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Cherimolin-1, New Selective Inhibitor of the First Energy-Coupling Site of the NADH:Ubiquinone Oxidoreductase (Complex I)

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The mechanism linking electron transport to proton translocation in the NADH:ubiquinone oxidoreductase (complex I of the mitochondrial respiratory chain) is still unclear. Inhibitors acting at different sites of the enzyme are powerful tools to clarify this mechanism. Up to now, a unique inhibitor, the Annonaceous acetogenin rolliniastatin-2, selectively blocks the most internal proton-translocation site. This study introduces cherimolin-1, a new acetogenin that inhibits the complex I with this special mode of action, which is more easily available from the plant material. Moreover, the mode of action of this scarce type of complex I inhibitor is further characterized. © 1997 Academic Press

Inhibitors of mammalian and bacterial NADH:ubiquinone oxidoreductase (complex I) acting by different ways are powerful tools to elucidate the mechanism of this complicate enzyme. Indeed, the NADH:ubiquinone oxidoreductase is the largest and most complex of the proton-translocating enzymes in the inner membrane of mammalian mitochondria. Although the knowledge of the enzyme has been greatly increased during the last years (1-7), many clues regarding the electron pathways from NADH to ubiquinone and the translocation of protons across the membrane are still unknown. Three controversial models to explain the mechanisms linking electron transport to vectorial proton translocation in complex I have been recently proposed (3-5). At this stage, selective inhibitors of each proposed event of the complicate sequence of both proton and electron transport reactions became absolutely necessary to validate these hypothesis.

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The ubiquinone binding sites within the transmembrane part of the complex I are inhibited by great variety of compounds with very different structures. Recent studies have suggested that there are at least three different sites where inhibitors can act. Two of them, namely A or N_A and B or P_I respectively, are linked to proton translocation through intermediate ubisemiquinone formation, whereas the other one, namely C or N_B, is associated to the final ubiquinol formation and release without proton translocation (4, 5). One of the two energy-coupling steps is blocked by the classical inhibitor rotenone and by many other inhibitors acting at site B or P_I (4, 5, 8-13). Some compounds, as piericidin A, are able to inhibit both energy-coupling sites (4, 5, 8-11). Nevertheless, a unique inhibitor, the Annonaceous acetogenin rolliniastatin-2, selectively blocks the most internal proton-translocation site (the first site, A or N_A), an oddity among the great number of complex I inhibitors (4, 10, 14-16).

Nevertheless, the restricted availability of rolliniastatin-2, isolated from rare tropical plants, have greatly limited the biochemical studies and the potentiality of this compound to clarify the intricate complex I mechanism. On the other hand, a great bulk of work on the enzyme structure and function is being done with complex I from several sources other than beef heart, mainly bacteria (13, 16-20). The affinity and potency of inhibitors differ substantially for such enzyme (12, 13, 18), therefore, it could be important the availability of alternative inhibitors with this special mode of action. In our research on the mechanism of complex I inhibition by the Annonaceous acetogenins, an important effort has been done to search new specific inhibitors of this relevant site (A or N_A) of the complex. This work introduces the cherimolin-1; an Annonaceous acetogenin isolated from the seeds of the "cherimoya" (fruit of *Annona cherimolia*), a commercial table fruit produced in the south of Spain (21, 22). This inhib-

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itor shows an hyperbolic titration curve, an uncompetitive behavior respect to the quinone substrate, and a binding site not overlapped with rotenone. These three features are only matched by rolliniastatin-2, whereas other complex I inhibitors differ by at least one of these characteristics.

MATERIALS AND METHODS

Cherimolin-1 was isolated and purified from seeds of the Spanish "cherimoya" (*Annona cherimolia*) as previously described (22). Other Annonaceous acetogenins used in this study were extracted from the corresponding plant material (see (23, 24) for review). Rotenone, antimycin A, decylubiquinone and other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions (2 mM in absolute ethanol) of the inhibitors were prepared and kept in the dark at −20 °C. Appropriate dilutions (5-50 μM) were made before the experiments (10).

Inverted submitochondrial particles (SMP) from beef heart were obtained by extensive ultrasonic disruption of frozen-thawed mitochondria to produce open membrane fragments where permeability barriers to substrates were lost (25). Beef-heart SMP were diluted to 0.5 mg/ml in 250 mM sucrose, 10 mM Tris-HCl buffer, pH 7.4, and treated with 300 μM NADH to activate complex I (26) before starting experiments.

The enzymatic activities were assayed at 22 °C in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA with the SMP diluted to 6 μg/ml in the cuvette. NADH:ubiquinone oxidoreductase was measured with 75 μM NADH and 30 μM decylubiquinone (DB) as soluble short-chain analogue of ubiquinone in presence of 2 μM antimycin and 2 mM potassium cyanide to block any reaction downstream the complex I (27). NADH oxidase was measured as the aerobic oxidation of 75 μM NADH in absence of the quinone substrate and other inhibitors of the respiratory chain.

Inhibitor titrations against both evaluations of complex I activity were made as previously described (10). Data from four titrations in the same conditions were pulled and fitted for graphics. The inhibitory concentration 50 (IC_{50}) was taken as the final compound concentration in the assay cuvette that yielded 50 % inhibition of the activity. Data from individual titrations were used to assess the means and standard deviations. Inhibition kinetics were evaluated against the NADH:ubiquinone oxidoreductase by varying the concentration of the ubiquinone analogue.

RESULTS AND DISCUSSION

Cerimolin-1 was first isolated from the seeds of the Spanish "cherimoya" (21, 22) and later from other species of *Annonaceae* (23, 24). This Annonaceous acetogenin belongs to the group of the nonadjacent bis-tetrahydrofuranic acetogenins, which basically differs, from both the adjacent bis-tetrahydrofuranic and the monofuranic acetogenins by the structure of the furanic core of the molecule. Figure 1 shows the chemical structure of the cherimolin-1 compared with that of the rolliniastatin-2; an adjacent bis-tetrahydrofuranic acetogenin characterized as the unique selective inhibitor, up to now, of the first site (A or N_A) involved in proton translocation within the complex I (4, 10, 14-16). Rolliniastatin-1, another adjacent bis-tetrahydrofuranic acetogenin that inhibits both energy coupling sites in the complex I and thus acts like piericidin A (10, 14, 28), differs from the rolliniastin-2 by the stereochemical

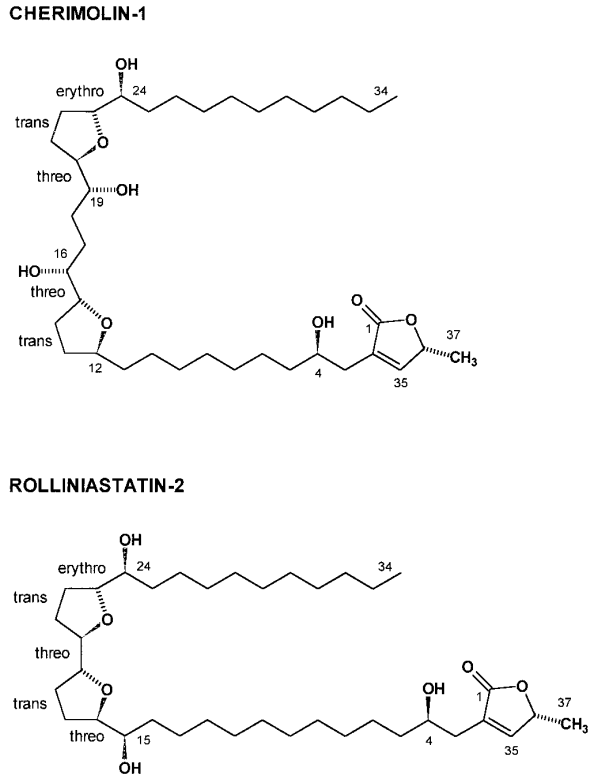

FIG. 1. Chemical structures of cherimolin-1 and rolliniastatin-2. Cherimolin-1 is a nonadjacent bis-tetrahydrofuranic acetogenin, whereas rolliniastatin-2 is an adjacent bis-tetrahydrofuranic acetogenin with the same number of C atoms. Cherimolin-1 presents a more expanded hydrophilic core and a shorter first aliphatic tail.

configuration of the tetrahydrofuranic rings (23, 24). Main differences between cherimolin-1 and rolliniastatin-2 are thus the more spacious hydroxylated tetrahydrofuranic system of the core, which it is thought to mimic the quinone ring of the ubiquinone, and the subsequent shorter length of the first aliphatic tail that contains the initial methyl-α,β-unsaturated-γ-lactone moiety.

Figure 2 shows the titration curve of cherimolin-1 against the NADH oxidase activity compared with those of rolliniastatin-2 and rotenone, the classical complex I inhibitor. All three inhibitors gave typical hyperbolic curves, although rolliniastin-2 approached the linearity. This behavior contrasts with the inhibitors of the two sites of the complex I involved in the proton translocation and energy coupling, as rolliniastatin-1 and piericidin A, that give sigmoidal titration curves (8, 28, 29). Cherimolin-1 was slightly less potent than rolliniastin-2 (IC_{50} 1.83 ± 0.26 nM and 0.51 ± 0.02 nM, respectively), although it was more potent than rotenone (IC_{50} 5.1 ± 0.9 nM). Similar titration curves were obtained against the NADH:ubiquinone oxidoreductase activity measured with decylubiquinone (DB) as externally added analogue. However, rol-

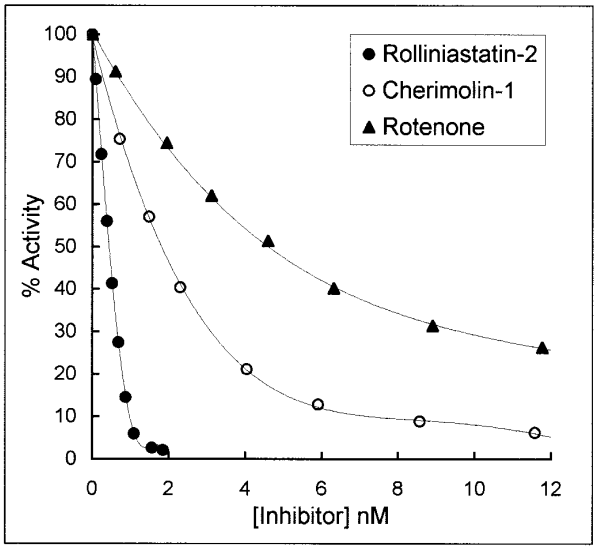

FIG. 2. Titrations against the NADH oxidase activity in bovine heart submitochondrial particles. Mitochondrial protein concentration was 6 μg/ml. Control activity was approximately 0.95 μmol.-min^{-1}.mg^{-1}. Data were obtained from four determinations for each product. IC_{50} are given in the text.

liniastin-2 was almost as potent as it was against the NADH oxidase activity (IC_{50} 0.61 $\pm$ 0.04 nM), cherimolin-1 was less potent (IC_{50} 5.2 $\pm$ 0.6 nM), and the greatest loss of potency was found for rotenone (IC_{50} 28.8 $\pm$ 1.5 nM). Inhibitors acting either at the first site of the complex I or simultaneously at the two sites are very effective against the decylubiquinone induced NADH oxidation, whereas inhibitors acting only at the second site, like rotenone, are much less potent against this latter activity compared with the potency against the aerobic NADH oxidation (unpublished observations). A reason for this behavior could be that inhibitors of the second site allow the formation of the first ubisemiquinone at site A (N_A) within the complex creating a non-physiological electron sink (rotenone insensitive) that reduces a part of the ubiquinone analogue (14, 15). Other reason could be that the ubiquinone analogue can penetrate into the second site (B or P_1) competing in some extend with the inhibitor, whereas it can not reach the first site (A or N_A), more internal, which only can be occupied by the endogenous trapped ubiquinone (4, 30). Anyway, this characteristic seems to indicate a similar behavior between cherimolin-1 and rolliniastatin-2, which is different from that of rotenone.

Moreover, compounds acting at the first site behave as uncompetitive inhibitors with respect to suitable ubiquinone analogues such as decyl- and undecylubiquinone (10, 30). This is a consequence of the inhibitor binding to a site upstream the substrate-binding site. Figure 3 shows the inhibition kinetics for rolliniastatin-2, cherimolin-1 and rotenone. Indeed, rolliniastatin-2 is an uncompetitive inhibitor, contrarily to that stated by Friedrich et al. (12). These authors described the rolliniastatin-2 (also called "annonin VI") as a partially competitive inhibitor. However, experiments were done with the homologue CoQ_2 (di-isoprenylubiquinone) that has been proved to be both a poor substrate and a competitive inhibitor for complex I (14, 15, 25, 27), and thus, their observations combined the effect of both, the inhibitor and the ubiquinone homologue. As shown in Figure 3, cherimolin-1 also behaved as uncompetitive inhibitor with respect to the DB substrate, whereas rotenone acted as noncompetitive inhibitor, a characteristic of the inhibitors acting only at the second binding site (B or P_1) of the complex I (10, 12, 30).

Nevertheless, compounds acting at both sites, as piericidin A and rolliniastatin-1, also behave as uncompetitive inhibitors due to the binding at the first site (10). Although they give sigmoidal titration curves (8, 28) as first indication of their dual inhibition, the relative binding site can be characterized by titrating each inhibitor in presence of each other to explore their mutual exclusivity. Figure 4 shows the Dixon plots of cherimolin-1 in presence of rotenone and rolliniastatin-2. At low concentration, two inhibitors mutually non exclusive should give plots that extrapolate at the same point of the x-axe representing the K_i of the titrated inhibitor, which is not modified by the presence of the second inhibitor. It was the case of cherimolin-1 that clearly showed lack of interference by rotenone (Figure 4A), as it was previously demonstrated for rolliniastin-

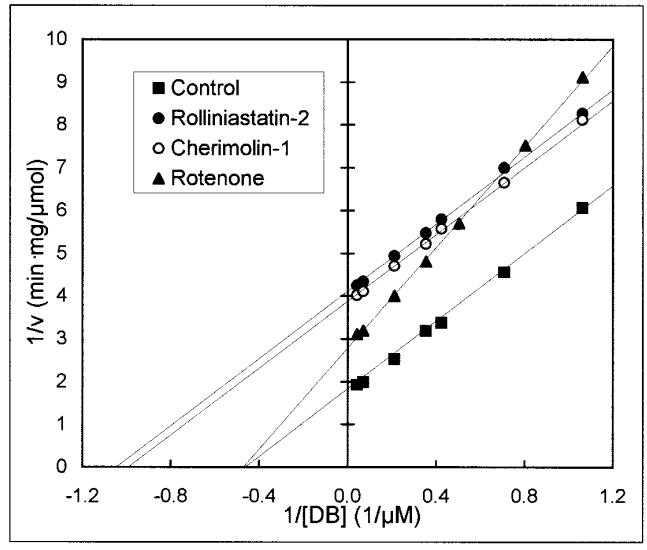

FIG. 3. Reciprocal plots of the NADH:ubiquinone oxidoreductase activity in the presence of inhibitors. NADH:ubiquinone oxidoreductase was measured with decylubiquinone (DB) as ubiquinone analogue. Mitochondrial protein concentration was 6 μg/ml. Inhibitor concentrations were 1.2 nM for rolliniastatin-2 (●), 3.8 nM for cherimolin-1 (○), and 18.5 nM for rotenone (▲).

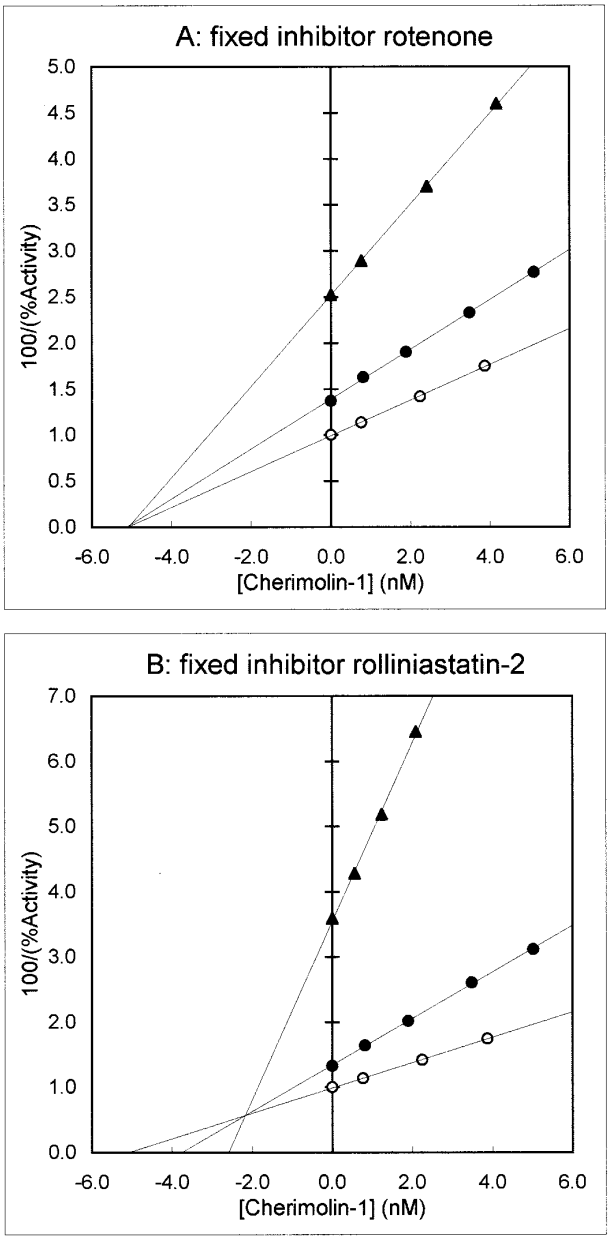

FIG. 4. Dixon plots of cherimolin-1 in the presence of rotenone and rolliniastatin-2. Relative activity refers to NADH:ubiquinone oxidoreductase under the conditions given in Figure 3. (A) Rotenone fixed concentrations were 0 nM (○), 26 nM (●), and 96 nM (▲). (B) Rolliniastin-2 fixed concentrations were 0 nM (○), 0.6 nM (●), and 1.2 nM (▲).

2 (10). In turn, titration of cherimolin-1 in presence of rolliniastatin-2 gave plots that crossed each other and extrapolated at different intercepts indicating mutual exclusivity (Figure 4B). Taking into account that dual inhibitors are mutually exclusive with rotenone due to the overlapping at the second site (4, 10), we can state that cherimolin-1 is a selective inhibitor of the first site of the complex I, like rolliniastatin-2.

It is important to note that other inhibitors mutually non-exclusive with rotenone as myxothiazol, stigmatellin, methyl-phenylpyridium (MPP^+) analogues, capsaicins and quinolones (9, 17, 26, 31), act in different mode. They probably bind a third site in the complex I (the site C or N_B) that is not involved in the mechanism of proton translocation and energy coupling (4, 5). These compounds generally are weaker inhibitors that behave in competitive manner (5, 9, 17, 31), they are not uncompetitive inhibitors, and they are also mutually non-exclusive with rolliniastatin-2 (9, 10).

Our results have demonstrated that cherimolin-1 belongs to a special and scarce class of inhibitors of complex I that are able to block selectively the first energy-coupling step of the enzyme, and thus, rolliniastatin-2 is not the unique member of this group. Taking into account the structural similarities between both inhibitors (see Figure 1), we suggest that the lactone moiety with the hydroxyl group at C-4 should be determinant for binding the first site (A or N_A) of the complex I. Indeed, other Annonaceous acetogenins with the same furanic structure and configuration that rolliniastatin-2, but lacking the C-4 hydroxyl, bind only the rotenone site (the second site, B or P_I) and they behave as non-competitive inhibitors (10), as well as otivarin, an acetogenin that resembles strongly cherimolin-1 but it differs by the position of the hydroxyl group (10). Moreover, dual inhibitors, as rolliniastatin-1, present the same chemical arrangement in the initial part of the molecule, although they differ by the configuration of the furanic core compared with rolliniastatin-2 (10, 28). Modification of this part (e.g., lack of the C-4 hydroxyl group) abolishes the dual behavior (28) indicating again that the ability of some Annonaceous acetogenins to inhibit the first site of the complex I could lie in this portion of the molecule.

The disposal of new inhibitors with this specific mode of action could be important to define the structural features of the site A (N_A) of the complex I that is involved in the first step of the energy coupling. Furthermore, cherimolin-1 could be a useful alternative for studies with bacterial and fungal complex I, which has shown different sensitivity to inhibitors compared to mammalian complex I (12, 13, 17). Finally, although cherimolin-1 is slightly less potent than rolliniastatin-2 to inhibit beef heart complex I, it presents the additional advantage that the plant source, a commercial table fruit, is easy to obtain and thus, it will expand the availability of this inhibitor to many complex I research groups.

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