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SCREENING OF DRUGS AGAINST MICROSPORIDIA

MD. AZIZUL HAQUE* AND ELIZABETH U. CANNING

Department of Biology, Imperial College of Science ,Technology and Medicine, London, SW7 2AZ, United Kingdom

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Thirteen drugs were tested against *Nosema bombycis* Naegeli in an *in vitro* system using *Spodoptera frugiperda* cell culture to investigate antimicrosporidial activities. Drugs tested were: fumagillin, benzimidazole, pentostam, thimerosal, ciprofloxacin, metronidazole, itraconazole, sulphadimidine, sulphapyridine, rimocidin sulphate, cerulenin, imidocarb dipropionate and compound 566C80 (atovaquone). Only five drugs namely, fumagillin, benzimidazole, pentostam, ciprofloxacin and thimerosal showed antimicrosporidial activity when infected cultures were exposed to the drug. Although thimerosal inhibited development of the microsporidia relative to controls, but it did not prevent an increase in the percentage of infected cells from the initial level. The other form drugs not only prevented further spread of infections between cells but also reduced the level of infection from the initial point. The minimum concentrations of the drugs which inhibited further spread of the parasite were: fumagillin at 5.0 µg/ml, benzimidazole at 250 µg/ml, pentostam at 2000 µg/ml and ciprofloxacin at 250 µg/ml.

Key words: *Nosema bombycis,* Drug screening, *In vitro.*

Introduction

Many species of microsporidia have been reported to affect laboratory colonies of insects either by destroying the colonies [1,2] or by temporarily interrupting the rearing schedules [3-5]. The response of insects from such diseased colonies to experimental treatments may be erratic and could result in misleading conclusions. Furthermore, in beneficial insects microsporidia may exert their effects by depressing the productivity of honey bees [6, 7] and silkworms [8,9] and by reducing the efficacy of insects predators [10] and parasitoids [11,12]. Therefore, it is important to investigate antimicrosporidial drug(s) to run laboratory colonies of insects free from microsporidian infections.

Until recently only fumagillin and benomyl have been found as effective antimicrosporidial drugs [13-15]. However, the doses required to completely eliminate the parasite were toxic to the host insects [5, 16 - 18]. Last year, sinefungin and albendazole have been added to the list of drugs with anti microsporidial activity [19- 21]. Although there are reports on the effects of thimerosal and itraconazole on the ultrastructure of microsporidian parasites [22,23], no further studies have been reported on these two drugs. The present study was, therefore, undertaken to screen 13 drugs for antimicrosporidial activity using an *in vitro* system. The knowledge gained may be used to prevent microsporidian incidence in laboratory colonies of insects.

*Department of Entomology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh.

Materials and Methods

Microsporidium. The microsporidium used in this study was a strain of the type species, *Nosema bombycis* Naegeli which was originally isolated from lawn grass cutworm, *Spodoptera depravata* by Professor R. Ishihara and Dr. H. Iwano of Nihon University, Japan.

Drugs tested. Thirteen drugs selected for testing against *N. bombycis* were: fumagillin, benzimidazole, pentostam, thimerosal, ciprofloxacin, metronidazole, itraconazole, sulphadimidine, sulphapyridine, rimocidin sulphate, cerulenin, imidocarb dipropionate and compound 566C80 (atovaquone).

Insect cell line. The cell line of the fall armyworm, *Spodoptera frugiperda* was used in this study. The cells were grown as monolayers in 25 cm^2 disposable plastic tissue culture flasks (Sterilin) containing 5 ml TC-100 medium (Gibco). The growth medium was TC-100 with 10% heat inactivated foetal calf serum (FCS) (Gibco) and the maintenance medium was TC-100 with 5%FCS. Penicillin, streptomycin (Gibco) and kanamycin (Sigma) were supplemented to both growth and maintenance media at concentrations of 100 I.U., 100 µg and 100 µg per ml, respectively. Cultures were incubated at 25°C and were subcultured every 5-7 days.

Infection of S. frugiperda cell culture. The *Antheraea eucalypti* cell line infected with *N. bombycis,* was used as the source of parasitized cells. The infection was transferred to the *S. frugiperda* cell line using the technique of Kurtti *et al.* [24]. For this purpose 1 ml of infected *A. eucalypti* cells was added to 2 ml of a suspension of *S. frugiperda* cells and the mixture

centrifuged at 150xg for 5 min. The pellet was resuspended in 3 ml of fresh growth medium and 1 ml of this suspension was introduced into each flask together with 4 ml of growth medium for incubation. The parasitized *S. frugiperda* cells were passaged at split ratios of one part infected cells to three to five parts of normal cells.

Preparation of cell cultures for drug testing. Infected and uninfected cells were mixed 1:10 and 1 ml (about $1x10^5$ cells) was placed into each well of a 24- well tissue culture plate (Nunc). It was found that *S. frugiperda* cells would not adhere to glass coverslips placed in the tissue culture trays. In order to prevent the cells adhering to the plastic base of the wells, glass coverslips were inserted, so that the cells remained in suspension. Cells were allowed to grow to establish infections for 3-5 days for drug treatment.

Preparation of drugs. All the drugs were obtained in powdered form except ciprofloxacin which was obtained as a sterile solution, from which a series of concentrations were prepared in maintenance medium. Powdered drugs were dissolved in specific solvents. The minimum amount of solvent was used just to dissolve the drug. These drug solutions were further diluted with TC-100 medium and sterilized by passing through 0.22µm millipore membrane filters. From these stock solutions, desired concentrations were made up by dilution in maintenance medium. In the case of itraconazole and compound 566C80 (atovaquone), the drugs dissolved in solvent precipitated out when added to medium and were partially redissolved by sonication. The concentrations of sonicated drug in the maintenance medium were 5.9 and 11.88 µg/ml for itraconazole and compound 566C80, respectively. Lower concentrations of these drugs were prepared as before by further dilution in maintenance medium. In each experiment the amount of solvent was kept constant in all the concentrations of drugs including the control (Table 1).

Assays for drug activity. N. bombycis infected *S. frugiperda* cell culture were exposed to medium containing drugs for 7 days, unless otherwise stated. The spent medium was removed carefully from the wells of the tissue culture plates and was replaced immediately by 0.5 ml of fresh medium containing the drug. The spent medium was centrifuged at 150xg for 5 mins. The supernatant was discarded, and the pellet was resuspended in 0.5 ml of drug treated medium which was returned to the original culture well. Thus, each culture well received a replacement of 1 ml of medium containing drug and infected cells. As controls, infected cultures were exposed to maintenance medium containing the same amount of solvent used in the drug treatments. There were 2 to 3 replicates for each treatment and the medium was renewed after a further 3 days by centrifuging the cells as before. Uninfected cell cultures were also included in the experiments to assess the effects of drug and solvent on host cells.

To assess the effect of drugs, cell suspensions harvested from 24 well tissue culture trays after exposure to drug were centrifuged at 150xg for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml fresh medium. The cells were spun on to glass slides using a cytospin centrifuge. The smears were air dried, fixed in methanol for 4-5 min and stained in 10% (v/v) Giemsa stain for 45 min. The effect of drug was assessed by estimating the percentage of infected cells. In order to remove bias, the number of infected cells was counted per 200 cells in each of 5-10 areas on the slide. These areas were marked by the vernier on the microscope stage and the cells in the same areas were examined for the control and drug treated samples, at each of the time intervals. The perentage data were transformed by angular transformation before statistical analysis.

Results and Discussion

Fumagillin. Fumagillin was tested at 6 concentrations, 5, 10, 25, 50, 100 and 200 µg/ml in culture medium added to *N. bombycis* infected cultures. Compared with control cultures receiving no drug, there were significant decreases in the percentage of infected cells from the initial level of 20% at all 6 concentrations of fumagillin (F test; Table 2). There were no

TABLE 1. DRUG SOLVENTS AND AMOUNT OF SOLVENT USED.

Drug	Solvent	Amount of solvent used
Rimocidin sulphate	Dimethyl sulfoxide	0.2%
Itraconazole	Dimethyl sulfoxide	0.05%
Compound 566C80 (atovaquone)	Dimethyl sulfoxide	0.09%
Sulphapyridine	2N NaOH	0.5%
Cerulenin	Absolute ethanol	Expt.I 0.2% Expt.II 0.02%

TABLE 2. NUMBER OF *S. FRUGIPERDA* CELLS INFECTED WITH *N. BOMBYCIS* BEFORE AND AFTER TREATMENT WITH FUMAGILLIN.

Concentration (µg/ml)	Percentage of infected cells (Mean ± S.E.)	
	0 days	7 days
0	20	50.7 ± 0.6 a
5	20	6.9 ± 0.4 b
10	20	5.8 ± 0.1 b
25	20	5.2 ± 0.3 b
50	20	5.4 ± 0.2 b
100	20	5.6 ± 0.3 b
200	20	6.2 ± 0.3 b

Means followed by the same letter are not significantly different from each other ($P<0.01$).

significant differences in the effects of the drug at any of the concentrations used from the lowest concentration (5 µg/ml) to the highest concentration (200 µg/ml), the percentage of infected cells ranging from 5.2 ± 0.3% to 6.9 ± 0.4%. In the control cultures the percentage of infected cells rose to 50.7% in the same period.

The results revealed that fumagillin had marked antimicrosporidial activity against *N. bombycis* in S.*frugiperda* cell culture. The drug significantly reduced the level of infection from the initial level. However, the drug failed to eliminate the infections from the cell culture completely. Bayne et al. [25] reported that fumagillin at 25-50 µg/ml of culture medium markedly reduced but failed to eliminate the infections of *Pleistophora*-like microsporidia completely from *Biomphalarea glabarata* cell culture even exposing the culture to the drug upto 15 weeks. Kurtti and Brooks [26] and Shadduck [27] concluded that fumagillin is microsporidia-static not microsporidicidal.

Benzimidazole. Benzimidazole was first tested at 10, 25, 50 and 100 µg/ml and the results are presented in Table 3. Doses of 10 µg/ml and 25 µg/ml did not produce significant changes in the numbers of infected cells and, at 50 µg/ml and 100 µg/ml, the differences were significant (F test) but minimal. In another experiment, benzimidazole was tested at 250, 500, 750 and 1000 µg/ml. Doses of benzimidazole which inhibited multiplication of the microsporidia were 250 µg/ml and 500 µg/ml: the level of infections were 27.6% and 17% compared with the initial level of 25.1%. In the controls, the infection level rose to 58.4% during the same period. Doses of 750 µg/ml and 1000 µg/ml were toxic to the host cells (Table 3).

The results showed that *In vitro* benzimidazole has potent antimicrosporidial activity. The drug at 50 µg/ml significantly reduced the percentage of infected cells and at 250 µg/ml prevented further spread of the parasite.

Pentostam. In one experiment, pentostam was tested at 4 concentrations, 100, 250, 500 and 1000 µg/ml (Table 4). The spread of infection from an initial level of 31.5% was not prevented at any of these concentrations, although growth was inhibited relative to control levels, when cells were exposed to 500 or 1000 µg/ml of drug (F test). Compared with the control no significant decreases in infection in the culture were observed with doses of 100 or 250 µg/ml. Pentostam was then tested for with 1500 antimicrosporidial activity at concentrations of 1500-3000 µg/ml and 2000 µg/ml of pentostam, the percentages of infected cells were 46.8% and 42%, respectively, which differed significantly from the control level of 82.8% (F test). Additionally, 2000 µg/ml maintained the infection at about the original level of 40% (Table 4). Concentrations of pentostam of 2500 and 3000 µg/ml gave rise to gross changes and death of the host cells even after 24h.

Thimerosal. The effects of thimerosal on microsporidian growth are presented in Table 5. At 5 µg/ml in culture medium the percentage of infected cells rose above the initial level of 25.1% but at 2 days was significantly lower than the control level (F test 35.6% compared with 55.6%). However, on day 3 cells in the cultures exposed to drug had begun to die,

TABLE 3.NUMBER OF *S. FRUGIPERDA* CELLS INFECTED WITH *N. BOMBYCIS* BEFORE AND AFTER TREATMENT WITH BENZIMIDAZOLE.

Concentration (µg/ml)	Percentage of infected cells (Mean ± S.E.)		Concentration (µg/ml)	Percentage of infected cells (Mean ± S.E.)	
	0 days	7 days		0 days	7 days
0	34	84.3 ± 0.2a	0	25.1	58.4 ± 1.2 a
10	34	82.5 ± 0.5a	250	25.1	27.6 ± 0.6 b
25	34	83.4 ± 0.3a	500	25.1	17.0 ± 0.7 b
50	34	75.2 ± 0.3b	750	25.1	Toxic
100	34	69.5 ± 0.4c	1000	25.1	Toxic

Means followed by the same letter are not significantly different from each other (P<0.01).

TABLE 4.NUMBER OF *S. FRUGIPERDA* CELLS INFECTED WITH *N. BOMBYCIS* BEFORE AND AFTER TREATMENT WITH PENTOSTAM.

Concentration (µg/ml)	Percentage of infected cells (Mean ± S.E.)		Concentration (µg/ml)	Percentage of infected cells (Mean ± S.E.)	
	0 days	7 days		0 days	7 days
0	31.5	63.6 ± 0.5a	0	40	82.8 ± 1.7 a
100	31.5	63.8 ± 1.9a	1500	40	46.8 ± 2.3 b
250	31.5	63.6 ± 2.4a	2000	40	42.0 ± 1.2 b
500	31.5	47.4 ± 1.0b	2500	40	Toxic
1000	31.5	39.4 ± 0.5b	3000	40	Toxic

Means followed by the same letter are not significantly different from each other (P<0.01).

TABLE 5.NUMBER OF *S. FRUGIPERDA* CELLS INFECTED WITH *N. BOMBYCIS* BEFORE AND AFTER TREATMENT WITH THIMEROSAL.

Concentration (µg/ml)	Percentage of infected cells (Mean ± S.E.)			Concentration (µg/ml)	Percentage of infected cells (Mean ± S.E.)	
	0 day	3 days	7 days		0 days	7 days
0	19.3	58.4 ± 1.2a	69.8 ± 0.3	0	25.1	55.6 ± 1.3 a
0.5	19.3	57.7 ± 1.5a	70.7 ± 0.8	5	25.1	35.6 ± 0.6 b
1.0	19.3	53.5 ± 1.7a	70.9 ± 0.8	10	25.1	Toxic
2.0	19.3	36.6 ± 1.6a	Toxic	20	25.1	Toxic

Means followed by same letter are not significantly different from each other (P<0.01).

indicating that the drug was at a toxic level. Higher doses were toxic at 2 days post exposure. In a 2nd experiment doses below 5 ug/ml were used. As expected these did not prevent an increase in the percentage of cells above the initial level (19.3%). Doses below 2 µg/ml did not affect the development of the microsporidia compared with the controls during the 7-day period of observation. At 2 µg/ml there was a significant decrease in the percentage of infected cells at 3 days but the toxic effects were such that the cultures were destroyed at 7 days.

Ciprofloxacin. The results of testing the exposure of cultures to ciprofloxacin ranging from 25 µg/ml to 500 µg/ml are presented in Table 6. Doses of 25 and 50 µg/ml had no effect on the percentage of infection. At 100µg/ml there was a significant reduction of infection to 71.6% compared to 85.3% in the controls (F test). However, the dose failed to reduce the level of infection from the initial point of 57%. Spread of infection was only prevented and reduced from the initial level to 54.2% and 49% by concentrations of 250 and 500 µg/ml, respectively. None of the concentrations of ciprofloxacin had adverse effects on the gross structure of the host cells.

Other drugs. Metronidazole (50-3000), itraconazole (1.0-5.9), sulphadimidine (100-900), sulphapyridine(50-1000), rimocidin sulphate (10-100), cerulenin (2.5-100), imidocarb dipropionate (100-1000) and compound 566C80 (3.04 - 11.88) µg/ml were tested against *N. bombycis* in *S. frugiperda* cell culture. But none of them showed any antimicrosporidial activity.

The results revealed that out of 13 drugs tested five drugs namely, fumagillin, benzimidazole, pentostam, ciprofloxacin and thimerosal showed potent antimicrosporiial activity. Except thimerosal, other four drugs not only prevented further spread of the parasite but also reduced the level of infection from the initial level. Like other antibiotics, these drugs may be used in the insect diet to prevent the incidence of microsporidian infection in the laboratory colonies of insects.

TABLE 6. NUMBER OF *S. FRUGIPERDA* CELLS INFECTED WITH *N. BOMBYCIS* BEFORE AND AFTER TREATMENT WITH CIPROFLOXACIN.

Concentration (µg/ml)	Percentage of infected cells (Mean ± S.E.)	
	0 days	7 days
0	57	85.3 ± 0.1 a
25	57	84.0 ± 0.2 a
50	57	84.1 ± 0.2 a
100	57	71.6 ± 1.5 b
250	57	54.2 ± 1.4 c
500	57	49.0 ± 1.3 c

Means followed by the same letter are not significantly different from each other (P<0.01).

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References

1. G. L. Finney, J. Econ. Entomol., **49,** 134 (1956).
2. H. Kamasaki, J. Econ. Entomol., **63,** 1353 (1970).
3. R. T. Gast, J. Econ. Entomol., **59,** 793 (1966).
4. G. L. Finney, S. E. Flanders and H. S. Smith, Hilgardia, **17,** 437 (1947).
5. H. M. Flint, J. Eaton and W. Klassen, Ann. Entomol. Soc. Amer., **65,** 942 (1972).
6. C. L. Farrar, J. Econ. Entomol., **40,** 333 (1947).
7. J. C. M. L. Arrivee, Canad. Agric., **11,** 24 (1966).
8. J. Veber and J. Jasic, J. Insect Pathol., **3,** 103 (1961).
9. R. H. Jafri, K. M. S. Khan and R. Iqbal, Pakistan J. Zool, **10,** 294 (1978).
10. O. G. Marti Jr. and J. J. Hamm, J. Agric. Entomol., **2,** 285 (1988).
11. T. G. Andreadis, J. Invertebr. Pathol., **35,** 229 (1980).
12. O. S. Own and W. M. Brooks, Environ. Entomol., **15,** 32 (1986).
13. E. Armstrong. J. Invertebr. Pathol., **27,** 363 (1976).
14. T. H. Hsiao and C. Hsiao, J. Invertebr. Pathol., **22,** 303 (1973).
15. G. G. Wilson, Can. Entomologist, **106,** 995 (1974).
16. R. E. Lynch and L. C. Lewis, J. Invertebr. Pathol., **17,** 243, (1971).
17. G. T. Harvey and P. M. Gaudet, Can. Entomologist, **109,** 987, (1977).
18. L. C. Lewis and R. E. Lynch, J. Invertebr. Pathol., **15,** 43, (1970).
19. C. Blanshard, D. S. Ellis, G. Torey, S. Dowell and B. G. Gazzard, AIDS, **6,** 311 (1992).
20. M. A. Haque, W. S. Hollister, A. Willcox and E.U. Canning, J. Invertebr. Pathol., **62,** 171 (1993).

21. M. Sichtova, M. A. Haque, J. Vavra, E. U. Canning and M. Robert-Gero, Folia Parasitol., **40,** 85 (1993).
22. T. P. Liu, Parasitol. Res., **74**, 492 (1988).
23. T. P. Liu, and G. R. Myrick, Parasitol. Res., **75,** 498 (1989).
24. T. J. Kurtti, K. R. Tsang and M. A. Brooks, J. Invertebr. Pathol., **30,** 652 (1983).
25. C. J. Bayne, A. Owczarzak and W. E. Noonan, Ann. N. Y. Acad., Sci., **226,** 513 (1975).
26. T. J. Kurtti and M. A. Brooks, J. Invertebr. Pathol., **29,** 126 (1977).
27. J. A. Shadduck, J. Protozool., **27,** 202 (1980).