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Complex I inhibitors as insecticides and acaricides [1]

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Abstract

Structurally diverse synthetic insecticides and acaricides had been shown to inhibit the proton-translocating NADH:ubiquinone oxidoreductase (complex I) activity. In addition, secondary metabolites from microbial and plant sources known to act on complex I exhibited biological activity against agricultural and environmental insect pests. Mechanistic studies indicated that these compounds interfered with ubiquinone reduction most likely at the same site(s) as the classical complex I inhibitors rotenone and piericidin A. Two approaches to characterize the mechanism of insecticidal/acaricidal complex I inhibitors were followed: enzyme kinetic studies and binding studies with radiolabeled inhibitors. Enzyme kinetic experiments were sometimes controversially interpreted regarding a competitive or non-competitive inhibitor mechanism with respect to the electron acceptor. In general, radioligand binding data with submitochondrial membranes were in line with the enzymological results but due to methodological drawbacks, saturation kinetic analyses were impossible. The main problems underlying many studies of inhibitor interaction with complex I were (i) the use of membrane-bound enzyme preparations and (ii) the physicochemical properties of the amphiphilic inhibitors with their strong tendency to accumulate in the membrane phase. A more recent approach to characterize inhbibitor interaction sites in complex I was the isolation of piericidin-resistant mutants of photosynthetic bacteria which produce a simpler homologue of mitochondrial NADH:Q oxidoreductase. © 1998 Elsevier Science B.V.

Keywords: NADH:ubiquinone oxidoreductase; Complex I; Inhibitor; Insecticide; Enzyme kinetics; Radioligand binding

1. Introduction

Following the United Nations' Food and Agriculture Organization (FAO), global human population will increase to about 7.2 billion by 2010 with an annual growth rate of 1.7% [1]. This population increment has to be accompanied by a substantial enhancement in food supply by a more productive agriculture at the global level.

Chemical insecticides, together with genetically modified insect-resistant crops and biological plant protection methods, prevent severe harvest losses due to phytophageous insects and mites.

A few important (in terms of market size) chemical insecticide classes show a broad spectrum of biological activity and have thus been used for years in agricultural applications. They are mainly neurotoxins directed against a limited range of receptors and enzymes: acetylcholinesterase (organophosphates, carbamates), the voltage-dependent sodium channel (DDT, pyrethroids, dihydropyrazoles), the GABA receptor/chloride channel (cyclodienes, phenylpyra-

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[1] Dedicated to the memory of Dr. Gerhard Salbeck.

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zoles) [2,3], and the nicotinic acetylcholine receptor (nitromethylenes) [4].

Resistances arising in insect field populations against almost all classes of chemical insecticides had been reported. Since target site insensitivity was identified as one of the predominant resistance mechanisms [5], the major objective for insecticide research is the identification of new chemical classes with new molecular modes of action.

During the last years, new insecticides/acaricides were developed interfering with mitochondrial electron transport, most of them with the proton-translocating NADH:ubiquinone oxidoreductase (complex I) [6–8]. It is worth mentioning that complex I is not a completely new insecticide target since one of the classical complex I inhibitors, rotenone, is known for its (weak and limited) insecticidal activity.

Present knowledge of complex I is primarily based on studies with bovine heart and *Neurospora crassa* enzymes, whereas little is known about the structures of insect NADH:ubiquinone oxidoreductases. It is noteworthy that insect flight muscles represent a rich source of electron transport enzyme activities [9]. Enzymatic and immunochemical evidence indicated a high degree of similarity to their mammalian and fungal counterparts [10].

Proton-translocating NADH:ubiquinone oxidoreductase is the first electron transport complex of the mitochondrial respiratory chain. It oxidizes NADH and transfers the electrons via a flavin mononucleotide cofactor and several iron–sulfur clusters to ubiquinone (Q). A 4 protons per 2 electrons stoichiometry is the widely accepted figure for the coupled vectorial proton translocation. So, complex I contributes to the proton-motive force that drives ATP synthesis. Presumably because of its high degree of structural complexity, NADH:Q oxidoreductase is the least understood of the respiratory chain enzymes [11].

A detailed discussion of structure and function of complex I is far beyond the scope of this review. Only selected structural aspects of immediate importance for the discussion of inhibitor mechanisms will be outlined here briefly. For a more comprehensive view, the reader is referred to recent reviews [12–15] and to the relevant contributions in this volume.

Complex I of bovine heart mitochondria consists of more than 40 subunits with a total molecular mass of about 900 kDa [15]. With the non-ionic detergent *N,N*-dimethyldodecylamine *N*-oxide (LDAO), bovine complex I is splitted into different subcomplexes [16]. Subcomplex I λ roughly corresponds to the peripheral arm of the L-shaped *N. crassa* enzyme (Fig. 1) [17]. It carries the FMN cofactor and all EPR–visible iron–sulfur clusters and catalyzes NADH oxidation with the isoprenoid Q analogue Q-1 as electron acceptor. Rotenone-sensitive ubiquinone (Q) reduction requires the hydrophobic integral membrane part of complex I presumably containing the physiological ubiquinone reduction site(s). These are believed to be relatively hydrophobic but access to the aqueous phase is necessary for proton uptake in the course of Q reduction [18]. However, this picture remains to be confirmed by structural data.

The mechanistic linkage between redox reactions and proton-translocation is still a matter of debate, but it has immediate relevance for the number of Q/inhibitor binding sites and the mechanism how inhibitors interfere with energy conservation. Different models have been proposed in the literature, for a recent synopsis see the review of Brandt [19].

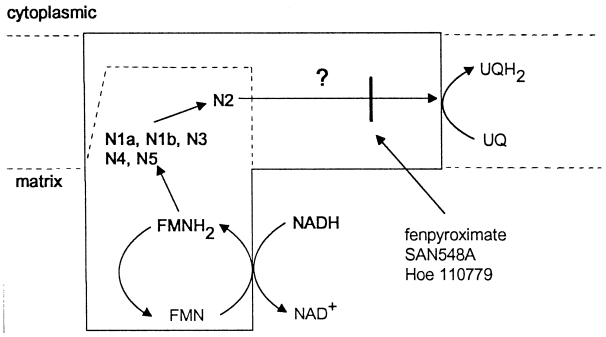

Fig. 1. Schematic diagram of complex I illustrating the molecular mode of action of selected insecticides/acaricides. Only the sequence of electron transfer reactions [11,13,14] is schematically depicted, leaving out the proton-translocation step. From the point of view of inhibitor mechanism, iron sulfur cluster N2 is most interesting because it has the highest midpoint potential [14,15] and is therefore the likely candidate for ubiquinone reduction [12,15]. In addition, N2 may be in direct contact with the membrane arm [12]. Considering the lack of precise structural and mechanic knowledge, it is presently sufficient to point out that the pyrazole and aminopyrimidine insecticides block electron transfer between cluster N2 and Q.

This review will summarize the present knowledge about the biochemical mechanisms of selected synthetic and natural complex I inhibitors from different chemical families showing insecticidal and/or acaricidal activity.

2. Insecticides and acaricides as inhibitors of complex I

Rotenone and piericidin A were known for a long time as high-affinity inhibitors of proton-translocating NADH:Q oxidoreductase. Piericidin A was isolated from cultures of *Streptomyces mobaraensis* [20] and rotenone was the active component in the insecticidal and fish-poisonous extract of *Derris spec.* (*Leguminosae*) roots.

During the last years, new potent insecticides/acaricides were shown to inhibit mitochondrial respiration at coupling site I. Further enzymatic analysis indicated that they specifically blocked ubiquinone-dependent NADH oxidation with high efficacy.

The synthetic insecticides/acaricides could be grouped in two main classes, the pyrazoles in the first class and substituted pyrimidines, pyridines and quinazolines in the second class. Experimental and

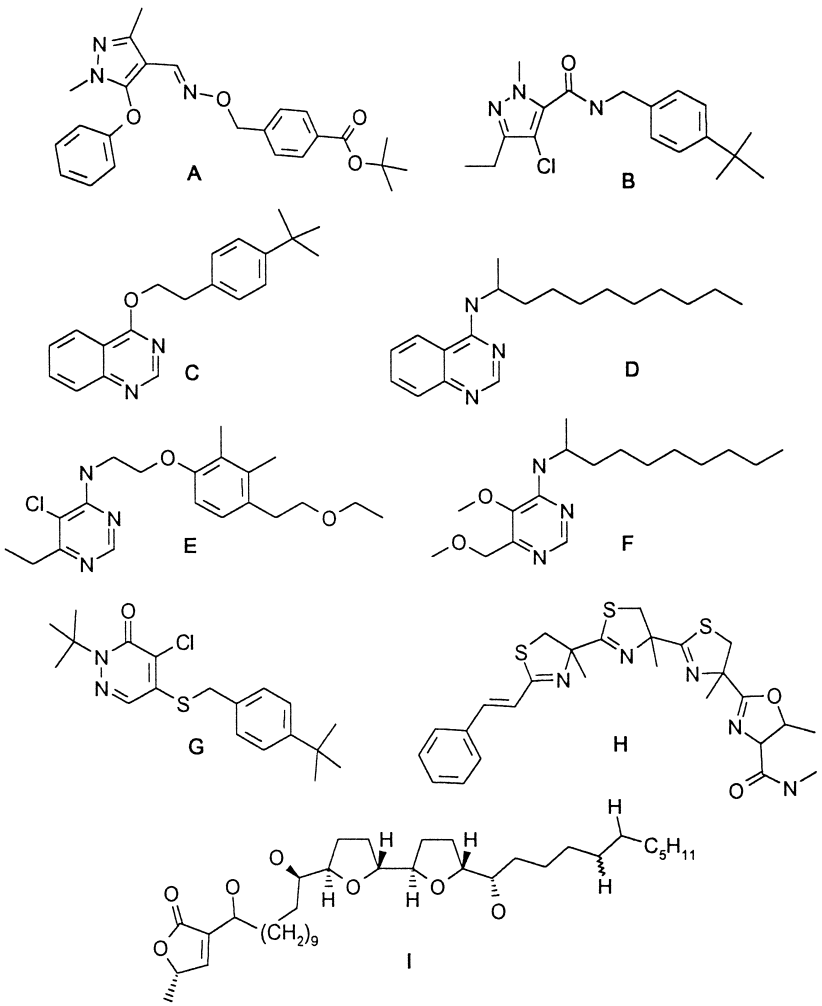

Fig. 2. Structures of insecticides/acaricides active on complex I. Synthetic compounds: (A) fenpyroximate (NNNI-850, Nihon Nohyaku); (B) tebufenpyrad (MK-239, Mitsubishi Kasei); (C) fenazaquin (EL-436, DowElanco); (D) SAN 548A (Sandoz, now Novartis); (E) pyrimidifen (SU-8801, Ube Industries); (F) Hoe 110779 (Hoechst, now AgrEvo); (G) pyridaben (NC-129, Nissan Chem.). Secondary metabolites: (H) thiangazole; (I) annonin VI (annonaceous acetogenin).

commercial insecticides representing the two synthetic classes as well as natural compounds with insecticidal activity will be presented in the following.

2.1. Pyrazoles

Members of the substituted pyrazole family were discovered as effective acaricides and introduced to the market by Japanese companies.

Fenpyroximate (NNI-850, Fig. 2A) was a 5-phenoxypyrazole commercialized by Nihon Nohyaku in 1991. After spraying with fenpyroximate, oxygen consumption and the total ATP content of adult spider mites *Tetranychus urticae* were drastically decreased [21]. Further biochemical analysis revealed that NADH:Q oxidoreductase activities in rat liver and *Tetranychus* mitochondrial membranes were inhibited with IC_{50} values of 0.4 and 0.08 μM, respectively. NADH:ferricyanide reductase was not affected by inhibitor concentrations up to 10 μM. Interestingly, electron microscopy revealed morphological changes of mitochondria isolated from different tissues indicating that the peripheral nerve cells appeared to be most sensitive to fenpyroximate. This might explain the characteristic intoxication symptomology like rapid knockdown and paralysis.

The related pyrazole-5-carboxamide acaricide tebufenpyrad (MK-239, Fig. 2B) from Mitsubishi Kasei controlled different spider mite species and also some sucking insects. Complex I was identified as the molecular target [7].

MK-239 inhibited NADH:decylubiquinone oxidoreductase activity in housefly flight muscle submitochondrial membranes with an IC_{50} of 2.0 nM (Lümmen, unpublished).

2.2. Pyridines, pyrimidines and quinazolines

This familiy contains *N*-heterocyclic compounds like pyridine, pyrimidine or quinazoline with a lipophilic side chain attached to them via a bridging atom.

Fenazaquin (EL-436, Fig. 2C) was discovered by DowElanco as an acaricide with good efficacy against motile forms and eggs of spider mites. Hollingworth et al. [8] showed that complex I inhibition was the molecular mode of action.

The experimental aminoquinazoline insecticide SAN 548A (Fig. 2D) inhibited bovine heart NADH:Q-1 oxidoreductase, but NADH:ferricyanide activity was not affected [8].

Substituted aminopyrimidines, including the Sankyo/Ube developmental product pyrimidifen (Fig. 2E), and the similar Hoe 110779 of Hoechst/AgrEvo (Fig. 2F) were effective insecticides and acaricides against a broad pest spectrum including aphids, lepidoptera, coleoptera and bugs [21]. Hoe 110779 completely blocked rotenone-sensitive, *n*-decylubiquinone-dependent NADH oxidation by membrane-bound complex I from a variety of biological sources like rat heart, housefly flight muscles, *N. crassa*, and the bacterium *Paracoccus denitrificans* (Ref. [22] and Lümmen, unpublished). On the contrary, NADH:ferricyanide oxidoreductase and the *Escherichia coli* NDH2-type NADH:Q oxidoreductase (non proton-translocating) were not inhibitied.

2.3. Pyridaben

The pyridazinone compound pyridaben (Fig. 2G) was introduced as an acaricide by Nissan Chemical . Complex I was inhibited in mitochondrial preparations from different insect species [7].

2.4. Annonaceous acetogenins

Plants from the family *Annonaceae* were known to contain potent bioactive secondary metabolites called annonins or acetogenins (see for example annonin VI: Fig. 2I) [23–25]. Biological activities including pronounced insecticidal and antiparasitic effects had been described. Acetogenins were active against aphids (*Aphis fabae*), flies (*Calliphora vicina*), the Mexican bean beetle (*Epilachna varivestis*) and the diamondback moth (*Plutella xylostella*).

The *Annona squamosa* annonins inhibited the NADH-, but not succinate-dependent cytochrome *c* reduction in submitochondrial particles from blowfly flight muscles [24]. Asimicin, an insecticidal acetogenin from *Asimina triloba*, blocked oxygen consumption in larval European corn borer mitochondria typical of a coupling-site I inhibitor [23]. Bullatacin, selected from a metabolite screening program of over

50 species of *Annonaceae*, affected cellular respiration in cultured *Spodoptera frugiperda* Sf9 cells and in mitochondrial preparations from the tobacco hornworm (*Manduca sexta*) measured with the Clark-type oxygen electrode [25].

2.5. Thiangazole

Thiangazole (Fig. 2H) was isolated from a *Polyangium* strain. Insecticidal, acaricidal and anthelmintic activity were claimed in a patent application [26]. Complex I inhibition was shown by Friedrich et al. [27].

2.6. Structure-activity relationships

A common structural feature could be seen in the *N*-heterocyclic ring substituted with a lipophilic side chain via a bridging nitrogen or oxygen atom. Little information had been published on structure-activity relationships with respect to the synthetic complex I-active insecticides. For the pyrazole carboxamide series (Fig. 2B), biological activity against spider mites was correlated with the chemical variations of the lead structure [28]. Certain substituent positions of the pyrazole ring including lower alkyl and halogen were essential for biological activity. In the lipophilic side chain, higher degrees of freedom were possible when the 4-alkyl substituent of the benzylic moiety was altered.

Obata et al. [29] systematically analyzed chemical variations in the aminopyrimidine series with respect to their biological activity. The most potent compounds were substituted by lower alkyl or halogen in the 5- and 6-positions of the pyrimidine ring. When lower alkyl substituents were introduced at the 1-position of the lipophilic side chain, an asymmetric carbon center was created. The (+) optical isomer was found to be more potent than the (−) isomer. As in the case of the pyrazoles, broad chemical variations including alkyl and diphenylether moieties were possible in the lipophilic side chain.

Although the presented structure-activity relations were not based on enzymatic measurements, it is reasonable to assume that the most active insecticides were also effective complex I inhibitors. By comparison with related compounds like 4-hydroxypyridines, 4-hydroxyquinolines [30] and 2-alkyl-3-methylquinolones [31], one could speculate about common structural determinants of inhibitor activity. For the piericidin-type 4-hydroxypyridines and 4-hydroxyquinolines, a π-electron interaction between the heteroaromatic ring and the binding site was suggested. The lipophilic side chain length played a role for inhibitor efficacy [30]. This was also true for the 2-alkylquinolones, where 9–12 carbon atoms in the *n*-alkyl chain gave maximum activity in vitro [31].

In summary, structure-activity relationships of the insecticidal *N*-heterocycles were largely speculative. Assuming that some thousands of compounds had been synthesized by different companies, this would form a good basis to systematically elaborate the structural determinants for complex I inhibition.

3. Molecular mechanism of complex I inhibition

In enzymatic assays with membrane-bound or solubilized NADH:Q oxidoreductase, the complex I-active insecticides closely resembled the classical inhibitors rotenone and piericidin which block electron transport between iron–sulfur cluster N2 and ubiquinone [6,8,22,27]. Recently, proposed models to explain the redox mechanism of complex I with its associated proton-pump implied two (or even three) Q binding sites. It was proposed that inhibitors from the structurally diverse chemical classes might specifically interact with different Q sites, thus providing the experimental tools to test the corresponding model [19,32,33]. Consequently, efforts have been made to define the number of Q sites by studying complex I inhibitor mechanisms and binding site specificity.

In the following, mechanistic analyses of complex I inhibitors, with emphasis on the insecticidally active compounds, are discussed in the context of complex I structure and function. The majority of information comes from enzyme kinetic studies mostly with membrane bound, but also solubilized and (partially) purified complex I.

Radioligand binding experiments represent a straightforward method to describe ligand–protein interactions directly: applications of these methods to the study of inhibitor binding to complex I are presented.

A start has been made to apply the powerful tools of molecular genetics on complex I inhibitor binding site analysis by characterizing inhibitor-resistant mutants of the bacterial proton-translocating NADH:Q oxidoreductase.

3.1. Enzyme kinetic studies

Friedrich et al. [34] characterized 12 structurally diverse inhibitors (10 natural, two synthetic compounds) in terms of Michaelis–Menten-type inhibition kinetics with membrane-bound and reconstituted NADH:Q oxidoreductase from *N. crassa*. They defined two inhibitor classes. The pyrazole acaricide fenpyroximate blocked ubiquinone-2 reduction in a partially competitive manner similar to piericidin A and annonin VI (class I). Class II inhibitors, represented for example by rotenone, exhibited non-competitive behaviour under the same experimental conditions.

In agreement with these results, Hollingworth et al. [8] reported that two other members of the quinazoline/pyrimidine family, fenazaquin and SAN 548A, were competitive inhibitors of NADH:Q-1 activity in bovine heart mitochondrial membranes and purified complex I preparations. Mutually exclusive binding of rotenone and the quinazoline/pyrimidine insecticides was postulated.

According to the criteria of Friedrich et al., the aminopyrimidine insecticide Hoe 110779 competitively inhibited membrane-bound complex I from housefly mitochondria measured with *n*-decylubiquinone [22].

Jewess tested a number of structurally not explicitly specified lipophilic insecticides/acaricides including examples from the pyrazole and the quinazoline/pyrimidine families [35]. Facing the problem of unstable solubilized insect complex I he used submitochondrial particles from blowfly (*Lucilia caesar*) flight muscles to measure Q-1-dependent NADH oxidation. Inhibition kinetics of all compounds tested showed largely non-competitive behaviour with respect to Q-1 and non-competitive behaviour with NADH.

The molecular mode of action of the insecticidal acetogenins had been studied by several groups. Londershausen et al. [24] suggested that annonins, rotenone and piericidin shared a common binding site. Inhibition of complex I by acetogenins was confirmed by Ahammadsahib et al. [25] for bullatacin. Degli Esposti et al. [36] analysed in depth the inhibitor mechanism of rolliniastatins against 6-undecyl-Q reductase. Rolliniastatin-1 and rolliniastatin-2 (possibly identical to bullatacin) were found to be the most effective complex I inhibitors so far with Ki values of 0.3 and 0.6 nM, respectively.

Contrary to Friedrich et al. [34], who identified annonin VI to be a competitive inhibitor of Q-1 reduction, Degli Esposti et al. [36] described rolliniastatin inhibition to be uncompetitive, i.e., the slope of double-reciprocal data transformations was not changed between inhibited and control activities. All rolliniastatins were mutually exclusive with piericidin and rotenone under steady-state conditions except for rolliniastatin-2, which did not interfere with rotenone binding. Consequently, an independent binding site for rolliniastatin-2 not overlapping the rotenone site was postulated.

So far, enzyme kinetics did not provide a clear picture regarding the inhibitory mechanisms and the number of inhibitor binding sites. The sometimes confusing, sometimes even contradictory results could be explained at least partially by the heterogeneous enzymatic assay conditions. Most remarkable was the discrepancy between the (partially) competitive behaviour of the pyrimidine/quinazoline insecticides against *Neurospora* [27,34] and bovine [8] membrane bound complex I and Jewess's [35] data stating non-competitive inhibition of the insect (*L. caesar*) enzyme. A functional uniqueness of the insect complex I in this respect is rather unlikely since housefly complex I activity, tested with submitochondrial membranes with *n*-decylubiquinone, was also competitively inhibited [22]. Possibly, the different Q analogues, Q-1 and decyl-Q, used in the activity assays could provide an explanation. Subtle differences between Q analogues in supporting the energy-conserving function of complex I were identified by Degli Esposti et al. [33]. The authors recommended decyl- or undecyl-Q which most closely resembled the functions of endogeneous ubiquinone. Finel et al. [16] observed that all the subcomplexes generated by differential solubilization with LDAO and lauroylsarcosine retained NADH-oxidation activity with Q-1 irrespective of their inhibitor sensitivity. However, rotenone sensitivity was correlated with

n-decylubiquinone-dependent NADH-oxidation indicating different Q reduction sites with particular inhibitor specificities in solubilized complex I.

Clearly, conclusions concerning the inhibitor mechanisms and the number of binding sites of membrane-bound *N. crassa* complex I presented by Friedrich et al. [34] were based on a Michaelis–Menten relationship between initial velocity and the Q-2 concentration. In contrast, membrane-bound complex I activity in *L. caesar* flight muscles as a function of Q-1 or Q-2 electron acceptor concentrations deviated from Michaelis–Menten kinetics [35]. Heterogeneous and possibly co-operative Q binding sites were derived from the sigmoidal curves.

Inhibition of the *Gluconobacter oxidans* glucose dehydrogenase was proposed as a second distinctive criterium between class I and II compounds [34]. The bacterial inner-membrane enzyme catalyzes the direct oxidation of extracellular glucose to gluconate employing a pyrroloquinoline quinone (PQQ) redox cofactor. Two electrons are transferred to ubiquinone linking periplasmic glucose oxidation to the respiratory electron transport chain [37]. Fenpyroximate inhibited glucose dehydrogenase-catalyzed Q reduction at higher concentrations, but class II inhibitors showed weak, if any, inhibition [34].

The ND1 subunit of complex I had been suggested to contribute at least in part to the binding site for rotenone. This was concluded from photoaffinity labeling experiments with [^{3}H]dihydrorotenone [38]. By sequence alignment with bacterial glucose dehydrogenase, Friedrich et al. [39] postulated a common sequence motif for ubiquinone binding. The relevance of this homology had been questioned [15]. Nevertheless, the specificity of Q-site inhibitors for different Q-dependent oxidoreductases may still provide valuable information on general structural and physicochemical features of quinone/inhibitor binding sites [18]. It is noteworthy in this context that high-affinity bc_1 complex inhibitors like stigmatellin and myxothiazol also block NADH:ubiquinone oxidoreductase at higher concentrations supporting the hypothesis of common structural features for Q site inhibition [40].

In summary, enzyme kinetics provided useful information on insecticide/acaricide molecular mode of action but the kinetics of complex I activity will have to be defined more precisely to understand the structural and mechanistic determinants of complex I inhibitors.

The particular physicochemical properties of complex I inhibitors should also be considered. The strong tendency of hydrophobic or, more precisely, amphiphilic inhibitors to accumulate in the small membrane volume in comparison with the aqueous phase under assay conditions substantially increases the actual inhibitor concentration at the target site. So, the apparent IC_{50} and K_i values do not only reflect the specific interaction between the inhibitor and its binding site, but to a significant extent, the entropy-driven, hydrophobic interaction with the membrane. So, comparisons of relative inhibitory efficacies have to be interpreted very carefully.

Solubilized and purified complex I preparations of maximum structural and functional integrity would resolve most of the uncertainties described above. Substantial progress has been made during the last years in obtaining purified complex I enzymes or their bacterial homologues for structural analyses [41–44]. Some of these enzyme preparations retained their inhibitor sensitivity and should be useful for enzyme kinetic studies.

3.2. Radioligand binding methods

In principle, radioligand binding experiments provide a direct method to quantitatively describe ligand-receptor interactions. Jewess [35] and Wood et al. [45] adopted this approach to study the binding site specificity of selected complex I-active insecticides with submitochondrial membranes from the blowfly *L. caesar* and bovine heart, respectively.

Piericidin, the pyrazole acaricide fenpyroximate and other (not structurally specified) insecticides inhibited specific [^{3}H]dihydrorotenone binding in a concentration-dependent fashion with IC_{50} values ranging from 30 to 100 nM [35]. The results were interpreted in favour of a direct competition between the insecticidal inhibitors (and also piericidin) at the same, or very close to, the binding site of dihydrorotenone. A more convincing conclusion would have required to show, for example by Scatchard analysis, that the maximum number of binding sites B_{max} was not altered when dihydrorotenone plus a competing ligand were present under steady state conditions.

In fact, saturation binding analysis was not possible, most likely prevented by the strong non-specific binding of the hydrophobic radioligand to the membrane phase.

Testing diverse Q analogues revealed weak, if any, dihydrorotenone displacement: the IC_{50} of Q-2 was determined 400 μM and the *n*-decyl-Q analogue was completely inactive. From these results, separate binding sites for Q and the inhibitors were postulated, apparently confirming the non-competitive enzyme inhibition kinetics [35]. The possibility that quinone concentrations tested were not sufficient to displace the high-affinity radioligand [^{3}H]dihydrorotenone ($K_D = 29$ nM; Lümmen, unpublished) could not be ruled out. It is known that quinones have weak binding constants allowing rapid movement on and off the binding site(s) [18]. So, significant displacement of a tight-binding ligand would necessarily require high quinone concentrations under steady state conditions. Performing the binding analyses with a radioligand of lower affinity could help to clarify the binding site specificity with respect to Q.

Wood et al. [45] studied the effect of rotenone, the quinazoline insecticide SAN 548A, the acaricide pyridaben, and the acetogenins asimicin and bullatacin on [^{3}H]fenazaquin binding in equilibrium binding experiments with bovine heart submitochondrial particles. Due to the high percentage of non-specific radioligand binding (58%), meaningful saturation kinetic analyses were not possible. Incorporation of BSA in the binding assays did not improve specific binding.

Selected insecticidal/acaricidal complex I inhibitors were tested for their ability to reduce specific fenazaquin binding. The acaricide pyridaden and the acetogenins asimicin and bullatacin inhibited 85% of fenazaquin binding at 30 nM. Rotenone and the quinazoline insecticide SAN 548A were less potent, showing 59% and 67% inhibition, respectively. A good correlation ($r^2 = 0.96$, $n = 18$) was found between inhibition of NADH:ubiquinone oxidoreductase and fenazaqin binding. The authors pointed out correctly, that it was not clear if all compounds competed for the same binding site or alternatively blocked fenazaquin binding in a non-competitive manner [45].

As discussed above in the context of enzyme kinetic studies, the use of submitochondrial membranes presented methodological problems in the analysis of hydrophobic ligand interaction with complex I. A solubilzed and purified enzyme preparation could presumably be suitable for Scatchard analysis of saturation binding data. If a competing inhibitor did not change the apparent B_{max} of the radioligand, a common, or at least overlapping, binding site could be postulated.

3.3. Inhibitor-resistant mutants

Genetic evidence for two inhibitor binding sites was presented by Darrouzet and Dupuis [46]. They isolated piericidin-resistant mutants from the photosynthetic bacterium *Rhodobacter capsulatus*. The apparent K_i values for piericidin measured with the bacterial complex I homologue, NADH dehydrogenase NDH-1, were increased by a factor of about 30 in two mutants compared to the wild-type control. Cross-resistance to rotenone was found, but the sensitivity to rolliniastatin-2 was not altered. Assuming that bacterial NDH-1 enzymes resemble mitochondrial complex I with respect to inhibitor mechanisms and binding sites, the characterization of resistance mutants could lead to the identification of enzyme subunits and the amino acid residues contributing to inhibitor binding.

4. Conclusions

Proton-translocating NADH:Q oxidoreductase was found to be the molecular target of new and structurally diverse insecticides of synthetic and natural origin. Convincing data in the literature on membrane-bound and purified complex I from different biological sources show that these compounds block ubiquinone reduction similar to the classical inhibitors piericidin and rotenone.

With respect to the inhibitor mechanisms some methodological problems remain to be solved. Using purified complex I preparations for systematic enzyme kinetic and radioligand binding studies would most probably resolve most of the ambiguities stated above.

Concerning the number of quinone and inhibitor binding sites, the picture is still far from being consistent. A large Q/inhibitor binding domain in which bound ubiquinones can easily exchange with the mo-

bile Q pool in the membrane would fit to the published data and could be a sensible working hypothesis for the study of inhibitor specificity. The binding domain should accomodate structurally heterogeneous inhibitor molecules which interact at overlapping (competitive, mutually exclusive), or at non-overlapping (non-competitive, mutually non-exclusive) binding sites, defined by particular amino acid residues in the binding domain. Clearly, at least part of the inhibitor binding is governed by hydrophobic interactions.

From the point of view of applied biochemistry, extended structural and mechanistic knowledge of complex I would help in the identification of new efficient inhibitors for agrochemical application. On the other hand, synthetic compounds of great structural variability will be valuable tools in basic research to adress the intriguing questions of complex I structure and reaction mechanism.

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