MECHANISM OF INHIBITION BY UBICIDIN: INHIBITOR WITH PIERICIDIN RING STRUCTURE AND UBIQUINONE SIDE CHAIN

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1. Introduction

Amytal, rotenone and piericidin A are inhibitors of the mitochondrial respiratory chain, blocking the oxidation of NADH-dehydrogenase by CoQ_{10} but not by $K_3Fe(CN)_6$ [1]. Piericidin A, the most potent inhibitor, reacts with the respiratory chain at various sites, from all but one it is removed by washing with 2% BSA leaving the inhibitor only at electron exit port of the NADH dehydrogenase [1], a site defined as the specific binding site [2]. It was later shown that there are two non-identical specific binding sites per membrane bound enzyme molecule [3], differentiated by their reactivity with mersalyl [4]. These two sites were attributed to the electron exit ports, each contributes differently to the overall flux and differ in the piericidin binding constant [3,4]. The average dissociation constant from the two sites was estimated as $K_d = 3.9 \cdot 10^{-10}$ M [5]. The site of inhibition is functionally close to Fe-S center 2 and does not prevent energy linked reaction in the enzyme [6,7]. Thus reverse electron transport can be observed isolated from redox reaction of CoQ_{10}. The similarity in structure of piericidin A and CoQ was always tempting to associate it with the inhibition mechanism. Yet, the different structure of rotenone and amytal, all inhibiting at the same site [2], exclude this possibility. Because of that, the competitive relationship between piericidin and CoQ_{10} was attributed to competition on the inhibitory and not on the substrate site [5].

The recently synthetic products, ubicidins, (table 1) of Professor H. Rappaport and his colleagues, University of California, Berkely, combining the piericidin ring structure and the isoprenoid side chain of CoQ, are an excellent tool for studying the quinone reducing side of NADH-dehydrogenase. In this preliminary note we shall show that they share the same site with piericidin, a site different from the CoQ_{10} reducing one. Future use of these inhibitors is discussed.

Abbreviations: ETP_H, Phosphorylating submitochondrial particles; CoQ, Ubiquinone; UC3, ubicidin 3; BSA, Bovine serum albumin; PA, piericidin A.

2. Materials and methods

Beef heart mitochondria were made according to Ringler et al. [8]. ETP_H according to Hansen and Smith [9]. Ubicidins were a generous gift of H. Rappaport, University of California, Berkely. The naming of ubicidins follows the nomenclature common for the quinone family denoting the number of isoprenoide units in the chain.

NADH oxidase was measured as described as before [3]. Labelling of specific sites carried by adding the inhibitor to ETP_H suspended in 0.25 M sucrose, 10 mM K-phosphate, pH 7.4, containing 2% bovine serum albumin/ml. After 30 min equilibrium at 30°C, the particles were spun down and washed again by the same buffer.

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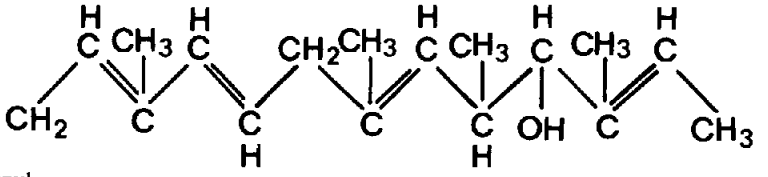
Table 1
Structure and inhibitory properties of ubicidines, 2 alkyl derivatives of 5,6 methoxy-3 methyl 4 pyridone

Name		Length of side chain in carbon atoms	I_{50}* mol/mg protein
Piericidin A	R = 	13	6×10^{-11}
Ubicidin 1	R = Prenyl	4	9×10^{-8}
Ubicidin 2	R = Geranyl	8	2.3×10^{-10}
Ubicidin 3	R = Farnasyl	12	1.7×10^{-10}
Ubicidin 4	R = Phytyl	16	2×10^{-8}
Ubicidin 10	R = Solanesyl	40	2×10^{-8}

*I_{50} values are taken from fig.1.

3. Results and discussion

3.1. *Inhibition by ubicidins*

In the absence of BSA the pyridone derivatives inhibit NADH oxidase in the order piericidin A > ubicidin 3 > ubicidin 2 >> ubicidin 4 = ubicidin 10 >> ubicidin 1. In order to avoid interaction with inhibitor at non-specific sites the titrations were repeated in the presence of 2% BSA [2]. It was noted that BSA lowered a little the inhibition level both for piericidin and the ubicidins, reflecting both inhibition at the non-specific sites and adsorption of inhibitor on BSA [3]. The results of titrations where only specific inhibition is measured are presented (as a Hill plot) in fig.1. As seen from the figure and documented also in table 1, ubicidin 3 and ubicidin 2 inhibits the enzyme to 50% (I_{50}) at an inhibitor/enzyme ratio very close to that of piericidin A. Shorter or longer analogues are 2 and 3 orders of magnitude less effective. Was the inhibition a result of interaction at the CoQ_{10} binding site one would expect ubicidin 10 with side-chain identical with CoQ_{10} to be the most potent inhibitor. The finding that inhibition potency peak at side-chain length which resembles that of piericidine A, suggests that the inhibitors share a site different from CoQ_{10} reducing sites. Being most potent, ubicidin 3 was employed in the rest of the experiments for comparison with piericidin A.

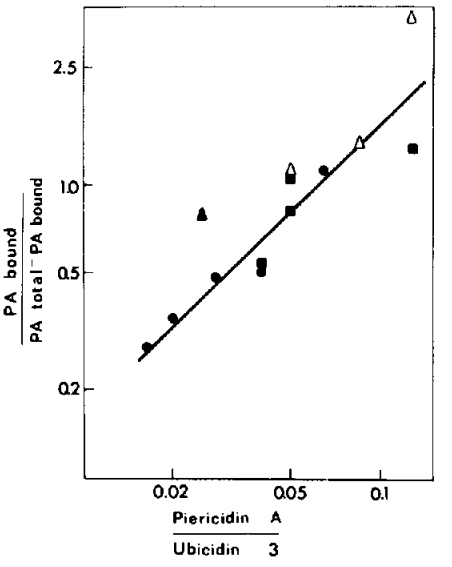


Fig.1. Hill plot for inhibition of NADH by piericidin A and its analoges. Determination of NADH oxidase activity and labeling at specific sites were carried out as described in Materials and methods. (△) Piericidin A; (○) ubicidin 3; (■) ubicidin 2; (●) ubicidin 4 (□) ubicidin 10; (▲) ubicidin 1.

3.2. *Additivity of inhibition*

Additivity of inhibition by ubicidin 3 and piericidin A was measured to determine whether the two inhibitors react with the same site. If both react in a mutually exclusive way with the same site, inhibition should be additive. Where the two inhibitors reacting independently with different sites, the resulting inhibition should be less than additive. As shown in table 2, the inhibition is additive. The values predicted for two

Table 2
Additivity of inhibition of NADH oxidase by piericidin A and ubicidin 3

Piericidin A nmol/mg	I_{PA}	Ubicidin 3 nmol/mg	I_{obs}	ΔI ($I_{obs} - I_{PA}$)	$I_{predict}$
40	23%	70	50%	27%	42%
60	43%	70	68%	25%	57%
80	51%	70	76%	25%	63%
120	57%	70	90%	23%	67%
–	–	70	25%	–	–

ETP_H were labeled at specific sites by increasing piericidin A concentration. After equilibration (30 min at 30°C) and determination of inhibition level (I_{PA}), constant amount of ubicidin 3 (70 mmol/mg) were added. The inhibition in presence of the two inhibitors (I_{obs}) was measured after additional equilibrium period. The inhibition predicted ($I_{predict}$) for two independent binding sites is: $I_{predict} = I_{PA} + I_{UC3} - I_{PA} \cdot I_{UC3}$ where I_{PA} and I_{UC3} are inhibition levels measured for Piericidin and ubicidin 3 respectively at the given concentration (I_{UC3} = 25%).

sites deviates markedly from I_{obs}. Thus we conclude that the two inhibitors react with the same site.

3.3. *Mutual displacement of piericidin and ubicidin 3*

In absence of radio-labelled ubicidin 3, the interaction of piericidin and ubicidin 3 with the enzyme was measured as binding of [^{14}C]piericidin and the effect of ubicidin 3 on the amount bound.

The most direct experiment is to displace [^{14}C]-piericidin by ubicidin 3 from the specific binding site. Even 5000 pmol ubicidin 3/mg (30 I_{50}/mg) for 30 min, at 30°C failed to release any [^{14}C]piericidin from the specific sites. This lack of displacement means either that different binding sites are involved or, that the lack of release is due to kinetic reasons. The dissociation constant of piericidin from its specific site ($3–9 \cdot 10^{-10}$ M) is small enough to make the dissociation extremely slow, essentially irreversible, reaction. In order to evaluate the kinetic factor, we tried to displace [^{14}C]piericidin by un-labelled piericidin (30 I_{50}/mg). No exchange was noted, after 30 min at 30°C, suggesting that if such a reaction proceeds, it must be extremely slow. This also accounts for the lack of displacement of piericidin A by ubicidin 3.

An analogous case should be mentioned: Klingenberg et al. could not measure any exchange between bound and free [^{35}S]carboxyatractilate ($K_d = 10^{-8}$ M), while the more loosely bound atractilate ($K_d = 5 \cdot 10^{-8}$ M) was suitable for such exchange [10].

3.4. *Estimation of binding constant of ubicidin 3*

To avoid kinetic difficulties, the ETP_H were first titrated by ubicidin 3 (30 min, 30°C) and then challenged by a constant amount of [^{14}C]piericidin. After additional equilibration period (30 min, 30°C) the particles were spun down, washed by BSA and [^{14}C]piericidin bound to specific sites was determined.

Assuming two independent equilibria of the two inhibitors with the same site,

$$K_{E.\,PA} = \frac{(E.\,PA_n)}{(E)\,(PA)^n}$$

$$K_{EUC3} = \frac{(E.\,UC3_m)}{(E)\,(UC3)^m}$$

than at saturating conditions where $E_{free} = 0$, it can be shown that,

$$\log \frac{(E.\,PA)_i}{(E.\,PA)_T - (E.\,PA)_i} = \log \frac{K_{E.\,PA}}{K_{E.\,UC3}} + \frac{n}{m} \log \frac{\text{piericidin}}{\text{ubicidin 3}}$$

($(E.\,PA)_T$ and $(E.\,PA)_i$ are the amounts of [^{14}C]-piericidin specifically bound at ubicidin 3 = 0 and ubicidin 3 = i, respectively).

The experimental results given in fig.2 fit a straight line with a slope of 1; meaning that there is no cooperativity in the binding of each of the two inhibitors.

The concentration ratio which lowered the piericidin binding to 50% and the slope of the line allows to estimate the value of $K_{E.\,PA}/K_{E.\,UC3}$. The ratio obtained is 0.06, taking $K_{E.\,PA} = 3–9 \cdot 10^{-10}$ M, we estimate $K_{E.\,UC3} = 0.5–1.5 \cdot 10^{-9}$ M. The number of piericidin binding sites per enzyme is 2. As ubicidin 3 can block more than half of the binding sites, we must conclude that it reacts with at least these two sites, most probably m = n = 2.

3.5. *Comparison of* I_{50} *and* K_d

There is a discrepancy between the ratios of inhibition ($\frac{I_{50}\ PA}{I_{50}\ UC3}$) and dissociation $\frac{K_{E.\,PA}}{K_{E.\,UC3}}$ constants being 3 and 16, respectively. This discrepancy is an apparent one. Even in the case of piericidin the correlation between binding and inhibition is sigmoidal [3]. As there is no cooperativity in inhibition (fig.1) or binding (fig.2) of the two inhibitors, the sigmoidal dependence of inhibition on binding (specific) is due to small differences in K_d and intrinsic electron flux of the two sites. Thus, the differences between binding and inhibition observed for ubicidin 3 are similar in nature to those reported for piericidin.

We can summarize that the newly synthesized inhibitor ubicidin 3 is very similar in nature to piericidin A: (1) It inhibits at molar ratio similar to piericidin; (2) The inhibition is not released by BSA washing; (3) the inhibition by piericidin and ubicidin 3 is additive and; (4) ubicidin 3 can prevent binding of piericidin.

The new family of inhibitors (table 1) displays a typical correlation between the length of the side chain and inhibition potency, reaching a maximum at

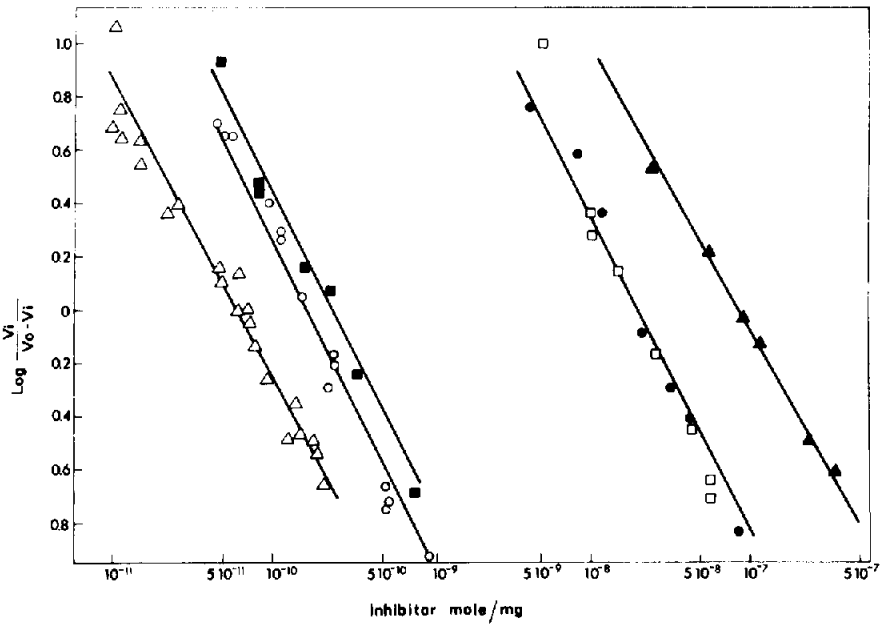


Fig.2. Effect of ubicidin 3 on binding of [^{14}C]piericidin A to specific binding sites. ETPH were treated by increasing ubicine 3 concentration in 2% BSA sucrose phosphate buffer 30 min 30°C. [^{14}C]piericidin A was added and after 30 min 30°C ETPH were spun down washed by BSA and [^{14}C]piericidin A content determined by scintillation counting. The function drawn in the figure is explained in the text. Different symbols are for different experiments.

chain-length resembling that of piericidin. The tightness of the binding of piericidin is so high that it is not suitable for observing minor changes in the binding site (to be monitored as exchange or immobilization of spin labelled derivatives). The new ubicidins offer a wide range of dissociation constants, one or few will be suitable for measuring conformational changes of the enzyme (or the lipid component of the site) [11], by one of the techniques described above. As the binding site is in close proximity to the first coupling site [7,8], a tool for close observation of enzyme–membrane interaction of specific loci in immediate proximity to coupling site might be at hand.

Finally we wish to propose a novel explanation to the bizarre effect of CoQ_3 on the respiratory system. On one hand it can restore NADH and succinate oxidase activity in CoQ-depleted preparations [12], on the other hand it inhibits NADH oxidase but not succinoxidase activity [13]. The site of inhibition must be very similar to piericidin as it blocks reduction of CoQ, but not of $K_3Fe(CN)_6$ [13]. If one regards the similarity between CoQ_3 and ubicidin 3 then these facts are self explained. Apparently, CoQ_3 reacts both with the piericidin, ubicidin sites as inhibitor and with the CoQ reducing site as electron acceptor. It is the superposition of these antagonistic effects which blurred the observations.

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