Natural substances (acetogenins) from the family *Annonaceae* are powerful inhibitors of mitochondrial NADH dehydrogenase (Complex I)

Mauro DEGLI ESPOSTI,*§ Anna GHELLI,* Marina RATTA,* Diego CORTES† and Ernesto ESTORNELL‡

*Dipartimento di Biologia, Università di Bologna, Via Irnerio 42, 40126 Bologna, Italy, †Departament de Farmacologia (Farmacognosia i Farmacodinamia) and ‡Departament de Bioquímica i Biologia Molecular, Universitat de València, València Spain

Natural products from the plants of the family *Annonaceae*, collectively called Annonaceous acetogenins, are very potent inhibitors of the NADH–ubiquinone reductase (Complex I) activity of mammalian mitochondria. The properties of five of such acetogenins are compared with those of rotenone and piericidin, classical potent inhibitors of Complex I. Rolliniastatin-1 and rolliniastatin-2 are more powerful than piericidin in terms of both their inhibitory constant and the protein-dependence of their titre in bovine submitochrondrial particles. These acetogenins could be considered therefore the most potent inhibitors of mammalian Complex I. Squamocin and otivarin also have an inhibitory constant lower than that of piericidin, but display a larger protein-dependence of the titre. Squamocin and otivarin, contrary to the other acetogenins, behave qualitatively like rotenone. Rolliniastatin-2 shows unique properties as its interaction, although mutually exclusive to that of piericidin, appears to be mutually non-exclusive to that of rotenone. It is the first time that a potent inhibitor of Complex I is found not to overlap the active site of rotenone.

INTRODUCTION

NADH–ubiquinone oxidoreductase (respiratory Complex I; EC 1.6.5.3) is the most complicated of the enzyme complexes the function of which is coupled to energy conservation (Wikström and Saraste, 1984; Ragan, 1985; Walker, 1992; Ohnishi, 1993). The mechanism of Complex I is much less well understood than that of the other energy-conserving complexes of mitochondria (Wikström and Saraste, 1984; Walker, 1992; Singer and Ramsay, 1992). However, the sequencing of all of the subunits of the bovine complex is now complete (Walker, 1992) and the gross structure and biogenesis of the *Neurospora* complex are known (Weiss et al., 1991). The molecular biology of some bacterial homologues of the complex is also at hand (Ohnishi, 1993; Sled et al., 1993). It is hoped that this structural knowledge will stimulate biochemical research to find correlations with function.

The increasing number of inhibitors of mammalian Complex I may provide clues to the enzyme mechanism. Very many substances inhibit the NADH–ubiquinone reductase activity and consequently the energy-conserving function of Complex I (Storey, 1980; Singer and Ramsay, 1992; Oettmeier et al., 1992; Degli Esposti et al., 1993; Friedrich et al., 1994a,b; Jewess, 1994, and references therein). Despite a wide variation in chemical structure, all such inhibitors act in a way that is essentially superimposable on the action of rotenone (Walker, 1992; Singer and Ramsay, 1992; Oettmeier et al., 1992; Ahammadsahib et al., 1993), the classic potent inhibitor of mammalian Complex I (Ernster et al., 1963; Singer, 1979; Singer and Ramsay, 1992). Different types of inhibitor are, however, needed to dissect functionally the pathway of electron and proton transport at the quinone junction of the complex (Walker, 1992; Singer and Ramsay, 1992).

We report here that natural substances isolated from the seeds of *Annona cherimolia* and *Rollinia membranacea* are more potent than rotenone and also piericidin [piericidin is the most powerful inhibitor of Complex I reported so far (Horgan et al., 1968; Singer, 1979; Gutman, 1980; Singer and Ramsay, 1992; Friedrich et al., 1994a,b)]. These compounds belong to a group of bis-tetrahydrofuran acetogenins which have also been extracted from other species of the *Annonaceae* family (Cortes et al. 1984, 1991a,b, 1993a,b; Hui et al., 1989; Rupprecht et al., 1990; Born et al., 1990; Londershausen et al., 1991; Ahammadsahib et al., 1993; Saez et al., 1993). Only recently has it been realized that compounds purified from different plant species, and named after them, could have the same chemical structure (Cortes et al., 1993a,b). For example, rolliniastatin-2 from *Rollinia mucosa* (Pettit et al., 1989) may be identical with bullatacin from *Annona bullata* (Hui et al., 1989; Ahammadsahib et al., 1993), 14-hydroxy-25-desoxyrollinicin from *Annona reticulata* (Etse and Waterman, 1986) and probably also annonin VI from *Annona squamosa* (Born et al., 1990). This compound with different names is of particular interest because it is very potent and has a mode of action that is non-exclusive with respect to rotenone.

MATERIALS AND METHODS

Preparations

Phosphorylating submitochondrial particles were prepared from bovine heart mitochondria as described by Hansen and Smith (1964). The particles were stored at −70 °C at 10–50 mg/ml (protein measured by the biuret method) in buffer comprised of 0.25 M sucrose and 20 mM Tricine/HCl, pH 7.6, containing 5 mM $MgCl_2$, 2 mM KATP, 2 mM potassium succinate and 2 mM GSH (Hansen and Smith, 1964). Crude Complex I was isolated as fraction R_4B from bovine heart mitochondria by the procedure of Hatefi and Stiggall (1978) and further fractionated twice with cholate and 40%-satd $(NH_4)_2SO_4$ to remove excess phospholipids and contaminating Complex II. This preparation

Abbreviations used: I/A, ratio between percentage inhibition and percentage residual activity; MOA, β-methoxyacrylate; UBQ, 2,3-dimethoxy-5-methyl-6-*n*-undecyl-*p*-benzoquinone.

§ To whom correspondence should be addressed.

contained 3.3 nmol of cytochrome *b* and about 0.5 nmol of FMN/mg of protein (biuret) as determined by conventional procedures (Hatefi and Stiggall, 1978; Degli Esposti et al., 1986).

Inhibitors

Seeds of *Annona cherimolia* and *Rollinia membranacea* were extracted with methanol and the various acetogenins were purified by chromatographic methods and spectroscopically characterized as described previously (Cortes et al., 1991a,b; 1993a,b; Saez et al., 1993). All the samples of acetogenins were more than 95% pure as judged by chromatographic criteria (their chemical structures are shown in Figure 1). These acetogenins, like all other inhibitors tested, were dissolved in ethanol at 0.05–3 mM. For routine evaluation of the concentration, the absorption coefficient in ethanol for rolliniastatin-2, rolliniastatin-1 and molvizarin was determined by weight to be 10.7 $mM^{-1} \cdot cm^{-1}$ at 207–208 nm. The concentrations of otivarin and squamocin were determined by weight only because of the blue shift in their u.v. absorption maxima which prevented an accurate evaluation of the absorption coefficient.

In this study we have used several other inhibitors not commercially available but obtained through the generosity of the following colleagues: piericidin A from T. Friedrich, University of Dusseldorf, Dusseldorf, Germany; methoxyacrylate (MOA) stilbene from P. Rich, Bodmin, Cornwall, U.K.; *sec*-butylacridone from W. Oettmeier, University of Bochum, Bochum, Germany; tridecyl stigmatellin from A. M. Colson, University of Louvain-la-Nueve, Louvain-la-Nueve, Belgium; mucidin from J. Subik, University of Bratislava, Bratislava, Slovakia; deguelin and other rotenoids from F. Delle Monache, Catholic University of Rome, Rome, Italy.

Inhibitor concentrations were determined spectrophotometrically (Degli Esposti et al., 1986, 1993).

Reagents

Reagents of analytical grade were all purchased from Sigma, St. Louis, MO, U.S.A. except for stigmatellin, which was from Fluka. Ubiquinone-1 and -2 were a generous gift from Eisai Co., Tokyo, Japan. 2,3-Dimethoxy-5-methyl-6-*n*-undecylbenzoquinone (UBQ) was kindly provided by E. Berry, University of California, Berkeley, CA, U.S.A. [see Berry et al. (1991) for the synthesis and use of the quinone]. All quinones were dissolved in ethanol and determined at 275 nm using an average absorption coefficient of 14.5 $mM^{-1} \cdot cm^{-1}$. Ubiquinols were prepared and determined as described previously (Degli Esposti et al., 1986).

Enzyme assays

Ubiquinol-2–cytochrome *c* reductase activity was assayed as described by Degli Esposti et al. (1986). NADH–ferricyanide reductase activity was assayed at 420–500 nm (using an absorption coefficient of 1 $mM^{-1} \cdot cm^{-1}$) at 30 °C with 2 mM ferricyanide as substrate, in the same buffer as used for the NADH–quinone reductase assay (described below) using a rapid mixing device. The specific activity of ferricyanide reductase was used to estimate the content of active Complex I in the submitochondrial particles by considering half of the turnover maximum [8×10^5 min^{-1} (Cremona and Kearnley, 1964)] because the concentration of ferricyanide was approximately equal to the K_m (Ragan, 1985). According to such calculations, Complex I content ranged between 0.041 and 0.047 nmol/mg of mitochondrial protein (measured by the biuret method) and was lower than the total FMN content as reported previously (Cremona and Kearnley, 1964; Gutman and Singer, 1970).

NADH–quinone reductase activity was usually assayed, at room temperature (20–23 °C), in either the dual-wavelength mode at 350–410 nm using an absorption coefficient of 5.5 $mM^{-1} \cdot cm^{-1}$ or at 340 nm using an absorption coefficient of 6.2 $mM^{-1} \cdot cm^{-1}$. In the latter case rapid mixing was not required and the initial rates were calculated from the linear portion of the traces by a computer program. The reaction was started by the addition of the quinone substrate to 2 ml of the assay mixture generally consisting of 50 mM potassium phosphate buffer, pH 7.6, 1 mM EDTA and 2–10 mM KCN (Yagi, 1990; Degli Esposti et al., 1993). The high concentration of KCN did not affect the rates significantly (Schatz and Racker, 1966) and was useful to induce uncoupling of the particles as well as to block cytochrome oxidase completely. Assays were also performed in sucrose/Tris buffers with or without KCN. Generally, disposable plastic cuvettes were used (Kartell 1937 model). The final concentration of submitochondrial particles was 0.02–0.1 mg/ml and that of NADH was 120–150 μM (Degli Esposti et al., 1993). The concentration of various quinones was kept at quasi-saturating values (25–40 μM) in the titrations of the inhibitors, or varied between 1 and 100 μM in the quinone titrations.

UBQ was selected as the standard quinone of the NADH–quinone reductase activity for the following reasons: (i) its maximum rates were even higher than the rotenone-sensitive rates with ubiquinone-1, which is usually regarded as the best substrate for Complex I (Schatz and Racker, 1966; Ragan, 1985); (ii) in contrast with the decyl analogue of ubiquinone (Estornell et al., 1993), the reaction was fairly linear with time; (iii) UBQ promoted proton translocation with a stoichiometry of about $4H^+$/NADH, in agreement with the current consensus on the proton-pumping capability of Complex I (Rottenberg and Gutman, 1977; Weiss et al., 1991; Walker, 1992); (iv) the rates were fully sensitive to rotenone or piericidin; (v) unlike other quinones (Degli Esposti et al., 1993; Estornell et al., 1993), UBQ essentially does not receive electrons from Complex III during NADH oxidation.

Succinate-driven reverse electron transfer was measured in buffer containing 0.25 M sucrose, 20 mM Tricine/HCl, pH 7.6, 5 mM $MgCl_2$, 2 mM KCN, 0.1 mg/ml submitochondrial particles, 1 mM NAD^+ and 5 mM succinate and was initiated by the addition of 1 mM KATP (Ernster and Lee, 1967). No activation of succinate dehydrogenase was performed.

Inhibitor titrations

Titrations of the inhibitors were performed as follows. Submitochondrial particles or crude Complex I were diluted to 3–5 mg/ml in 0.25 M sucrose/30 mM Tris/HCl, pH 7.6, and treated with 40–100 μM NADH (Singer, 1979), 2 mol of antimycin/mol of cytochrome *b* and 2–3 mol of MOA stilbene or mucidin/mol of cytochrome *b* to block any reactions with Complex III (Degli Esposti et al., 1993). The particles were then transferred in graduated glass test tubes and treated with increasing concentrations of the ethanolic solution (ethanol never exceeded 2% of the total volume) of an inhibitor, with about 5 min incubation on ice between each addition. Several controls with and without ethanol were performed at the beginning and end of the titration. IC_{50} values were the final inhibitor concentrations in the assay cuvette that yielded 50% inhibition of the NADH–UBQ reductase activity averaged among the controls. When ubiquinone-1 or ubiquione-2 were used as substrates, the rates in the presence of 1 μM rotenone or piericidin were subtracted (Degli Esposti et al., 1993; Estornell et al., 1993).

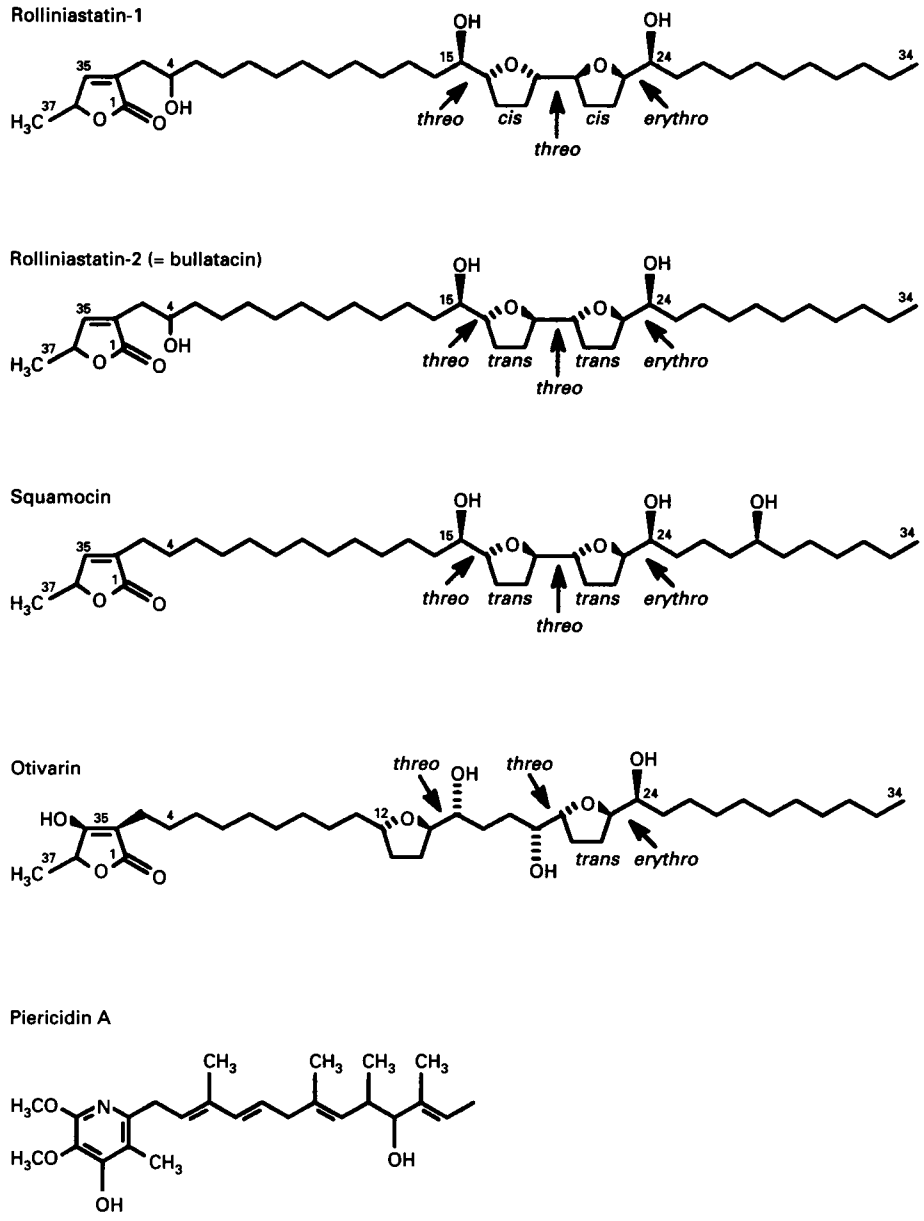

Figure 1 Molecular structure of some annonaceous acetogenins and piericidin A

Molvizarin differs from rolliniastatin-2 in that only 11 of the 13-CH_2- groups separate the unsaturated γ-lactone from the bis-tetrahydrofuran structure (Cortes et al., 1993a).

When the K_i of the inhibitor was measured via protein-dependence of the IC_{50} (Tischer and Strotmann, 1977), small samples of the particles treated as above were incubated with different concentrations of the inhibitor for at least 2 h on ice before their activity was assayed. The same long incubation time was generally used when the mutual exclusivity of two inhibitors was evaluated by the procedure of Chou and Talalay (1981) (Degli Esposti et al., 1994) and in the quinone titrations.

RESULTS AND DISCUSSION

Enzymic assay of Complex I with UBQ

We routinely used UBQ as the quinone substrate for NADH–quinone reductase activity of Complex I both *in situ* in submitochondrial particles and in mitochondrial fractions enriched in Complex I. In submitochondrial particles the rate of NADH oxidation stimulated by 30 μM UBQ (a concentration yielding about 85% of the maximal rate) ranged between 0.3 and 1.0 μmol/min per mg of protein under the conditions described in the Materials and methods section. Most frequently, the reductase activity of frozen–thawed particles was around 0.5 μmol/min per mg of protein and was close to the rate of NADH oxidation in buffers without KCN in the absence of uncouplers. Crude Complex I (fraction R_4B) had an NADH–UBQ reductase activity of 0.5–0.7 μmol/min per mg of protein in the absence of exogenous phospholipids.

The rates obtained with UBQ were comparable with those obtained with ubiquinone-1 under the same experimental conditions. UBQ was preferred as the quinone substrate, however,

because of its properties described in the Materials and methods section.

Annonaceous acetogenins as Complex I inhibitors

The compounds studied here, which are collectively called annonaceous acetogenins (Rupprecht et al., 1990; Cortes et al., 1993a,b; Ahammadsahib et al., 1993), are shown in Figure 1; their original names have been retained as reviewed by Cortes et al. (1993a). These acetogenins represent different chemical classes. Rolliniastatin-1 differs from rolliniastatin-2 in the stereochemistry of the central bis-tetrahydrofuran structure. Squamocin differs from rolliniastatin-2 and molvizarin [which has only 11 of the 13 CH_2 groups separating the unsaturated γ-lactone from the bis-tetrahydrofuran structure (Cortes et al., 1991a)] in the change of a hydroxy substituent from position 4 to position 28 along the hydrocarbon tail (Figure 1). Otivarin is rather different from the above acetogenins because of modifications in the γ-lactone 'head' and, in particular, in the structure of the non-adjacent bis-tetrahydrofurans [Figure 1 and Cortes et al. (1993b)].

Annonaceous acetogenins act primarily as inhibitors of Complex I (Londershausen et al., 1991; Ahammadsahib et al., 1993). The reported studies have utilized inhibitor titration in mitochondria of different sources but not in bovine submitochondrial particles, which is the best preparation in which to study the native properties of NADH–quinone reductase (Singer, 1979; Singer and Ramsay, 1992). Figure 2 shows titration of NADH–UBQ reductase activity in bovine mitochondrial particles against the annonaceous acetogenins shown in Figure 1. All were more potent than rotenone, and the two rolliniastatins were also more potent than piericidin (Figure 2b). Bullatacin, which is probably identical with rolliniastatin-2 (Cortes et al., 1993a), has been reported to be over 20-fold more potent than rotenone in isolated bovine Complex I (Ahammadsahib et al., 1993). Similar results have been obtained here for the effect of rolliniastatin-2 on NADH–UBQ reductase activity of crude Complex I. This acetogenin was also more potent than piericidin in the same preparation (results not shown). Of the acetogenins tested, only molvizarin affected the ubiquinol–cytochrome *c* reductase activity of the mitochondrial particles, and even then only at high concentrations (IC_{50} about 15 μM).

In the experiment shown in Figure 2 the inhibitors were incubated for a relatively short time with the particles. However, rotenone and piericidin generally show a pronounced time lag in the development of their most potent action on NADH–quinone reductase (cf. Horgan et al., 1968; Singer, 1979). As the same phenomenon has been found for the acetogenins examined here (see also Ahammadsahib et al., 1993), accurate titrations of the most potent Complex I inhibitors were performed by using long incubation times. IC_{50} for inhibitors obtained in this way were measured at different particle concentrations and the value of K_i was extrapolated from the intercept at zero particle concentration (Tischer and Strotmann, 1977; Oettmeier et al., 1992). The dependence of IC_{50} on particle concentration was usually linear and extrapolated to different K_i values, the lowest of which was that for rolliniastatin-1 (Figure 3 and Table 1).

Table 1 summarizes the quantitative aspects of inhibition of NADH–UBQ reductase by the annonaceous acetogenins as compared with piericidin A and rotenone under the same conditions. The K_i values obtained here for rotenone and piericidin are in good agreement with earlier data (Ernster et al., 1963; Horgan et al., 1968; Gutman and Singer, 1970; Singer, 1979). The rotenone titre normalized to the protein concentration

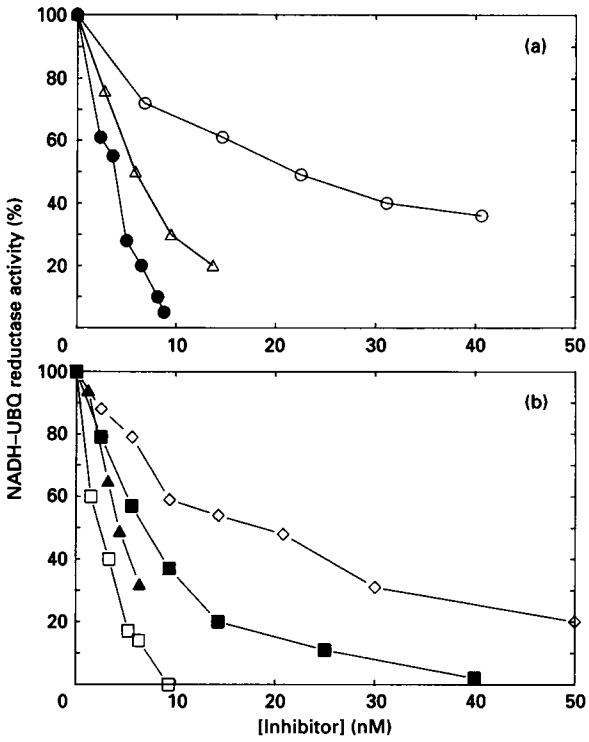

Figure 2 Titrations of inhibitors against NADH–UBQ reductase activity of bovine heart submitochondrial particles

The concentrations of NADH and UBQ were 140 and 30 μm respectively, and the protein concentration of the particles was 0.066 mg/ml in (**a**) and 0.04 mg/ml in (**b**). Control activities were 0.32 and 0.4 μmol/min per mg of protein in (**a**) and (**b**) respectively. (**a**) ○, rotenone; △, molvizarin; ●, rolliniastatin-2; (**b**) ◇, otivarin; ■, squamocin; ▲, piericidin; □, rolliniastatin-1.

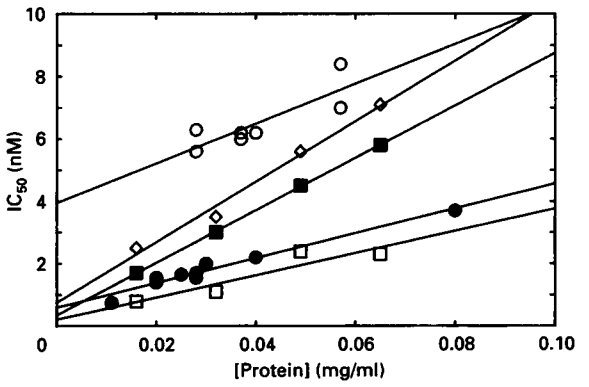

Figure 3 Protein-dependence of IC_{50} for various Complex I inhibitors

NADH–UBQ reductase activity of submitochondrial particles was assayed as described in the legend of Figure 2, but the particles were incubated for over 2 h with the inhibitors. Control activities were 0.33–0.5 μmol/min per mg of protein. Note that IC_{50} for rotenone decreased on increasing the incubation time to more than 4 h. ○, Rotenone; ◇, otivarin; ■, squamocin; ●, rolliniastatin-2; □, rolliniastatin-1.

was also in excellent agreement with the value of 62 pmol/mg of protein reported by Horgan et al. (1968) using specific binding to the radioactive inhibitor. Rolliniastatin-1 had a K_i threefold smaller than that of piericidin, and thus could be considered the most potent Complex I inhibitor of mammalian mitochondria.

Table 1 Quantitative parameters of the potent inhibitors of Complex I

NADH–UBQ reductase activity was measured after prolonged (> 2 h) incubation with bovine heart submitochondrial particles (see the Materials and methods section and Figure 3). Complex I concentration was estimated from the activity of NADH–ferricyanide reductase as described by Cremona and Kearnley (1964); 1 nM Complex I corresponded to about 0.025 mg/ml of particle protein. Protein-dependence was measured as the slope of the protein-dependence of the IC_{50} value (Figure 3; cf. Tischer and Strotmann, 1977).

Inhibitor	K_i (nM)	IC_{50} at 1 nM Complex I (nM)	Protein-dependence (pmol/mg of protein)
Piericidin A	1.0	2.8	77
Rolliniastatin-1	0.3	1.2	32
Rolliniastatin-2	0.6	1.5	37
Molvizarin	1.0	1.7	30*
Squamocin	0.4	2.5	83
Otivarin	0.8	3.4	95
Rotenone	4.0	5.5	63

* Approximate value with less than 0.05 mg/ml protein; at higher protein concentrations the IC_{50} values increased non-linearly.

The K_i values of squamocin and rolliniastatin-2 were also smaller than that of piericidin. Although otivarin had an extrapolated K_i similar to that of pericidin, its protein-dependence was more pronounced (Table 1). Consequently, the experimental IC_{50} values, which were normally measured at a particle protein concentration of 0.02 mg/ml, were always higher for otivarin than for piercidin (Figure 2).

Interestingly, the protein-dependence of IC_{50} for the rolliniastatins was about half that of piericidin and retenone (Figure 3 and Table 1). This dependence, obtained as the slope in plots such as those of Figure 3, is theoretically an expression of the concentration of half of the inhibitor-binding sites (Tischer and Strotmann, 1977). Although the values obtained here were within the concentration range of active Complex I in the preparations (43 pmol/mg of protein on the average), it was found that factors such as temperature also influenced the slope parameter. Moreover, the presence of multiple binding sites could also complicate the interpretation of the K_i values obtained in these experiments.

NADH–ferricyanide activity was not affected by concentrations of acetogenins that completely inhibited quinone reductase activity (results not shown). This agrees with previous suggestions that annonaceous acetogenins, like rotenone, act at the quinone junction of Complex I (Londerhausen et al., 1991; Ahammadsahib et al., 1993).

Mutual exclusivity of the potent Complex I inhibitors

As the annonaceous acetogenins appear to act in the same way as piercidin and rotenone, but with increased potency (Table 1), we investigated whether all such inhibitors share common interaction sites in Complex I. Previously, the action sites of some of these acetogenins could not be distinguished from that of rotenone on the basis of NADH–quinone reductase inhibition at quasi-saturating levels of the substrates (Londershausen et al., 1991; Ahammadsahib et al., 1993). However, there are qualitative features of quinone reductase activity that may be influenced in different ways by rotenone and piericidin (Gutman and Singer, 1970; Gutman, 1980; Singer and Ramsay, 1992; Friedrich et al., 1994a,b).

Friedrich et al. (1994a,b) reported that piericidin and an annonaceous acetogenin [annonin VI (Born et al., 1990)] behaved

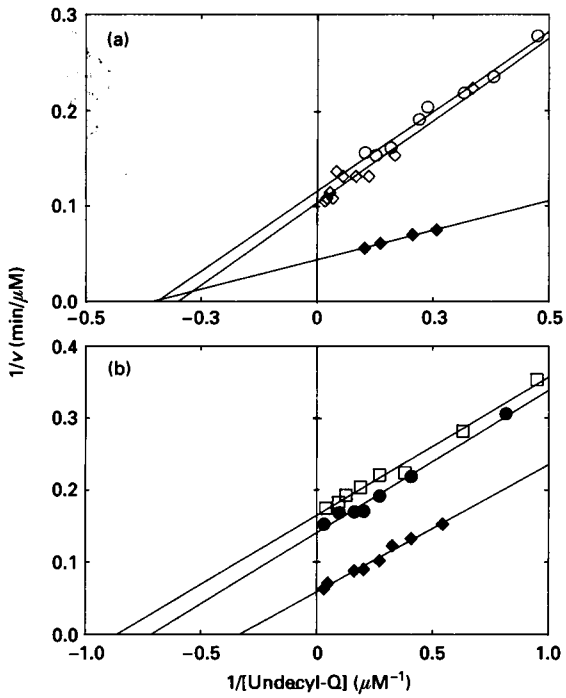

Figure 4 Titration of the UBQ substrate in the presence of various inhibitors

Protein concentration was around 0.04 mg/ml in both (**a**) and (**b**) and the maximal activity of the controls (◆) was 0.56 μmol/min per mg of protein in (**a**) and 0.45 μmol/min per mg of protein in (**b**). The concentration of the inhibitors, incubated for about 2 h, was 10 nM for rotenone (○), 8 nM for otivarin (◇) and 2.5 nM for both rolliniastatin-1 (□) and rolliniastatin-2 (●).

competitively with respect to ubiquinone-2 in mitochondria from various sources, whereas rotenone and others inhibitors behaved non-competitively with the same substrate. Rotenone also behaved non-competitively with UBQ, and essentially the same behaviour was exhibited by otivarin (Figure 4a) and also by squamocin (results not shown). On the other hand, the rolliniastatins (Figure 4b), molvizarin and piericidin behaved uncompetitively (i.e. with no change in the slope of the double-reciprocal plot) with respect to the UBQ substrate. This uncompetitive behaviour was seen also with ubiquinone-1 and ubiquinone-2 (results not shown). Conversely, the other inhibitors tested (deguelin, amytal, stigmatellin, myxothiazol, *sec*-butylacridone, demerol, casaicin and the product undecylubiquinol) modified both the slope and intercepts in double-reciprocal plots of any quinone substrate, thus behaving, like rotenone, as general non-competitive inhibitors (M. Degli Esposti and A. Ghelli, unpublished work). Therefore it appears that the uncompetitive pattern of the rolliniastatins and molvizarin is a property that associates these acetogenins more with the piericidin mode of action than with that of rotenone.

Another property shared by piericidin A and the rolliniastatins involves their different potencies in the quinone reductase and the reverse quinol oxidase function of Complex I in coupled submitochondrial particles. These acetogenins were about twice as potent in inhibiting succinate-driven reverse electron transfer to NAD^+ than in inhibiting NADH–UBQ reductase (results not shown). Similar results were obtained with piericidin, in accordance with previous observations (Gutman and Singer, 1970; Gutman, 1980). In contrast, rotenone was less potent in inhibiting

Table 2 Mutual exclusivity of potent inhibitors of Complex I

NADH–UBQ reductase activity was measured in the presence of inhibitor concentrations usually yielding over 50% inhibition after prolonged incubation, as described in the Materials and methods section. Control activities ranged between 0.3 and 0.7 μmol/min per mg of protein. I/A is the ratio between percentage inhibition and percentage residual activity (at IC_{50}, I/A = 1). For mutually exclusive inhibitors, the experimental value of I/A in the presence of both inhibitors should approach the sum of the individual values of I/A (Chou and Talalay, 1981). The numbers underlined are the theoretical values that best approach the experimental values. For mutually non-exclusive inhibitors, i.e. inhibitors interacting with sites that do not overlap each other, the theoretical value of I/A in the presence of both inhibitors should approach a number resulting from the sum of the individual I/A plus the factor I1I2/A1A2 (Chou and Talalay, 1981). In other words, the experimental value of I/A is much higher for two non-exclusive inhibitors than for two exclusive inhibitors as expression of the increased additivity of their inhibitory power on the enzymic activity. The numbers underlined are the theoretical values that best approach the experimental values.

Inhibitor 1	I/A (1)	Inhibitor 2	I/A (2)	I/A (1+2)	Exclusive	Non-exclusive
Rotenone	1.50	Piericidin	1.99	2.75	3.49	6.49
Rotenone	1.66	Otivarin	0.88	1.72	2.54	4.00
Rolliniastatin-1	1.50	Rotenone	1.66	2.33	3.17	5.67
Rolliniastatin-1	1.73	Piericidin	1.99	2.50	3.72	7.16
Rolliniastatin-1	1.50	Otivarin	0.88	1.72	2.37	3.70
Rolliniastatin-1	1.50	Squamocin	1.14	2.00	2.60	4.35
Rolliniastatin-2*	4.00	Rotenone	1.50	8.09	3.61	6.29
Rolliniastatin-2†	0.85	Rotenone	1.58	3.50	2.42	3.76
Rolliniastatin-2†	0.85	Piericidin	3.80	5.56	4.66	7.89
Rolliniastatin-2	2.89	Otivarin	0.76	5.67	3.66	5.87
Squamocin	2.00	Piericidin	1.99	4.00	4.00	7.99
Otivarin	0.76	Piericidin	1.99	2.33	2.76	4.28
Squamocin	2.00	Otivarin	0.76	2.75	2.77	4.30

* Control activity of 0.33 μmol/min per mg of protein.
† Control activity of 0.70 μmol/min per mg of protein.

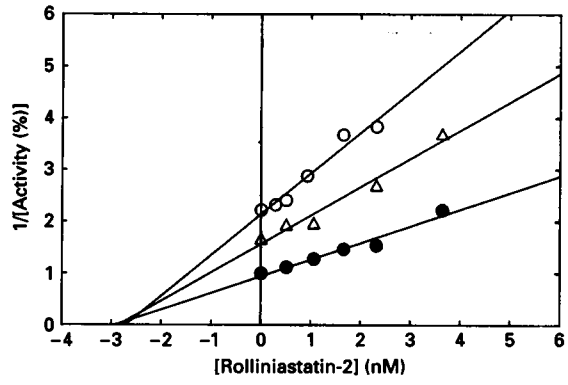

Figure 5 Dixon plot of rolliniastatin-2 at fixed levels of rotenone

The protein concentration was 0.046 mg/ml with a control activity of 0.36 μmol/min per mg of protein. Other conditions were as in Figure 2. Rotenone was incubated for about 1 h at a final concentration of 5.7 nM (△) and 9.5 nM (○). ●, Titration of rolliniastatin-2 in the absence of rotenone.

reverse electron transfer than in inhibiting quinone reductase in coupled submitochondrial particles (Kotlyar and Gutman, 1992). This would indicate that the rolliniastatins do not act in a manner qualitatively superimposable on that of rotenone.

Degli Esposti et al (1994) have applied the procedure of additivity of inhibition of steady-state (Chou and Talalay, 1981) to evaluate the mutual overlapping in the action site of different inhibitors of Complex I and III. With this procedure it was possible to discern differences in the interaction sites of inhibitors that have similar functional effects or even displace each other in binding studies, e.g. myxothiazol and stigmatellin in complex III (Degli Esposti et al., 1994). The same approach has been extensively applied here to the NADH–UBQ reductase of submitochondrial particles in the presence of various annonaceous acetogenins and other inhibitors of Complex I (Table 2). All the acetogenins were mutually exclusive to piericidin, which was in turn mutually exclusive to rotenone, in accordance with binding studies (Horgan et al., 1968; Gutman and Singer, 1970; Singer, 1979). The same acetogenins were also mutually exclusive to rotenone or other rotenoids (e.g. deguelin) except for rolliniastatin-2 which was consistently non-exclusive to rotenoids and otivarin (Table 2 and results not shown). This different behaviour of rolliniastatin-2 has been found in eight different preparations of submitochondrial particles exhibiting various control activities, and also with ubiquinone-1 as substrate.

In order to determine the mutual exclusivity of Complex I inhibitors, the procedure introduced by Marth et al. (1986) was also employed (Figure 5). This approach is based on the pattern of Dixon plots of one inhibitor titrated at different fixed levels of another inhibitor of the same enzyme. Mutually non-exclusive inhibitors are expected to yield Dixon plots that cross each other, ideally with a common intercept at the x-axis, as this parameter represents the apparent K_i which should not be modified by the presence of the other inhibitor binding to a different site (Marth et al., 1986). Indeed, rolliniastatin-2 gave Dixon plots that crossed at a common intercept when titrated against NADH–UBQ reductase at different fixed levels of rotenone (Figure 5). This was not the case for rolliniastatin-1 (results not shown).

The different functional approaches employed here indicate that rolliniastatin-2 and rotenone have interaction sites that are independent from each other. This is in agreement with the recent data showing that the inhibitory potency of bullatacin does not correlate with its ability to displace radioactive dihydrorotenone from blowfly mitochondrial particles (Jewess, 1993, 1994).

Conclusions and perspectives

The first conclusion that emerges from this work is that rolliniastatin-1, an annonaceous acetogenin extracted from the

seeds of *Rollinia membranacea* (Saez et al., 1993), is the most potent inhibitor of mammalian Complex I. Rolliniastatin-1 is closely followed in potency by rollinastatin-2 and squamocin, which both have K_i values lower than that of piericidin A. Moreover, all the annonaceous acetogenins tested here have a K_i value that is about one order of magnitude lower than that of rotenone measured under the same conditions (Table 1). The potency of compounds that could be structurally equivalent to rolliniastatin-2 has been underestimated previously because the specific NADH–quinone reductase activity was not measured properly in mitochondrial membranes. Factors such as the use of intact mitochondria (where the catalytic sites of Complex I are not exposed), the presence of BSA in the medium and/or the utilization of poor quinone substrates such as ubiquinone-2 (Estornell et al., 1993) have led to overestimated titres of acetogenins in previous studies (Londershausen et al., 1991; Ahammadsahib et al., 1993; Friedrich et al., 1994a,b).

Qualitatively, the annonaceous acetogenins tested here could be divided into three groups (Tables 1 and 2): (i) those that act like piericidin (rolliniastatin-1 and possibly molvizarin); (ii) those that act like rotenone (otivarin and squamocin); (iii) those that act in a way that is mutually exclusive to piericidin but not exclusive to rotenone (rolliniastatin-2). The latter mode of action is an oddity with respect to that of potent inhibitors of Complex I (Degli Esposti et al., 1994), and could reflect a specific interaction with one of the two binding sites of piericidin within the native mitochondrial membrane (Gutman and Singer, 1970; Gutman, 1980; Singer and Ramsay, 1992). This site could be tentatively identified as that which is rate-limiting in reverse electron transfer to NAD^+ in coupled submitochondrial particles (Gutman, 1980) because both piericidin and rolliniastatin-2 were more powerful as inhibitors of this function.

The fact that rolliniastatin-1 is more potent than its stereoisomer rolliniastatin-2 and also mutually exclusive to rotenone (Tables 1 and 2) could be rationalized by assuming that this acetogenin binds to both piericidin sites simultaneously. The resemblance of the basic structure of the rolliniastatins to a fusion of two quinone-like compounds which is about twice as big as piericidin (Figure 1) supports this idea. However, extensive structure–activity investigations are needed to understand the quantitative and qualitative differences in the action of annonaceous acetogenins on Complex I function. Studies in this direction are under way and will exploit several analogues of the inhibitors tested here (Cortes et al., 1993a).

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REFERENCES

Ahammadsahib, K. I., Hollingworth, R. M., McGovern,, J. P., Hui, Y.-H. and McLaughlin, J. L. (1993) Life Sci. **53**, 1113–1120
Berry, E. A., Huang, L. and DeRose, V. (1991) J. Biol. Chem. **266**, 9064–9077
Born, L., Lieb, F., Lorentzen, J. P., Moeschler, H., Nonfon, M., Sollner, R. and Wendisch, D. (1990) Planta Med. **56**, 312–316
Chou, T. C. and Talalay, P. (1981) Eur. J. Biochem. **115**, 207–216
Cortes, D., Rios, J. L., Villar, A. and Valverde, S. (1984) Tetrahedron Lett. **25**, 3199
Cortes, D., Myint, S. H. and Hocquemiller, R. (1991a) Tetrahedron **38**, 8195–8202
Cortes, D., Myint, S. H., Leboeuf, M. and Cavé, A. (1991b) Tetrahedron Lett. **32**, 6133–6134
Cortes, D., Figarede, B. and Cavé, A. (1993a) Phytochemistry **32**, 1467–1473
Cortes, D., Myint, S. H., Dupont, B. and Davoust, D. (1993b) Phytochemistry **32**, 1475–1482
Cremona, T. and Kearnley, E. B. (1964) J. Biol. Chem. **239**, 2328–2334
Degli Esposti, M., Avitabile, E., Barilli, M., Montecucco, C., Schiavo, G. and Lenaz, G. (1986) Comp. Biochem. Physiol. **85B**, 543–552
Degli Esposti, M., Ghelli, A., Crimi, M., Estornell, E., Fato, R. and Lenaz, G. (1993) Biochem. Biophys. Res. Commun **190**, 1090–1096
Degli Esposti, M., Crimi, M. and Ghelli, A. (1994) Biochem. Soc. Trans. **22**, 209–213
Ernster, L. and Lee, C. P. (1967) Methods Enzymol. **10**, 729–738
Ernster, L., Dallner, G. and Azzone, G. F. (1963) J. Biol. Chem. **238**, 1124–1131
Estornell, E., Fato, R., Pallotti, F. and Lenaz, G. (1993) FEBS Lett. **332**, 127–131
Etse, J. T. and Waterman, P. G. (1986) J. Nat. Proc. **49**, 684–686
Friedrich, T., Ohnishi, T., Forche, E., Kunze, B., Jansen, R., Trowitzsch, W., Hofle, G., Reichenbach, H. and Weiss, H. (1994a) Biochem. Soc. Trans., in the press
Friedrich, T., Van Heek, P., Leif, H., Ohnishi, T., Forche, E., Kunze, B., Jansen, R., Trowitzsch, W., Hofle, G., Reichenbach, H. and Weiss, H. (1994b) Eur. J. Biochem. **219**, 691–698
Gutman, M. (1980) Biochim. Biophys. Acta **594**, 53–84
Gutman, M. and Singer, T. P. (1970) J. Biol. Chem. **245**, 1992–1997
Hansen, M. and Smith, A. L. (1964) Biochim. Biophys. Acta **81**, 214–222
Hatefi, Y. and Stiggall, D. L. (1978) Methods Enzymol. **53**, 5–21
Horgan, D. J., Ohno, H., Singer, T. P. and Casida, J. E. (1968) J. Biol. Chem. **243**, 5967–5976
Hui, Y. H., Rupprecht, J. K., Liu, Y. M., Anderson, J. E., Smith, D. L., Chang, C. J. and McLaughlin, J. L. (1989) J. Nat. Prod. **52**, 463–477
Jewess, P. (1994) Biochem. Soc. Trans. **22**, in the press
Kotlyar, A. B. and Gutman, M. (1992) Biochim. Biophys. Acta **1140**, 169–174
Londershausen, H., Leicht, W., Leib, H. M. and Weiss, H. (1991) Pestic. Sci. **33**, 427–438
Marth, G., Falchuk, K. H., Auld, D. S. and Vallee, B. L. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 2836–2840
Oettmeier, W., Masson, K. and Soll, M. (1992) Biochim. Biophys. Acta **1099**, 262–266
Ohnishi, T. (1993) J. Bioenerg. Biomembr. **25**, 325–329
Pettit, G. R., Riesen, R., Leet, J. E., Polonsky, J., Smith, C. R., Schmidt, J. M., Dufresne, C., Schaufelberger, D. and Moretti, C. (1989) Heterocycles **28**, 213–217
Ragan, C. I. (1985) in Coenzyme Q (Lenaz, G., ed.), pp. 315–336, Wiley, London
Rottenberg, H. and Gutman, M. (1977) Biochemistry **16**, 3220–3227
Rupprecht, J. K., Hui, Y. H. and McLaughlin, J. L. (1990) J. Nat. Prod. **53**, 237–278
Saez, J., Sahpaz, S., Villaescusa, L., Hocquemiller, R., Cave, A. and Cortes, D. (1993) J. Natl. Prod. **56**, 351–356
Schatz, G. and Racker, E. (1966) J. Biol. Chem. **241**, 1429–1438
Singer, T. P. (1979) Methods Enzymol. **55**, 454–462
Singer, T. P. and Ramsay, R. R. (1992) in Molecular Mechanisms in Bioenergetics (Ernster, L., ed.), pp. 145–162, Elsevier, Amsterdam
Sled, V. D., Friedrich, T., Leif, H., Weiss, H., Meinhardt, S. W., Fukumori, Y., Calhoun, M. W., Gennis, R. B. and Ohnishi, T. (1993) J. Bioenerg. Biomembr. **25**, 347–355
Storey, B. T. (1980) Pharmacol. Ther. **10**, 399–406
Tischer, W. and Strotmann, H. (1977) Biochim. Biophys. Acta **460**, 113–123
Walker, J. (1992) Q. Rev. Biophys. **25**, 253–324
Weiss, H., Friedrich, T., Hofhaus, G. and Preis, D. (1991) Eur. J. Biochem. **197**, 563–576
Wikström, M. and Saraste, M. (1984) in Bioenergetics (Ernster, L., ed.), pp. 49–94, Elsevier, Amsterdam
Yagi, T. (1990) Arch. Biochem. Biophys. **281**, 305–311

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