Microcalorimetric Evaluation of the Biological Activity of Polyene Complexes with Divalent Metal Ions: Mg(II), Ca(II), Ni(II), Cu(II) and Zn(II)

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Received June 20, 1985

Abstract

Polyene complexes with Mg(II), Ca(II), Ni(II), Cu(II) and Zn(II) have been prepared and evaluated for biological activity in a flow microcalorimetric study. The bioactivities are all lower per g of complex than is the bioactivity of the parent polyene, nystatin. However extrapolation of the linear bioassay data suggests that because of enhanced solubilities the metal ion complexes may be able to yield higher overall bioactivity than can nystatin alone.

Introduction

In the preceding papers [1, 2] we have described the preparation, characterisation and structures of some metal ion (Mg(II), Ca(II), Cu(II), Zn(II), Ni(II)) complexes of nystatin and amphotericin B (polyene antibiotics). Interest in such complexes stems from their possible involvement in mode-of-action theories [3] and from possible improvements in clinical efficacy through increase in solubility and/or biological potency.

Attempts to improve therapeutically achievable concentrations for polyene antibiotics have been made previously. For example improved solubility although with reduced bioactivity was attained for the N-acetyl derivative of nystatin [4, 5]. Ca(II) is known to be involved in yeast cell wall and membrane structure in a manner which leads to increased rigidity *i.e.* it is implicated in mechanisms of permeation [6, 7] Ca(II) is a regulator for glycogenesis and gluconeogenesis and is involved with the control of deoxyribonuclease, phospholipase and a wide range of proteolytic enzymes [8]. Mg(II) is also important in maintaining the integrity of the yeast cell membrane [9] and in activating phosphate transferases and decarboxylases [10]. Mg(II) and Zn(II) are co-factors of several vital enzymic reactions in yeast cells [11, 12]. Cu(II), Zn(II) and Fe(II) can restore the enzymic activities of yeast aldolases [13] and in the presence of iron, copper induces increased activity in cytochromes and in the citric acid cycle.

Pretreatment with Ca(II) and Mg(II) has been shown to protect yeast membranes from polyene damage [14]. Both divalent metal ions form complexes at the C_3-hydroxyl group of sterols [16, 19].

Nystatin and amphotericin B have been assayed, in this laboratory, on interaction with *Saccharomyces cerevisiae* by a flow microcalorimetric technique [20]. The advantages cited [20] for this technique include improvements in sensitivity, reproducibility and time per test. (It is striking however that very little quantitative data exists for the bioassay of antibacterials using this technique.) A further advantage noted was that in the assay of nystatin in both raw and formulated forms the concentration of DMF (N,N′ dimethylformamide) required to ensure polyene antibiotic solubility was 1% (cf. 10% in agar plate diffusion procedures) [21]. Thus in comparison with *in vivo* situations where aqueous solubility (to achieve higher therapeutic levels) is important this is a considerable advantage.

Whilst there is little evidence of the appearance of resistance in organisms exposed to these antifungal antibiotics [22] it is possible to prepare such organisms for use in mode-of-action studies [23]. This paper reports an examination of the biological activity of the Mg(II), Ca(II), Cu(II), Zn(II) and Ni(II) complexes of nystatin by flow microcalorimetry. The assay procedures involved interaction with sensitive and resistant *Saccharomyces cerevisiae* cells. The preparation of the resistant cells [23] will be described elsewhere. Both cell preparations were stored in liquid nitrogen to ensure consistency and reproducibility [24].

Experimental

Materials and Methods

Inocula

Saccharomyces cerevisiae. NCYC 239 was grown in a defined medium containing (g l^{-1} unless other-

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0020-1693/85/$3.30

wise stated) glucose, 40; NaCl, 20; citric acid, 6.62; Na_3 citrate, 17.21; deinonised water to 900 cm^3; sterilised at 115 °C for 15 min. Immediately prior to use 100 cm^3 of yeast nitrogen base, membrane filtered, was added aseptically to 900 cm^3 of the medium. The growth regime, storage and recovery of inocula were as previously described [21]. Resistant cells [23] were grown, stored and recovered in a similar manner.

Nystatin and nystatin–metal complexes

Solutions. The metal ion complexes were prepared as described previously [1, 2]. Appropriate concentrations of each compound studied were dissolved in DMF such that the required applied concentration in the test could be achieved through addition of 1 cm^3 of DMF solution (or DMF alone as control) to the incubation mixture.

Calorimetry. The procedures were as described previously [25].

Results and Discussion

Both sensitive and resistant yeast cell preparations (2×10^7 cells ml^{-1}) suspended at 30 °C in phthalate buffer (pH 4.5) containing glucose at 10 mM concentration resulted in p–t (power–time) curves which corresponded to simple zero-order kinetic processes.

The addition of nystatin to both sensitive and resistant cells gave a power output rate which first exceeded the maximum of that of the control but eventually fell below it when concentrations of nystatin of <1 μg ml^{-1} were used. At higher concentrations of antibiotic (1.5–5 μg ml^{-1}) this phenomenon was not observed. Beezer *et al.* [21] reported similarly and suggested that low concentrations of nystatin modified the yeast cell membrane in a way that permitted easier transport of glucose from the medium, thus giving rise to a temporary maximum power higher than that for yeast-cell controls.

A linear relationship was found between log of the nystatin dose and the time required for the signal to rise and fall from the first calorimetric response to some arbitrary level above the baseline. Table I and Fig. 1 show the relationship between log dose and response of sensitive and resistant yeast cells. For resistant cells (which are smaller than the parent wild type strain [23]):

(i) same cell number was adopted as that for sensitive cells (2×10^7 cells ml^{-1});

(ii) cell number was increased 4% (2.08×10^7 cells ml^{-1}) to that required to equal surface area of sensitive cells.

There was essentially no difference between the results from these different experimental designs

TABLE I. Relationship between Dose of Nystatin and Response of Sensitive and Resistant Yeast Cells in Phthalate Buffered Glucose (2×10^7 cells ml^{-1}).

Nystatin concentration (μg ml^{-1})	Deflection (%)[a]		
	Sensitive	Resistant	
		Same cell no.	Same surface area[b]
1.0	103.3	107.7	102.1
1.5	64.5		
2.0	50.2	75.5	79.3
2.5	31.7		
3.0	15.4	50.6	51.0
4.0	12.3	32.4	31.7
5.0	5.9	14.3	14.5

[a]Mean of at least triplicate experiments; reproducibility ±3%. [b]Cell number was increased 4% (2.08×10^7 cells ml^{-1}) to that required to equal surface area of sensitive cells; see text.

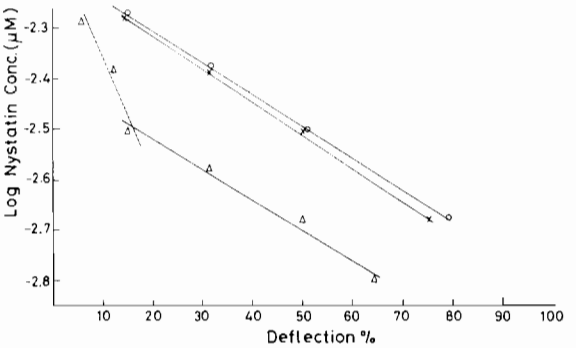

Fig. 1. Relationship between log[nystatin] and response of sensitive cells and of resistant cells. △ sensitive (2×10^7 cells ml^{-1}); × resistant (2×10^7 cells ml^{-1}); ○ resistant (same surface area as sensitive cells).

in response in microcalorimetry. This means that biological consequences of nystatin interaction are not terribly sensitive to cell size or area but of course physical consequences such as diffusion through the cell wall and the reduction of metabolic activity do depend on these variables. Sensitive and resistant cells show the same slope in plots of log dose *vs.* response *i.e.* same relative sensitivity to nystatin; resistant cells, however, required greater concentrations of nystatin to achieve the same reduction in deflection. The complexes are somewhat labile and on dissolution in the citrate containing medium may generate ternary M(II) nystatin citrate species [26]. This possibility was not examined.

Sensitive cells show a break in the log dose *vs.* response curve *i.e.* there appears to be 2 distinct regions; above and below 2–3 μg ml^{-1}. This is almost equivalent to the MIC (1–2 μg); hence 2 different modes of action may be indicated which depend on concentration.

Figure 2 and Table II show the relationship between log dose of nystatin–metal complex and response fo sensitive yeast cells. It can be seen that the responses of Ca(II) and Mg(II) nystatin complexes are distinctly different from those of Zn(II) and Ni(II) nystatin complexes. Perhaps this reflects the role of Ca(II) and Mg(II) in the cell structures. Ca(II) and Mg(II) are known to be released from yeast cells on interaction of nystatin and to protect yeast from nystatin interaction. All the plots, except for Zn(II) nystatin complex, of log dose *vs.* response are linear and thus may be analytically useful. The reason for the curvature in the Zn(II) nystatin curve is unknown but presumably reflects a more complex interaction of this salt with yeast cells. The Cu(II) nystatin complex showed low activity in the range of concentration examined (*i.e.* response appeared at >10 μg ml^{-1}).

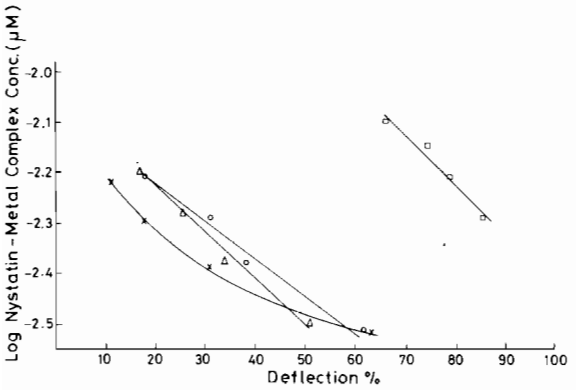

Fig. 2. Relationship between log[nystatin–metal complex] and response of sensitive yeast cells. □ Ni; × Zn; ○ Ca; △ Mg.

TABLE II. Relationship between Dose of Nystatin–metal Complex and Response of Sensitive Yeast Cells in Phthalate Buffered Glucose (2×10^7 Cells ml^{-1}).

Nystatin–metal complex	Concentration of nystatin–metal complex (μg ml^{-1})	Amount of nystatin in nystatin–metal complex (μg ml^{-1})	Deflection (%)	Apparent nystatin concentration (μg ml^{-1})
Ni(II)	5.0	4.72	85.2	1.19
	6.0	5.66	78.6	1.29
	7.0	6.60	74.0	1.38
	8.0	7.54	65.5	1.54
Zn(II)	3.0	2.80	63.4	1.55
	4.0	3.74	30.7	2.24
	5.0	4.67	17.4	2.86
	6.0	5.61	11.3	4.64
Ca(II)	3.0	2.87	61.4	1.60
	4.0	3.83	37.9	2.22
	5.0	4.79	30.7	2.42
	6.0	5.75	17.4	2.90
Mg(II)	3.0	2.95	51.0	1.87
	4.0	3.89	33.8	2.34
	5.0	4.87	25.6	2.61
	6.0	5.85	16.9	2.93
Cu(II)	10.0	9.36	60.0	1.45

If the relationship of the concentrations of added metal–nystatin complex and apparent nystatin concentrations holds throughout the whole concentration range *i.e.* up to saturating concentration, then the apparent nystatin activity of a saturated solution of metal–complex can be evaluated. This apparent activity is shown in Table III and this is to be compared with the maximum aqueous phase activity achievable with nystatin itself of 50 μg ml^{-1}. Thus the Zn(II), Mg(II) and Ca(II) complexes are able to sustain a greater active concentration of nystatin in aqueous solution than can be achieved with nystatin alone. These results may have clinical interest as it is likely that the required Zn(II), Mg(II) and Ca(II) concentrations do not have serious adverse clinical consequences.

The metals more commonly found in pharmaceutical preparations, Ca(II) and Mg(II), have fortunately enhanced activity and should therefore be capable of raising biological fluid concentrations of nystatin.

TABLE III. Comparison of Saturating Solubility in Aqueous Solution of Nystatin and Nystatin–Metal Complex.

Metal complex	Saturating solubility (mg ml^{-1})	Apparent[a] nystatin concentration at saturation (mg ml^{-1})
Ni(II)	0.51	0.09
Zn(II)	0.64	0.63
Ca(II)	0.72	0.19
Mg(II)	0.75	0.26
Cu(II)	0.47	

[a]Maximum nystatin concentration in aqueous solution is 0.05 mg ml^{-1}.

References

1 A. E. Beezer, P. O'Brien and W. L. Sham, *Inorg. Chim. Acta, 108*, 115 (1985).
2 A. E. Beezer, P. O'Brien and W. L. Sham, *Inorg. Chim. Acta, 108*, 121 (1985).
3 J. M. T. Hamilton-Miller, *Bact. Rev., 37*, 166 (1973).
4 W. Mechlinski and C. P. Schaffner, *J. Antibiot. (Tokyo), 25*, 256 (1972).
5 A. E. Beezer and B. Z. Chowdhry, *Talanta, 27*, 1 (1980).
6 J. F. Mannery, *Fed. Proc., 25*, 1804 (1966).
7 E. Bozler, *J. Gen. Physiol., 49*, 37 (1965).
8 F. L. Seigel, in J. D. Dunitz (ed.), 'Structure and Binding, Vol. 17', Springer, Berlin, 1973.

9 P. J. Romero, *B.B.A., 339*, 116 (1974).
10 H. J. M. Bowen, 'Trace Elements in Biochemistry', Academic Press, London, 1966.
11 H. Suomalainen and E. Oura, in H. A. Rose and J. S. Harrison (eds.), 'The Yeasts', Academic Press, London, 1971.
12 A. Sols, C. Gaucedo and G. de la Fueute, in H. A. Rose and J. S. Harrison (eds.), 'The Yeasts', Academic Press, London, 1971.
13 F. F. Nord and S. Weiss, in A. H. Cook (ed.), 'The Chemistry and Biology of Yeasts', Academic Press, London, 1968.
14 A. E. Beezer and P. B. Sharma, *Microbios, 31*, 7 (1981).
15 J. O. Lampen and P. Liras, *B.B.A., 374*, 159 (1974).
16 S. M. Hammond and B. N. Kliger, *J. Appl. Bact., 41*, 59 (1976).
17 P. Venables and A. D. Russell, *Microbios, 6*, 239 (1972).
18 I. Berdicevsky and N. Grosswicz, *Antimicrob. Agents Chemother., 2*, 1 (1972).
19 P. Marfey and H. Chessin, *B.B.A., 337*, 136 (1974).
20 B. Z. Chowdhry, A. E. Beezer and E. J. Greenhow, *Talanta, 30*, 209 (1983).
21 A. E. Beezer, R. D. Newell and H. J. V. Tyrrell, *Anal. Chem., 49*, 34 (1977).
22 D. C. E. Speller, 'Antifungal Chemotherapy', Wiley, Chichester, 1980.
23 W. B. Park, *Ph.D. Thesis*, University of London, 1984.
24 R. F. Cosgrove, A. E. Beezer and R. J. Miles, *J. Pharm. Pharmacol., 31*, 171 (1979).
25 B. F. Perry, A. E. Beezer and R. J. Miles, *Microbios, 29*, 81 (1980).
26 H. Sigel, *Angew. Chem., Int. Ed. Engl., 14*, 394 (1975).