●) SHAM OPERATING, UNINFECTED TREATED WITH PBS OR 1.5 mg/kg/dose, (o) INFECTED UNTREATED. TESTS: INFECTED AND TREATED WITH: (◘) 0.25 mg/kg/dose, (x) 0.5 mg/kg/dose, (□) 0.75 mg/kg/dose, (*) 1.0 mg/kg/dose AND (⊙) 1.5 mg/kg/dose.

OF NA, RESULTED IN COMPLETE SURVIVAL. C. NEOFORMANS WAS NOT CULTURED FROM ANY ORGAN (FIGURE 13). THE CD-50 OF THIS DRUG WAS 100 μg/kg/dose.

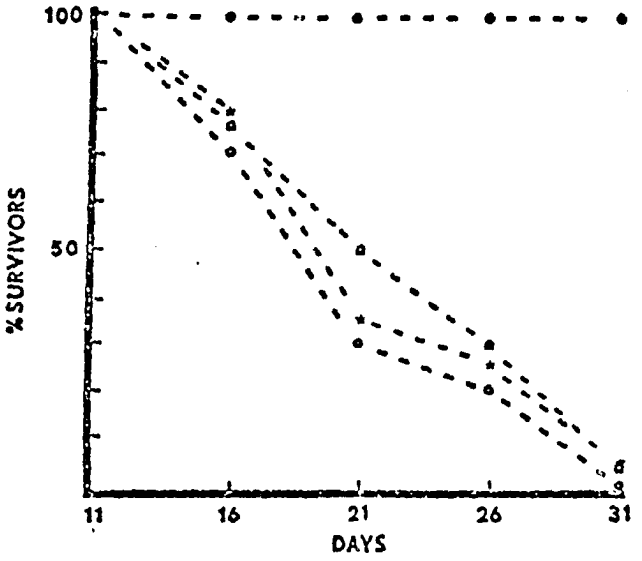


FIGURE 10. PERCENTAGE OF SURVIVORS OF MICE INFECTED WITH C. NEOFORMANS AND TREATED ORALLY WITH DIFFERENT CONCENTRATIONS OF Qu OVER PERIOD OF 31 DAYS. CONTROLS: (●) UNINFECTED TREATED WITH 10 mg/kg/dose, (○) INFECTED UNTREATED. TESTS: INFECTED AND TREATED WITH (▲) 500 µg/kg/dose, (■) 1 mg/kg/dose AND (*) 10 mg/kg/dose.

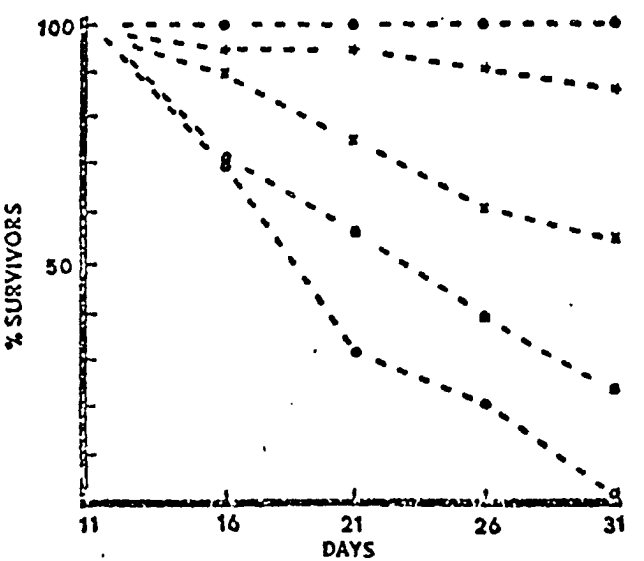


FIGURE 11. PERCENTAGE OF SURVIVORS OF MICE INFECTED WITH C. NEOFORMANS AND TREATED IP WITH DIFFERENT CONCENTRATIONS OF Qu OVER PERIOD OF 31 DAYS. CONTROLS: (●) A-SHAM OPERATING, B-UNINFECTED TREATED WITH PBS, AND C-UNINFECTED TREATED WITH 800 mg/kg/dose,(○) INFECTED UNTREATED. TESTS: INFECTED AND TREATED WITH: (▲) 100 µg/kg/dose, (x) 200 µg/kg/dose, (■) 400 µg/kg/dose AND (*) 800 µg/kg/dose.

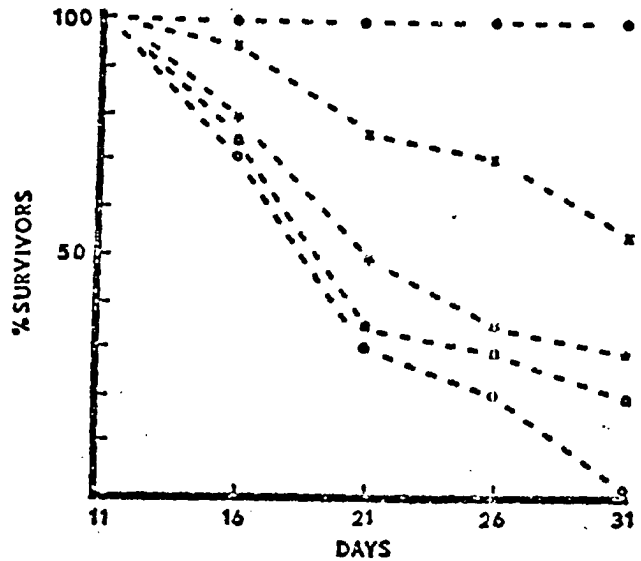


FIGURE 12. PERCENTAGE OF SURVIVORS OF MICE INFECTED WITH C. NEOFORMANS AND TREATED ORALLY WITH DIFFERENT CONCENTRATIONS OF MA OVER PERIOD OF 31 DAYS. CONTROLS: (•) UNINFECTED TREATED WITH 1 mg/kg/dose, (o) INFECTED UNTREATED. TESTS: INFECTED AND TREATED WITH (▪) 125 µg/kg/dose, (*) 250 µg/kg/dose, (x) 500 µg/kg/dose AND (●) 1 mg/kg/dose.

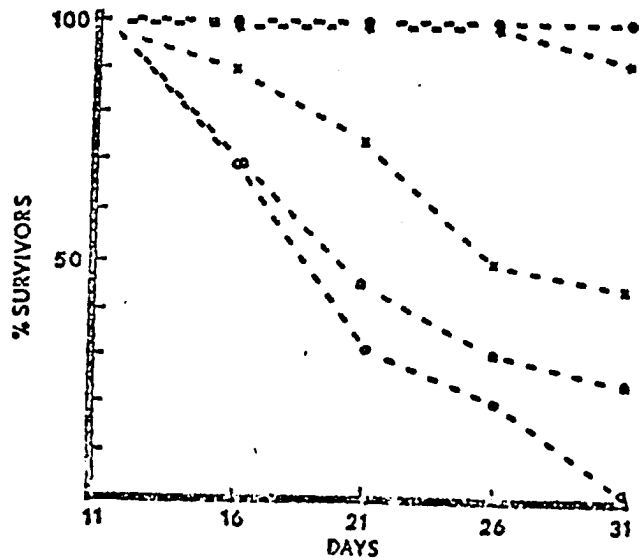


FIGURE 13. PERCENTAGE OF SURVIVORS OF MICE INFECTED WITH C. NEOFORMANS AND TREATED ORALLY WITH DIFFERENT CONCENTRATIONS OF NA OVER PERIOD OF 31 DAYS. CONTROLS: (•) UNINFECTED TREATED WITH 400 µg/kg/dose, (o) INFECTED UNTREATED. TESTS: INFECTED AND TREATED WITH (▪) 50 µg/kg/dose, (x) 100 µg/kg/dose, (*) 200 µg/kg/dose AND (●) 400 µg/kg/dose.

CHAPTER IV

DISCUSSION

In our investigations, which included several screening procedures, three chemicals showed promise as new antifungal agents, particularly against C. neoformans.

Quinazoline derivatives have been used extensively against eucaryotic parasites especially Plasmodium species (45,46). However, this particular derivative was found to lack any activity against Plasmodium species. Subsequent in vitro antimicrobial screening against several yeasts, revealed that this compound displayed antifungal properties. The MIC was in the range of 6-8.25 µg/ml against C. neoformans but fungistatic dose varied slightly as the time of incubation increases.

Mn derivative of 1-phenethylimidazole, exhibited inhibitory activity against bacteria and fungi (13) and oral administration reduced tetracycline-induced fungal overgrowth in the feces. Mn was absorbed readily from the gastro-intestinal tract (6). In our investigations, Mn demonstrated inhibitory activities against several yeast cells including C. neoformans with an MIC value of approxi-

MATELY 0.5 µg/ml. DOSAGES UP TO THIS CONCENTRATION PROVED NOT TO BE INHIBITORY TO MACROMOLECULES BIOSYNTHESIS.

NA HAS BEEN SHOWN TO BE EFFECTIVE AGAINST PROCARYOTIC CELLS (20), IN THAT IT INHIBITS DNA SYNTHESIS IN BACTERIA (15). THE EFFECTS OF NA ON EUKARYOTIC ORGANISMS HAVE BEEN STUDIED AND IN TWO INVESTIGATIONS THIS AGENT INHIBITED THE GROWTH OF EUGLENA GRACILIS BY BLOCKING CHLOROPLASTS REPLICATION (9,30). IN OUR INVESTIGATION, WE FOUND THAT PARTIAL INHIBITION OF TOTAL CELL DNA, RNA AND PROTEIN SYNTHESIS WAS ACHIEVED, USING CONCENTRATIONS BELOW THE MIC. CRYPTOCOCCUS NEOFORMANS WAS INHIBITED BY NA AT 60 µg/ml ON THE FIRST DAY INCREASING TO 140 µg/ml BY THE SEVENTH DAY.

THE ABOVE DATA INDICATES THAT THE MIC'S WERE DIRECTLY PROPORTIONAL TO THE PERIOD OF EXPOSURE TO Qu AND NA, WHILE THE MIC'S OF Amb AND Mn WERE INDEPENDENT OF TIME. THIS SUGGESTS THAT Qu AND NA WERE PROBABLY FUNGISTATIC AND THAT Amb AND Mn WERE PROBABLY FUNGICIDAL. THE IN VITRO FUNGISTATIC OR FUNGICIDAL PROPERTIES OF MOST DRUGS DEPEND ON MANY FACTORS SUCH AS TIME, TEMPERATURE, ORGANISM, AND DOSAGE. AN INCREASE IN DOSE OF Qu OR NA ABOVE THEIR MIC'S, EVENTUALLY LEADS TO FUNGICIDAL LEVELS.

THIS CONCEPT SHOULD BE CONSIDERED WHEN APPLYING A DRUG THERAPEUTICALLY IN THE MAMMALIAN SYSTEM WHICH MAY LIMIT THE UTILIZABLE DOSE. THESE LIMITATIONS INCLUDE ADVERSE EFFECTS ON THE MAMMALIAN SYSTEM IN RESPONSE TO INCREASED DOSAGES, ALTHOUGH THERAPEUTIC ACTIVITY MAY BE ENHANCED.

An example of this phenomenon can be seen with Amb which exhibits dose dependent toxicity to the host. Lowering the dosage eliminates or diminishes side effects of this drug, as well as its antifungal properties.

Amb and other polyene antibiotics (5,31,35) bind with sterols which are present in the cell membrane, causing alterations in their permeability and impairment of the transport system of sensitive organisms (22,23). Individual polyene antibiotics differ in their affinity to certain lipids, for example, filipin binds to ergosterol and cholesterol, while Amb has mainly an affinity to ergosterol (51). These differences account in part for the particular antimicrobial spectrum and toxicity of individual polyenes. Polyenes possessing the broadest spectrum of lipid affinity have proved the most toxic to mammalian cells.

The mechanism of Amb action on model membranes (24) probably facilitates the passage of other drugs through the cells membrane and increases their availability in the cytosol.

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 another antifungal agents having a different mode of action than Amb. This has proved feasible and an example was shown in our studies using either Qu or NA along with Amb. Actually, combinations led to potentiated anti-

FUNGAL EFFECTS ON THE YEAST CELLS WHEN COMPARED TO THE USE OF EITHER DRUG ALONE. POTENTIATION WAS NOT OBSERVED USING Mn IN COMBINATION WITH Amb AT EITHER THE CELLULAR OR MOLECULAR LEVEL. Mn ALONE DOES NOT EXHIBIT ANY INHIBITORY EFFECTS ON MACROMOLECULAR BIOSYNTHESIS WHILE Qu OR NA DO INHIBIT MACROMOLECULAR BIOSYNTHESIS.

BROAD SPECTRUM ANTIBACTERIAL AGENTS HAVE BEEN USED TO POTENTIATE THE EFFECTS OF ANTIFUNGAL AGENTS IN VITRO (36). THIS CAN NOT BE PRACTICALLY APPLIED TO FUNGAL INFECTIONS IN MAMMALIAN SYSTEM AS IT LACKS THERAPEUTIC VALUE FOR SEVERAL REASONS. LONG TERM BROAD SPECTRUM ANTIBACTERIAL THERAPY UPSETS THE BALANCE BETWEEN BACTERIAL AND FUNGAL FLORA, DEPLETING THE BACTERIAL POPULATION THUS FACILITATING THE ESTABLISHMENT OF A FUNGAL INFECTION (19,55). THEREFORE, EACH DRUG SHOULD BE ESTABLISHED AS AN ANTIFUNGAL AGENT PRIOR TO ITS USE IN COMBINATION WITH OTHER AGENTS.

IN VITRO INVESTIGATION OF Qu, Mn, AND NA INDICATED THAT THEY WERE PROMISING ANTIFUNGAL AGENTS AND TO OUR KNOWLEDGE, THEIR USE IN THE TREATMENT OF EXPERIMENTAL CRYPTOCOCCOSIS IN MICE HAS NOT BEEN PREVIOUSLY REPORTED. OUR RESULTS WITH THIS IN VIVO MOUSE MODEL INDICATED A STATISTICALLY SIGNIFICANT DOSE RELATED THERAPEUTIC EFFECT OF ALL FOUR DRUGS. IT WAS ENCOURAGING TO OBSERVE THAT NO APPARENT DEATHS OCCURRED DUE TO DRUG TOXICITY AT THESE 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 GASTRO-INTESTINAL TRACT. INTERAPERITONEAL INJECTIONS OF INFECTED MICE WITH Qu RESULTED IN COMPLETE RECOVERY FROM THE DISEASE.

FURTHER EVALUATIONS AND INVESTIGATIONS OF THESE CHEMICAL COMPOUNDS ARE NEEDED TO PROVIDE SUFFICIENT INFORMATION REQUIRED TO ALLOW SAFE APPLICATION OF THESE AGENTS AGAINST MYCOTIC INFECTIONS. AREAS OF INVESTIGATIONS MUST INCLUDE CYTOLOGICAL AND PATHOLOGICAL EFFECTS OF THE AGENTS ON MAMMALIAN TISSUE, THE ROUTE OF ELIMINATION OR EXCRETION FROM THE BODY AND TARGET ORGANS OR TISSUES OF DRUG ACTIVITY. HOPEFULLY, THE CONTINUATION OF OUR STUDIES WILL LEAD TO ANSWERS TO THE ABOVE AND OTHER QUESTIONS AND RESULT IN THE FINDING OF AGENTS TO MORE SUCCESSFULLY TREAT MYCOTIC INFECTIONS.

CHAPTER V

SUMMARY

THE MINIMUM INHIBITORY CONCENTRATIONS OF QUINAZOLINE DERIVATIVE, MICONAZOL NITRATE, NALIDIXIC ACID, AND AMPHOTERICIN B WERE DETERMINED BY THE TUBE DILUTION METHOD FOR *CRYPTOCOCCUS NEOFORMANS*, STRAIN 184.

THE EFFICIENCY OF Qu, Mn, OR NA ALONE AND IN COMBINATION WITH Amb WAS INVESTIGATED *IN VITRO*. AMPHOTERICIN B WAS FOUND TO POTENTIATE THE ANTIFUNGAL EFFECTS OF Qu AND NA, BY ALTERATION OF THE FUNGAL CYTOPLASMIC MEMBRANE PERMEABILITY PERMITTING INCREASED PENETRATION OF THESE CHEMICAL COMPOUNDS. ON THE OTHER HAND, COMBINATION OF Amb AND Mn DID NOT RESULT IN SYNERGISM.

THE EFFECTS OF CHEMICAL AGENTS ON MACROMOLECULAR METABOLISM ON *C. NEOFORMANS* WERE STUDIED. IT WAS FOUND THAT Amb AND Mn AT CONCENTRATIONS UNDER THE MIC VALUES DO NOT EFFECT THE SYNTHESIS OF MACROMOLECULES. HOWEVER, Qu AND NA INHIBITED THE INCORPORATIONS OF LABELED PRECURSORS INTO RNA AND PROTEIN OF *C. NEOFORMANS*. IN ADDITION THERE WAS A DECLINE IN RATE OF THYMIDINE UPTAKE IN THE CELLS TREATED WITH NA. WHEN Qu OR NA WAS USED IN COMBINATION WITH Amb THIS

INHIBITORY EFFECT OF CHEMICAL COMPOUNDS ON MACROMOLECULAR BIOSYNTHESIS WAS ENHANCED.

A MOUSE MODEL INFECTION WITH C. NEOFORMANS WAS ESTABLISHED, AFTER WHICH THE ANIMALS WERE GIVEN IP OR ORAL ADMINISTRATION OF VARIOUS CONCENTRATIONS OF EXPERIMENTAL DRUGS. THE CURING DOSE 50 (CD-50) WAS DETERMINED ON THE BASIS OF ISOLATION OF C. NEOFORMANS FROM THE ORGANS OF ANIMALS DURING OR AT THE TERMINATION OF THE EXPERIMENTS. INFECTED MICE RESPONDED TO DRUG TREATMENTS WITH THE EXCEPTION OF ORAL ADMINISTRATION OF Qu AND THE PERCENTAGE OF SURVIVING MICE INCREASED PROGRESSIVELY WITH INCREASING DRUG DOSAGE.

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