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Research paper

Ligand-based design, synthesis and biochemical evaluation of potent and selective inhibitors of *Schistosoma mansoni* dihydroorotate dehydrogenase

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ABSTRACT

Schistosomiasis ranks second only to malaria as the most common parasitic disease worldwide. 700 million people are at risk and 240 million are already infected. Praziquantel is the anthelmintic of choice but decreasing efficacy has already been documented. In this work, we exploited the inhibition of *Schistosoma mansoni* dihydroorotate dehydrogenase (SmDHODH) as a strategy to develop new therapeutics to fight schistosomiasis. A series of quinones (atovaquone derivatives and precursors) was evaluated regarding potency and selectivity against both SmDHODH and human DHODH. The best compound identified is **17** (2-hydroxy-3-isopentylnaphthalene-1,4-dione) with $IC_{50} = 23 \pm 4$ nM and selectivity index of 30.83. Some of the new compounds are useful pharmacological tools and represent new lead structures for further optimization.

1. Introduction

On the bright side, globalization can be considered a set of processes that afford easy access to knowledge, economic stability, and social equality [1]. However, it has also contributed to spread some of the deadliest infectious diseases across wide geographic areas. Malaria, for instance, has crossed the African borders and became global as humans migrated to other continents and parasites adapted to different mosquito species that were evolutionarily distant from African vectors [2]. Ebola virus outbreak, which killed more than 11,000 people and infected at least 28,000, is a stark reminder of the world health security fragility [3]. A recent example of the negative impact of globalization is the spread of Zika virus from Uganda in 1947 [4,5] to other African countries within a few years, then to the Micronesia in 2007, to finally arrive at Americas late December 2015 [6]. In Brazil, for instance, more than 1.5 million cases may have occurred, being the largest outbreak of Zika in human history [7]. Despite the fact that Zika has drawn attention to flavivirus infections, which were largely forgotten by the pharmaceutical industry, the scenario for other neglected diseases remains unchanged. To make matters worse, neglected tropical diseases, previously found in developing countries, also became widespread posing a serious challenge to the health systems of developed countries that receive immigrants and refugees from endemic areas [8,9]. Schistosomiasis, for instance, is a chronic, debilitating disease caused by blood-dwelling trematodes of the genus *Schistosoma*. It

Abbreviations: DHODH, Dihydroorotate dehydrogenase; DHO, Dihydroorotate; ORO, Orotate; SmDHODH, *Schistosoma mansoni* dihydroorotate dehydrogenase; HsDHODH, *Homo sapiens* dihydroorotate dehydrogenase; DCIP, Dichlorophenolindophenol; IC_{50}, Concentration responsible to inhibit 50% of the enzymatic reaction; CoQ_0, Coenzyme Q_0; TLC, Thin layer chromatography; NMR, Nuclear magnetic resonance; HRMS, High resolution mass spectrometry; TOF, Time-of-flight; ESI, Electrospray ionization; GC, Gas chromatography; EI, Electron impact ionization.

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affects over 200 million people in more than 70 countries, including sub-Saharan Africa, the Middle East, Southwest Asia and parts of South America [10,11]. Although this disease is not endemic in other regions, cases have already been reported in Scotland [12], France, Germany, Italy [13,14] and China [15]. The only drug available to treat the patients (praziquantel) has been on the market for over 50 years and parasite resistance is becoming a significant issue in some areas [16,17]. Thus, the development of novel drugs to fight schistosomiasis is of utmost importance.

In order to accomplish this goal, one can take advantage of drug repositioning and/or drug repurposing strategies. Drug repositioning consists of evaluating and/or using existing drugs for the treatment of diseases other than those for which they were originally developed [18]. In addition, drug repurposing involves the re-exploration of existing compounds that failed approval for therapeutic indications [19]. The fact that these compounds have already been through regulatory clinical trials suggests that the drug development timeline, from bench to market, can be not only shortened but characterized by smaller attrition rates [20].

In this work, we intend to take advantage of the current knowledge and investment in the development of antimalarial drugs to search for alternative strategies to combat schistosomiasis. In particular, we focused on the enzyme dihydroorotate dehydrogenase, a validated target against malaria, and its current known inhibitors, as a starting point for the development of anti-schistosomiasis therapeutics.

Dihydroorotate dehydrogenase (DHODH) is a flavin-dependent enzyme and a validated target for anti-inflammatory, immunosuppressive, anti-proliferative and anti-parasitic drug development [21–23]. This enzyme catalyzes the stereospecific oxidation of *S*-dihydroorotate (DHO) to orotate (ORO) in the fourth of the six conserved enzymatic steps involved in the *de novo* pyrimidine biosynthetic pathway.

DHODHs can be categorized into two classes according to their structural features and cellular location [24]. DHODH from humans, *S. mansoni, P. falciparum,* and *Escherichia coli* belong to class 2 DHODHs [25]. Such enzymes are monomeric proteins that are bound to the mitochondria inner membrane of eukaryotes [26] and cytosolic membrane of some prokaryotes, such as the Gram-negative bacteria *E. coli* [27], and require quinones as their physiological oxidizing agent.

As expected, class 2 DHODHs are inhibited by quinone derivatives [28,29]. Interestingly, chemically similar compounds also display cercaricidal activity [30]: Plumbagin, a naphthoquinone isolated from *Plumbago scandens,* [10] norobtusifolin and kwanzoquinone E, anthraquinones isolated from *Hemerocallis fulva* [31,32], resulted in either the mortality or the immobilization of *S. mansoni* cercariae. Likewise, lapachol and isolapachol derivatives have shown activity against different life cycle stages of *S. mansoni* [33].

The relevance of DHODH in the *de novo* pyrimidine biosynthesis, the coupling of class 2 DHODH catalytic reaction to ubiquinone reduction, which links the pyrimidine biosynthetic pathway to the mitochondrial respiratory chain, along with the previous findings regarding the sensitivity of *Schistosoma* species to quinone derivatives points this enzyme as a potential druggable target for the development of new therapies against schistosomiasis. In the present study, we synthesized and evaluated 34 quinone derivatives as putative SmDHODH inhibitors.

2. Results and discussion

Chemistry. A group of 34 compounds was evaluated as possible inhibitors of SmDHODH and HsDHODH enzymes. The compounds were clustered into five series (named A – E) according to their chemical similarity (Fig. 1): A – controls are well-known DHODH inhibitors, B – 2-hydroxy-3-amine-naphthoquinones were designed by molecular simplification and ring isosterism to atovaquone, C – 1,4-naphthoquinones derivatives were designed to evaluate the importance of 2-OH, D – Lapachol and its derivatives, and E – Benzoquinones. Compounds from groups A and E, as well as Lawsone **10**, were commercially available. The other compounds were synthesized as described in this work.

Considering atovaquone **2** as the starting structure, a previously described strategy of ring bioisosterism and molecular simplification [34] was applied to synthesize quinoidal derivatives (Scheme 1) and evaluate the different amine substitution at the quinoidal ring. We initially synthesized epoxide **5** to carry out a ring-opening reaction with several amines toward substituted 2-hydroxynaphthoquinones. Compounds **6a-e** synthesis was carried out in water with addition of Lewis acid to catalyze the reaction. Compounds **6f-o** were obtained in good yields only when isopropyl alcohol was used as solvent and additional time was given to complete conversion of **5**. In both conditions, anilines with electron withdrawing group at *ortho* and *meta* positions (**6c**, **6e**, **6i**, and **6k**) afforded poor yields in comparison to the other electron-rich amine groups used.

Serie C (Fig. 1) was designed to evaluate the importance of the hydroxyl group at C-2 position regarding DHODH inhibition. For these compounds, 3-amino-naphthoquinone derivatives were synthesized from 2,3-dichloro-1,4-naphthoquinone **7** (Scheme 2). Compound **8**, after several attempts, was obtained in low yields from the reaction of **7** and 2-chloro-aniline, in water, when zinc chloride was added to the reaction mixture. Refluxing **7** in dimethylformamide (DMF) unexpectedly produced the amino derivative **9**. DMF is known to suffer thermolysis or hydrolysis in high temperature to give dimethylamine (DMA) [5] that substitutes the halogen at C-2 of **7**. Radical demethylation catalyzed by light is suggested to be responsible for the formation of **9**, according to previously described findings [35].

Compounds **11** and **12** of Series C were synthesized from Lawsone **10** (Scheme 3) in good yields, following previously described methods [36,37]. Lawsone was also used to synthesize Lapachol **13** and isoLapachol **14** (Scheme 3), in moderate yields, using prenyl bromide and isovaleraldehyde, respectively, as previously described [38,39]. Lapachol derivatives were synthesized as depicted in Scheme 3. Efficient substitutions at C-2-hydroxyl group were carried out in good yields when tosyl chloride (**15**) and iodoacetonitrile (**16**) were employed. Reduced Lapachol **17** and alpha-Lapachol **18** were also obtained from Lapachol using palladium catalyzed reductive hydrogenation and acid-mediated heterocyclization, respectively.

SmDHODH Inhibition Assay. Activity measurements were carried out by indirect assay, monitoring DCIP reduction at 610 nm [40,41]. The choice of this method was due to the fact that most compounds absorb close to orotate maximum absorption wavelength (300 nm). Initial screening of approximately 200-compounds (data not shown) in single dose concentration (50–500 μM, according to compounds solubility limit) allowed us to select a subset of derivatives that inhibit SmDHODH (Table 1).

Compounds that reduced SmDHODH activity by more than 60% in the single concentration assays had their IC_{50} values (Table 2) determined against SmDHODH and its human homologue enzyme HsDHODH. All IC_{50} graphs are present in the supplementary material (Figs. S1 to S49).

Among the tested compounds, **2**, **6c**, **6e**, **6i**, **6j**, **6k**, **6l**, **6m**, **6n**, **6o**, **13**, and **17** showed nanomolar IC_{50} values against SmDHODH. Both Atovaquone **2** ($IC_{50} = 432 \pm 21$ nM) and **6m** ($IC_{50} = 227 \pm 24$ nM) have aliphatic rings (cyclohexane and piperazine ring, respectively) directly attached to the quinoidal moiety and terminal p-Cl substituted aromatic ring. As both compounds have similar steric

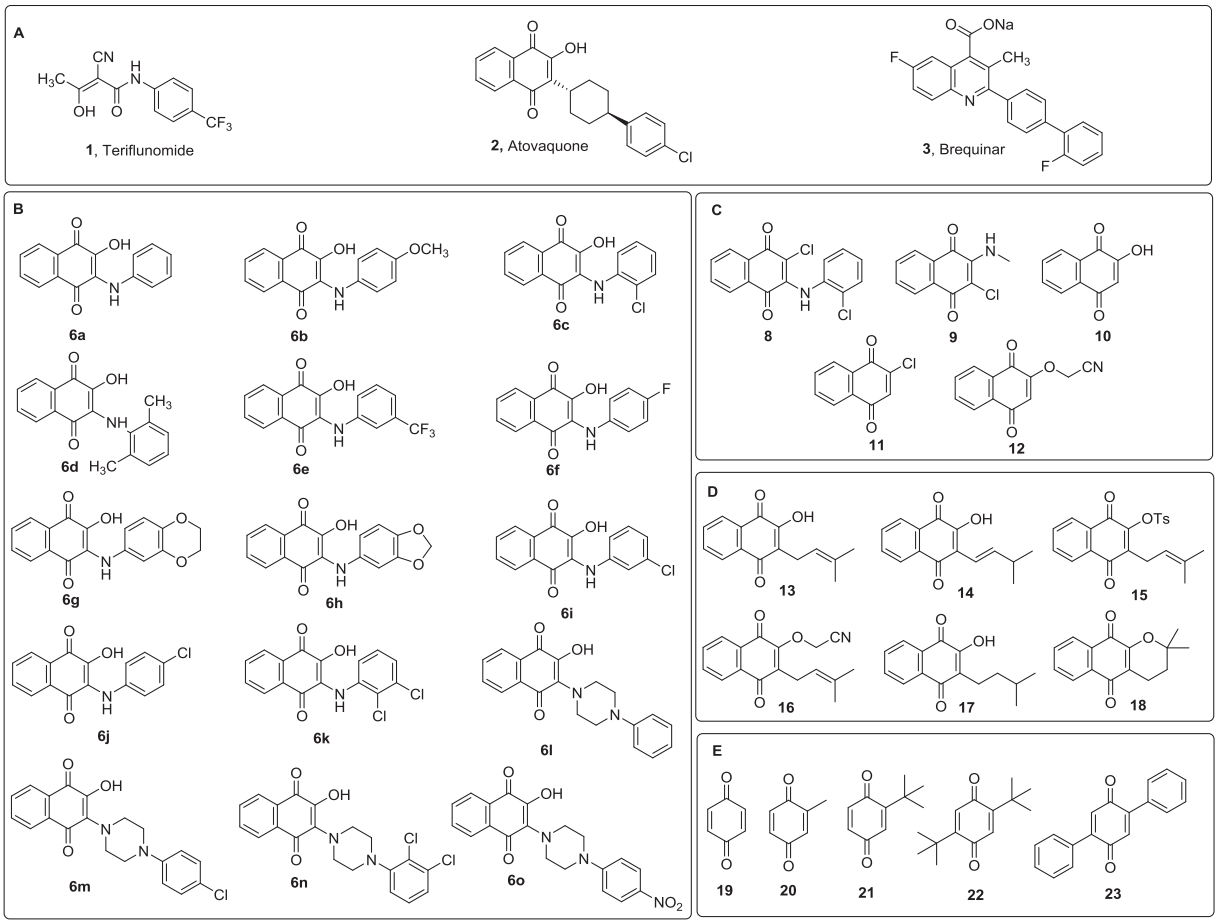


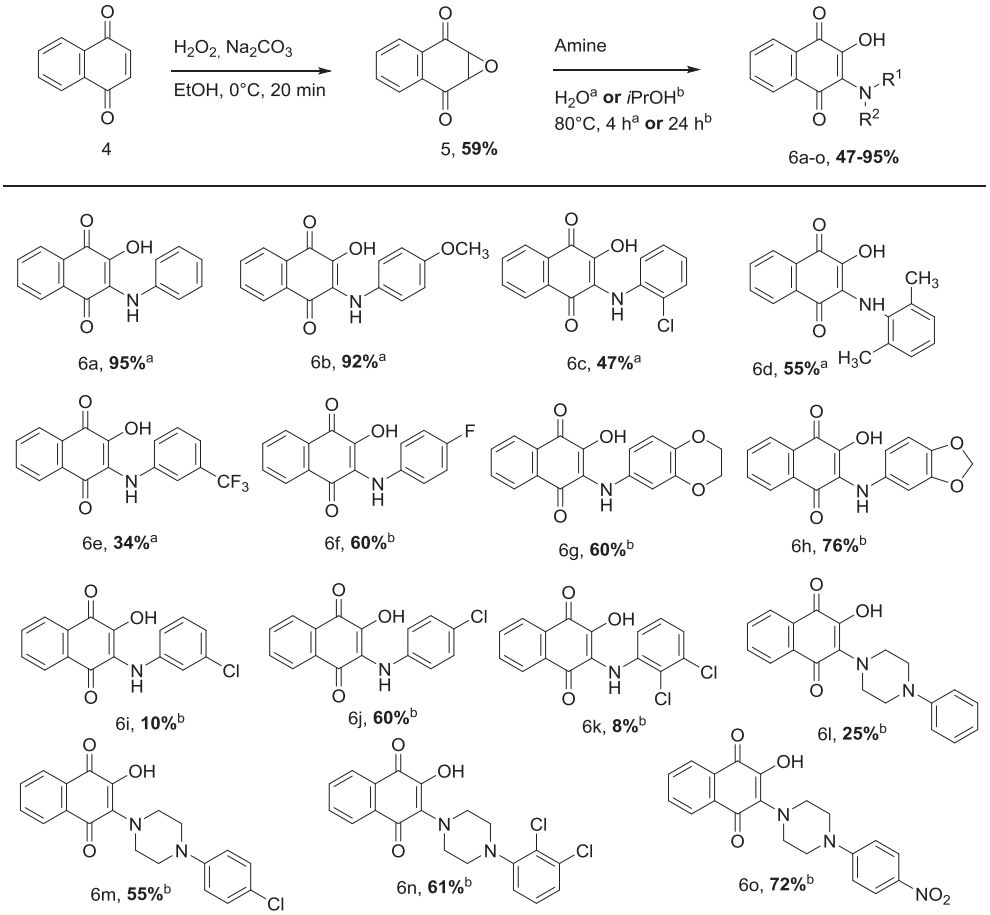
Fig. 1. Compounds tested as Sm/HsDHODH inhibitors grouped according to their chemical scaffold. A – Known DHODH inhibitors. B – 2-hydroxy-3-amine-naphthoquinones. C – 1,4-naphthoquinones derivatives. D – Lapachol and its derivatives. E – Benzoquinones.

features, it seems that electronic properties are responsible for the potency gain (approx. 2X) of **6m**. However, lack of substituents in the aromatic ring (**6l** $IC_{50} = 711 \pm 23$ nM) seems to be detrimental to potency. This result suggests that steric complementarity towards SmDHODH is best with chlorine at *para* position (compare **6m** *vs* **6n** or **6m** *vs* **6o**). In order to further investigate the steric requirements for SmDHODH inhibition, 2-hydroxy-3-amine-naphthoquinone derivatives **6a-k** were evaluated. Although nitrogen spacing group lead to a 10-fold reduction in potency (**6a** $IC_{50} = 2100 \pm 200$ nM), chlorine substitution at *ortho* (**6c** $IC_{50} = 78 \pm 9$ nM) or *meta* (**6i** $IC_{50} = 129 \pm 10$ nM) yield simplified and yet more potent compounds than **6m**. In fact, the additive effect of these substituents leads to the most potent compound of this series (**6k** $IC_{50} = 19 \pm 2$ nM). Interestingly, electron donor groups at *para* position are detrimental to potency, (**6b** $IC_{50} = 4700 \pm 400$ nM) whereas electron withdrawing groups retain potency (**6j** $IC_{50} = 1850 \pm 10$ nM). This result also supports that electronic features play a key role in SmDHODH inhibition. It is possible that the increased potency is due to activation of quinoidal ring by nitrogen electron donating effect. However, the potency drops due to the substitution of quinoidal OH by Cl (**8** -SmDHODH activity $= 63.1 \pm 0.5\%$ at 50 μM *vs* **6c** $IC_{50} = 78 \pm 9$ nM), suggesting that other features also play key roles in SmDHODH inhibition. Moreover, the presence of non-substituted OH in position 3 affords only weak SmDHODH inhibitors. For instance, lawsone **10**, a natural hydroxynaphthoquinone, shows $IC_{50} = 29{,}000 \pm 3000$ nM. This result suggests that the inhibitory activity reported herein cannot be explained by an irreversible mechanism of action due to the Michael addition to the quinoidal ring.

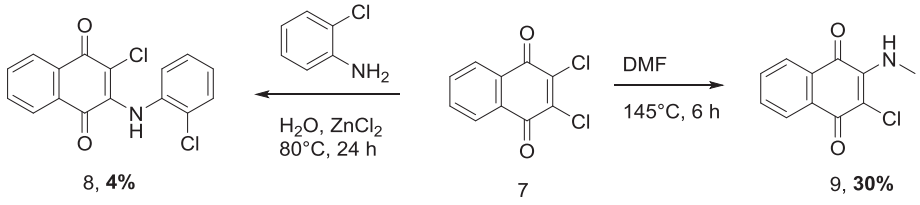
It is also reasonable to assume that changes in the potency, among compounds of series 6, might be due to the improved hydrophobic interaction of the aromatic ring, instead of piperazine ring, within SmDHODH binding site. In order to evaluate this hypothesis, series D compounds were assayed against SmDHODH. When the quinoidal OH is protected, or not available for hydrogen bonding, only micromolar inhibition of SmDHODH is achieved (**15** $IC_{50} = 31{,}400 \pm 900$ nM and **16** $IC_{50} = 2500 \pm 100$ nM). On the other hand, both **13** ($IC_{50} = 19 \pm 2$ nM) and **17** ($IC_{50} = 23 \pm 4$ nM) are as potent SmDHODH inhibitors as **6k** ($IC_{50} = 19 \pm 2$ nM).

Lapachol derivatives, with constrained side-chain, show reduced potency against SmDHODH (**14** $IC_{50} = 1200 \pm 100$ nM and **18** SmDHODH activity $= 68.1 \pm 0.8\%$ at 500 μM). Although it is tempting to assume that rigid analogs fail to adopt the bioactive conformation, it is also possible that electronic features are responsible for this decrease in potency: the double bond in the side chain of isolapachol **14** is conjugated to the quinoidal ring, whereas the double bond of Lapachol **13** is not. Therefore, both **13** and **17** have more electron deficient side chains than **14**. Furthermore, the hydrophobicity of these side-chains is similar to the one found in ubiquinone (SmDHODH substrate).

HsDHODH Inhibition Assay and Selectivity. When the macromolecular target is also present in the host, drug development efforts must balance potency and selectivity if a high-quality lead compound is desired. Accordingly, all compounds with IC_{50} values



Scheme 1. Synthesis of 2-hydroxy-3-amino-1,4-naphthoquinones series. [a]reaction performed in water (10 mL) using **5** (1 mmol), $ZnCl_2$ (1 equiv) at 80 °C for 4 h [b]reaction performed in *i*PrOH (10 mL) using **5** (1 mmol) at 80 °C for 24 h.

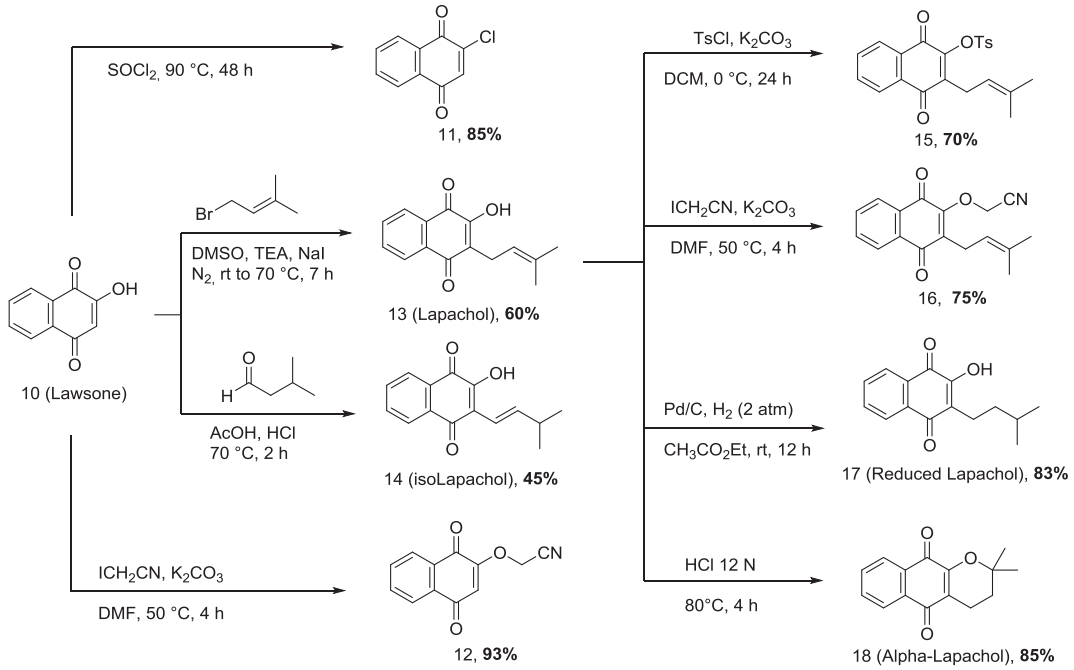


Scheme 2. Synthesis of 2-chloro-3-amino-1,4-naphthoquinones series.

against SmDHODH lower than 50 μM, in previous assays, had their inhibition profile against HsDHODH investigated. The IC_{50} values (Table 2) against the human enzyme show that 2-hidroxy-3-amine-naphthoquinone derivatives **6f** and **6g** have low selectivity for the human enzyme, whereas **6a**, **6b**, **6d**, **6e**, **6h**, and **6o** inhibit both parasitic and human DHODH likewise. The most potent compound against SmDHODH (**6k**- SI = 6.47) is less selective than either **6c** (SI = 10.36) or **6m** (SI = 20.26). In fact, **6c** is approximately 66% more selective and 5 fold more potent than atovaquone **2**. Then, this compound can be considered the most promising lead compound from series B.

Among lapachol derivatives (series D), only tosylated lapachol shows a minor selectivity index (SI) towards HsDHODH, whereas the reduced lapachol **17** presented the largest SI (30.83). The comparison of **13** and **17** IC_{50} values for HsDHODH (100 ± 7 nM vs 709 ± 38 nM, respectively) shows that one double bond is responsible for an approximately 6-fold increase in selectivity. As none of these compounds have a side chain conjugated to the quinoidal ring, it is reasonable to conclude that side-chain flexibility is crucial to the selective inhibition of SmDHODH. This hypothesis is in good agreement with **6m** higher selectivity than **6c**: Intra-molecular hydrogen-bonding in **6c** might restrain its conformational space, whereas **6m** would explore the torsional space around the single bond that links the piperazine ring (chair conformation) to the quinoidal ring. Thus, the *para* substituted phenyl ring can be predicted positioning in dissimilar pockets in human or parasitic DHODH, providing a higher selectivity profile. Overall, **17** was considered the best lead compound for SmDHODH selective inhibition.

Inhibition mode determination. Despite the chemical similarity among the described inhibitors, there was no proof, up to this point, that all of them have a similar binding profile towards their



Scheme 3. Synthesis of Lawsone and Lapachol derivatives.

Table 1
Results from single-concentration screening assays against SmDHODH, compounds codes, concentration and percent of activity.

Code	Concentration (μM)	% Activity	Code	Concentration (μM)	% Activity
1 (Teriflunomide)	500	6.1 ± 0.2	**6o**	250	1.80 ± 0.01
2 (Atovaquone)	500	1.0 ± 0.5	**8**	50	63.1 ± 0.5
3 (Brequinar)	500	4.4 ± 0.4	**9**	250	31 ± 1
6a	500	1.64 ± 0.05	**10**	500	9.7 ± 0.3
6b	250	3.8 ± 0.2	**11**	250	46 ± 1
6c	250	2.0 ± 0.6	**12**	500	54 ± 1
6d	500	6 ± 2	**13**	250	0.0 ± 0.2
6e	50	1.5 ± 0.4	**14**	62.5	2.9 ± 0.2
6f	50	1.8 ± 0.2	**15**	500	7.9 ± 0.2
6g	50	2.6 ± 0.1	**16**	500	4.0 ± 0.2
6h	50	3.5 ± 0.2	**17**	125	2 ± 1
6i	50	1.8 ± 0.6	**18**	500	68.1 ± 0.8
6j	50	1.4 ± 0.7	**19**	500	−1.3 ± 0.8
6k	50	1.2 ± 0.5	**20**	500	3.6 ± 0.3
6l	50	3.3 ± 0.3	**21**	500	20.6 ± 0.5
6m	50	2.7 ± 0.2	**22**	500	94 ± 1
6n	50	1.9 ± 0.2	**23**	500	86 ± 3

macromolecular target. In order to further investigate this matter, one compound from each class (Group A – **2**, B – **6c**, C – **10**, D – **17** and E – **19**) had its mode of inhibition studied. Double reciprocal plots were employed to analyze the effect of increasing inhibitor concentrations over *Sm*DHODH K_M and V_{max} (Supplementary Material, Figs. S50–S55). When increasing concentrations of compound **19** are added to the reaction, the substrate affinity towards SmDHODH decreases, whereas k_{cat} remains constant. This result suggests a competitive inhibition mechanism, which is expected due to the high structural similarity between this inhibitor and the ubiquinone substrate CoQ_0 (a benzoquinone, such as compound **19**). On the other hand, double reciprocal plot for compounds **6c**, **17**, and **10** show that substrate affinity towards SmDHODH remains unchanged, whereas the slope of the curve increases, suggesting a non-competitive inhibition mechanism. Compound **2** shows a mixed-type inhibition, a mixture of both competitive and non-competitive events, where both k_{cat} and substrate affinity changes with increasing inhibitor concentration. Aiming to evaluate if this behavior is observed with the human DHODH, compound **17** (best lead compound for SmDHODH selective inhibition) was tested against that enzyme; it presented the same mechanism of inhibition as the parasitic enzyme, suggesting that despite differences between human and parasitic DHODHs, the inhibitors' binding mode might be similar.

In order to explain the non-competitive inhibition mechanism identified for the majority of the compounds, analysis of class 2 DHODH overall structure was undertaken. The analysis of the tridimensional structures available for other class 2 DHODHs, reveals that the tunnel created by the two N-terminal helices, reported to be the class 2 inhibitor binding site and where CoQ_0 is expected to bind, is deep. Small compounds, such as compound **19**, are able to go through all the way and get in close proximity to the FMN prosthetic group, which explains its competitive mechanism of inhibition against CoQ_0. The other non-competitive compounds might interact in the entrance of the tunnel, without reaching CoQ_0 binding site. A second hypothesis could rely on the existence of a

Table 2
IC_{50} values and selectivity index obtained for both SmDHODH and HsDHODH.

Compound	SmDHODH IC_{50} (nM)	*Hs*DHODH IC_{50} (nM)	Selectivity Index
1 (Teriflunomide)	50,000 ± 2000	312 ± 27	160 (Hs)
2 (Atovaquone)	432 ± 21	2700 ± 200	6.25 (Sm)
3 (Brequinar)	20,000 ± 1000	37 ± 2	540.54 (Hs)
6a	2100 ± 200	2600 ± 500	–
6b	4700 ± 400	n.d.	–
6c	78 ± 9	808 ± 62	10.36 (Sm)
6d	9900 ± 600	10,000 ± 1000	–
6e	111 ± 6	108 ± 8	–
6f	1000 ± 40	330 ± 12	3.03 (Hs)
6g	2100 ± 100	1050 ± 40	2.00 (Hs)
6h	5500 ± 100	3600 ± 100	–
6i	129 ± 10	911 ± 34	7.06 (Sm)
6j	185 ± 10	541 ± 24	2.92 (Sm)
6k	19 ± 2	123 ± 11	6.47 (Sm)
6l	711 ± 23	6800 ± 100	9.56 (Sm)
6m	227 ± 24	4600 ± 100	20.26 (Sm)
6n	436 ± 19	1800 ± 100	4.13 (Sm)
6o	375 ± 27	n.d.	–
9	79,000 ± 14,000	n.p.	–
10	29,000 ± 3000	n.d.	–
13	19 ± 2	100 ± 7	5.26 (Sm)
14	1200 ± 100	10,000 ± 900	8.33 (Sm)
15	31,400 ± 900	7900 ± 900	3.97 (Hs)
16	2500 ± 100	n.d.	–
17	23 ± 4	709 ± 38	30.83 (Sm)
19	8800 ± 400	34,000 ± 2000	3.86 (Sm)
20	57,000 ± 2000	n.p.	–
21	768,000 ± 679,000	n.p.	–

n.d. – not detected at the highest tested concentration; n.p. – not performed; selectivity index of each compound was calculated by IC_{50}/IC_{50} (higher/smaller), when above 2.00.

second binding pocket created by an extended loop present in SmDHODH but not in other class 2 DHODHs [41]. The crystallization of SmDHODH bound to these compounds, the evaluation of different quinones, and different constructs (deletion and mutations of residues) are currently in progress to evaluate this matter.

3. Conclusion

In conclusion, a series of atovaquone precursors and derivatives were synthesized and evaluated as inhibitors of the enzyme dihydroorotate dehydrogenase from *Schistosoma mansoni* and *Homo sapiens*. In addition, commercially available compounds were investigated. The most potent compounds against SmDHODH exhibited IC_{50} values of 19 nM (2-((2,3-dichlorophenyl)amino)-3-hydroxynaphthalene-1,4-dione, **6k**), 19 nM (2-hydroxy-3-(3-methylbut-2-en-1-yl)naphthalene-1,4-dione, **13**), 23 nM (2-hydroxy-3-isopentylnaphthalene-1,4-dione, **17**), and 78 nM (2-((2-chlorophenyl)amino)-3-hydroxynaphthalene-1,4-dione, **6c**).

Selectivity was also evaluated by using inhibition tests against the human homologue enzyme (HsDHODH). Some compounds, such as **6f**, **6g**, and **15**, present low selectivity for the human enzyme, whereas **6a**, **6b**, **6d**, **6e**, **6h**, and **6o** inhibit both parasitic and human enzymes. On the other hand, compounds **2**, **6c**, **6i**, **6j**, **6k**, **6l**, **6m**, **6n**, **6o**, **13**, and **17** display selectivity index towards the parasitic enzyme, of which compound **17** presented the largest SI (30.83). The comparison of **13** and **17** IC_{50} values for HsDHODH (100 ± 7 nM *vs* 709 ± 38 nM, respectively) shows that one double bond is responsible for approximately 6-fold increase in selectivity. Our findings bring an important contribution for the development of lead compounds against schistosomiasis.

4. Experimental section

General Remarks for Chemistry. Compounds **1** (Purity 98%), **2** (Purity 98%), **3** (Purity 97%), **10** (Purity 97%), **19** (Purity 98%), **20** (Purity 98%), **Fe-20** (Purity 98%), **21** (Purity 98%), **22** (Purity 99%), and **23** (Purity 98%) were acquired from Sigma-Aldrich. Other commercially available reagents and solvents were used in the reactions without further purification. Melting points were determined in open capillary tubes using an electronic apparatus. Yields refer to isolated and purified products, unless otherwise noted. Reactions were monitored by thin layer chromatography (TLC) and visualized under UV (Ultraviolet) light at 254 and 365 nm. Column chromatography was performed using silica gel 60 (70–230 mesh). ^1H and ^{13}C NMR spectra were recorded at 300, 400 or 500 MHz and 75, 101 or 126 MHz, respectively. Chemical shifts were referenced to the deuterated solvent (i.e., for $CDCl_3$, $\delta = 7.26$ and 77.16; for DMSO-d_6, $\delta = 2.50$ and 39.52, for ^1H and ^{13}C NMR, respectively) and are reported in parts per million (ppm, δ). Coupling constants (J) are stated in Hz using the splitting abbreviations: s, singlet; d, doublet; t, triplet; m, multiple. High-resolution mass spectra (HRMS) were measured by a TOF spectrometer, using electrospray ionization (ESI). The purity of synthesized compounds was determined by gas chromatography (GC) analyses performed on a GC system coupled to a mass-selective detector with electron impact ionization (EI) and the samples showed more than 95% of purity.

1a,7a-dihydronaphtho[2,3-b]oxirene-2,7-dione (**5**): To a solution of 1,4-naphthoquinone (4.0 g, 25.3 mmol, 1 equiv) in ethanol (120 mL) at 0 °C was dropwise added hydrogen peroxide 35% in water (8 mL, 92 mmol, 3.6 equiv) followed by dropwise addition of sodium carbonate (2.68 g, 25.3 mmol, 1 equiv) solution in water (8 mL). The mixture was stirred at 0 °C for 20 min followed by addition of water (150 mL). The precipitated compound was filtered, taken up with ethyl acetate (100 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel with hexane/ethyl acetate (9:1) to afford the desired product as white crystals (2.61 g, 15 mmol, 59%); mp 134–136 °C; ^1H NMR (300 MHz, DMSO-d_6)

δ 7.95–7.85 (m, 4H), 4.17 (s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 190.7, 134.7, 131.4, 126.5, 55.3. The spectral data are in accordance with those reported in the literature [42].

General procedure for reaction of 5 with amines in water: To a suspension of **5** (174 mg, 1 mmol, 1 equiv) in water (10 mL) was added the amine (1.2 mmol, 1.2 equiv) and $ZnCl_2$ (136 mg, 1 mmol, 1 equiv). The mixture was stirred at 80 °C for 4 h. After cooling, the precipitated compound was filtered, taken up with ethyl acetate (100 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel with hexane/ethyl acetate (9:1) to afford the desired product.

2-hydroxy-3-(phenylamino)naphthalene-1,4-dione (**6a**): 252 mg (95%); dark purple solid, mp 216–218 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (ddd, J = 6.7, 2.7, 1.4 Hz, 2H), 7.68 (dt, J = 7.5, 1.7, 2H), 7.35–7.28 (m, 2H), 7.12–7.06 (m, 1H), 7.04–6.98 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.5, 179.6, 138.9, 136.4, 134.1, 133.7, 130.9, 130.5, 128.4, 126.8, 126.1, 124.9, 123.4, 121.6. HRMS (+ESI) m/z: $[M + H]^+$ calcd for $C_{16}H_{12}NO_3^+$: 266.0812; Found: 266.0805.

2-hydroxy-3-((4-methoxyphenyl)amino)naphthalene-1,4-dione (**6b**): 271 mg (92%); dark purple solid, mp 140–142 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.93 (s, 1H), 7.93 (dd, J = 6.8, 1.8 Hz, 2H), 7.90 (s, 1H), 7.81–7.68 (m, 2H), 6.86 (d, J = 8.9 Hz, 2H), 6.78 (d, J = 8.9 Hz, 2H), 3.70 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 182.1, 178.6, 154.2, 139.4, 134.1, 133.8, 133.2, 130.7, 130.6, 126.7, 125.6, 125.2, 121.9, 113.0, 55.2. HRMS (+ESI) m/z: $[M + H]^+$ calcd for $C_{17}H_{14}NO_4^+$: 296.0917; Found: 296.0918.

*2-((2-chlorophenyl)amino)-3-hydroxynaphthalene-1,4-dion**e*** (**6c**) 151 mg (47%); dark purple solid, mp 154–155 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.61 (s, 1H), 8.00–7.95 (m, 2H), 7.84–7.75 (m, 2H), 7.40 (dd, J = 7.9, 1.2 Hz, 1H), 7.20 (t, J = 7.7 Hz, 1H), 7.08 (s, 1H), 6.95 (td, J = 7.9, 1.4 Hz, 1H), 6.86 (d, J = 8.1 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 219.2, 217.2, 180.1, 175.4, 171.5, 171.4, 168.3, 168.1, 166.4, 164.5, 163.4, 163.2, 162.6, 161.1, 160.0, 159.4; HRMS (+ESI) m/z: $[M + H]^+$ calcd for $C_{16}H_{11}ClNaNO_3^+$: 322.0241; Found: 322.0241.

2-((2,6-dimethylphenyl)amino)-3-hydroxynaphthalene-1,4-dione (**6d**) 161 mg (55%); dark purple solid, mp 150–152 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.91 (s, 1H), 7.92–7.84 (m, 2H), 7.71 (pd, J = 7.5, 1.7 Hz, 2H), 7.64 (s, 1H), 6.99 (s, 3H), 2.14 (s, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.6, 177.9, 138.8, 136.4, 135.2, 134.0, 132.9, 130.7, 130.2, 129.9, 127.1, 125.4, 124.9, 18.4. HRMS (+ESI) m/z: $[M + H]^+$ calcd for $C_{18}H_{16}NO_3^+$: 294.1125; Found: 294.1124.

2-hydroxy-3-((3-(trifluoromethyl)phenyl)amino)naphthalene-1,4-dione (**6e**); 112 mg (34%); dark purple solid, mp 219–221 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.72 (s, 1H), 8.33 (s, 1H), 8.02–7.93 (m, 2H), 7.80–7.77 (m, 2H), 7.37 (t, J = 7.9 Hz, 1H), 7.16 (s, 1H), 7.12 (d, J = 7.8 Hz, 1H), 7.07 (d, J = 8.2 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 182.3, 180.1, 143.7, 142.8, 134.2, 134.1, 131.3, 130.9, 129.1 (d, J = 31.3 Hz), 129.1, 126.1, 125.9, 125.0, 124.8 (d, J = 272.3 Hz), 122.7, 116.3 (d, J = 4.2 Hz), 115.3 (d, J = 3.9 Hz); HRMS (-ESI) m/z: $[M - H]^-$ calcd for $C_{17}H_9F_3NO_3^-$: 332.0540; Found: 332.0534.

General procedure for reaction of 5 with amines in isopropyl alcohol: To a suspension of **5** (174 mg, 1 mmol, 1 equiv) in isopropyl alcohol (10 mL) was added the amine (1,2 mmol, 1.2 equiv) and the mixture was stirred at 80 °C for 24 h. After cooling, the mixture reaction was concentrated to dryness and the residue was purified by column chromatography on silica gel with hexane/ethyl acetate (8:2) to afford the desired product.

2-((4-fluorophenyl)amino)-3-hydroxynaphthalene-1,4-dione (**6f**); 169 mg (60%); dark purple solid, mp 201–203 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.21 (s, 1H), 8.03 (s, 1H), 7.97–7.93 (m, 2H), 7.80–7.72 (m, 2H), 7.01 (dt, J = 12.3, 2.8 Hz, 2H), 6.89 (ddd, J = 6.9, 5.3, 2.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 182.0, 179.1, 157.0 (d, J = 236.9 Hz), 140.8, 137.5, 133.8, 133.4, 130.7, 130.6, 126.0, 125.6, 125.3, 121.2 (d, J = 7.9 Hz), 114.2 (d, J = 22.3 Hz). HRMS (-ESI) m/z: $[M - H]^-$ calcd for $C_{16}H_9FNO_3^-$: 282.0572; Found: 282.0564.

2-((2,3-dihydrobenzo[b][1,4]dioxin-6-yl)amino)-3-hydroxynaphthalene-1,4-dione (**6g**); 192 mg (60%); dark purple solid, mp 180–182 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.98 (s, 1H), 7.95–7.91 (m, 2H), 7.82 (s, 1H), 7.79–7.70 (m, 2H), 6.67 (d, J = 8.5 Hz, 1H), 6.43 (dd, J = 8.5, 2.6 Hz, 1H), 6.40 (d, J = 2.4 Hz, 1H), 4.18 (q, J = 4.8 Hz, 4H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 182.1, 178.8, 142.4, 140.1, 138.1, 134.9, 133.8, 133.3, 130.7, 126.3, 125.6, 125.2, 115.8, 113.7, 109.2, 64.1, 63.9; HRMS (-ESI) m/z: $[M - H]^-$ calcd for $C_{18}H_{12}NO_5^-$: 322.0721; Found: 322.0718.

2-(benzo[d][1,3]dioxol-5-ylamino)-3-hydroxynaphthalene-1,4-dione (**6h**): 233 mg (76%); dark purple solid, mp 162–164 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.02 (s, 1H), 7.95–7.92 (m, 2H), 7.89 (s, 1H), 7.79–7.71 (m, 2H), 6.75 (d, J = 8.3 Hz, 1H), 6.53 (d, J = 2.2 Hz, 1H), 6.41 (dd, J = 8.3, 2.2 Hz, 1H), 5.94 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 182.0, 178.9, 146.6, 141.7, 140.1, 135.8, 133.7, 133.3, 130.7, 126.4, 125.6, 125.2, 113.0, 107.2, 102.6, 100.6; HRMS (+ESI) m/z: $[M + H]^+$ calcd for $C_{17}H_{12}NO_5^+$: 310.0710; Found: 310.0705.

2-((3-chlorophenyl)amino)-3-hydroxynaphthalene-1,4-dione (**6i**): 30 mg (10%); dark purple solid, mp 209–211 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.61 (s, 1H), 8.17 (s, 1H), 7.96 (s, 2H), 7.78 (s, 2H), 7.17 (t, J = 7.0 Hz, 1H), 6.84 (d, J = 7.5 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.9, 179.7, 143.3, 143.1, 133.7 (2), 132.4, 130.9, 130.5, 129.2, 125.7, 125.4, 124.6, 119.4, 118.2, 117.4; HRMS (+ESI) m/z: $[M + H]^+$ calcd for $C_{16}H_{11}ClNO_3^+$: 300.0422; Found: 300.0416.

2-((4-chlorophenyl)amino)-3-hydroxynaphthalene-1,4-dione (**6j**): 180 mg (60%); dark purple solid, mp 226–228 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.13 (s, 1H), 7.95 (d, J = 2.1 Hz, 2H), 7.77 (d, J = 3.8 Hz, 2H), 7.20 (d, J = 8.5 Hz, 2H), 6.85 (d, J = 8.6 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.9, 179.5, 142.4, 140.5, 133.7, 133.6, 130.9, 130.5, 127.5 (2), 125.7, 125.4, 125.2, 123.8, 120.7 (2); HRMS (+ESI) m/z: $[M + H]^+$ calcd for $C_{16}H_{11}ClNO_3^+$: 300.0422; Found: 300.0417.

2-((2,3-dichlorophenyl)amino)-3-hydroxynaphthalene-1,4-dione (**6k**): 27 mg (8%); dark purple solid, mp 201–203 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.84 (s, 1H), 7.98 (dd, J = 8.3, 5.2 Hz, 2H), 7.80 (dd, J = 5.4, 3.1 Hz, 2H), 7.23 (s, 1H), 7.17 (t, J = 7.6 Hz, 2H), 6.81 (dd, J = 7.3, 1.9 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.3, 180.0, 144.4, 140.4, 134.0, 133.8, 131.2, 130.8, 130.5, 127.3, 125.8, 125.6, 124.4, 122.1, 121.2, 119.6; HRMS (+ESI) m/z: $[M + H]^+$ calcd for $C_{16}H_{10}Cl_2NO_3^+$: 334.0032; Found: 334.0032.

2-hydroxy-3-(4-phenylpiperazin-1-yl)naphthalene-1,4-dione (**6l**): 83 mg (25%); dark purple solid, mp 78–80 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.01 (d, J = 6.4 Hz, 2H), 7.70–7.58 (m, 2H), 7.30 (dd, J = 8.2, 7.5 Hz, 2H), 6.98 (d, J = 8.4 Hz, 2H), 6.91 (t, J = 7.2 Hz, 1H), 3.76 (s, 4H), 3.40–3.33 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 180.6, 180.5, 151.2, 133.9, 133.1, 132.4, 130.16, 130.10, 129.3 (2), 126.6, 126.0, 125.8, 120.5, 116.7 (2), 50.5 (2), 50.1 (2); HRMS (+ESI) m/z: $[M + H]^+$ calcd for $C_{20}H_{19}N_2O_3^+$: 335.1390; Found: 335.1397.

2-(4-(4-chlorophenyl)piperazin-1-yl)-3-hydroxynaphthalene-1,4-dione (**6m**): 202 mg (55%); dark purple solid, mp 172–174 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.92 (dd, J = 15.3, 7.4 Hz, 2H), 7.73 (dt, J = 22.0, 6.8 Hz, 2H), 7.26 (d, J = 8.8 Hz, 2H), 7.00 (d, J = 8.9 Hz, 2H), 3.62 (s, 4H), 3.41 (s, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.8, 177.8, 149.3, 134.1, 134.0, 132.59, 132.50, 130.4, 128.7 (2), 126.7, 125.7, 125.4, 122.8, 117.2 (2), 49.6 (2), 47.3 (2); HRMS (+ESI) m/z: $[M + H]^+$ calcd for $C_{20}H_{18}ClN_2O_3^+$: 369.1000; Found: 369.1001.

2-(4-(2,3-dichlorophenyl)piperazin-1-yl)-3-hydroxynaphthalene-1,4-dione (**6n**); 244 mg (61%); dark purple solid, mp 208–209 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.94 (s, 1H), 7.95 (d, J = 7.3 Hz, 1H), 7.90 (d, J = 7.2 Hz, 1H), 7.77 (t, J = 7.0 Hz, 1H), 7.70 (t, J = 7.3 Hz, 1H), 7.34 (d, J = 4.6 Hz, 2H), 7.19 (t, J = 4.7 Hz, 1H), 3.66 (s, 4H), 3.23 (s, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.7, 178.2, 156.6, 150.7, 134.0, 132.7, 132.55, 132.51, 130.3, 128.6, 127.3, 126.1, 125.7, 125.4, 124.7, 119.7,

50.27, 50.21; HRMS (+ESI) *m/z*: $[M + H]^+$ calcd for $C_{20}H_{17}Cl_2N_2O_3^+$: 403.0611; Found: 403.0609.

2-hydroxy-3-(4-(4-nitrophenyl)piperazin-1-yl*)naphthalene-1,4-dione* (**6o**): 273 mg (72%); dark purple solid, mp 208–209 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.08 (d, $J = 9.3$ Hz, 2H), 7.91 (t, $J = 7.6$ Hz, 2H), 7.80–7.66 (m, 2H), 7.09 (d, $J = 9.4$ Hz, 2H), 3.70 (s, 4H), 3.56 (s, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.2, 179.4, 154.4, 137.17, 137.13, 133.9, 132.7, 132.2, 130.3, 130.2, 128.8, 125.7, 125.3, 112.9, 49.2, 46.2; HRMS (+ESI) *m/z*: $[M + H]^+$ calcd for $C_{20}H_{18}N_3O_5^+$: 380.1241; Found: 380.1242.

2-chloro-3-((2-chlorophenyl)amino)naphthalene-1,4-dione (**8**): To a suspension of 2,3-dichloro-1,4-naphthoquinone (226 mg, 1 mmol, 1 equiv) in water (10 mL) was added the 2-chloroaniline (126 μL, 1,2 mmol, 1.2 equiv) and $ZnCl_2$ (136 mg, 1 mmol, 1 equiv). The mixture was stirred at 80 °C for 24 h. After cooling, the mixture reaction was concentrated to dryness and the residue was purified by column chromatography on silica gel with hexane/ethyl acetate (9:1) to afford the desired product as an orange solid (14 mg, 0.04 mmol, 4%); mp 140–142 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.19 (s, 1H), 8.02 (dd, $J = 7.5$, 1.3 Hz, 2H), 7.87 (td, $J = 7.5$, 1.3 Hz, 1H), 7.80 (td, $J = 7.4$, 1.2 Hz, 1H), 7.51–7.47 (m, 1H), 7.38–7.33 (m, 2H), 7.33–7.25 (m, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 179.4, 176.5, 143.7, 136.6, 134.9, 133.3, 131.7, 130.0, 129.9, 129.0, 128.5, 127.6, 127.0, 126.5, 126.1, 113.1; HRMS (+ESI) *m/z*: $[M + H]^+$ calcd for $C_{16}H_{10}Cl_2NO_2^+$: 318.0083; Found: 318.0077.

2-chloro-3-(methylamino)naphthalene-1,4-dione (**9**): A solution of 2,3-dichloro-1,4-naphthoquinone (452 mg, 2 mmol, 1 equiv) in DMF (20 mL) was stirred at 145 °C for 6 h. After cooling, the mixture reaction was concentrated to dryness and the residue was purified by column chromatography on silica gel with hexane/ethyl acetate (19:1) to afford the desired product as red solid (132 mg, 0.6 mmol, 30%); mp 67–69 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.16 (dd, $J = 7.7$, 1.0 Hz, 1H), 8.03 (dd, $J = 7.6$, 1.1 Hz, 1H), 7.74 (td, $J = 7.6$, 1.4 Hz, 1H), 7.63 (td, $J = 7.5$, 1.3 Hz, 1H), 6.13 (s, 1H), 3.46 (d, $J = 5.8$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 180.6, 177.0, 144.9, 135.1, 132.8, 132.5, 129.8, 126.96, 126.90, 32.7; HRMS (+ESI) *m/z*: $[M + H]^+$ calcd for $C_{11}H_9ClNO_2^+$: 222.0316; Found: 222.0311.

2-chloronaphthalene-1,4-dione (**11**) A solution of Lawsone (870 mg, 5 mmol, 1 equiv) in thionyl chloride (25 mL) was stirred at 90 °C for 48 h. After cooling, the mixture reaction was verted in water (200 mL). The precipitated compound was filtered, taken up with chloroform (100 mL), washed with solution of sodium bicarbonate 10% in water (100 mL). The organic layer was dried over $MgSO_4$, and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel with hexane/ethyl acetate (13:1) to afford the desired product as yellow solid (816 mg, 4.25 mmol, 85%), mp 93–95 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21–8.15 (m, 1H), 8.12–8.06 (m, 1H), 7.83–7.75 (m, 2H), 7.23 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.8, 178.1, 146.4, 136.0, 134.6, 134.3, 131.8, 131.4, 127.6, 126.9. The spectral data are in accordance with those reported in the literature [36].

2-((1,4-dioxo-1,4-dihydronaphthalen-2-yl)oxy)acetonitrile (**12**): To a solution of Lawsone (174 mg, 1 mmol, 1 equiv) in DMF (20 mL) was added potassium carbonate (138 mg, 1 mmol, 1 equiv). After 20 min of stirring the misture reaction at 50 °C, was added iodoacetonitrile (180 μL, 2.5 mmol, 2.5 equiv). The mixture reaction was stirred at 50 °C for more 4 h. After cooling, the mixture reaction was concentrated to dryness and the residue was taken up in ethyl acetate (50 mL) and washed with solution of sodium bicarbonate 10% in water (3 × 50 mL). The organic layer was dried over $MgSO_4$, and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel with hexane/ethyl acetate (9:1) to afford the desired product as yellow solid (198 mg, 93%); mp 131–133 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19–8.04 (m, 2H), 7.82–7.73 (m, 2H), 6.33 (s, 1H), 4.89 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 184.0, 178.9, 157.1, 134.9, 134.0, 131.7, 130.9, 127.0, 126.6, 112.8, 112.1, 53.42; HRMS (+ESI) *m/z*: $[M + H]^+$ calcd for $C_{12}H_8NO_3^+$: 214.0499; Found: 214.0511.

2-hydroxy-3-(3-methylbut-2-en*-1-yl*)naphthalene-1,4-dione (**13**): To a solution of Lawsone (6.97 g, 40 mmol, 1 equiv) in DMSO (50 mL) was added 3,3-dimethylallyl bromide (51.2 mL, 44 mmol, 1.1 equiv), triethylamine (5.85 mL, 42 mmol, 1.05 equiv), and sodium iodide (6 g, 40 mmol, 1 equiv). The mixture was stirred at nitrogen atmosphere and room temperature for 1 h and then at 70 °C for 6 h. After cooling, the mixture reaction was verted in water (200 mL), extracted with dichloromethane, and washed with solution of sodium bicarbonate 10% in water (3 × 100 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel with hexane/ethyl acetate (9:1) to afford the desired product as yellow solid (5.8 g, 24 mmol, 60%), mp 117–118 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.04 (s, 1H), 7.97–7.73 (m, 2H), 7.86–7.71 (m, 2H), 5.10 (t, $J = 7.2$ Hz, 1H), 3.13 (d, $J = 7.2$ Hz, 2H), 1.70 (s, 3H), 1.61 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 184.2, 181.1, 155.1, 134.5, 133.1, 132.0, 131.9, 129.9, 125.7, 125.6, 122.9, 120.7, 25.5, 22.0, 17.8; HRMS (+ESI) *m/z*: $[M + H]^+$ calcd for $C_{15}H_{15}O_3^+$: 243.1016; Found: 243.1027.

(E)-2-hydroxy-3-(3-methylbut-1-en*-1-yl*)naphthalene-1,4-dione (**14**): To a solution of Lawsone (2.0 g, 11.5 mmol, 1 equiv) in acetic acid (35 mL) was added hydrochloric acid 37% (2 mL, 230 mmol, 20 equiv) and isovaleraldehyde (5 mL, 46.5 mmol, 4 equiv). The mixture was stirred at 70 °C for 2 h. After cooling, the mixture reaction was verted in water (200 mL). The oily layer was taken up in ethyl ether (100 mL) and washed with solution of sodium bicarbonate 10% in water (100 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel with hexane/ethyl acetate (9:1) to afford the desired product as orange solid (1.25 g, 5.17 mmol, 45%); mp 94–95 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.12 (dd, $J = 7.7$, 1.0 Hz, 1H), 8.06 (dd, $J = 7.5$, 1.0 Hz, 1H), 7.77 (s, 1H), 7.73 (td, $J = 7.5$, 1.3 Hz, 1H), 7.67 (td, $J = 7.4$, 1.3 Hz, 1H), 7.04 (dd, $J = 16.3$, 7.3 Hz, 1H), 6.59 (dd, $J = 16.3$, 1.0 Hz, 1H), 2.53 (dq, $J = 13.5$, 6.6 Hz, 1H), 1.13 (s, 3H), 1.11 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 184.5, 181.5, 151.4, 150.6, 135.0, 133.1, 132.8, 129.5, 127.1, 126.0, 118.9, 116.0, 33.6, 22.3; HRMS (+ESI) *m/z*: $[M + H]^+$ calcd for $C_{15}H_{15}O_3^+$: 243.1016; Found: 243.1031.

3-(3-methylbut-2-en*-1-yl*)-1,4-dioxo-1,4-dihydronaphthalen-2-yl 4-methylbenzenesulfonate. (**15**): To a solution of lapachol (242 mg, 1 mmol, 1 equiv) in DCM (10 mL) was added potassium carbonate (138 mg, 1 mmol, 1 equiv) and tosyl chloride (381 mg, 2 mmol, 2 equiv). The mixture was stirred at 0 °C for 24 h. The mixture reaction was concentrated to dryness and the residue was purified by recrystallization in ethanol to afford the desired product as yellow crystals (277 mg, 70%): mp 110–111 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14–8.04 (m, 2H), 8.01 (d, $J = 8.4$ Hz, 2H), 7.80–7.68 (m, 2H), 7.43 (d, $J = 8.1$ Hz, 2H), 5.09 (t, $J = 7.3$ Hz, 1H), 3.36 (d, $J = 7.3$ Hz, 2H), 2.50 (s, 3H), 1.72 (s, 3H), 1.65 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 184.6, 178.9, 148.8, 145.9, 141.3, 135.6134.3, 134.1, 133.8, 132.0, 130.8, 129.9, 128.6, 126.9, 126.7, 118.3, 25.9, 24.7, 21.9, 18.1. HRMS (+ESI) *m/z*: $[M + H]^+$ calcd for $C_{22}H_{21}O_5S^+$: 397.1104; Found: 397.1124.

3-(3-methylbut-2-en*-1-yl*)-1,4-dioxo-1,4-dihydronaphthalen-2-yl)oxy)acetonitrile (**16**): was synthesized with the same procedure for compound **12** but using Lapachol instead of Lawsone. The desired product was obtained after purification as pale yellow solid (210 mg, 75%); mp 53–55 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14–8.00 (m, 2H), 7.79–7.66 (m, 2H), 5.21 (s, 2H), 5.10 (t, $J = 7.3$ Hz, 1H), 3.35 (d, $J = 7.3$ Hz, 2H), 1.79 (s, 3H), 1.69 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 184.6, 181.4, 153.4, 137.1, 134.8, 134.4, 133.8, 131.9, 131.0, 126.7, 126.4, 119.0, 115.0, 57.2, 25.9, 23.4, 18.1; HRMS

(+ESI) *m/z*: $[M + H]^+$ calcd for $C_{17}H_{16}NO_3^+$: 282.1125; Found: 282.1124.

2-hydroxy-3-isopentylnaphthalene-1,4-dione (**17**): To a solution of lapachol (242 mg, 1 mmol, 1 equiv) in ethyl acetate was added Pd/C (10 mg) and the mixture was stirred under H_2 atmosphere (2 atm) at room temperature for 12 h. The mixture reaction was concentrated to dryness and the residue was purified by column chromatography on silica gel with hexane/ethyl acetate (95:5) to afford the desired product as yellow solid (195 mg, 83%), mp 81–82 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.11 (dd, $J = 7.7$, 1.1 Hz, 1H), 8.07 (dd, $J = 7.5$, 1.3 Hz, 1H), 7.75 (td, $J = 7.5$, 1.4 Hz, 1H), 7.67 (td, $J = 7.5$, 1.3 Hz, 1H), 7.29 (s, 1H), 2.64–2.56 (m, 2H), 1.69–1.54 (m, 1H), 1.47–1.34 (m, 2H), 0.97 (s, 3H), 0.94 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 184.8, 181.6, 153.0, 134.9, 133.1, 133.0, 129.5, 126.8, 126.2, 125.2, 37.3, 28.4, 22.5, 21.5. HRMS (+ESI) *m/z*: $[M + H]^+$ calcd for $C_{15}H_{17}O_3^+$: 245.1172; found: 245.1189.

2,2-dimethyl-3,4-dihydro-2H-benzo[g]chromene-5,10-dione (**18**): A solution of lapachol (242 mg, 1 mmol, 1 equiv) in 12 N hydrochloric acid (25 mL) was stirred at 80 °C for 4 h. After cooling, the mixture reaction was verted in water (25 mL), extracted with chloroform, and washed with solution of sodium bicarbonate 10% in water (3 × 50 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel with hexane/ethyl acetate (9:1) to afford the desired product as yellow solid (205 mg, 85%); mp 98–100 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08–8.04 (m, 2H), 7.72–7.62 (m, 2H), 2.61 (t, $J = 6.6$ Hz, 2H), 1.81 (t, $J = 6.6$ Hz, 2H), 1.42 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 184.5, 180.1, 154.7, 133.9, 133.0, 132.1, 131.2, 126.4, 126.0, 120.2, 78.2, 31.5, 26.6, 16.8; HRMS (+ESI) *m/z*: $[M + H]^+$ calcd for $C_{15}H_{15}O_3^+$: 243.1016; found: 243.1046.

Enzymatic Inhibition Assays. The experiments were performed in a 96-well microplate reader, containing the buffer 60 μM DCIP, 50 mM Tris pH 8.15, 150 mM KCl, 0.1% Triton X-100, 500 μM DHO, 100 μM CoQ_0 and varied inhibitor concentrations. To start the reaction, 5 μL of SmDHODH or HsDHODH were added to a final concentration of 40 nM and 20 nM, respectively. As a control, 5 μL of each enzyme were added to 200 μL buffer, without the presence of the inhibitors. The reaction was monitored each 4 s at 600 nm over a period of 60 s, in triplicate, for each concentration of inhibitor. The IC_{50} was determined through the graph of percent of inhibition versus log of the inhibitor concentration. The data fitting used was a sigmoid curve obtained in the software OriginPro 8.

Inhibition Mechanism Assays. The experiments were performed in a 96-well microplate reader, containing the buffer 50 mM Tris pH 8.15, 150 mM KCl, 0.1% Triton X 100, 500 μM DHO, CoQ_0 varying from 0 to 1000 μM and varied inhibitor concentrations. To start the reaction, 5 μL of SmDHODH or HsDHODH were added to a final concentration of 90 nM and 80 nM, respectively. As a negative control, 5 μL of each enzyme were added to 200 μL buffer, without the presence of the inhibitors. The reaction of orotate (ORO) formation was monitored each 4 s at 300 nm over a period of 60 s, in triplicate, for each concentration of substrate and each tested compound. The inhibition mechanism was determined through the Michaelis-Menten graph of k_{obs} versus CoQ_0 concentration using hyperbolic data fitting in the software SigmaPlot 11.0 and also through the Lineweaver-Burk graph of $1/k_{obs}$ versus $1/CoQ_0$ concentration using linear data fitting in the software OriginPro 8.

Associated content

Supporting **Information**. A PDF file containing the IC_{50} of all compounds assayed either against SmDHODH or HsDHODH, the graphs for the inhibition mechanism of the six compounds assayed, and ^{1}H and ^{13}C NMR spectra for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

Author contributions

F.A.C., J.S.D., and M.C.N. developed the *in vitro* assays. E.R.C.C., F.F., R.B.M., F.S.E., F.H.L., and M.S.C. designed and synthesized compounds. All authors contributed to data analysis and wrote the manuscript. All authors have given approval to the final version of the manuscript.s.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2019.02.018.

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