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# Effects of new ubiquinone-imidazo[2,1-*b*]thiazoles on mitochondrial complex I (NADH-ubiquinone reductase) and on mitochondrial permeability transition pore

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**Abstract**—In this work we describe the synthesis of a series of imidazo[2,1-*b*]thiazoles and 2,3-dihydroimidazo[2,1-*b*]thiazoles connected by means of a methylene bridge to $CoQ_0$. These compounds were tested as specific inhibitors of the NADH:ubiquinone reductase activity in mitochondrial membranes. The imidazothiazole system when bound to the quinone ring in place of the isoprenoid lateral side chain, may increase the inhibitory effect (with an $IC_{50}$ for NADH-$Q_1$ activity ranging between 0.25 and 0.96 μM) whereas the benzoquinone moiety seems to lose the capability to accept electrons from complex I as indicated by very low maximal velocity elicited by the compounds tested. Moreover the low rotenone sensitivity for almost all of these compounds suggests that they are only partially able to interact with the physiological ubiquinone-reduction site. The compounds were investigated for the capability of increasing the permeability transition of the inner mitochondrial membrane in isolated mitochondria. Unlike $CoQ_0$, which is considered a mitochondrial membrane permeability transition inhibitor, the new compounds were inducers.


## 1. Introduction

Mitochondrial NADH-Coenzyme Q (CoQ, ubiquinone) oxidoreductase (EC 1.6.99.3, complex I) is the most complicated and the least understood of the respiratory chain complexes. Despite recent progress in structural studies,[1,2] there is no structural information about complex I comparable with the crystallographic data available for other respiratory complexes. This lack of information is principally due to the complexity of the system: the mammalian enzyme consists of up to 46 different subunits reaching a total mass of almost 1000 kDa.[3] Nevertheless, there seems to be a general agreement on the presence of ubisemiquinones as intermediates for the electron transfer to the bulky quinone and on the proton pumping stoichiometry of $4H^+/2e^-$.[4–9] Another typical feature of complex I is related to the high number of its inhibitors: over sixty different families of compounds are known to inhibit complex I,[10] most of which are commonly used as pesticides, including insecticides, miticides, piscicides, and ascaricides.

The discovery that mitochondrial DNA mutations are at the basis of a number of human pathologies has opened a new and extremely active chapter about mitochondrial research.[11,12] A lesion in a structural gene, such as that due to a point mutation, would interfere with the function of the polypeptide encoded by that gene, leading to decreased enzyme activity. Indeed this is often found in such kind of cytopathies, such as a form of Leber's hereditary optic neuropathy (LHON) where in spite of normal complex I activity, the flux over the entire respiratory chain is significantly decreased.[13] It is predictable that the quinone-binding sites are involved in a defective energy conservation present in this form of the disease, since they take part in the proton translocation activity of complex I.[14] Moreover, in the last decades, the interest in this NADH-ubiquinone reductase is increasing, due to its possible involvement in the pathogenesis of human neurodegenerative diseases[15,16] such as

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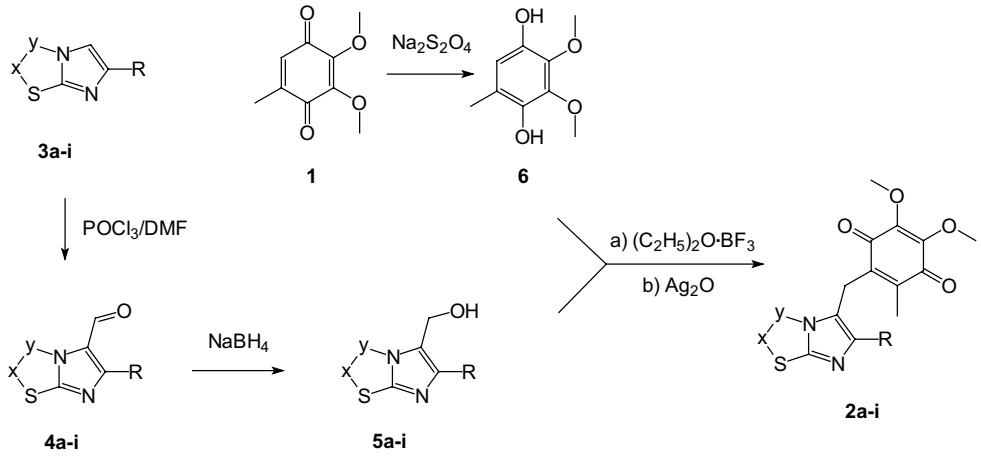


**Scheme 1.** x, y, R see Table 1.

Alzheimer's and Parkinson's diseases, in diabetes and in aging.[17] On the other hand rotenoids acting as NADH-ubiquinone oxidoreductase inhibitors induce ornithine decarboxylase activity and are evaluated as candidate cancer chemopreventive agents.[18,19]

The identification and characterization of specific quinones involved in the inhibition of complex I, represent a significant advance to understand the functional mechanism of the complex, to define the structural and functional features of the ubiquinone-reduction site of bovine heart mitochondrial complex I[20–25] and also contributes to develop drugs specifically acting on mitochondria when complex I deficits are implicated in human diseases. Some quinones are already known as complex I inhibitors. The inhibitory action of $CoQ_2$ and other short chain isoprenoid homologs (but not $CoQ_1$) is well documented in beef heart mitochondria.[10,21] In bovine heart submitochondrial particles (SMP) only $CoQ_1$ and 6-decyl-$Q_0$ (DB) were found to elicit rates comparable to those calculated for reduction of endogenous $CoQ_{10}$ and are used as electron acceptors substitutes of the physiological ubiquinones. Also $CoQ_0$ and the tetramethylbenzoquinone analogue, duroquinone (DQ), are electron acceptors.[10,21,26] All these substitutes of the ubiquinones interact with the physiological site(s), in place of the endogenous CoQ and their reduction is almost completely inhibited by rotenone. On the other hand, the quinone 2,3-dimethoxy-5-methyl-1,4-benzoquinone ($CoQ_0$ or ubiquinone$_0$, **1**, Scheme 1) is known to be a potent inhibitor of the so called permeability transition pore (PTP),[27,28] a cyclosporin A-sensitive channel of the inner mitochondrial membrane.[29] Other quinones show different behaviors being inducers or inhibitors.[28] The PTP has been studied as a target for mitochondria dysfunction in vivo and although the physiological function of the pore is obscure, more recently the PTP has been considered as a potential mediator in the mechanism of programmed cell death.[30,31] Furthermore a relation between PTP and complex I activity has been suggested.[32]

In a previous study[33–35] it was shown that thienylimidazo[2,1-*b*]thiazoles were able to inhibit complex I acting in a non-competitive way with the ubiquinone substrate and interacted with a site, which is mutually exclusive with that of rotenone but non-exclusive with that of piericidin and several inhibitors of NADH dehydrogenase. Founded on those data, we have synthesized a new series of different imidazothiazoles (**2a**–**i**, Scheme 1) linked to a $CoQ_0$ moiety, in order to evaluate the effect on complex I and on PTP of the hybrid ubiquinone and to elucidate the ambiguous quinone specificity in the interaction with the enzyme.

## 2. Chemistry

Compounds **3**–**5** were prepared according to the literature,[36–42] except **3i**, **4i**, and **5h**–**i**, which were synthesized according to the Scheme 1; details for the synthesis are described under Section 5. Compound **3i** was synthesized by treating 2-amino-5-chlorothiazole hydrochloride with chloroacetone. The aldehyde **4i** was prepared by means of the Vilsmeier reaction on the imidazothiazole **3i** and the hydroxymethyl derivatives **5h**–**i** were prepared by reduction of the aldehydes with sodium borohydride. 2,3-Dimethoxy-5-methyl-1,4-benzoquinone **1** was converted to 2,3-dimethoxy-5-methylhydroquinone **6** by a conventional reductive step employing sodium dithionite as the reducing agent. In the subsequent step, compound **6** was reacted with the appropriate substituted 5-hydroxymethylimidazo[2,1-*b*]thiazole **5a**–**i**. The condensation was carried out in dioxane at room temperature and under nitrogen in order to prevent oxidation of the hydroquinone to the corresponding benzoquinone. Boron trifluoride etherate was used as the acidic condensing agent.[43] The hydroquinones were not characterized but used as such for a mild oxidation with silver(I)oxide, which afforded the substituted 6-imidazo[2,1-*b*]thiazolylmethyl-2,3-dimethoxy-5-methyl-benzoquinones **2a**–**i**. All the new derivatives **2a**–**i** are reported in Tables 1 and 2.

## 3. Biological results

The biological activity of compounds **2a**–**i** on mitochondrial NADH dehydrogenase activity has been

**Table 1.** Compounds **2a–i**

| Compounds | x–y | R | Starting **4/5** ref. | Formula | $M_w$ | Mp, °C |
|---|---|---|---|---|---|---|
| **2a** | CH=CH | Cl | 36/36 | $C_{15}H_{13}ClN_2O_4S$ | 352.8 | 135–138 (a) |
| **2b** | CH=CH | $CH_3$ | 37/42 | $C_{16}H_{16}N_2O_4S$ | 332.4 | 145–150 (b) |
| **2c** | CH=CH | $C_6H_5$ | 37/42 | $C_{21}H_{18}N_2O_4S$ | 394.4 | 135–140 (b) |
| **2d** | $CH_2$–$CH_2$ | Cl | 38/42 | $C_{15}H_{15}ClN_2O_4S$ | 354.8 | 156–157 (b) |
| **2e** | $CH_2$–$CH_2$ | $CH_3$ | 38/42 | $C_{16}H_{18}N_2O_4S$ | 334.4 | >300 (b) |
| **2f** | $H_3C$C=CH | Cl | 39/39 | $C_{16}H_{15}ClN_2O_4S$ | 366.8 | 125–130 (c) |
| **2g** | $H_3C$C=CH | $CH_3$ | 40/39 | $C_{17}H_{18}N_2O_4S$ | 346.4 | 115–117 (b) |
| **2h** | ClC=CH | $C_6H_5$ | 41/exp. | $C_{21}H_{17}ClN_2O_4S$ | 428.9 | 70–72 (d) |
| **2i** | ClC=CH | $CH_3$ | exp./exp. | $C_{16}H_{15}ClN_2O_4S$ | 366.8 | 154–156 (b) |

Purified by: crystallisation with (a) ethyl ether/esane or by chromatography with the following eluents: (b) acetone/petroleum ether 60–80 °C; (c) ethyl ether; (d) ethyl acetate/petroleum ether 60–80 °C.

**Table 2.** IR and $^1$H NMR of compounds **2a–i**

| Compounds | IR: $\nu_{max}$, $cm^{-1}$ | $^1$H NMR:[a] $\delta$, ppm in DMSO-$d_6$ |
|---|---|---|
| **2a** | 1650, 1605, 1265, 715 | 2.06 (3H, s, $CH_3$), 3.86 (3H, s, $OCH_3$), 3.89 (3H, s, $OCH_3$), 4.01 (2H, s, $CH_2$), 7.39 (1H, d, th, $J$ = 4.5), 7.91 (1H, d, th, $J$ = 4.5) |
| **2b** | 1655, 1635, 1265, 770 | 2.01 (3H, s, $CH_3$), 2.13 (3H, s, $CH_3$), 3.85 (3H, s, $OCH_3$), 3.87 (3H, s, $OCH_3$), 3.95 (2H, s, $CH_2$), 7.16 (1H, d, th, $J$ = 4.5), 7.66 (1H, d, th, $J$ = 4.5) |
| **2c** | 1640, 1605, 1260, 770 | 1.67 (3H, s, $CH_3$), 3.81 (6H, s, $OCH_3$), 4.20 (2H, s, $CH_2$), 7.29 (1H, d, th, $J$ = 4.6), 7.36 (3H, m, ar), 7.49 (2H, m, ar), 7.81 (1H, d, th, $J$ = 4.6) |
| **2d** | 1660, 1635, 1600, 1265 | 2.00 (3H, s, $CH_3$), 3.72 (2H, s, $CH_2$), 3.84 (2H, t, thn, $J$ = 7.3), 3.88 (3H, s, $OCH_3$), 3.89 (3H, s, $OCH_3$), 4.12 (2H, t, thn, $J$ = 7.3) |
| **2e** | 1675, 1645, 1600, 1100 | 1.97 (3H, s, $CH_3$), 2.01 (3H, s, $CH_3$), 3.67 (2H, s, $CH_2$), 3.79 (2H, t, thn, $J$ = 7.2), 3.87 (3H, s, $OCH_3$), 3.88 (3H, s, $OCH_3$), 3.97 (2H, t, thn, $J$ = 7.2) |
| **2f** | 1660, 1640, 1600, 1260 | 2.03 (3H, s, $CH_3$), 2.42 (3H, d, $CH_3$, $J$ = 1.4), 3.85 (3H, s, $OCH_3$), 3.88 (3H, s, $OCH_3$), 3.94 (2H, s, $CH_2$), 7.67 (1H, q, th, $J$ = 1.4) |
| **2g** | 1690, 1655, 1610, 1265 | 2.04 (3H, s, $CH_3$), 2.19 (3H, s, $CH_3$), 2.41 (3H, s, $CH_3$), 3.85 (3H, s, $OCH_3$), 3.88 (3H, s, $OCH_3$), 3.94 (2H, s, $CH_2$), 7.56 (1H, s, th) |
| **2h** | 1640, 1605, 1260, 765 | 1.70 (3H, s, $CH_3$), 3.78 (3H, s, $OCH_3$), 3.79 (3H, s, $OCH_3$), 4.17 (2H, s, $CH_2$), 7.33 (3H, m, ar), 7.43 (2H, m, ar), 8.26 (1H, s, th) |
| **2i** | 1640, 1610, 1265, 1025 | 2.03 (3H, s, $CH_3$), 2.11 (3H, s, $CH_3$), 3.86 (3H, s, $OCH_3$), 3.89 (3H, s, $OCH_3$), 3.95 (2H, s, $CH_2$), 8.11 (1H, s, th) |

[a] Abbreviations: th = thiazole, thn = thiazoline, ar = aromatic.

initially tested to evaluate if they were electron acceptors. The electron acceptors most commonly employed as substitutes of the physiological ubiquinones are short chain CoQ homologues (the quinone most used as acceptor is $CoQ_1$) and analogues (such as decyl-ubiquinone). The experiments were carried out using submitochondrial particles from beef heart. By the kinetic analysis of the redox reactions, two parameters were determined, $K_m$ and $V_{max}$. As shown in Table 3 it is possible to divide the compounds in two groups.

The first group consists of **2a**, **2b**, **2d**, **2f**, **2i**, which cannot be considered electron acceptors, because they have a low affinity for complex I ($K_m$ range from 220 to 417 μM, whereas $Q_1$ displays $K_m$ = 24 μM), even though they have the highest values of $V_{max}$ (from 0.134 to 0.255 $\mu mol\,mg^{-1}\,min^{-1}$): these values are however low compared with that elicited by the best acceptor $CoQ_1$ ($V_{max}$ = 0.66 $\mu mol\,mg^{-1}\,min^{-1}$). The second group consists of **2c**, **2e**, **2g**, and **2h**, which are weak electron acceptors as shown by the low values of $V_{max}$ (from 0.026 to 0.0805 $\mu mol\,mg^{-1}\,min^{-1}$), but they have a greater affinity for the complex, indeed they show a low $K_m$ (ranging from 16.8 to 43.4 μM). Moreover it could be considered that all compounds tested show a very low rotenone sensitivity, suggesting that they are not able to interact with the physiological CoQ reduction site. In Table 3 is also reported the values calculated for the ratio $k_{cat}/K_m$ that is taken as index for the complex I specificity versus the **2a–i** derivatives. $k_{cat}$ was calculated dividing the $V_{max}$ values by the complex I concentration calculated by the ferricyanide assay as indicated in Fato et al.[26] From the results listed in Table 3 it appears that complex I has a specificity for almost all the derivatives tested that is more than one order of magnitude lower with respect to that one elicited for $CoQ_1$. Compound **2h** is capable to induce the highest $k_{cat}/K_m$ value suggesting that even if it is a very poor substrate it is able to interact with the physiological CoQ reduction site as confirmed also by the high rotenone sensitivity of the NADH-**2h** reductase activity. It should be remembered that in the compound **2h** the positions 2 and 6 of the imidazothiazole group are substituted by a chlorine and a phenyl group, respectively. These substitutions make the compound very hydrophobic indicating that the hydrophobicity is an important parameter for ensuring the interaction with complex I. In general, the benzoquinone moiety when condensed with the imidazothiazole system seems to lose

**Table 3.** Summary of the kinetic parameters of **2a–i** in bovine complex I[a]

| Compounds | $K_m$ (μM) | $V_{max}$ (μmol mg$^{-1}$ min$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | Rotenone sensitivity (%)[b] | log $P$[c] |
|---|---|---|---|---|---|---|
| **2a** | 274.10 | 0.196 | 62.82 | $2.29 \times 10^5$ | 50 | 1.973 |
| **2b** | 359.81 | 0.255 | 81.73 | $2.27 \times 10^5$ | 14 | 1.691 |
| **2c** | 41.72 | 0.074 | 23.88 | $5.72 \times 10^5$ | 43 | 3.040 |
| **2d** | 414.35 | 0.198 | 63.46 | $1.53 \times 10^5$ | 22 | 1.210 |
| **2e** | 43.38 | 0.026 | 8.33 | $1.92 \times 10^5$ | 45 | 0.683 |
| **2f** | 417.01 | 0.238 | 76.28 | $1.83 \times 10^5$ | 0 | 2.742 |
| **2g** | 33.48 | 0.035 | 11.22 | $3.35 \times 10^5$ | 25 | 2.190 |
| **2h** | 16.83 | 0.080 | 25.80 | $15.30 \times 10^5$ | 80 | 3.754 |
| **2i** | 220.59 | 0.134 | 42.95 | $1.95 \times 10^5$ | 5.6 | 2.405 |
| **$Q_0$** | 65.00 | 0.180 | 57.69 | $8.88 \times 10^5$ | 90 | 0.131 |
| **$Q_1$** | 24.00 | 0.660 | 211.54 | $88.10 \times 10^5$ | 90 | 2.152 |

[a] The $K_m$ and $V_{max}$ values were obtained from at least three independent experiments. The concentration of SMP protein used ranged between 20 and 40 μg/mL. $k_{cat}$ values were obtained by division of the $V_{max}$ by the concentration of complex I of 0.052 nmol mg$^{-1}$ calculated by ferricyanide assay.[26]
[b] % of NADH-**2a–i** activity inhibited by 2 μM rotenone.
[c] CLOGP; 4.3 ed. BioByte Corp. Claremont, CA.

the capability of electron acceptor from complex I as indicated by the very low maximal velocity elicited by the compounds tested.

Natural CoQ homologues are hydrophobic molecules and their membrane/water partitioning has to be taken into account in any consideration concerning their specificity and kinetics of interaction. For this reason, we calculated the octanol/water partition coefficient of the derivatives **2a–i** (see Table 3). The calculated log $P$ values were estimated with the CLOGP program (CLOGP; 4.3 ed. BioByte Corp. Claremont, CA). There is almost no relationship between the affinity of these compounds for complex I ($K_m$) and their partition coefficients (correlation coefficient $r^2 = 0.18$). This result brings to conclude that even if hydrophobicity is a property essential to influence the affinity of **2a–i** for the complex, it may not be the only one involved. By contrast if we calculate the correlation coefficient excluding the most deviating compounds (**2e** and **2g**) the $r^2$ value is higher: 0.79. This observation strengthens the idea that both hydrophobicity and some other structural parameters have to be considered to explain the interaction of the complete series of products with complex I. Moreover the low rotenone sensitivity of complex I activity for almost all these compounds (see Table 3) suggests that they are only partially able to interact with the physiological CoQ-reduction site.

Since not all the compounds investigated are electron acceptors, we tested if they inhibited the NADH-ubiquinone reductase activity of complex I using submitochondrial particles. We assayed the NADH-$O_2$ activity for **2a–i** by measuring their inhibition of the overall electronic flux determined by the rates of NADH oxidation (NADH-$O_2$ oxidation activity) and the NADH-$CoQ_1$ activity (NADH-Coenzyme Q reductase) using $Q_1$ as electron acceptor while blocking complex III (ubiquinol cytochrome c reductase) with antimycin and complex IV with KCN (see Table 4). Compounds **2c**, **2e**, and **2h** were not inhibitors. The presence of an aromatic ring in position 6 of the imidazothiazole moiety (**2c** and **2h**) or the saturation in 2,3 position (**2e**) brings to lack of the inhibitory effect. On the contrary **2a**, **2b**, **2d**, **2f**, **2g**, and **2i** inhibit the NADH dehydrogenase but with different features (see Table 4).

Compounds **2b**, **2d**, **2f**, and **2g** appear to be inhibitors of complex I only, since they exert similar extent of inhibition on NADH-$CoQ_1$ and NADH-$O_2$ activities. Compound **2a** has an $IC_{50}$ for NADH-$O_2$ ($0.24 \pm 0.07$ μM) lower with respect to $IC_{50}$ for NADH-$CoQ_1$ ($0.96 \pm 0.17$ μM); for this derivative a titration of the inhibitory effects on the reductase activity showed that it acts prevalently as competitive inhibitor of $CoQ_1$. Compound **2i** inhibits only NADH-$O_2$ activity suggesting that its preferential target is one of the other redox

**Table 4.** NADH-$CoQ_1$ and NADH-$O_2$ activity in submitochondrial particles (SMP) of beef heart mitochondria in the presence of compounds **2a–i**

| Compounds | $IC_{50}$ (μM)[a] for NADH-$CoQ_1$ activity | $IC_{50}$ (μM)[a] for NADH-$O_2$ activity | Concentration inducing 50% swelling (PTP-activating) |
|---|---|---|---|
| **2a** | $0.960 \pm 0.170$ | $0.240 \pm 0.070$ | 2.83 μM |
| **2b** | $0.280 \pm 0.200$ | $0.570 \pm 0.260$ | 4.40 μM |
| **2c** | No inhibition | No inhibition | 18.7 μM |
| **2d** | $0.650 \pm 0.210$ | $0.570 \pm 0.040$ | 4.50 μM |
| **2e** | No inhibition | No inhibition | 42.5 μM |
| **2f** | $0.250 \pm 0.040$ | $0.210 \pm 0.001$ | 5.70 μM |
| **2g** | $0.770 \pm 0.090$ | $1.420 \pm 0.530$ | 4.60 μM |
| **2h** | No inhibition | No inhibition | 3.60 μM |
| **2i** | No inhibition | $0.680 \pm 0.220$ | 2.80 μM |
| **$Q_0$** | — | — | PTP-inhibiting[b] |

[a] The $IC_{50}$ values were averaged from three to six independent experiments.
[b] See Ref. 28.

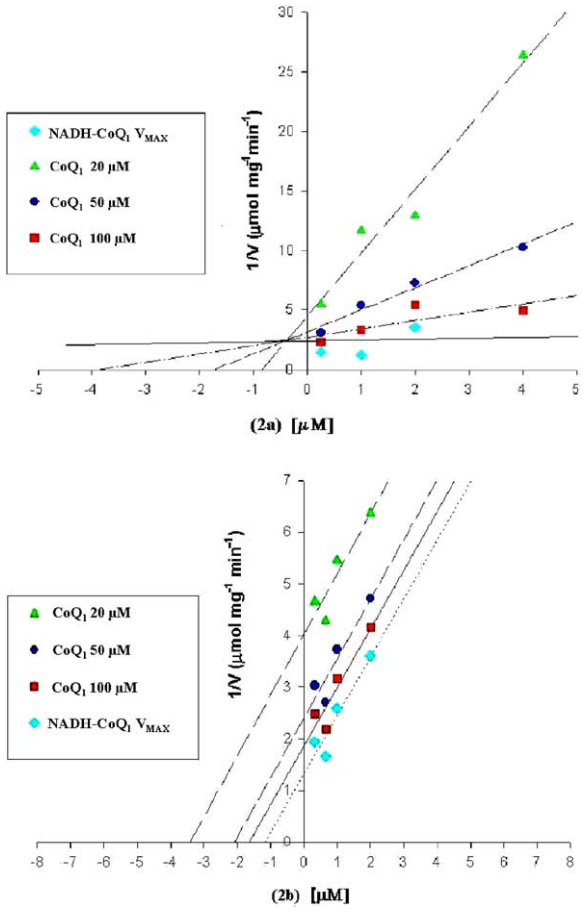


**Figure 1.** Dixon plot of NADH-$CoQ_1$ activity in SMP at varying concentrations. *Up*: compound **2a**: the straight line refers to the NADH-$CoQ_1$ activity in presence of a large excess of $CoQ_1$. This behavior refers to a competitive mechanism of inhibition. *Down*: compound **2b**: the dotted line refers to the NADH-$CoQ_1$ activity in presence of a large excess of $CoQ_1$. This behavior refers to a uncompetitive mechanism of inhibition.

complexes of the respiratory chain located downstream the complex I. With respect to the mechanism of inhibition, it appears that **2a** behaves as a competitive inhibitor of $CoQ_1$, whereas **2b** has an uncompetitive behavior as shown by titration (see Fig. 1); the other show an intermediate mechanism of inhibition.

In the literature there are many reports dealing with the PTP of isolated mitochondria,[27–29,44,45] a field expanded by the observation that the mitochondria soon after isolation begin to swell, lose their ability to phosphorylate and release matrix contents unless $Ca^{2+}$ ions are excluded from the suspension. More recently the PTP has been considered a calcium dependent cyclosporine A-sensitive channel.[29] $CoQ_0$ is a PTP-inhibitor as demonstrated by an analysis of quinone with structural variants useful to identify the features required for the regulation of the PTP, whereas $CoQ_1$ is a PTP-binding analogue that has no effects per se, but counteracts the effects of both inhibitors and inducers of PTP. Moreover, the structure–activity correlations indicate that minor modifications in isoprenoid side chain can turn a PTP-inhibitor into an activator.[27] For the above reasons we tested our derivatives, which have an imidazothiazole system in place of an isoprenoid side chain, on permeability transition of isolated rat liver mitochondria. They turned out to be PTP openers since they were able to induce mitochondrial swelling independently from their inhibitory effect on complex I. As shown in Table 4 the tested derivatives induced 50% mitochondrial swelling at 2.83–5.70 μM with the exception of **2c** and **2e**, which present lower capacity to induce swelling (50% swelling at 18.7 and 42.5 μM, respectively).

In order to explain the remarkably different biological behaviors displayed by compounds **2a**–**i**, we undertook some studies on the geometrical conformations and electrostatic properties of the molecules. In particular, a conformational analysis was carried out on all compounds and, for each of the low energy conformers, we calculated both the energy of their frontier orbitals (HOMO and LUMO) and the two dihedral angles formed by the planes containing the two cyclic moieties (the quinone ring and the imidazothiazole system). From the results obtained (data not shown) no apparent relationship emerged between the calculated stereoelectronic parameters and the biological profile of compounds **2a**–**i** as determined by the above reported assays. This suggests that compounds interact with different binding subsites on the complex target system or, alternatively, that a seemingly unspecific effect (driven at least in part by the hydrophobicity of the compounds) is at the basis of the biological response.

## 4. Conclusion

Most complex I inhibitors are hydrophobic or amphipathic compounds and it seems that they generally act as ubiquinone antagonists. Kinetic studies suggested that these inhibitors can be grouped into three classes represented by piericidin (class I/A-type), rotenone (class II/B-type), and capsaicin (C-type).[10] Direct competition experiments with inhibitors from different classes revealed that they all share one common binding domain with overlapping sites.[24] Radioligand binding and photoaffinity labeling studies confirmed that a wide variety of structurally different inhibitors act at a common binding domain in complex I.[25,46] Since imidazothiazoles also inhibit complex I,[33] we are prompted to imagine that also compounds **2a**–**i** might bind in the large amphipathic pocket, which is thought to accept a plethora of chemically different compounds and to contain the ubiquinone binding site(s) of complex I. The interactions of the different functional groups in the imidazothiazole system with the binding environment of several subunits outlining a cavity-like structure, determine the variation of inhibitory profile from a prevalently competitive inhibition to an almost uncompetitive behavior with respect to the acceptor substrate, $CoQ_1$.

This study shows that when the substituent in position 6 is a phenyl group (**2c**, **2h**) the compounds are no inhibitors and seem to be very poor electron acceptors, but they appear to be able to reach the physiological site(s)

even if the amount of these compounds that can interact with this site is very low as indicated by the low $V_{max}$ induced. Chlorine has the opposite effect since compounds **2a**, **2d**, and **2f** are the best inhibitors of the series and they are also quite weak electron acceptors. In compounds **2b**, **2e**, **2g**, and **2i**, bearing the same substituent in position 6, the behavior is quite different and determined by the substituents at the 2–3 positions.

The polar 1,4-benzoquinone ring of ubiquinones (CoQs) is directly involved in redox reactions. Molecular orbital calculation[47,48] demonstrated that the conformations of the methoxy groups in the 2- and 3-positions affect the electrical potential of the oxidized form of CoQs or semiquinone radicals through conformational interconversion. The presence of this polar ring in compounds **2a**–**i** is responsible for their electron acceptor activity. $k_{cat}/K_m$ ratio shows that complex I has the same 'affinity' for all compounds **2a**–**i** indicating that the higher $V_{max}$ values elicited by some of them are due to their interaction with a non-physiological site. Only compound **2h** seems to be more specific for complex I, in fact its $k_{cat}/K_m$ value is the highest, moreover it is the only compounds showing an 80% of sensitivity to rotenone. From the study of their electrostatic properties, it is evident that the benzoquinone part of the new molecules could be directly involved in redox reactions. In any case, the substitution of the hydrophobic isoprenyl tail of ubiquinones with the hydrophobic imidazothiazole system causes loss of capability to be electron acceptors from complex I, so the tail in the 6 position of the ubiquinone series seems not only to increase the hydrophobicity and to facilitate lateral diffusion in biomembranes[49] but it is also indispensable to ensure a correct interaction with the active site of the enzyme. Our results are in good agreement with the concept of a large and rather unspecific inhibitor binding pocket that could provide an amphipathic 'ramp' guiding ubiquinone from the membrane domain into the catalytic site located in the hydrophilic domain of complex I. In this kind of picture it is possible to guess that the imidazothiazole system interacts with initial part of that amphipathic 'ramp' preventing the interaction of the benzoquinone moiety with the catalytic site (perhaps the methylene bridge is too short). The benzoquinone ring guides the imidazothiazole group to the complex I active site as demonstrated by the lower $IC_{50}$ of these compounds in comparison with that induced by the imidazothiazole system without the quinone ($IC_{50}$ = 0.2–1.5 μM vs 75–80 μM).[34] On the other hand the imidazothiazole system may prevent the reduction of the benzoquinone ring. The molecular skeleton allows for structural modifications that are presently under study to find the distance between the imidazothiazole group and the benzoquinone ring, which reverses the inhibitory effect. This should give us information about the distance between the inhibition and the electron acceptor sites.

From this study it is difficult to say if these new quinone inhibitors **2a**–**i** are able to mediate the observed swelling of mitochondria through a binding to a specific site or to an alteration of the membrane potential. Preliminary results seem to support the hypothesis of a direct interaction with the PTP: in fact all the compounds tested are PTP inducer irrespective of their effect on complex I.

## 5. Experimental

### 5.1. Chemistry

The melting points are uncorrected. Analyses (C, H, N) were within ±0.4% of the theoretical values. Bakerflex plates (silica gel IB2-F) were used for TLC. Kieselgel 60 (Merck) was used for column chromatography. The IR spectra were recorded in Nujol on a Nicolet Avatar 320 E.S.P.; $\nu_{max}$ is expressed in $cm^{-1}$. The $^1H$ NMR spectra were recorded in $(CD_3)_2SO$ on a Varian Gemini (300 MHz); the chemical shift (referenced to solvent signal) is expressed in $\delta$ (ppm) and $J$ in Hz. 2,3-Dimethoxy-5-methyl-1,4-benzoquinone and chloroacetone were purchased from Aldrich. 2-Amino-5-chlorothiazole hydrochloride was purchased from Lancaster. Other chemicals were commercial products of analytical grade.

**5.1.1. Synthesis of 2-chloro-6-methylimidazo[2,1-*b*]thiazole 3i.** 2-Amino-5-chlorothiazole hydrochloride (2.56 g, 15 mmol) was dissolved in water (80 mL) and the solution was cautiously basified by dropwise addition of 15% $NH_4OH$. The obtained precipitate was collected by filtration and dissolved in THF (30 mL). The solution was added of chloroacetone (1.4 mL, 15 mmol) and $NaHCO_3$ (1.2 g, 15 mmol). The reaction mixture was stirred and kept under reflux for 20 h. After cooling THF was evaporated under reduced pressure, the crude product was suspended in water (80 mL) and extracted with $CHCl_3$ (3 × 30 mL). The organic phase was dried over anhydrous sodium sulfate, filtered, and the filtrate was concentrated under reduced pressure to afford an oil (1.4 g) that was used without further purification in the next step. $C_6H_5ClN_2S$ (172.6). $^1H$ NMR: 2.21 (3H, d, $CH_3$, $J$ = 1.2), 7.48 (1H, q, H-5, $J$ = 1.2), 8.15 (1H, s, H-3).

**5.1.2. Synthesis of 2-chloro-6-methylimidazo[2,1-*b*]thiazole-5-carboxaldeyde 4i.** The Vilsmeier reagent was prepared at 0–5 °C by dropping $POCl_3$ (1.8 mL, 20 mmol) into a stirred solution of DMF (1.9 mL, 25 mmol) in $CHCl_3$ (5 mL). Compound **3i** (2 g, 10 mmol) in $CHCl_3$ (60 mL) was added dropwise to the Vilsmeier reagent while maintaining stirring and cooling. The reaction mixture was kept for 3 h at room temperature and under reflux for 8 h. Chloroform was removed under reduced pressure and the resulting oil was poured onto ice. The crude aldehyde **4i** thus obtained was extracted with chloroform and crystallized from ethanol with a yield of 65%. $C_7H_5ClN_2OS$ (200.6) mp 120–122 °C. IR: 1645, 1305, 1260, 885. $^1H$ NMR: 2.55 (3H, s, $CH_3$), 8.53 (1H, s, H-3), 9.83 (1H, s, CHO).

**5.1.3. General procedure for the synthesis of the substituted 5-hydroxymethylimidazo[2,1-*b*]thiazoles 5h–i.** The appropriate aldehyde **4h**–**i** (3 mmol) was dissolved in methanol (250 mL) and treated portionwise, under cooling and stirring, with sodium borohydride (226.9 mg,

6 mmol). Stirring at room temperature was maintained for 3 h, then the reaction mixture was refluxed for 4 h. Methanol was evaporated under reduced pressure and by addition of water the crude hydroxymethyl derivative was obtained (by filtration for compound **5h** and by extraction with chloroform for compound **5i**). These compounds were crystallized from methanol with a yield of 65% for **5h** and 74% for **5i**.

Compound **5h** $C_{12}H_9ClN_2OS$ (264.7) mp 168–171 °C; IR: 3170, 1010, 1000, 765; $^1$H NMR: 4.76 (2H, d, $CH_2$, $J = 5.5$), 5.46 (1H, t, OH, $J = 5.5$), 7.33 (1H, t, ar, $J = 7.3$), 7.45 (2H, t, ar, $J = 7.3$), 7.74 (2H, d, ar, $J = 7.3$), 8.31 (1H, s, H-3).

Compound **5i** $C_7H_7ClN_2OS$ (202.7) mp 167–168 °C dec; IR: 3100, 1305, 995, 790; $^1$H NMR: 2.20 (3H, s, $CH_3$), 4.58 (2H, d, $CH_2$, $J = 5.4$), 5.12 (1H, t, OH, $J = 5.4$), 8.13 (1H, s, H-3).

**5.1.4. General procedure for the synthesis of the substituted 6-imidazo[2,1-*b*]thiazolylmethyl-2,3-dimethoxy-5-methyl-benzoquinones 2a–i.** 2,3-Dimethoxy-5-methyl-1,4-benzoquinone **1** (332.5 mg, 2.1 mmol) was dissolved in 120 mL of diethyl ether and shaken with sodium dithionite (2.78 g, 16 mmol) in 70 mL of water until the solution becomes colorless. The mixture was extracted with diethyl ether. The organic phase was dried over anhydrous sodium sulfate, filtered, and evaporated. The hydroquinone **6**, obtained as colorless crystals, was dissolved in 30 mL of dry dioxane. This solution was added by a syringe in a three necked flask containing a solution of the appropriate 5-hydroxymethylimidazo[2,1-*b*]thiazole **5a**–**i** (2.1 mmol) in 20 mL of dry dioxane stirred in a nitrogen atmosphere at room temperature. The resulting solution was treated with 0.4 mL of boron trifluoride etherate (3.1 mmol) in 10 mL of dry dioxane over 20 min by means of a dropping funnel. The mixture was stirred for 10 h at room temperature, treated with 50 mL of water, and extracted with diethyl ether. The organic phase was dried over anhydrous sodium sulfate, treated with silver(I)oxide (486.65 mg, 2.1 mmol), and stirred for 3 h. The solution was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography or by crystallization (see Table 1). Compounds **2a**–**i** were obtained as orange/red solids with a yield of 15–35%.

### 5.2. Enzyme assays

Submitochondrial particles (SMP) were prepared from beef heart mitochondria (BHM) by sonic irradiation of the frozen and thawed BHM.[50] The particles were essentially broken membrane fragments.[51] Protein was evaluated by the Biuret method of Gornall et al.[52] with addition of 10% sodium deoxycholate and using bovine serum albumine (BSA) as standard. NADH-CoQ reductase was assayed essentially as described by Yagi[53] and modified by Degli Esposti et al.[54] in presence of 2 mM KCN and 2 μM antimycin A to block complex IV and III, respectively. Determination of the kinetic constants was accomplished at quasi saturating concentration of NADH (75 μM) and varying ubiquinone concentrations following the decrease in absorbance in a double wavelength spectrophotometer at 340 − 380 nm and using an extinction coefficient of $3.5\,mM^{-1}cm^{-1}$. NADH-$O_2$ reductase activity was assayed essentially in the same conditions avoiding only KCN and antimycin A in the assay mixture.

### 5.3. Measurement of mitochondrial swelling

Mitochondria were prepared from the livers of male albino Wistar rats (Charles-River, Calco, LC, Italy), weighing 150–175 g essentially as previously described.[55] Rat liver coupled mitochondria permeabilization to sucrose (swelling) was determined by monitoring absorbance changes at 540 nm with a Jasco V-550 spectrophotometer equipped with magnetic stirring and thermostatic control. The incubation medium contained 0.2 M sucrose, 1 mM $KH_2PO_4$, 10 mM Tris–Mops, pH 7.4; 5 mM succinate–Tris, 10 μM EGTA–Tris, $1\,\mu g\,mL^{-1}$ rotenone, $1\,\mu g\,mL^{-1}$ oligomycin; final volume 2 mL at 25 °C. The assay was performed in presence of 0.5 mg mitochondrial protein pre-loaded with 10 μM $Ca^{2+}$ pulse followed by the addition of 0.5 mM EGTA to prevent the redistribution of $Ca^{2+}$ released after partial mitochondrial swelling, then the pore opening was tested monitoring the decrease in absorbance at 540 nm due to the addition of FCCP (carbonylcyanide *p*-trifluorometoxyphenylhydrazone)[56] that induces membrane depolarization; the loss of swelling in presence of $1\,\mu g\,mL^{-1}$ cyclosporin A, a specific inhibitor of the pore opening, is indicative of the involvement of the permeability transition pore in the swelling process. The experiment allows a calibration of the absorbance as a function of the fraction of swollen mitochondria, where zero is the value obtained from mitochondria incubated in the absence of $Ca^{2+}$ and 100 is the value obtained after all of the mitochondria have undergone the transition induced by 200 nM FCCP according to Petronilli et al.[57,58] The fraction of mitochondria with open pore was indicated as $\phi$. The effect of the different compounds on the pore opening was evaluated comparing the fraction of swollen mitochondria, induced by addition of **2a**–**i** to the assay mixture, with that obtained by the addition of 200 nM FCCP.

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