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High-performance liquid chromatographic determination of dihydroorotate dehydrogenase of *Plasmodium falciparum* and effects of antimalarials on enzyme activity

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ABSTRACT

A reversed-phase high-performance liquid chromatographic technique for the determination of dihydroorotate dehydrogenase in *Plasmodium falciparum* was developed. The assay was applied to the evaluation of the effects of several antimalarial drugs on the enzyme. Treatment of both the asexual and gametocyte stages of *P. falciparum* in culture with menoctone, primaquine or the primaquine derivative WR 238605 led to depression of the enzyme activity, although the drugs did not appear to inhibit the enzyme directly.

INTRODUCTION

Dihydroorotate dehydrogenase (DHO-DH) (L-5,6-dihydroorate:oxygen oxidoreductase; EC 1.3.3.1) catalyses the oxidation of L-5,6-dihydroorotic acid to orotic acid, the fourth sequential step in the biosynthesis of pyrimidine nucleotides *de novo*. The enzyme activity has been assayed by both direct measurement of the difference in absorbance of L-dihydroorotic and orotic acids [1,2] and indirect assay of the reduction of 2,6-dichloroindophenol (DCIP) via superoxide [3]. Radiometric assays for DHO-DH have been described using [4-^{14}C]-, [6-^{14}C]- and L-[5,6-^{3}H]dihydroorotate as substrate [4–8]. Peters *et al.* [9] developed a method to assay DHO-DH of rat liver mitochondria by high-performance liquid chromatography (HPLC) using an anion-exchange column (Partisil-SAX) with UV detection.

This paper reports the development of a sensitive reversed-phase HPLC assay method for DHO-DH. The method was used to measure the enzyme activity in *Plasmodium falciparum* and to measure the effects of treatment of the parasites with some antimalarials such as primaquine or a

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novel primaquine derivative WR 238605 (1), which may work through their action on the parasite mitochondria, where the enzyme is presumably located.

EXPERIMENTAL

Materials

PIC A reagent was obtained from Waters Assoc., dihydroorotic acid, orotic acid, uridine 5′-monophosphate (UMP), 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), sodium hydrogencarbonate, potassium hydroxide and ammonium dihydrogenphosphate from Sigma, powdered RPMI-1640 from Gibco, methanol from J. T. Baker and [^{3}H]hypoxanthine from Amersham. Antimalarials were obtained from the Walter Reed Army Institute of Research (Washington, DC, USA).

Parasite preparation

P. falciparum was cultivated in type O red cells according to the method described by Trager and Jensen [10] using RPMI-1640 supplemented with 10% (v/v) of human O serum, 25 m*M* HEPES and 32 m*M* $NaHCO_3$ containing 50 μg/ml hypoxanthine (dissolved in $NaHCO_3$) to enhance the growth of the parasite. Some cultures were synchronized by sorbitol lysis as described by Lambros and Vanderberg [11] when most of the parasites were at the ring stage and when parasitaemia was about 10–20%. The parasites were harvested at the late trophozoite stage when the parasitaemia was about 20–40%. The parasitized cells were obtained by centrifugation at 1000 *g* for 5 min (Tomy centrifuge). The packed parasitized cells were lysed by addition of saponin. The intact parasites were then resuspended in 10 m*M* HEPES–KOH buffer (pH 8.0) at a dilution of 1:3 and kept in ice before use immediately for DHO-DH analysis.

Gametocytes used in this study were provided by Dr. Katchrinnee Pavanand (Department of Immunology, Armed Forces Research Institute of Medical Science, Bangkok, Thailand).

Protein determination

Protein was determined by a method modified for membrane and lipoprotein samples in the presence of sodium dodecyl sulphate [12]. Albumin was used as a standard.

Assay of dihydroorotate dehydrogenase (DHO-DH)

The reaction mixture consisted of 180 μl of DHO (180 μ*M*) in 10 m*M* HEPES–KOH buffer (pH 8.0) and 20 μl of intact parasites. The reaction mixture was incubated at 37°C for 25 min, then the reaction was stopped by boiling in a water-bath for another 10 min. The mixture was centrifuged at 7000 *g* for 5 min (Tomy centrifuge) and the supernatant was subjected to HPLC analysis. Blanks were performed simultaneously but the intact parasites were added to the reaction mixture just before boiling. The chromatographic system consisted of Waters Model 510 pumps connected with a Rheodyne Model 7125 injector, Waters Model 680 automated gradient controller, JASCO UVIDEC-100 IV detector and LDC Milton-Roy integrator. The column used was a pre-packed Waters μBondapak C_{18} (10 μm) reversed-phase column (100 mm × 3.9 mm I.D.) with a Corasil μBondapak C_{18} (27–40) guard column. The mobile phase consisted of 3 m*M* PIC A in 5 m*M* ammonium dihydrogenphosphate (pH 6.0) containing 5% of methanol. Separation was by isocratic elution at a flow-rate of 1.0 ml/min. Both dihydroorotate and orotate were monitored by UV absorption at 230 nm.

The molarity of the components of the mobile phase, ammonium dihydrogenphosphate, PIC A and methanol, was determined on the basis of capacity factor (k') and response factor by assay of parasite DHO-DH at various concentrations of each component. The final appropriate molarity was a compromise between the highest values of both k' and the response factor of each component.

In some experiments the DHO-DH activity of the malaria parasite was determined by a modification of the method described by Gero *et al.* [13].

Effects of antimalarials on asexual parasites and gametocytes

The cultured parasitized cells, synchronized at the ring stage, 20–30% parasitaemia, were resuspended to about 2% haematocrit with prewarmed complete RPMI-1640 medium. The cell suspension, 5 ml each, was added to plastic culture plates (10 × 30 mm) containing the various concentrations of each antimalarial or inhibitor. The cell suspension was then cultured in a candle jar as described above at 38°C for 24 h. The parasitized cells were harvested and then DHO-DH activities determined. In some experiments, the exposure time of the parasites to inhibitors was 4 h. This was performed as described above but the parasitized cells were synchronized at the trophozoite stage.

Gametocyte cultures, enriched for stage 5 and mature forms, were exposed to the antimalarials for 4 or 24 h. Further experiments were performed as described above for asexual parasites.

In vitro assay of P. falciparum viability by incorporation of [^{3}H]hypoxanthine

Parasite viability was measured by a modification of the method of Desjardins *et al.* [14]. For 24-h drug exposure, the parasitized cells, synchronized at the ring stage, with 20–30% parasitaemia, were diluted with complete RPMI-1640 medium to about 1.25% haematocrit. The diluted cell suspensions were transferred to 24-well sterile plastic plates (1 ml per well). Various concentrations of drugs were added to each well before incubation in a candle jar at 38°C for 24 h. After 24 h, the medium was carefully removed from the cell layer in each well. The cells were then resuspended in a new RPMI-1640 medium containing [^{3}H]hypoxanthine to about 2% haematocrit before aliquots were taken and placed in a flat-bottomed, 96-well plate (200 μl per well) and cultured for 4 h before harvest. The cells were lysed with distilled water and nucleic acids were trapped on glass filters (Whatman 934 AH). Each filter was placed in a microfuge tube and dried at 50°C for 1 h. Scintillation fluid, containing 0.35% (w/v) 2,5-diphenyloxazol (PPO) and 0.05% (w/v) 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) in toluene, was added to each tube (0.6 ml each). The microfuge tube was placed in a plastic vial and the radioactivity was measured with a Beckman Model LS-1801 liquid scintillation counter.

For 4-h drug exposure, the parasitized cells, synchronized at mature trophozoite stage, were incubated in RPMI-1640 medium containing various concentrations of drugs for 4 h before incorporation of [^{3}H]hypoxanthine as described above.

RESULTS

As shown in Fig. 1A, under the HPLC conditions developed for the DHO-DH assay, standard dihydroorotate and orotate were clearly

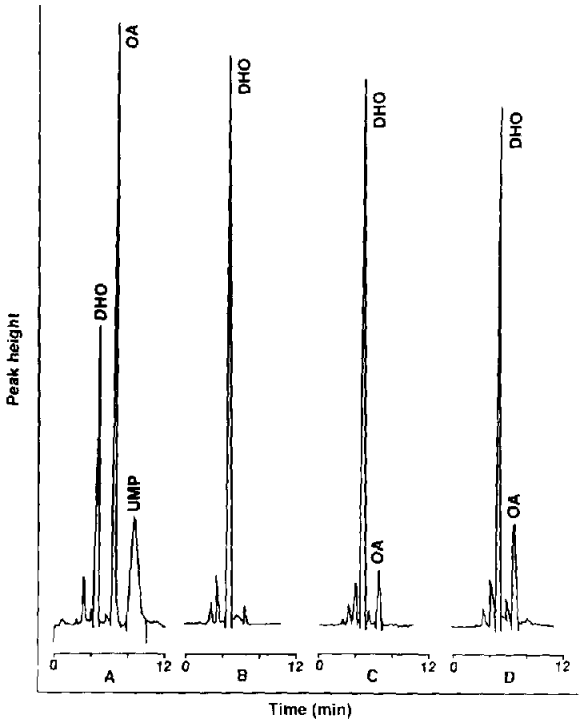

Fig. 1. HPLC profile at 230 nm of (A) authentic dihydroorotic acid (DHO), orotic acid (OA) and uridine monophosphate (UMP) and (B–D) decreasing amount of substrate (DHO) and increasing of the product (OA) during assay of parasite DHO-DH at (B) 0, (C) 10 and (D) 30 min. Column, μBondapak C_{18}; mobile phase, 3 m*M* PIC A–5 m*M* ammonium dihydrogenphosphate–5% methanol; flow-rate 1.0 ml/min.

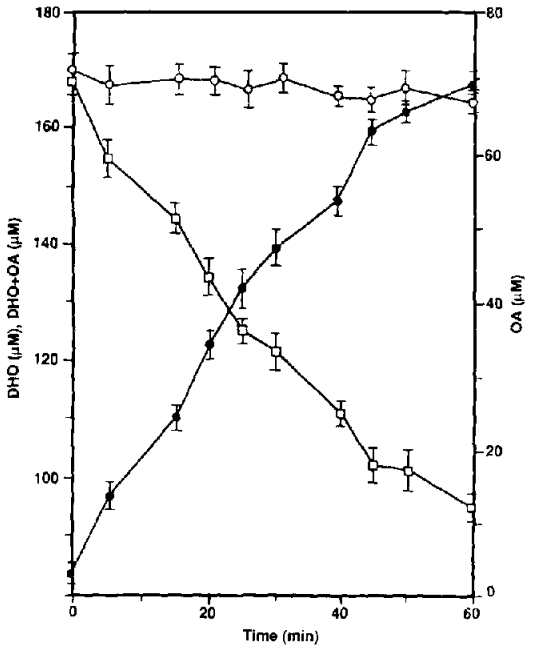

Fig. 2. Relationship between the amounts of substrate (dihydroorotic acid; DHO) and product (orotic acid; OA) at various time intervals during determination of parasite DHO-DH activity. The data shown are the average values of duplicate experiments, with bars indicating individual values. □ = DHO; ● = OA; ○ = sum of DHO and OA.

separated (dihydroorotate 4.9 min, orotate 6.8 min). Further, UMP, the end product of subsequent catalyses by orotate phosphoribosyltransferase and OMP decarboxylase, was also clearly separated (8.9 min). Using these conditions, DHO-DH from intact parasites was determined over a duration of 30 min. Fig. 1B, C and D show the levels of decreasing dihydroorotate and increasing orotate as functions of time. UMP was not found in the reaction. When the concentrations of both dihydroorotate and orotate in the assay mixture were measured at time intervals of 0–60 min, decreasing levels of dihydroorotate corresponded to increasing levels of orotate and the sums of dihydroorotate and orotate levels were approximately constant and equal to the original dihydroorotate level (Fig. 2).

Fig. 3A shows a linear correlation between parasite protein concentration and DHO-DH activity. The DHO-DH activity of the parasite also increased linearly with percentage parasitaemia (Fig. 3B), indicating that the enzyme activity detected was indeed of parasite origin.

The DHO-DH activities at various asexual stages and the gametocyte stage were determined by the newly developed HPLC method. The results in Table I show that the DHO-DH activity was highest in schizonts, followed by trophozoites and ring forms. Gametocytes contain approximately the same level of enzyme activity as trophozoites. The kinetic parameters of parasite DHO-DH were assessed using synchronized parasites at the mature trophozoite stage (Fig. 4). The Michaelis constant (K_m) of parasite DHO-DH was calculated to be 12.1 ± 1.7 mM and maximum velocity (V_{max}) was 75 nmol/h · mg protein.

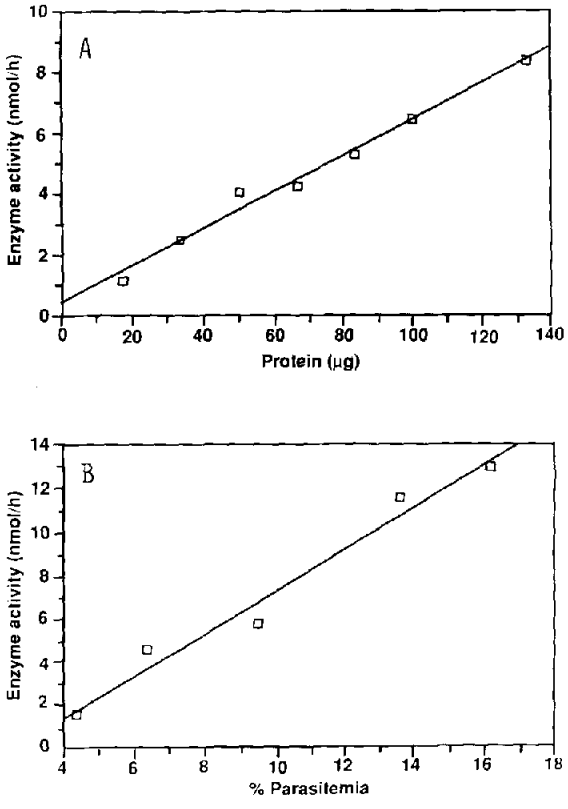

Fig. 3. Correlation between (A) DHO-DH activity and parasite protein concentration and (B) DHO-DH activity and percentage parasitaemia. Data are average values of duplicate experiments.

TABLE I

DHO-DH ACTIVITY OF *P. FALCIPARUM* AT VARIOUS MATURATION STAGES

Parasite stage		DHO-DH activity (nmol/h · mg protein)
Uninfected erythrocytes		Not detectable
Ring forms	(n = 5)	24.7 ± 2.3[a]
Trophozoites	(n = 5)	66.9 ± 13.4[a]
Schizonts	(n = 5)	93.6 ± 7.1[a]
Gametocytes	(n = 2)	70.3 ± 8.9[b]

[a] Mean ± S.D.
[b] Average ± range.

Using this HPLC assay, the effects of several antimalarials on DHO-DH activity were tested. As shown in Table II, there was no significant direct effect on isolated DHO-DH activity of these antimalarials at concentrations inhibitory to the parasites.

In order to study the time sequence of the effects of drugs on the parasite DHO-DH activity and their relation to parasite viability, the time courses of the enzyme inhibition were compared with those for hypoxanthine incorporation into the parasite. Fig. 5 shows that, with asexual stages after 4-h exposure, menoctone at concentrations up to 10^{-4} *M* effectively inhibited the DHO-DH whereas hypoxanthine incorporation was little affected. Pyrimethamine showed little effect on DHO-DH activity or viability except at high concentration. Primaquine and I showed up to 100% inhibition of DHO-DH activity at 10^{-4} *M*. Fig. 6 shows the effect after 4 h of these agents on the sexual (gametocyte) stage of *P. falciparum*. All agents showed a dose-dependent inhibition over the range of concentrations tested. I was more active than primaquine, and both 8-aminoquinolines showed greater inhibition than the diaminopyrimidine pyrimethamine.

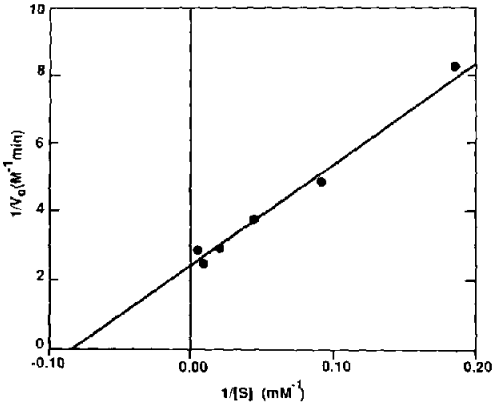

Fig. 4. Lineweaver–Burk plot for the determination of K_m and V_{max} of DHO-DH from *P. falciparum* (trophozoite stage).

DISCUSSION

The proposed HPLC method for the assay of DHO-DH compares favourably with previously published methods. Early methods for the measurement of the enzyme from eukaryotic sources [1,2] employed the difference in UV absorbance of dihydroorotate and orotate, but were hampered by low sensitivity. An indirect assay was later developed based on dichloroindophenol reduction [3]. Radiometric assays have also been described, using either [4-^{14}C]dihydroorotate or [6-^{14}C]dihydroorotate as substrate and following the formation of radioactive orotate after separation either by electrophoresis [4,5] or chromatography [6,7]. Another radiometric assay [8] used [5,6-^{3}H]dihydroorotate as substrate and followed the release of tritiated water from its oxidation. Peters *et al.* [9] described an assay based

TABLE II

EFFECTS OF ANTIMALARIALS AND CYANIDE ON PARASITE DHO-DH ACTIVITY

Drug[a]	Concentration (M)	Specific activity (nmol/h · mg protein)	Activity (%)
None	–	65.04 ± 7.8	100
Mefloquine	$1.2 \cdot 10^{-7}$	67.8 ± 5.1	104.2
Halofantrine	$1.5 \cdot 10^{-8}$	63.78 ± 7.37	98.1
Primaquine	$1.1 \cdot 10^{-5}$	64.6 ± 2.73	99.32
I	$9.6 \cdot 10^{-7}$	66.34 ± 5.7	102.0
Chloroquine	$1.6 \cdot 10^{-6}$	65.5 ± 7.2	100.7
Cyanide	$1.0 \cdot 10^{-3}$	26.1 ± 6.15	40.13[b]

[a] $n = 5$.

[b] $p < 0.002$.

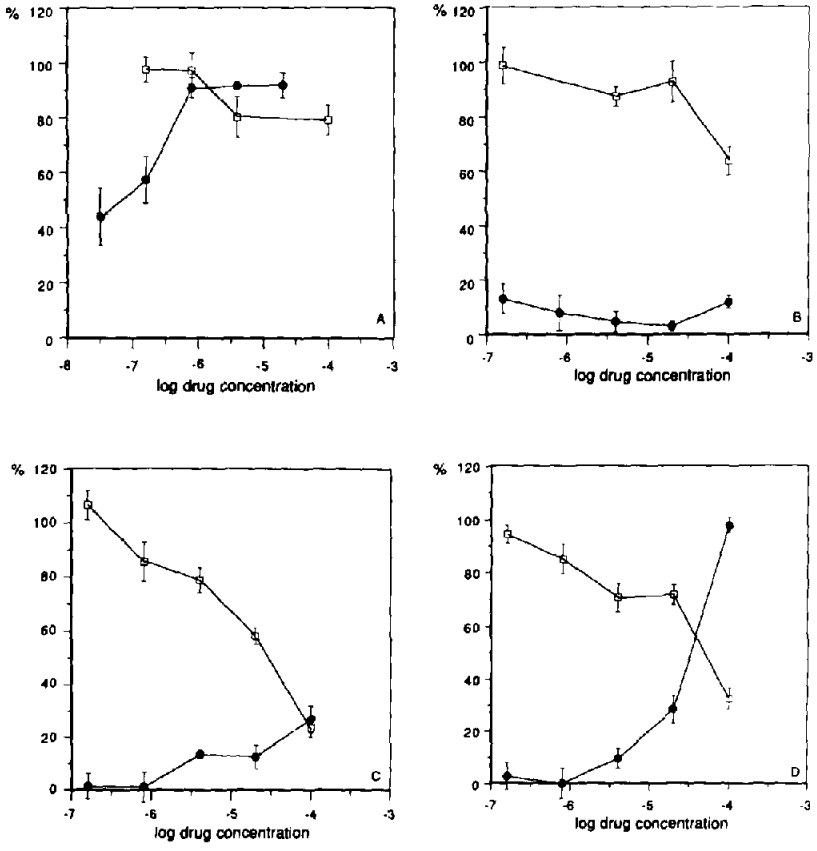

Fig. 5. Inhibition of DHO-DH activity and of hypoxanthine incorporation of *P. falciparum* (asexual intraerythrocytic stage) after cultivation for 4 h in the presence of (A) menoctone, (B) pyrimethamine, (C) primaquine and (D) I. Data are averages ± range of duplicate experiments. (□ = percentage hypoxanthine incorporation; ● = percentage DHO-DH inhibition.

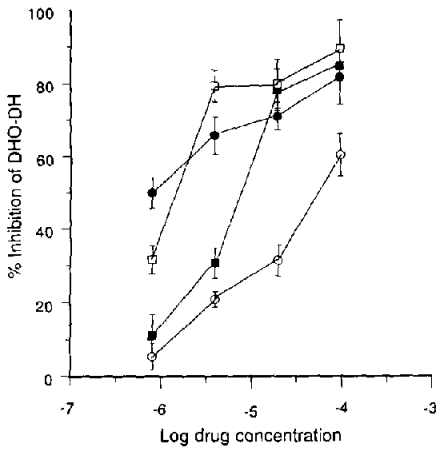

Fig. 6. Inhibition of DHO-DH activity of the gametocyte stage of *P. falciparum* after cultivation for 4 h in the presence of menoctone (●), pyrimethamine (○), primaquine (■) and I (□). Data are averages ± range of duplicate experiments.

on HPLC separation of the reaction mixture on an anion-exchange column and measurement of absorption of orotate at 280 nm. The method described here uses a simple and sensitive procedure, is rapid and reproducible and is suitable for measurement of low enzyme activities such as that present in the malarial parasites. By following both the formation of orotate and the disappearance of dihydroorotate at 230 nm, it was shown that the sum of the two remained constant over a long incubation time (Fig. 2), hence confirming the validity of the assay and ruling out complications of possible subsequent reactions catalysed by other enzymes which might be present in the reaction mixture. Failure to detect UMP, the end-product of the pyrimidine pathway, also strengthens this conclusion.

The detected DHO-DH activity in *P. falciparum*-infected erythrocytes can be ascribed to the parasite only, as the activity was linearly correlated with parasite protein concentration and percentage parasitaemia (Fig. 3) and was undetectable in uninfected erythrocytes. The activity was dependent on the stage of maturation of the asexual parasite, reaching a maximum at the schizont stage (Table I). The activities reported here are comparable with those previously reported from *P. berghei* [13,15,16] and *P. falciparum* [17], and the K_m value for dihydroorotate (12.1 m*M*) for the trophozoite stage of *P. falciparum* is comparable to the values of 23 m*M* [15] and 7.9 m*M* [16] reported for *P. berghei*.

With the proposed method it was possible to examine the effect of antimalarials on DHO-DH activity. Although incubation of the parasite extract with many antimalarials (Table II), as previously shown with tetracycline [17], resulted in no inhibition of the enzyme, treatment of the parasite in culture with some of these antimalarials led to depression of its activity. Fig. 5 shows that treatment with I or, to a lesser extent, primaquine at therapeutic concentrations led to a substantial decrease in DHO-DH activity, as also did menoctone, which had been shown to work through inhibition of this enzyme [18]. The decrease in enzyme activity occurred concurrently with a decrease in hypoxanthine incorporation into the parasites, which was a measure of their viability. In contrast, pyrimethamine treatment led to a much lower decrease in the enzyme activity. It is noteworthy that the depression of DHO-DH activity on incubation of the parasites with primaquine and I occurred to a much greater extent with gametocytes (Fig. 6) than with asexual forms (Fig. 5), an effect which may be related to the previous observation that 8-aminoquinolines have a greater killing effect on gametocytes than on the asexual forms [19].

It has been proposed previously that primaquine and derivatives act primarily on the mitochondria of the parasite [20], and that the action of 8-aminoquinolines may be related to parasite mitochondrial electron transport [21]. As DHO-DH is a mitochondrion-associated enzyme [13,15,16], our observation that treatment of the parasites with primaquine and I led to depression of the enzyme activity lends further support to the hypothesis that these drugs may be targeted to the mitochondria of the malarial parasites. Although the drugs do not inhibit DHO-DH directly, impairment of mitochondrial integrity might lead subsequently to the reduction of the enzyme activity, as previously observed for tetracycline [17].

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