EFFECTS OF NETROPSIN ON YEAST MITOCHONDRIA

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SUMMARY

Netropsin binds tightly to AT rich regions of DNA and correspondingly is an efficient inhibitor of mitochondrial DNA replication in Saccharomyces cerevisiae. Netropsin treatment does not cause formation of large populations of petite cells. However, a large portion of cells grown in cultures with ethanol as carbon source are killed by 1 μg/ml netropsin. When petite induction by berenil or ethidium bromide is carried out in the presence of netropsin, the petite cells are killed. This appears to be an effect of netropsin action on the cells during the process of petite formation.

Netropsin is a basic polypeptide antibiotic which is produced by Streptomyces netropsis. It has been shown to inhibit multiplication of animal viruses and to have antibacterial and antifungal activities (1). Zimmer et al. (2,3) demonstrated that netropsin binds with high specificity the A and T regions of DNA. It is non-intercalating but does cause a considerable modification of the DNA structure. Furthermore, netropsin does not bind to G-C base pairs (4). The antibiotic inhibits both DNA polymerase and RNA polymerase activities. Recently Keilman et al. (5) demonstrated inhibition of sporulation of Bacillus subtilis by netropsin.

Mitochondrial DNA (mtDNA) from the yeast Saccharomyces cerevisiae has a very high A-T content with large segments containing greater than 95% A-T (6). Netropsin may be expected to bind efficiently to this DNA and to interfere with its replication and transcription. Two other molecules which bind to A-T rich regions of DNA with somewhat less specificity interfere with mtDNA function in Saccharomyces. Berenil, which has structural features in common with netropsin, blocks replication of mtDNA, rapidly induces petite mutations, but has no immediate effects on respiration (7,8). Ethidium bromide blocks mtDNA replication, induces petites and also induces a rapid breakdown of mtDNA into small fragments

and total loss of mtDNA (9,10,11,12,13). Neither of these compounds causes a loss of viability of cells. We have found that netropsin is even more effective in inhibition of mtDNA replication than is ethidium bromide but differs from the other two inhibitors in that it does not give rise to viable petite colonies. A comparison of the actions of netropsin with those of berenil, ethidium bromide and guanidinium hydrochloride on yeast mitochondria provides an insight into the steps involved in petite formation.

MATERIALS AND METHODS

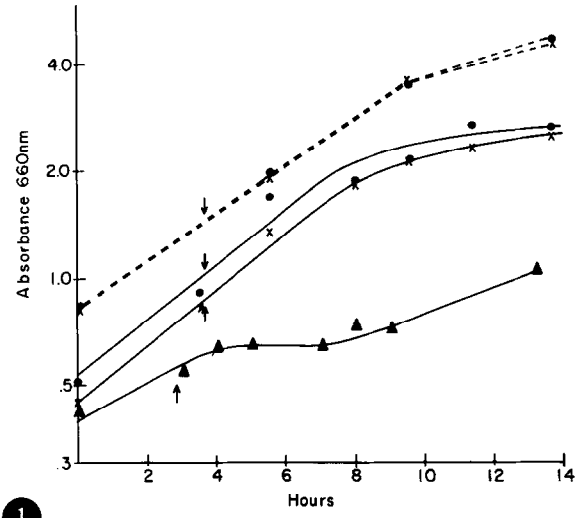
The yeast strain used in all of these studies was Saccharomyces cerevisiae strain D253-3C (14). Cultures were grown in flasks at 30° with continuous shaking in solutions containing 1% Difco Peptone, 1% Difco Yeast Extract and either 1% glucose or 2% ethanol. Analysis of petite formation was carried out as described previously (14) using ethidium bromide at 20 µg/ml, berenil at 40 µg/ml and varying levels of netropsin. Cells were plated on either glucose plates (1% peptone, 1% yeast extract, 2% agar, 2% glucose) or on petite plates differing in that they contained only 0.1% glucose and 2% ethanol. The fraction of petite colonies was then determined by analysis of colony size or by over-laying with tetrazolium chloride (15). Cell viability was analyzed from counts of total colonies on these plates.

Respiration in cultures of yeast cells was measured using an oxygen electrode (16). Synthesis of mtDNA was observed by pulse labeling cells with ^{3}H-adenine in the presence of 100 µg/ml cycloheximide using the methods of Goldring et al. (10). Nuclear DNA synthesis was measured in a similar fashion with no cycloheximide present. Potential inhibitors of the DNA synthesis were added to the cell cultures 20 min prior to addition of ^{3}H-adenine.

The netropsin used in these studies was a gift from Dr. E.L. Patterson, Lederle Laboratories. ^{3}H-Adenine, 0.5 mc/ml was purchased from Schwartz/Mann. Berenil was a gift from Dr. A.W. Linnane. Ethidium bromide was obtained from Calbiochem Corporation.

RESULTS

The growth rate of Saccharomyces cerevisiae strain D253-3C in glucose is not affected greatly by netropsin, as illustrated in Figure 1. In contrast, growth in medium with ethanol as substrate is inhibited by netropsin concentrations as low as 1 µg/ml. After several hours of exposure to netropsin, some of the cells in ethanol medium again resume growth. This apparently takes place following degradation of the netropsin in solution as growth may be inhibited once more by a fresh addition of the antibiotic. Inhibition of growth of cells on ethanol is not due to a direct effect on respiration as netropsin at concentrations of up to 10 µg/ml has no measurable effect on respiration as



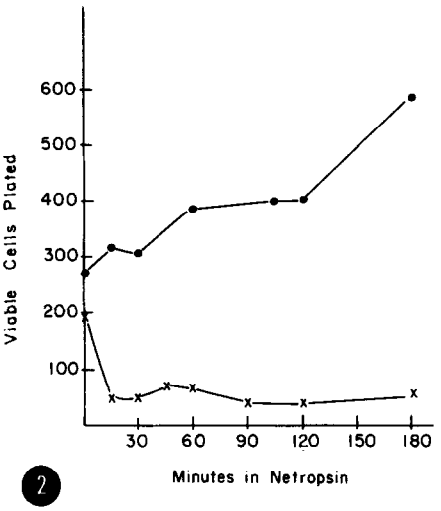


Fig. 1. Effects of netropsin on growth and respiration of yeast cells. Netropsin was added to a final concentration of 1 μg/ml to cultures of yeast cells growing in lag phase at the times indicated by the arrows. Cell growth and respiration were followed in the presence and absence of netropsin in glucose medium. Respiration was measured directly on aliquots of cells using an oxygen electrode. Cell cultures were diluted 1:10 in 0.9% KCl for absorbance measurement. Respiration without (-●--●-) and with netropsin (-X--X-) are shown. Cell growth was determined by absorbance at 660 nM for cultures without (-●-●-) and with (-X-X-) added netropsin. Cell growth was also measured in ethanol medium (-▲-▲-).

Fig. 2. Cell viability in netropsin treated cell cultures. Aliquots of logarithmically growing cells were diluted with water following various times of exposure to 1 μg/ml netropsin and then plated on glucose-agar plates. The viable cell colonies on the plates were counted after 3 days incubation at 30°C. Cells grown on glucose (-0-0-); cells grown on ethanol (-X-X-).

illustrated in Figure 1. Treatment of growing cell cultures in glucose medium with 1 μg/ml netropsin for times up to 1.5 hrs causes only a small viability loss (∿15-20%) when cells are subsequently plated on glucose agar plates. Interestingly, there appears to be no killing of cells when 10 μg/ml netropsin is used. In contrast, treatment of cells in ethanol medium causes nearly 70% loss of viability as shown in Figure 2, even when the treated cells are subsequently diluted and plated on glucose medium at the times indicated.

Netropsin inhibition of mtDNA replication was studied in the presence of cycloheximide. Replication of nuclear DNA is inhibited by cycloheximide under conditions which allow continued replication of mtDNA at a near normal rate

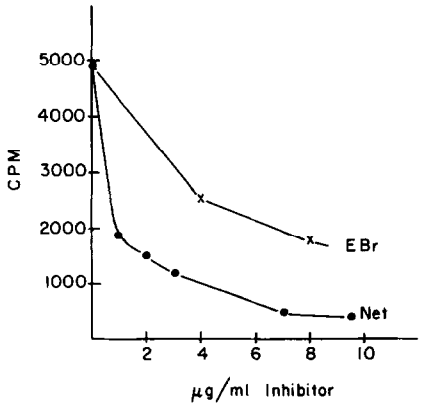


Fig. 3. Inhibition of mtDNA synthesis by netropsin and ethidium bromide. Lagarithmetically growing cells were treated with 200 μg/ml cycloheximide for 20 min to block nuclear DNA replication, then the inhibitors were added at the concentrations indicated. After 10 more min, ^{3}H-adenine was added and incubated for 2-1/2 hr at 30°. Incorporation into DNA was assayed by the method of Goldring et al. (10). Cells with added netropsin (-●-●-); cells with ethidium bromide (-X-X-).

(10). Cells were, therefore, exposed to 200 μg/ml cycloheximide for 20 min prior to addition of ^{3}H-adenine to allow measurement of incorporation of adenine specifically into mtDNA. Replication of mtDNA is sensitive to low levels of netropsin, with greater than 50% inhibition of replication at less than 1 μg/ml netropsin (Fig. 3). By way of comparison 4-5 μg/ml of ethidium bromide was required to obtain a similar inhibition.

Netropsin at a concentration of 1 μg/ml does not measurably inhibit nuclear DNA replication. This was studied using whole cells both with and without added cycloheximide to compare inhibition of the nuclear and mitochondrial systems. In addition, cell samples were treated with ethidium bromide, berenil and guanidinium hydrochloride for comparison. The results are shown in Table I. Note that radioactive adenine was added to samples tested for mitochondrial DNA replication at 10X the concentration used in analysis of incorporation into total DNA. Netropsin, ethidium bromide and berenil all markedly inhibit incorporation of ^{3}H-adenine into mtDNA with little or no effect on nuclear DNA. Guanidinium hydrochloride, which is an effective petite inducing agent (18)

TABLE I. Inhibition of Synthesis of Mitochondrial and Total Cellular DNA by Netropsin and Petite Inducing Agents.

	CPM ^{3}H-Adenine in:	
	Total DNA	Mitochondrial DNA
Control	9,104	9,726
Netropsin (1.0 µg/ml)	8,666	4,800
Guanidinium hydrochloride (700 µg/ml)	11,253	9,346
Ethidium bromide (10 µg/ml)	9,068	3,171

For determination of synthesis of total DNA, 0.015 ml of ^{3}H-adenine (0.5 mc/ml) was added to 30 ml of log phase cells. Mitochondrial DNA synthesis was analyzed in the presence of 200 µg/ml cycloheximide using 0.15 ml of added ^{3}H-adenine.

TABLE II. Petite Production by Treatment with Netropsin.

Netropsin Concentration	% Petite colonies formed during 2 hr treatment of cells growing on:	
	2% Glucose	2% Ethanol
0 (control)	1.1	0.8
0.1 µg/ml	0.3	0.5
1.0	0.9	0.7
8.0	1.1	1.0

has little effect on either system at the levels generally employed for petite formation.

As a number of compounds which bind to DNA and inhibit mtDNA replication are also efficient petite inducing agents in yeasts, netropsin was tested as a petite mutagen. Netropsin was added to logarithmetrically growing cultures of cells in 2% glucose to a final concentration of 5 µg/ml. After varying times of exposure, cells were diluted and plated on agar plates with glucose and also with ethanol as carbon source. As shown in Table II, petite frequency among viable cells plated is not increased above the spontaneous background level by treatment with netropsin.

While the results of Table II superficially suggest that netropsin is not

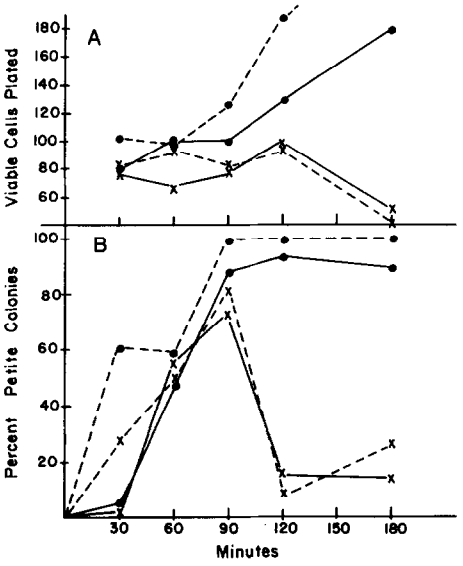


Fig. 4. Petite induction and cell viability studies in the presence and absence of netropsin. The fraction of cell colonies with petite phenotype following exposure of logarithmically growing cell cultures to ethidium bromide and to berenil both in the presence and absence of netropsin was measured and compared with changes in viable cell count. The concentration of ethidium bromide used was 20 μg/ml, berenil was 40 μg/ml, and netropsin was 1 μg/ml. Petite induction and determination were carried out as described previously (12). In both parts A and B the curves represent: ethidium bromide (-●--●-); ethidium bromide plus netropsin (-X--X-); berenil (-●-●-); berenil plus netropsin (-X-X-).

effective in inducing petites in either glucose or ethanol media, analysis of cell viability data and comparison with Fig. 2 indicates that many cells treated with netropsin in ethanol fail to survive. One possible explanation for these results is that petite formation may take place in ethanol media but only to a very limited extent in glucose media and that the petites formed may not survive. This possibility was examined by determining the effects of netropsin on petite induction by berenil and ethidium bromide. The results of these studies are shown in Fig. 4. Netropsin has no significant effect on the initial rate of petite formation by either berenil or ethidium bromide in 1% glucose media. However, when cell cultures were treated with both netropsin and one of these compounds, a sharp decrease in the fraction of colonies with petite phenotype was noted following a 90 min treatment (Fig. 4b). There was

also a parallel decrease of total viable cells in these cultures, thus suggesting a selective killing of petite cells by the netropsin (Fig. 4a). When stable petite cultures are treated with netropsin during growth on glucose, however, 1 μg/ml netropsin appears to have no effect on growth or viability.

DISCUSSION

Several reagents which specifically block mtDNA synthesis in yeast also act as potent petite inducing agents. Recent studies have suggested, however, that these two processes may be separated (8,19). Ethidium bromide blocks DNA replication and rapidly induces petite mutants but, in the presence of cycloheximide or nalidixic acid, synthesis may be blocked without petite formation (20). Nagley and coworkers (8) showed that the levels of berenil and ethidium bromide causing induction of petites are lower than those required for blocking replication. These authors concluded that the target site for mutagenesis is different from the mtDNA replication inhibitor site.

A number of treatments of yeast cells have been shown to increase petite mutation with little immediate effect on mtDNA replication. For example, heating cells or treatment with detergents enhances petite formation. Guanidinium hydrochloride has recently been established as a potent inducer of cytoplasmic petites in Saccharomyces. The studies reported here illustrate that concentrations of guanidinium hydrochloride sufficient to cause rapid petite induction have no measurable effect on mtDNA replication.

Netropsin falls into a separate category from the two classes of inhibitors listed above. As is the case with berenil and ethidium bromide, netropsin does bind very tightly to DNA and has a high specificity for dA-dT rich regions. It is a potent inhibitor of mtDNA replication with no measurable effect on nuclear DNA replication, but it does not generally induce petite formation. Netropsin also does not have a direct effect on respiration when added to cells at levels as high as 10 μg/ml. Thus, it is somewhat surprising that growth of cells on 2% ethanol is rapidly blocked and a large portion of the cells are killed. Growth of cells in glucose media is only marginally affected over the time periods studied.

The reasons for this behavior are not clear, but studies using netropsin in combination with petite inducing agents show that killing by netropsin accompanies the formation of petite cells. This effect must be directed at some intermediate step in petite formation, since established petite clones seem unaffected by netropsin. Thus, the simplest interpretation of the loss of viability when ethanol grown cells are treated with netropsin would be to postulate that petites can be induced by netropsin in these circumstances and that during petite formation the cells are killed. This interpretation suggests both a specific effect of netropsin on the mitochondria of cells grown in an obligatory aerobic substrate, which differs from that in glucose grown cells, and further that netropsin has a specific lethal effect on some intermediate in petite cell formation. This antibiotic should, therefore, be a useful probe in further analysis of the events involved in petite formation.

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