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Quinolones and their *N*-oxides as inhibitors of mitochondrial complexes I and III

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Abstract

4(1H)-quinolones (2-alkyl- (**1**), 2-alkyl-3-methyl- (**2**), 2-methyl-3-alkyl- (**3**), 1-hydroxy-2-methyl-3-alkyl- (**4**) and 1-hydroxy-2-alkyl- (**5**)) with *n*-alkyl side chains varying from C_5 to C_{17} have been synthesized and tested for biological activity in mitochondrial complexes. Whereas all quinolones were efficient inhibitors of electron transport in the cytochrome b/c_1-complex from either beef heart or *Rhodospirillum rubrum*, in complex I from beef heart quinolones **1** and **2** only were highly active. In a Quantitative Structure-Activity Relationship (QSAR) inhibitory activity in the cytochrome b/c_1-complexes could be correlated to the physicochemical parameters lipophilicity π and/or to STERIMOL *L*. Maximal inhibitory potency was achieved at a carbon chain length of 12–14 Å. Oxidant-induced reduction of cytochrome *b* established that some quinolones are inhibitors of the Q_p rather than the Q_n site.

Keywords: NADH:ubiquinone oxidoreductase; Cytochrome bc_1 complex; Q_p-site inhibitor; Oxidant-induced reduction; Structure–activity relationship; (Beef heart); (*Rhodospirillum rubrum*)

1. Introduction

The mitochondrial NADH:ubiquinone oxidoreductase (complex I) transfers electrons and protons as well accross the inner membrane. It is one of the most complicated membrane complexes. In mammalians, it consists of at least 41 protein subunits [1]. Though all their sequences are known, there is little information available about the function of the proteins and the binding of the cofactors (FMN and several iron-sulfur clusters) to them (for a recent review, see [2]).

Inhibitors can serve as useful tools to elucidate structural and mechanistic aspects of enzyme reactions. The inhibitors of complex I, known so far, are mostly of natural origin, like piericidins and rotenone. Most recently, a series of other natural substances, like annonins, aurachins, and thiangazole, to quote only a few, have been found to be potent inhibitors of complex I [3]. The most powerful inhibitors of complex I known so far are natural substances (acetogenins) from the family Annonaceae, which in their biological activity are even better then piericidin or rotenone [4]. The inhibitors mentioned so far have at least two [3], possibly even three though overlapping binding sites [4].

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Ubiquinol, reduced at complex I, is reoxidized at the cytochrome b/c_1-complex (complex III) under reduction of cytochrome *c*. Compared to complex I, complex III is relatively simple and contains only three redox carriers: cytochrome *b*, cytochrome *c* and the Rieske iron-sulfur protein. Ubiquinol and its oxidized species (ubiquinone, -semiquinone) bind to two different loci, designated as Q_n and Q_p. Inhibitors of both sites are well known, for example for the Q_p-site strobilurin, myxothiazol, UHDBT (undecylhydroxydioxobenzothiazole) and stigmatellin and for the Q_n-site antimycin, funicolusin and HQNO (heptyl-hydroxyquinoline-*N*-oxide; 1-hydroxy-2-heptyl-4(1H)-quinolone) (for review, see [5]).

Though the biological activity of some 2-alkyl-4(1H)-quinolones, isolated from the culture filtrates of *Pseudomonas aeroginosa* is known since 40 years [6], the influence of the substitution pattern and the length of the alkyl chain on the inhibitory potency of the quinolones and their *N*-oxides has not been investigated. Only HQNO has been thoroughly studied (for example [7]), because it is commercially available. In addition, the effect of some 2- and 3-arylalkyl-4(1H)quinolones on mitochondrial electron transport has been examined, though their site of action was not specified [8].

We wish to report here on the inhibitory activities of alkyl- and methyl-alkyl-4(1H)-quinolones (Fig. 1, **1**–**3**) and their corresponding *N*-oxides (Fig. 1., **4**–**5**) on mitochondrial complexes. The inhibitory potency of the quinolones reached a maximum at a chain length of the alkyl group of about 14–15 Å and decreased if the chain length was further increased. Furthermore, for the first time a QSAR (Quantitative Structure-Activity Relationship) of quinolone inhibitors in the cytochrome b/c_1-complex will be presented.

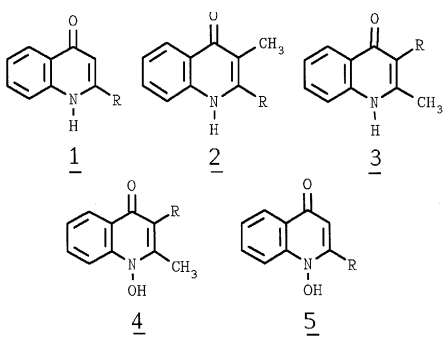

Fig. 1. Structures of 2-alkyl-4(1H)-quinolone (**1**), 2-alkyl-3-methyl-4(1H)-quinolone (**2**), 2-methyl-3-alkyl-4(1H)-quinolone (**3**), 1-hydroxy-2-methyl-3-alkyl-4(1H)-quinolone (**4**), and 1-hydroxy-2-alkyl-4(1H)quinolone (**5**).

2. Materials and methods

2.1. Chemicals

2.1.1. Synthesis of quinolones

*2.1.1.1. 2-alkyl- (**1**) and 2-alkyl-3-methyl-4(1H)-quinolones (**2**).* The 2-alkyl- (**1**) and 2-alkyl-3-methyl-4(1H)-quinolones (**2**) were prepared by alkylation of the corresponding dianions, generated by addition of two equivalents of *n*-butyl-lithium in tetrahydrofuran at −20°C, with alkyl-halogenides.

*2.1.1.2. 3-alkyl-2-methyl-4(1H)-quinolones (**3**).* The 3-alkyl-2-methyl-4(1H)-quinolones (**3**) were obtained by the Conrad-Limpach cyclization procedure [9]. Aniline is reacted with a suitable substituted acetylacetic acid ester to yield the corresponding β-anilino crotonic acid ester, which undergoes ring closure in diphenylether at 260°C.

*2.1.1.3. 3-alkyl-1-hydroxy-2-methyl-4(1H)-quinolones (**4**).* For the synthesis of the 3-alkyl-1-hydroxy-2-methyl-4(1H)-quinolones (**4**) the keto group in the 4-position was protected by conversion into the *p*-methoxybenzylether, the corresponding ether was converted into the 1-hydroxy compound by oxidation with 3-chloroperbenzoic acid. Finally, the *p*-methoxybenzl protecting group was removed by catalytic hydrogenation.

*2.1.1.4. 2-alkyl-1-hydroxy-4(1H)-quinolones (**5**).* 2-alkyl-1-hydroxy-4(1H)-quinolones (**5**) were synthesized according to Cornforth and James [10]. The keto group in the 4-position was protected by conversion into the *O*-ethoxycarbonyl-quinoline. Oxidation to the 1-hydroxy compound was achieved with 3-chloroperbenzoic acid. The protecting group was removed by alkaline hydrolysis.

2.2. Biochemical methods

The preparation of mitochondria and submitochondrial particles is described in [11] and that of ubiquinol:cytochrome *c* oxidoreductase (complex III, cytochrome b/c_1-complex) from beef heart or *Rhodospirillum rubrum* in [12]. The biological activities of the quinolones in the above complexes were determined using protocols given in [11] or [12], respectively.

Oxidant-induced reduction of cytochrome *b* in submitochondrial particles was measured in a dual-wavelength spectrophotometer at 562 versus 577 nm. The reaction system in 2 ml contained 25 mM phosphate (pH 7.2), 125 mM saccharose, 0.1 mM EDTA, 0.1 mg/ml dodecyl-β-D-maltoside, 0.1 mM KCN and cytochrome *b* at a concentration of 1.5 μM. Ubiquinol-2 was added at a concentration of 100 μM and ferricyanide at 16 μM. The concentrations of antimycin, myxathiazol and the quinolones were 2 μM. They were incubated for 15 s.

2.3. QSAR calculations

Regression equations were calculated on a Power Macintosh 7200/90 using the statistical package Minitab 10 Xtra from Minitab, State College, PA, USA. The π-values were taken from [13], the *L*-values from [14]. Values for the longer alkyl chains had to be extrapolated.

3. Results

3.1. NADH:ubiquinone-oxidoreductase (complex I)

The pI_{50}-values for inhibition of electron transport through mitochondrial complex I from beef heart are given in Table 1. It should be noted that all 2-methyl-3-alkyl-4(1H)-quinolones (**3**) tested proved to be completely inactive (data not shown). 1-Hydroxy-2-methyl-3-alkyl- (**4**) and 1-hydroxy-2-alkyl-4(1H)-quinolones (**5**) were only moderate inhibitors, whereas the 2-alkyl- (**1**) and 2-alkyl-3-methyl-4(1H)-quinolones (**2**) exhibit high inhibitory potency, the latter being so far the best quinolone-type inhibitors of complex I (pI_{50}-value of 7.70 for 2-*n*-undecyl-3-methyl-4(1H)-quinolone; Table 1). It is noteworthy, that the inhibitory activity of all quinolones increases with the chain length of the alkyl group until a maximum is reached at 12 carbon atoms which corresponds to a length of about 14 Å (Table 1 and Fig. 2). Further increase of the chain length lowers the activity (Table 1).

In order to gain more insight on the mode of action of quinolones on mitochondrial complex I, 2-*n*-undecyl-3-methyl-4(1H)-quinolone was studied in more detail. The kinetics of NADH-reduction at the expense of ubiquinol-1 was examined at various concentrations of this quinolone. The Lineweaver-Burk plot of the kinetic data is shown in Fig. 3. As is evident from the regression lines, they share a com-

Table 1
pI_{50} values for inhibition of electron transport through complex I from beef heart by quinolones

	1	**2**	**4**	**5**
4(1H)-quinolone	2-alkyl-	2-alkyl-3-methyl-	1-hydroxy-2-methyl-3-alkyl-	1-hydroxy-2-alkyl-
n-pentyl	4.61			< 4
n-hexyl				< 4
n-heptyl	5.09	6.57	< 4	< 4
n-octyl		6.81	4.07	< 4
n-nonyl	6.03	7.49	4.15	4.37
n-decyl		7.65	4.38	4.60
n-undecyl	6.66	7.70	4.42	4.99
n-dodecyl		7.10	4.24	4.72
n-tridecyl	5.93	6.98	4.22	4.65
n-tetradecyl			4.19	4.38
n-hexadecyl			< 4	
n-heptadecyl				< 4

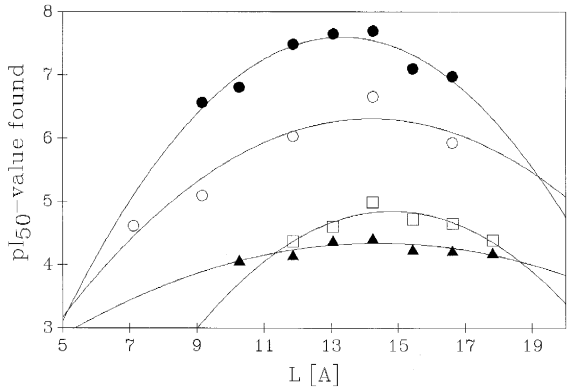

Fig. 2. Plot of Verloop's STERIMOL parameter L (Å) of the alkyl substituent vs. the pI_{50}-value of quinolones in beef heart mitochondrial complex I. ○, **1**; ●, **2**; ▲, **4**; □, **5**.

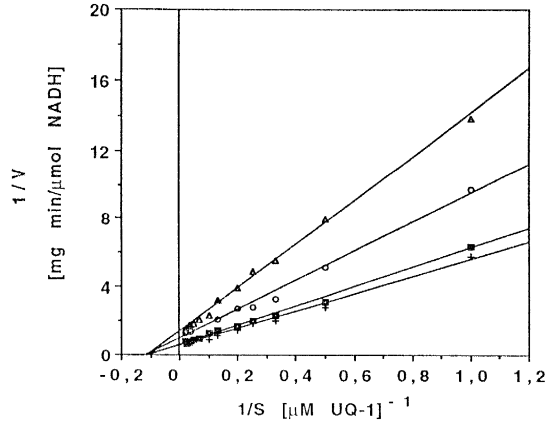

Fig. 3. Lineweaver-Burk plot of the kinetics of NADH-reduction by submitochondrial particles from beef heart in the presence of 3-methyl-2-*n*-undecyl-4-(1H)-quinolone. +, control; ■, 10^{-8} M; ○, $3 \cdot 10^{-8}$ M; △, $5 \cdot 10^{-8}$ M.

mon *x*-intercept, but have different *y*-intercepts. This indicates a noncompetitive displacement behaviour.

3.2. Ubiquinol:cytochrome c-oxidoreductase (cytochrome b / c_1-complex)

All quinolones tested so far are also potent inhibitors of electron transport through the mitochondrial complex III from beef heart (Table 2). The highest activity is found for 2-alkyl-3-methyl-4(1H)-quinolones (**2**), where 2-*n*-undecyl-3-methyl-4(1H)-quinolone with a pI_{50} value of 8.05 is the most active compound. The other quinolones are only slightly less active. Biological activity peaks again at a length of the carbon alkyl chain between 10 and 14 carbon atoms (Fig. 4) which corresponds to a chain length of 14–18 Å.

Similarily, the quinolones were also efficient inhibitors of electron transport in the cytochrome b / c_1 complex from the photosynthetic bacterium *R. rubrum* (Table 3). Basically, the inhibition pattern is the same as in complex III from beef heart (Fig. 5). However, the pI_{50}-values of the quinolones in the cytochrome b / c_1-complex from *R. rubrum* in some cases are more than one order of magnitude higher as compared to complex III from beef heart. The most active inhibitor is 1-hydroxy-2-*n*-tetradecyl-4(1H)-quinolone (**5**), which exhibits a pI_{50}-value of 9.19 (Table 3).

Table 2
pI_{50}-values for inhibition of electron transport through complex III from beef heart by quinolones

	1	**2**	**3**	**4**	**5**
4(1H)-quinolone	2-alkyl-	2-alkyl-3-methyl-	2-methyl-3-alkyl-	1-hydroxy-2-methyl-3-alkyl-	1-hydroxy-2-alkyl-
n-pentyl	4.64		5.60		4.47
n-hexyl			5.84	5.81	5.09
n-heptyl	5.66	6.12	6.88	6.04	5.82
n-octyl		6.44	7.05	6.33	6.43
n-nonyl	6.31	7.10	7.33	6.52	6.88
n-decyl		7.44	7.56	6.81	7.01
n-undecyl	7.00	8.05	7.46	7.26	7.25
n-dodecyl		7.44	7.58	7.07	7.36
n-tridecyl	6.80	7.30	7.60	6.95	7.57
n-tetradecyl			7.90	6.74	7.44
n-hexadecyl			6.71	6.51	
n-heptadecyl					6.49

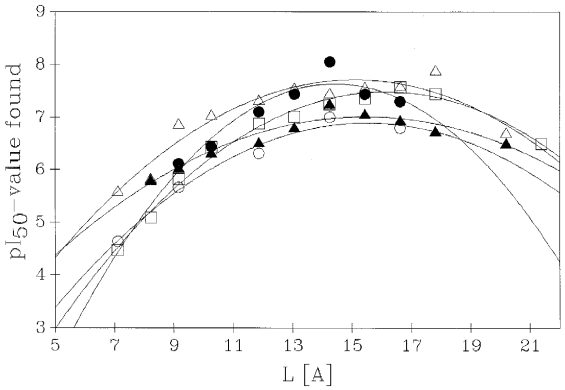

Fig. 4. Plot of Verloop's STERIMOL parameter L (Å) of the alkyl substituent versus the pI_{50}-value of quinolones in beef heart mitochondrial complex III., ○, **1**; ●, **2**; △, **3**; ▲, **4**; □, **5**.

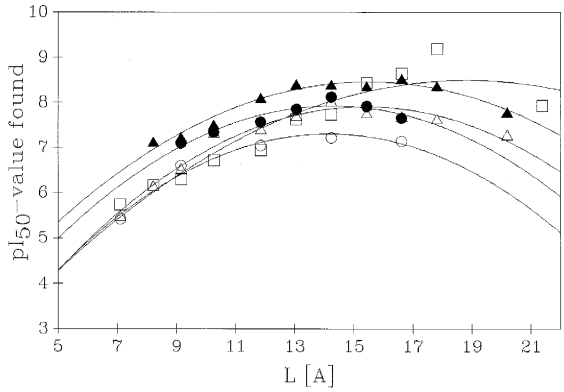

Fig. 5. Plot of Verloop's STERIMOL parameter L (Å) of the alkyl substituent versus the pI_{50}-value of quinolones in the cytochrome b/c_1-complex from *R. rubrum*. ○, **1**; ●, **2**; △, **3**; ▲, **4**; □, **5**.

In order to decide which quinone binding site is affected by the quinolones, i.e. the Q_n- or Q_p-site (for review, see [15]), their influence on the 'oxidant-induced reduction' of cytochrome *b* in the cytochrome b/c_1-complex in submitochondrial particles from beef heart has been investigated. If cytochrome c_1 and the Rieske iron-sulfur protein are both reduced in the complex and the Q_n-site is in addition inhibited by antimycin A, a reduction of cytochrome *b* by ubiquinol cannot occur. However, if cytochrome c_1 and the Rieske iron-sulfur protein are kept oxidized by ferricyanide, cytochrome *b* will be rapidly reduced [5]. This is demonstrated in Fig. 6. Trace a serves as a control and no extrareduction of cytochrome *b* is observed. In contrast, in the presence of antimycin A a fast reduction of cytochrome *b* occurs (trace b). Traces c to f demonstrate that quinolones HMUQ (for abbreviations see legend of Fig. 6), HTQ, MTQ and MUQ do not elicit a response similar to that of antimycin A. Therefore, the quinolones cannot be considered as Q_n-site inhibitors. On the other hand, the 'oxidant-induced' reduction of cytochrome *b* is blocked by inhibitors of the Q_p-site, like myxothiazol (trace g). In the presence of myxothiazol, antimycin A is no longer capable to induce a reduction of cytochrome *b* ('double kill'; trace h). As is evident from traces j and k, HTQ and MTQ behave exactly like myxothiazol and can be classified as true Q_p-type inhibitors. Contrary HMUQ (trace i) and MUQ (trace l) allow for an extrareduction of cy-

Table 3
pI_{50} values for inhibition of electron transport through the cytochrome b/c_1 complex from *R. rubrum* by quinolones

	1	**2**	**3**	**4**	**5**
4(1H)-quinolone	2-alkyl-	2-alkyl-3-methyl-	2-methyl-3-alkyl-	1-hydroxy-2-methyl-3-alkyl-	1-hydroxy-2-alkyl-
n-pentyl	5.42		5.49		5.74
n-hexyl			6.16	7.13	6.16
n-heptyl	6.60	7.10	6.54	7.23	6.30
n-octyl		7.34	7.34	7.51	6.73
n-nonyl	7.05	7.57	7.42	8.10	6.95
n-decyl		7.85	7.71	8.41	7.63
n-undecyl	7.22	8.12	8.04	8.40	7.74
n-dodecyl		7.92	7.77	8.37	8.44
n-tridecyl	7.15	7.66	7.70	8.53	8.65
n-tetradecyl			7.64	8.37	9.19
n-hexadecyl			7.29	7.79	
n-heptadecyl					7.93

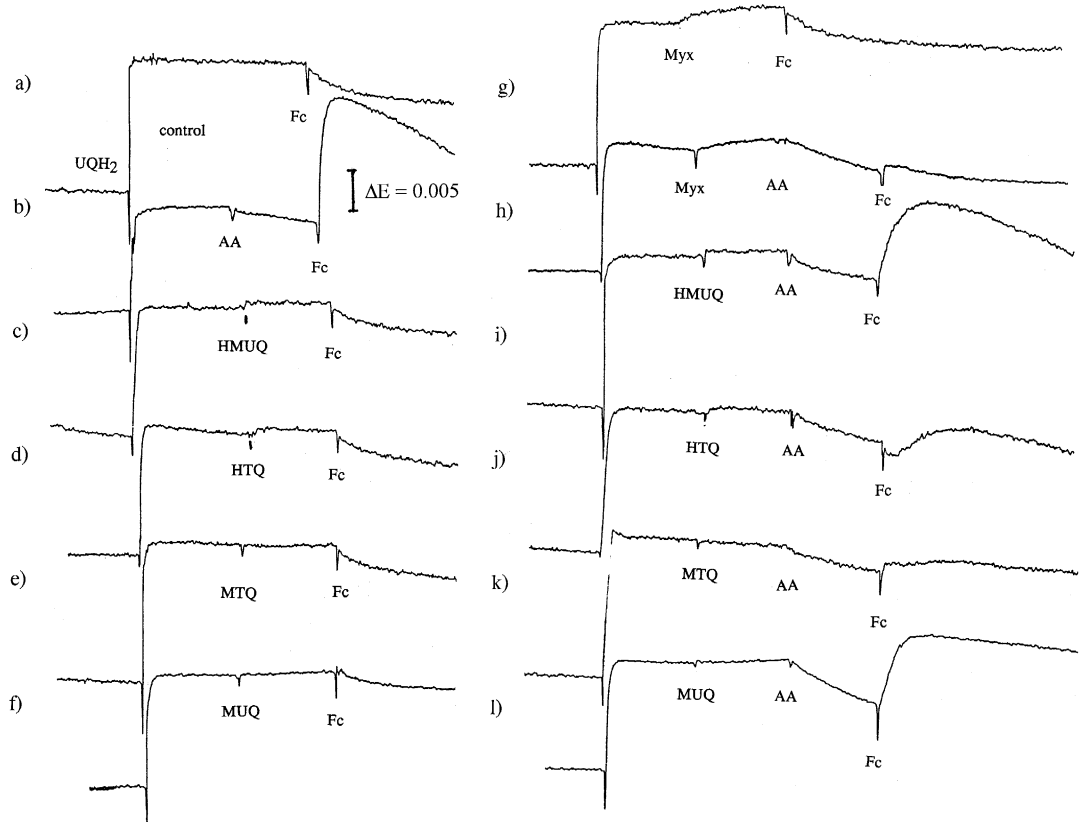

Fig. 6. Oxidant-induced reduction of cytochrome *b* in isolated complex III from beef heart (UQH_2, ubiquinol-2; AA, antimycin; Fc, ferricyanide; Myx, myxothiazole; HMUQ, 1-hydroxy-2-methyl-3-*n*-undecyl-4(1H)-quinolone (**4**); HTQ, 1-hydroxy-2-*n*-tridecyl-4-(1H)-quinolone (**5**); MTQ, 2-methyl-3-*n*-tetradecyl-4-(1H)-quinolone (**3**); MUQ, 3-methyl-2-*n*-undecyl-4-(1H)-quinolone (**2**)). For conditions, see Section 2. Trace a, control; traces b–l, addition of various inhibitors, see text for details.

tochrome *b* in the presence of antimycin A. In that behaviour they are different from myxothiazol.

4. Discussion

Quinolones are produced by the bacteria *Pseudomonas aeruginosa* and *Stigmatella aurantiaca* (‘aurachins’) and were shown to be inhibitors of respiratory and photosynthetic electron transport chains as well [6,16]. They were taken as ‘lead substances’ for synthesis and evaluation of biological activity of a series of 2-alkyl- (**1**), 2-alkyl-3-methyl- (**2**), 2-methyl-3-alkyl-4(1H)-quinolones (**3**) and 1-hydroxy-2-methyl-3-alkyl- (**4**) and 1-hydroxy-2-alkyl-4(1H)-quinolones (**5**). The length of the alkyl side chain was varied from five to seventeen carbon atoms.

In complex I of the mitochondrial respiratory chain 2-alkyl-4(1H)-quinolones (**1**) showed pronounced activity, which could be substantially increased by an additional methyl substituent in the 3-position. Conversly, the 2-methyl-3-alkyl-4(1H)-quinolones (**3**), where alkyl and methyl group have changed positions, are completely inactive. This indicates that the optimal position for a hydrophobic substituent is the 2- and not the 3-position. Furthermore, substitution of the nitrogen with a hydroxyl instead of a hydrogen is also detrimental for biological activity. 2-Alkyl- (**1**) and 2-alkyl-3-methyl-4-(1H)-quinolones (2) reached maximal activity at a length of the alkyl chain of eleven carbon atoms which corresponds to about 14 Å. A similar situation is found for 4-alkyl-or 4-alkoxy-acridones, which were recently found by us as efficient inhibitors of electron transport through mitochondrial complex I [11]. In their case, inhibitory activity peaked at a chain length of about 10 Å. If one takes into account that the acridones as compared to the quinolones bear an additional aromatic moiety,

the overall distance from the imino group to the end of the carbon chain is about the same for both types of compounds. This further corroborates the notion that the hydrophobic binding pocket of the inhibitor binding protein can only accommodate an alkyl side chain up to an optimal length.

The kinetic data of ubiquinone-1 reduction by NADH in the presence of 2-*n*-undecyl-3-methyl-4(1H)quinolone have indicated that the quinolone and ubiquinone-1 displace each other in a noncompetitive manner. This does not necessarily mean that their binding sites are completely different. It is known that rotenone is a noncompetitive inhibitor for ubiquinone at complex I, but rotenenone isomers, notably capsaicin and ist analogues, act as competitive inhibitors at high ubiquinone concentrations and noncompetitive inhibitors at low ubiquinone concentrations [17–20]. It is conceivable, therefore, that the quinolones and ubiquinone share identical or overlapping binding sites.

All quinolones **1**-**5** synthesized so far are efficient inhibitors of the cytochrome b/c_1-complexes from beef heart and *R. rubrum* as well. As already stressed, the pI_{50}-values of the quinolones are always higher in the *R. rubrum* complex. The same observation was made for 2- and 4-substituted acridones as inhibitors of cytochrome b/c_1 complexes, which have been recently reported by us [12]. This discrepancy has been discussed extensively in [12]. The notion about the optimal length of the alkyl side chains in quinolones compared to 2-alkyl- and 4-alkyl-acridones, as made above, also applies for their inhibitory activity in cytochrome b/c_1 complexes.

As is evident from the 'oxidant-induced reduction' of cytochrome b, all quinolones testet so far are definitely no Q_n-site inhibitors, because they behave different from antimycin (compare traces b and c to f in Fig. 6). An alternate explanation for the inhibitory activity of quinolones would be that they are inhibitors of the other quinone binding site, the Q_p-site. This is definitely true for HTQ and MTQ, which behave exactly like the Q_p-site inhibitor myxothiazol (compare trace h and j and k in Fig. 6). However, HMUQ and MUQ induce an extra reduction of cytochrome *b* in the presence of antimycin and in this respect are different from myxothiazol (see traces h and l in Fig. 6). If the structures of the two 1-hydroxy-quinolones HTQ and HMUQ and the two 1-hydrogen-quinolones MTQ and MUQ are compared, it is noted that in both groups the bulky alkyl side chains have changed positions from the 2- to the 3-position and vice versa. It is possible that 1-hydroxy- vs. 1-hydrogen-quinolones have a different orientation within their binding niche. It is possible that the Q_p-site cannot accomodate HMUQ and MUQ and that they bind to a not yet known quinone binding site which is not identical to the Q_p-site but may be possibly located adjacent. Further work will be needed to classify the exact mechanism of action of inhibitors like HMUQ and MUQ.

A QSAR for complex I inhibitors was not possible because either the data sets were to small or the variations in pI_{50}-values were too low to allow for a calculation. However, a QSAR could be performed for all quinolones in both cytochrome b/c_1-complexes from beef heart and *R. rubrum*. As physicochemical parameters, the lipophilicity π [13] and Verloop's STERIMOL parameter *L* [14] of the alkyl side chain in positions 2 (R^2) and 3 (R^3) have been used. The quinolones have been grouped in two classes: those bearing a hydrogen at the nitrogen (**1**–**3**) and those bearing a hydroxyl group at the nitrogen (**4**–**5**). The following equations have been obtained:

4.1. Cytochrome b/c_1-complex from beef heart

Quinolones **1**–**3**

$$pI_{50} = 1.86 + 1.57\pi(R^2) - 0.13\pi^2(R^2) + 1.88\pi(R^3) - 0.19\pi^2(R^3) \quad (1)$$

$n = 23;\ r = 0.847;\ s = 0.365;\ F = 24.91.$

Quinolones **4**–**5**:

$$pI_{50} = -0.71 + 2.93\pi(R^2) - 0.26\pi^2(R^2) + 2.50\pi(R^3) - 0.26\pi^2(R^3) \quad (2)$$

$n = 21;\ r = 0.963;\ s = 0.168;\ F = 104.23.$

4.2. Cytochrome b/c_1-complex from R. rubrum

Quinolones **1**–**3**:

$$pI_{50} = -1.33 + 2.73\pi(R^2) - 0.28\pi^2(R^2) + 1.05L(R^3) - 0.04L^2(R^3) \quad (3)$$

$n = 23;\ r = 0.935;\ s = 0.208;\ F = 64.70.$

Quinolones **4**–**5**:

$$pI_{50} = -2.76 + 1.05L(R^2) - 0.03L^2(R^2) + 1.18L(R^3) - 0.04L^2(R^3) \quad (4)$$

$n = 21$; $r = 0.906$; $s = 0.314$; $F = 38.48$.

In Eqs. (1)–(4), n is the number of compounds, r the correlation coefficient, s the SD and F denotes the F-test of significance.

In Eqs. (1)–(4) different physicochemical parameters have been used. Whereas in Eq. (1) and Eq. (2) the lipophilicity parameter π has been applied for both substituents, π (R^3) in Eq. (3) has been replaced by L (R^3) and in Eq. (4) L (R^2) and L (R^3) have been used exclusively. It should be noted that lipophilicity π and the STERIMOL parameter L (length of the substituent) are highly intercorrelated. This is due to the fact that the lipophilicity π increases linearly with the chain length. In the multiple linear regressions all combinations of parameter sets have been calculated and the equations with the highest correlation coefficient r and statistical significance have been selected. For instance, if in Eq. (3) L (R^3) is replaced by π (R^3), r drops from 0.935 to 0.721. Similarly, replacement of L (R^2) and L (R^3) in Eq. (4) by π (R^2) and π (R^3) leads to a decrease in r from 0.906 to 0.698. The use of different parameter sets does not allow for comparison of the numerical values of the variables and the constant. Furthermore, as already stressed, pI_{50}-values of quinolones in the cytochrome b/c_1-complex of *R. rubrum* are significantly higher than in the complex from beef heart. Compared to the QSAR of acridone inhibitors [12], Eqs. (1)–(4) are very simple and indicate that biological activity is only governed by the lipophilicity π and/or the length of the alkyl substituent, or the squares of them, respectively. The parabolic dependency from STERIMOL parameter L is already evident from Figs. 4 and 5.

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