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Research paper

Anti-tubercular profile of new selenium-menadione conjugates against *Mycobacterium tuberculosis* H37Rv (ATCC 27294) strain and multidrug-resistant clinical isolates



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ABSTRACT

Tuberculosis (TB) is one of the most fatal diseases and is responsible for the infection of millions of people around the world. Most recently, scientific frontiers have been engaged to develop new drugs that can overcome drug-resistant TB. Following this direction, using a designed scaffold based on the combination of two separate pharmacophoric groups, a series of menadione-derived selenoesters was developed with good yields. All products were evaluated for their *in vitro* activity against *Mycobacterium tuberculosis* H37Rv and attractive results were observed, especially for the compounds **8a**, **8c** and **8f** (MICs 2.1, 8.0 and 8.1 μ M, respectively). In addition, **8a**, **8c** and **8f** demonstrated potent *in vitro* activity against multidrug-resistant clinical isolates (CDCT-16 and CDCT-27) with promising MIC values ranging from 0.8 to 3.1 μ M. Importantly, compounds **8a** and **8c** were found to be non-toxic against the Vero cell line. The SI value of **8a** (>23.8) was found to be comparable to that of isoniazid (>22.7), which suggests the possibility of carrying out advanced studies on this derivative. Therefore, these menadione-derived selenoesters obtained as hybrid compounds represent promising new anti-tubercular agents to overcome TB multidrug resistance.

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1. Introduction

Tuberculosis (TB) is a major public health concern worldwide representing the principal cause of death from a single infectious agent, *Mycobacterium tuberculosis*. Each year, approximately 10

million people become ill with TB, with the prevailing incidence in Southeast Asia and Africa. The risk of developing TB is high, considering an estimated 1.7 billion people globally infected with these mycobacteria [1,2]. In 2018, 7 million of new cases and 11.1 million of the estimated incident cases were reported. Therefore, it is important to highlight the gap between underreporting and underdiagnoses cases between the new and the estimated incident cases. This observed gap is due to the global range of people with TB without access to health care [2].

TB represents a difficult challenge with multiple aggravating factors. The drug resistance in TB, the poor tolerability and efficacy of therapeutic regimen together with the difficulty in accessing therapy and incomplete treatment increase the complexity of

Abbreviations: NMR, nuclear magnetic resonance; HMRS, high resolution mass spectrometry; HR-ESIMS, High-resolution electrospray ionisation mass spectrometry; TB, tuberculosis; MDR-TB, multidrug-resistant TB; GPx, glutathione peroxidase; MIC, minimum inhibitory concentration; SI, selectivity index; CC₅₀, cytotoxic concentration.

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disease resolution [3]. The spread of infection by *M. tuberculosis* strains resistant to first and second-line anti-TB drugs through the population is another factor that makes it difficult to fight the disease. World Health Organization (WHO) published, in 2018 there were approximately half a million new cases of resistance to rifampicin (RR-TB) worldwide, of which 78% had multidrug-resistant TB (MDR-TB) [2]. The emergence and spread of multidrug-resistant (MDR; defined as strains resistant to at least rifampicin and isoniazid) and extensively drug-resistant (XDR; as MDR plus additional resistance to at least one fluoroquinolone and 1 s-line injectable drug) *M. tuberculosis* strains are associated with the ineffectiveness of the available treatments for TB and are a major public health concern. MDR and XDR strains are often associated with low cure and high mortality rates [1].

The treatment of drug-susceptible TB is complicated due to the use of drug cocktails composed of first-line drugs (rifampicin, isoniazid, pyrazinamide and ethambutol) and more toxic, less efficacious and more expensive second- or third-line agents (aminoglycosides, fluoroquinolones, clofazimine, amoxicillin plus clavulanate) [4,5]. At the same time, cases of extensively drug-resistant TB and drug-resistant tuberculosis (TDR-TB) have been reported [5–7]. Consequently, the development of new drugs for the treatment of TB appears to be extremely important and natural products may be a valid resource for drug discovery.

In this context, naphthoquinones are a characteristic group of quinones that exist as secondary metabolites largely present in plants, lichens and microorganisms. Some of them can be extracted and used in traditional medical practices and folk medicine [8]. Naphthoquinones present a chemical structure of naphthalene with two ketone groups at position C-1 and C-2 (1,2-naphthoquinones) or C-1 and C-4 (1,4-naphthoquinones) [9]. The considerable interest in these compounds is based on their pharmacological activities, especially as antimicrobial [8–11] and anti-tumor agents [12–15]. Most recently, 1,4-naphthoquinones, such as menadione (vitamin K3), have aroused a lot of interest due to relevant anticancer activity [16]. With specific attention on the anti-tubercular activity, studies have reported on the potential of using naphthoquinones as active agents against drug-resistant *M. tuberculosis* strains, although their mechanism of action has yet to be unveiled [17–19].

Since the 1970s, the identification of different selenoproteins and their involvement in mammalian biochemistry have motivated intense studies in the synthesis and biological properties of organoselenium compounds [20,21]. Notably, many studies have shown the potential applications of selenium compounds for the treatment of infectious diseases. The antimicrobial efficacy of selenium compounds has been tested against a great variety of microorganisms, demonstrating their activity as antibacterial [22], antifungal [23], antiviral [24] and antiparasitic agents [25]. Ebselen (**1**) is the most well-known and well-studied seleno-derivative, especially for its antioxidant effects, GPx-like activity [26] and antimicrobial activity [27]. Antitubercular activities of ebselen (**1**) and its derivatives have consistently been demonstrated and considering the capacity of ebselen (**1**) to act as an allosteric inhibitor of the *M. tuberculosis* antigen 85 complex [28,29], confirming the potential use of selenium compounds in MDR-TB. Recently, ebselen (**1**) was described among 10,000 molecules as the best non-reversible inhibitor of the SARS-CoV-2 encoded protease (Mpro), showing again the biological relevance of organoselenium compounds [30].

Considering the important chemical interest and potential biological applications of 1,4-naphthoquinones and chalcogen derivatives, different research groups have concentrated their efforts to develop new compounds possessing these two structural moieties [31]. A method for the synthesis of selenonaphthoquinones (**2**) by phenylselenide ions has been reported in the literature by

Sakakibara [32], and others chalcogenium-naphthoquinones were successively explored by Jacob to obtain new redox agents (**3-5**) as anti-cancer drugs [33–36], as shown in Fig. 1.

In the last three years, synthetic methodologies to coupling selenium groups involving direct selenilation of the quinone ring have been described through Rh-catalyzed C-H phenylselenation, as shown in Fig. 2. For example, using *N*-(phenylseleno)-phthalimide or phenyl hypochloroselenoites as an electrophile, the C-2 selenation of naphthoquinone can be performed, as well as the Rh-catalyzed selenation of benzoquinone (Fig. 2A) [37–39].

We herein report on the synthesis of a new series of naphthoquinone derivatives **8a-i** by selenofunctionalization of menadione (**7**) and their evaluation against the drug-susceptible *M. tuberculosis* H37Rv strain and multidrug-resistant clinical isolates (CDCT-16 and CDCT-27) (Fig. 2B).

2. Experimental section

2.1. Chemistry

The reagents were purchased from Sigma-Aldrich Brazil and were used without further purification. Column chromatography was performed with silica gel 60 (Merck 70–230 mesh). Analytical thin layer chromatography was performed with silica gel plates (Merck, TLC silica gel 60 F₂₅₄), and the plates were visualized using UV light. The indicated yields refer to homogeneous materials purified by chromatography and confirmed by spectroscopic techniques. Melting points were obtained on a Thermo scientific 9100 apparatus and were uncorrected. Infrared spectra were collected using KBr pellets on a PerkinElmer model 1420 FT-IR spectrophotometer, and the spectra were calibrated relative to the 1601.8 cm⁻¹ absorbance of polystyrene. ¹H and ¹³C Nuclear magnetic resonance (NMR) were recorded at room temperature using a Varian VXR Unity 300 or 500 MHz, in the DMSO-*d*₆. Chemical shifts (δ) are given in ppm and coupling constants (*J*) in Hertz. The chemical shift data were reported in units of δ (ppm) downfield from solvent, and the solvent was used as an internal standard; coupling constants (*J*) are reported in hertz and refer to apparent peak multiplicities. High-resolution mass spectra (HRMS) were recorded on a MICROMASS Q-TOF mass spectrometer (Waters) and the deviation of the measured mass from the theoretical mass (Δ) was inserted in ppm.

X-ray diffraction data for cubebin was carried out with radiation MoK α ($\lambda = 0.71073 \text{ \AA}$) in Bruker D8 Venture diffractometer at room temperature. The data collection and cell refinement were carried out using APEX3 (ref: Bruker APEX3, SAINT and SADABS Bruker AXS Inc., Madison, Wisconsin, USA). The structure was solved using Intrinsic Phasing and refinement package using Least Squares Methods with the SHELX programs [40]. Non-hydrogen atoms were refined with anisotropic displacement parameters, and the hydrogen atoms were positioned geometrically using the riding model. Molecular graphics draw using SHELXL [41].

The protocol for preparing 2-(chloromethyl)-3-methylnaphthalene-1,4-dione (**9**) was performed as described by Ferreira and cols [42].

2.2. General procedure for the synthesis of selenonaphthoquinones **8a-i**

To a two-necked round-bottom flask, under an argon atmosphere, containing Se⁰ (1.5 mmol) in 5 mL of ethanol was added portion wise to sodium borohydride (2.0 mmol). Then, the appropriate acyl chloride (1.5 mmol) was added and the reaction mixture was kept under stirring for 30 min. After this, 2-(chloromethyl)-3-methylnaphthalene-1,4-dione (1.0 mmol) was added and the mixture was left under stirring for 24 h and extracted with ethyl

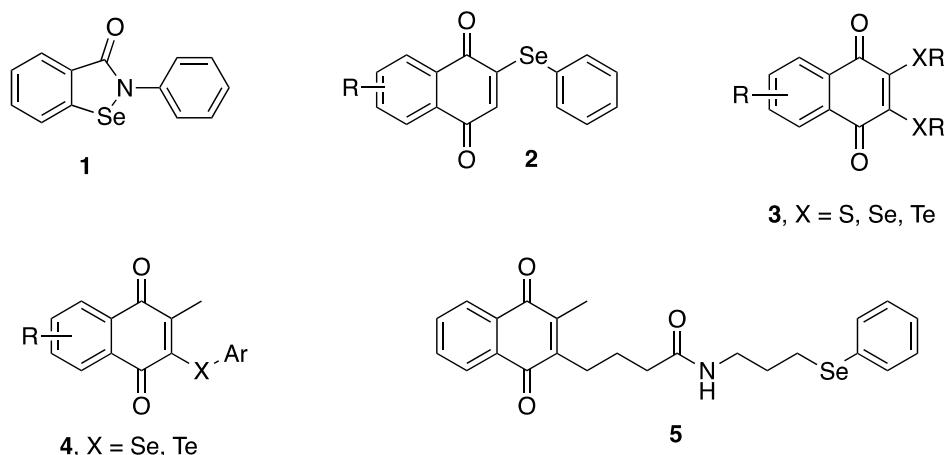
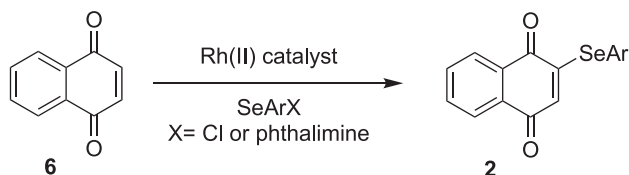


Fig. 1. Chemical structures of already known chalcogenium-naphthoquinones (1-5).

(A) **Previous works:** C-H phenylselenation Rh-Catalyzed of naphthoquinones and quinones



(B) **This work:** Selenofunctionalization of naphthoquinones to obtain menadione-derived selenoesters

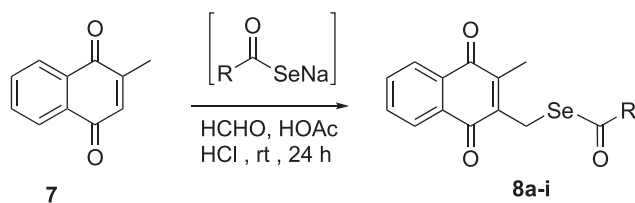


Fig. 2. Overview of the different synthetic methodologies to selenofunctionalization of quinones and naphthoquinones, through Rh-catalyzed C-H bond activation (Panel A) and to obtain menadione-derived selenoesters (Panel B).

acetate (3 x 15 mL), dried over anhydrous sodium sulfate and concentrated under vacuum. The crude product was purified by flash chromatography using gradient ethyl acetate:hexane as the eluent.

2.2.1. *Se-((3-methyl-1,4-dioxo-1,4-dihydronafaleno-2-yl)methyl)-benzoselenoate (8a)*

Yellow solid, m.p. 105–107 °C, yield 75%. ¹H NMR (300.00 MHz, DMSO-*d*₆) δ ppm: 8.03–7.99 (m, 2H), 7.89–7.82 (m, 4H), 7.73–7.70 (m, 1H), 7.58–7.55 (m, 2H), 4.28 (s, 2H), 2.23 (s, 3H). ¹³C NMR (75.00 MHz, DMSO-*d*₆) δ ppm: 193.32, 183.92, 183.27, 143.53, 143.11, 137.62, 134.30, 133.96, 133.81, 131.55, 131.20, 129.22, 126.77, 125.91, 125.77, 19.81, 12.59. HR-ESIMS [M+H]⁺ *m/z* calcd. for C₁₉H₁₅O₃Se: 371.0181. Found: 371.0192. Δ 3.0 ppm.

2.2.2. *Se-((3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-4-methylbenzoselenoate (8b)*

Yellow solid, m.p. 109–111 °C, yield 58%. ¹H NMR (500.00 MHz, DMSO-*d*₆) δ ppm: 8.10–8.05 (m, 2H), 7.77 (d, *J* 1.5 Hz, 2H), 7.72–7.66

(m, 2H), 7.23 (d, *J* 1.5 Hz, 2H), 4.30 (s, 2H), 2.38 (s, 3H), 2.32 (s, 3H). ¹³C NMR (75.00 MHz, CDCl₃) δ ppm: 193.43, 185.04, 184.42, 145.15, 144.47, 144.25, 136.07, 133.84, 133.70, 132.40, 132.10, 129.75, 127.58, 126.66, 126.57, 21.92, 20.14, 13.22. HR-ESIMS [M+Na]⁺ *m/z* calcd. for C₂₀H₁₆NaO₃SeNa⁺: 407.0157. Found: 407.0173. Δ 3.7 ppm.

2.2.3. *Se-((3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-2-methylbenzoselenoate (8c)*

Yellow solid, m.p. 125–127 °C, yield 32%. ¹H NMR (300.00 MHz, DMSO-*d*₆) δ ppm: 8.15–8.09 (m, 2H), 7.79–7.71 (m, 3H), 7.46–7.40 (td, *J* 3.0 and 9.0 Hz, 1H), 7.30–7.27 (td, *J* 3.0 and 9.0 Hz, 2H), 4.32 (s, 2H), 2.53 (s, 3H), 2.37 (s, 3H). ¹³C NMR (75.00 MHz, CDCl₃) δ ppm: 195.77, 184.88, 184.23, 144.36, 144.00, 138.40, 136.18, 133.68, 133.55, 132.22, 132.20, 131.92, 131.83, 128.78, 126.49, 126.41, 126.05, 20.98, 20.72, 13.04. HR-ESIMS [M+Na]⁺ *m/z* calcd. for C₂₀H₁₆NaO₃SeNa⁺: 407.0157. Found: 407.0150. Δ 2.1 ppm.

2.2.4. *Se*-((3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-4-ethylbenzoselenoate (**8d**)

Yellow solid. m.p. 95–97 °C, yield 25%. ¹H NMR (300.00 MHz, CDCl₃) δ ppm: 8.10–8.05 (m, 2H), 7.79 (d, *J* 1.5 Hz, 2H), 7.71–7.66 (m, 2H), 7.25 (d, *J* 1.5 Hz, 2H), 4.30 (s, 2H), 2.67 (q, *J* 7.6 Hz, 2H), 2.32 (s, 3H), 1.23 (t, *J* 7.6 Hz, 3H). ¹³C NMR (75.00 MHz, CDCl₃) δ ppm: 193.51, 185.10, 184.49, 151.35, 144.53, 144.28, 133.88, 132.43, 132.13, 131.94, 128.83, 128.62, 127.71, 126.70, 126.61, 29.23, 20.16, 15.29, 13.25. HR-ESIMS [M+Na]⁺ *m/z* calcd. for C₂₁H₁₈NaO₄SeNa⁺: 421.0313. Found: 421.0326. Δ 2.7 ppm.

2.2.5. *Se*-((3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-4-methoxybenzoselenoate (**8e**)

Yellow solid, m.p. 118–120 °C. Yield 24%. ¹H NMR (300.00 MHz, CDCl₃) δ ppm: 8.03–8.00 (m, 2H), 7.78 (d, *J* 8.9 Hz, 2H), 7.64–7.62 (m, 2H), 6.84 (d, *J* 8.9 Hz, 2H), 4.23 (s, 2H), 3.78 (s, 3H), 2.26 (s, 3H). ¹³C NMR (75.00 MHz, CDCl₃) δ ppm: 191.77, 184.90, 184.28, 164.22, 144.39, 144.02, 133.65, 133.51, 132.23, 131.93, 131.22, 129.62, 126.47, 126.38, 114.08, 55.58, 19.90, 13.03. HR-ESIMS [M+Na]⁺ *m/z* calcd. for C₂₀H₁₆NaO₄SeNa⁺: 423.0110. Found: 423.0106. Δ 0.6 ppm.

2.2.6. *Se*-((3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-4-fluorobenzoselenoate (**8f**)

Orange solid, m.p. 124–126 °C, yield 51%. ¹H NMR (500.00 MHz, DMSO-*d*₆) δ ppm: 8.00–7.92 (m, 4H), 7.84–7.80 (m, 2H), 7.39–7.30 (m, 2H), 4.28 (s, 2H), 2.22 (s, 3H). ¹³C NMR (75.00 MHz, DMSO-*d*₆) δ ppm: 192.42, 184.53, 183.86, 166.08 (d, *J* 253.1 Hz), 144.27, 143.68, 135.07, 134.52, 134.38, 132.24, 131.90, 131.41 (dd, *J* 9.7 Hz), 126.50, 126.36, 116.41 (dd, *J* 22.2 Hz), 20.66, 13.11. HR-ESIMS [M+Na]⁺ *m/z* calcd. for C₁₉H₁₃FN₃O₃SeNa⁺: 410.9906. Found: 410.9923. Δ 4.0 ppm.

2.2.7. *Se*-((3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-4-chlorobenzoselenoate (**8g**)

Yellow solid, m.p. 158–160 °C, Yield 26%. ¹H NMR (500.00 MHz, DMSO-*d*₆) δ ppm: 8.04–8.01 (m, 2H), 7.75 (d, *J* 1.6 Hz, 2H), 7.65–7.62 (m, 2H), 7.36 (d, *J* 1.6 Hz, 2H), 4.26 (s, 2H), 2.27 (s, 3H). ¹³C NMR (75.00 MHz, CDCl₃) δ ppm: 192.99, 184.99, 184.42, 144.48, 144.09, 140.57, 136.98, 133.95, 133.80, 132.42, 132.07, 129.45, 128.78, 126.74, 126.62, 20.62, 13.29. Δ = 1.1 ppm. HR-ESIMS [M+Na]⁺ *m/z* calcd. for C₁₉H₁₃ClNaO₃SeNa⁺: 426.9611. Found: 426.9609. Δ 0.2 ppm.

2.2.8. *Se*-((3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-pentaneselenoate (**8h**)

Yellow oil, yield 24%. ¹H NMR (500.00 MHz, CDCl₃) δ ppm: 8.98–7.94 (m, 2H), 7.61–7.58 (m, 2H), 4.02 (s, 2H), 2.55 (t, *J* 10.0 Hz, 2H), 2.16 (s, 3H), 1.57 (q, *J* 10.0 Hz, 2H), 1.28 (sextet, *J* 10.0 Hz, 2H), 0.82 (t, *J* 10.0 Hz, 3H). ¹³C NMR (125.00 MHz, CDCl₃) δ ppm: 200.92, 184.70, 183.99, 144.22, 143.78, 133.60, 133.47, 132.12, 131.81, 126.39, 126.30, 47.25, 27.45, 21.98, 19.79, 13.68, 12.83. HR-ESIMS [M+Na]⁺ *m/z* calcd. for C₁₇H₁₈NaO₃SeNa⁺: 373.0313. Found: 373.0329. Δ 4.0 ppm.

2.2.9. *Se*-((3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-2-methylpentanoselenoate (**8i**)

Yellow oil, yield 37%. ¹H NMR (500.00 MHz, CDCl₃) δ ppm: 7.99–7.96 (m, 2H), 7.61–7.59 (m, 2H), 4.02 (s, 2H), 2.63 (sextet, *J* 6.9 Hz, 1H), 2.16 (s, 3H), 1.64–1.60 (m, 1H), 1.32–1.25 (m, 3H), 1.09 (d, *J* 6.9 Hz, 3H), 0.81 (t, *J* 7.3 Hz, 3H). ¹³C NMR (126.00 MHz, CDCl₃) δ ppm: 205.64, 184.79, 184.06, 144.41, 143.78, 133.60, 133.48, 132.16, 131.86, 126.41, 126.33, 51.74, 35.98, 20.23, 19.45, 17.18, 13.97, 12.81. HR-ESIMS [M+Na]⁺ *m/z* calcd. for C₁₈H₂₀NaO₃SeNa⁺: 387.0470. Found: 387.0483. Δ 3.1 ppm.

2.3. Biological evaluation

Vero cell line (ATCC N° CCL-81) were purchased from the Rio de Janeiro Cell Bank, Brazil. Dulbecco's Modified Eagle's Medium (DMEM), Hank's Balanced Salt Solution, fetal bovine serum (FBS), antibiotic solution (10,000 U/mL penicillin, 10 mg/mL streptomycin), antimycotic solution (25–30 µg/mL amphotericin B), trypsin-ethylenediaminetetraacetic acid (EDTA) solution (2.5 mg/mL trypsin, 0.2 mg/mL EDTA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were all supplied by Sigma-Aldrich (São Paulo, Brazil). Dimethyl sulfoxide (DMSO) and other reagents were analytical grade.

2.3.1. *Mycobacterium tuberculosis* inhibition assay

The minimum inhibitory concentration (MIC) for each tested compound was determined by the resazurin reduction microplate assay (REMA), as thoroughly described before [43,44]. Isoniazid (INH, control drug) and compound solutions were solubilized at 1 mg/mL in neat DMSO, followed by dilution in Middlebrook 7H9 broth, supplemented with 10% ADC (albumin, dextrose, and catalase) and 5% DMSO, to a concentration of 50 µg/mL. Ten-point, two-fold serial dilutions were prepared directly in 96-well plates, giving a concentration range of 25.0 to 0.05 µg/mL. It is important to mention that all dilutions formed real solutions. A growth control (without compounds) and a sterility control (without bacterial inoculum) were included in each plate. Compounds were tested against the drug-sensitive *M. tuberculosis* H37Rv strain (ATCC 27294) and two drug-resistant clinical isolates (named CDCT-16 and CDCT-27). CDCT-16 is a multidrug-resistant clinical isolate resistant to isoniazid, ethionamide, rifampicin, streptomycin and ethambutol, while CDCT-27 is resistant to isoniazid and ethambutol only. The MIC was considered as the lowest drug concentration that prevented a color change from blue (no growth) to pink (growth). Three independent experiments were carried out, and MIC values reported here were observed in at least two experiments, or were the highest value observed among the three assays.

2.3.2. Cell line culture conditions

Vero cells were maintained in DMEM supplemented with 4.5 mg/mL glucose, 0.1 mg/mL penicillin, 0.14 mg/ml streptomycin and 10% inactivated FBS. Cultured cells were maintained at 37 °C in an atmosphere containing 95% air and 5% CO₂. Cells were subcultivated every 48 h using trypsin-EDTA solution.

2.3.3. Toxicity to the Vero cell line using the MTT assay

Metabolically active cells were assessed using the MTT reduction colorimetric assay, as previously reported by Mosmann (1983) [45] and Alley (1988) [46]. Cells were seeded in 96-well plates (Corning) at density of 30,000 cells/well, distributed in a total volume of 200 µL/well. Plates were kept in the incubator at 37 °C and 5% CO₂ for 24 h. After incubation, cells were put in contact with the samples (0–50 µM) for 24 h. 10% FBS DMEM containing 1% DMSO was used as the control treatment. Isoniazid (INH) was used as the comparative treatment. The samples were kept in contact with the cells for 3 h and then aspirated and treated with sterile MTT reagent solution (0.5 mg/mL in HBSS) by adding 125 µL per well. The plates containing the cells were incubated with MTT for 3 h at 37 °C and 5% CO₂. At the end of the incubation time, MTT was aspirated and the cells were washed with phosphate buffered saline (PBS) solution (pH 7.4). The phosphate buffer was then aspirated and 100 µL/well of DMSO was added to disrupt cell membranes and solubilize formazan crystals. The absorbance readings were performed on an iMARK™ Microplate Absorbance Reader (Bio-Rad Laboratories Srl, Segrate, Italy), with reference to 570 nm–690 nm after vigorous shaking for 30 s.

Cytotoxicity was expressed as the percentage of cells surviving in relation to untreated cells. Drug concentration required to inhibit growth by 50% (CC_{50}) and selectivity index (SI) were calculated with GraphPad Prism 5 (version 5.00; GraphPad Software, Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Chemistry

Our strategy was based on obtaining a series of novel menadione-derived selenoesters using a designed scaffold formed by two fragments through the combination of two separate pharmacophoric groups with analogous activity into one compound. The combination of 1,4-naphthoquinone and a selenium group in the same molecule was an interesting strategy to obtain hybrid compounds with synergistic effects as potent anti-tubercular agents, especially with the aim of overcoming TB multidrug resistance (Fig. 3). This selenofunctionalization of menadione is described for the first time in the literature and the methodology that we have developed is economical and fast compared with the other procedures used in the selenilation of naphthoquinones.

The synthetic approach used to obtain selenium-containing menadione derivatives **8a-i** initially provided some synthetic steps described in the literature [42] and was based on a two-step pathway entailing the use of commercial menadione (**7**). The synthetic route started with the first reaction of the insertion of the $-CH_2Cl$ group in menadione, in excellent yield (95%), through the reaction with formaldehyde, glacial acetic acid and dry hydrogen chloride, obtaining 2-(chloromethyl)-3-methylnaphthalene-1,4-dione (**9**) (Fig. 3).

Then, we promoted the selenofunctionalization of **9** through the reaction with the respective selenocarboxylate that was generated *in situ* from benzoyl chloride and Se^0 (Fig. 3). Using different acyl chlorides and benzoyl chlorides, a series of menadione-derived selenoesters in moderate to good yields ranged between 24 and 75% was obtained, as reported in Fig. 3.

For example, compound **8b**, with *p*-methylbenzoyl chloride, was obtained in 58% yield, while the best yield was represented by compound **8a** with 75% (Fig. 3). Exchanging the aromatic for aliphatic selenocarboxylate led to a decrease in the yield, as shown in Fig. 3. Additionally, we found that a change in the electron-

donating group in the aromatic ring to an electron withdrawer group did not produce significant variation in the product yield. The synthetic approach chosen shows that overall returns ranged from 23 to 71%.

The structure of all products synthesized was confirmed by 1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and HRMS spectrometry experiments. The 1H NMR experiment shows that the typical signals of hydrogens of the methyl group at C-3 of quinoidic rings are registered in the range of *ca.* 2.21–2.43 ppm and 2.17–2.19 ppm as singlets, integrating for three hydrogens, in case of benzoselenoates and pentaneselenoates, respectively. Another typical signal is represented by the singlet of two hydrogens of methyl group that connected the 1,4-dioxo-1,4-dihydronaphthalen core with the selenoate moiety. These protons are influenced by the presence of the selenium atom and they appear between 4.24–4.30 ppm and 4.04–4.05 ppm for benzoselenoates and pentaneselenoates, respectively. The presence of these signals confirmed the actual effectiveness of our synthetic method to obtain the desired selenonaphthoquinones [47–49].

The aromatic protons of 1,4-dihydronaphthalen nucleus respect the characteristic range of signals reported in literature.50 The ^{13}C NMR spectra shows the typical signal of the carbonyl selenoester moiety that appears between 191.74 and 195.75 ppm for benzoselenoates and between 200.89 and 205.86 ppm for pentaneselenoates [50].

Structure of **8d** was confirmed by X-ray diffraction and Ortep-3 diagram of the product is shown in Fig. 4. Bond lengths and angles are available in supporting information, being that all the

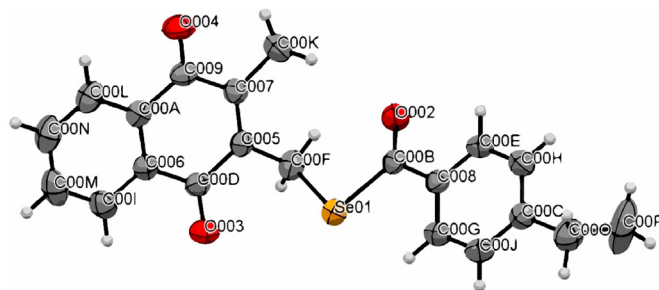


Fig. 4. ORTEP-3 representation of compound **8d** (CCDC 2006682).

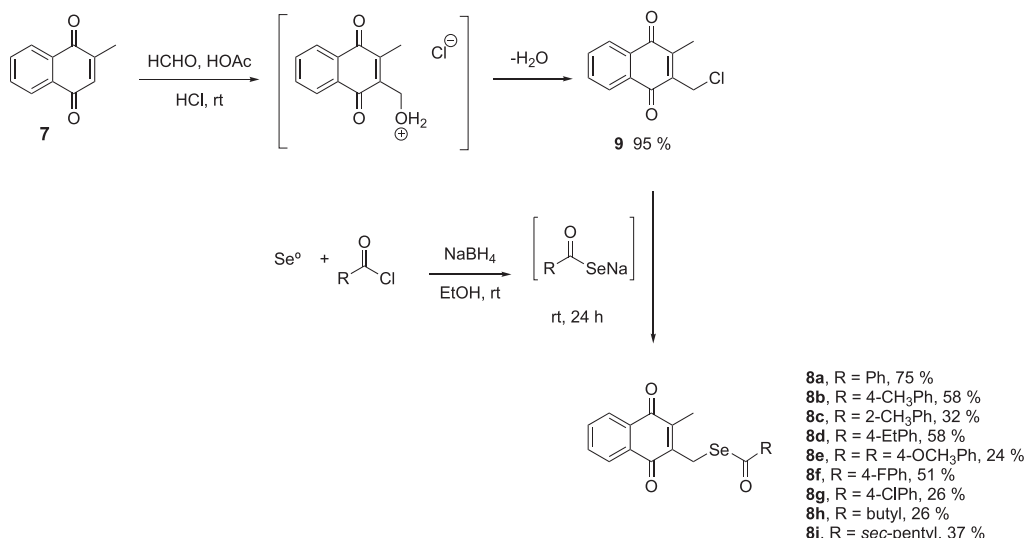


Fig. 3. Synthesis of menadione-derived selenoesters **8a-i**.

interatomic distances and angles are within the expected values for similar chemical bondings [51].

Table 1 lists main crystallographic parameters. For compound **8d** a total of 54362 reflections to a maximum 2θ of 25.4° were measured. The crystal structure for compound was solved by direct methods and refined anisotropically with full matrix least square on F^2 using SHELXL-97 program [52]. The bond angles between atoms are $121.4(2)^\circ$ for SeO1–C00B–O002 and $115.4(2)^\circ$ for SeO1–C00B–C008. All H atoms were located by geometric considerations placed (C–H = 0.93–0.97 Å).

Crystallographic data for compound **8d** have been deposited with the Cambridge Crystallographic Data Center as Supplementary Publication No. CCDC 2006682. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CH21EZ, UK (fax: þ44 1223 336 033 or e-mail: deposit@ccdc.cam.ac.uk).

3.2. Biological evaluation

3.2.1. Mycobacterium tuberculosis inhibition assay

Considering the emergence and spread of MDR and XDR-TB, new drug candidates have been proposed for treating these phenomena, as such as bedaquiline, delamanid and pretomanid, but possible cardiotoxicity and resistance to these new treatments were also rapidly reported [53]. Herein, we evaluated the effect of new selenium-containing menadione derivatives on the growth of drug-sensitive and -resistant strains of *M. tuberculosis*. As shown in Table 2, the compounds exhibited inhibitory activity for the H37Rv *M. tuberculosis* strain from 2.1 to 71.4 μM . Compound **8a** exhibited comparable activity to that of the related compound isoniazid; in fact, the MIC obtained for isoniazid under these experimental

Table 1
Crystallographic parameters for **8d**.

Empirical formula	C ₂₁ H ₁₈ O ₃ Se
Formula weight	397.3
Temperature (K)	296
Crystal system	Monoclinic
Space group	P 2 ₁ /c
a (Å)	12.2127
b (Å)	18.5981
c (Å)	8.3327
α (°)	90
β (°)	109.622 (1)
γ (°)	90
Volume (Å ³)	1782.72 (11)
Z	4
Calculated density (Mg/m ⁻³)	1.480
μ (mm ⁻¹)	2.12
F (000)	808.0
Crystal size (mm)	0.16 × 0.14 × 0.07
Radiation (Å)	Mo K α ($\lambda = 0.71073$)
hkl range	–14 ≤ h ≤ 14, –22 ≤ k ≤ 22, –10 ≤ l ≤ 10 k = –22, 22 l = –10, 10
θ (°)	2.6–25.4
Reflections collected	54362
Independent reflections	3281
R(int)	0.038
Final R indices	R ₁ = 0.033
[I > 2sigma(I)]	wR ₂ = 0.079
S	1.07
Largest difference peak and hole (e/Å ³)	0.52/–0.48

conditions was 2.2 μM , while that one determined for **8a** analog was 2.1 μM . Compounds **8c** and **8f** also demonstrated significant inhibitory activity, with MICs of 8.1 and 8.0 μM , respectively. These more active compounds show the phenyl group as a substituent of the carbonyl attached to selenium. The results show that the phenyl group at this position has an important role in the biological activity since the exchange of substituents significantly alter the results. Interestingly, only exchanging the fluor atom at the C-4 position of the phenyl group with a chlorine atom generated a molecule less active (compound **8f** versus **8g**).

Compounds with MIC <10 μM for the drug-sensitive strain (**8a**, **8c** and **8f**) were evaluated against two multidrug-resistant clinical isolates (CDCT-16 and CDCT-27). CDCT-16 is an MDR-TB strain that holds mutations in the *rpoB* (D516V) and *katG* (S315T) genes, as well as in the *inhA* promoter sequence [C(-15)T]. CDCT-27 carries the same mutation in the *katG* gene, which confers cross-resistance to isoniazid [54]. As shown in Table 2, none of these clinical isolates exhibited resistance to compounds **8a**, **8c** and **8f**, with MIC values ranging from 0.8 to 3.1 μM . These data suggest a possible capacity of menadione derivatives to evade the most common mechanisms of resistance and the potential interest to study these compounds in pre-clinical tests to develop new antimycobacterial agents. Finally, the absence of a resistant phenotype by the two clinical isolates suggests that these compounds do not share a mechanism of action similar to the first-line drugs isoniazid and rifampicin. However, how these compounds act to disrupt the mycobacterial growth and to inhibit their molecular target(s), as well as the contribution of each pharmacophoric group to the biological activity, has yet to be elucidated.

According to the importance of estimating the distribution and diffusion of drugs to across the cell membrane and tissue and their solubilization in an aqueous medium, the CLogP was calculated. In particular, it has been well established that the lipophilicity of anti-TB drug candidates is positively correlated with their ability to permeate through *M. tuberculosis* cell wall [55]. As reported in Table 2, the compounds **8a–i** showed CLogP values in the range of 4.31–6.88. Notably, steric and/or conformational properties seem to be directly related to the antimycobacterial activity exhibited by the compounds rather than the physicochemical parameters such as ClogP. Changing the methyl group from 4- to 2-position of the benzene ring greatly changed the activity of naphthoquinones **8b** and **8c** while their CLogP values are identical. It is noteworthy that the most effective compounds (**8a**, **8c**, and **8f**) present ClogP values consistent with the classic rule of five [56]. In addition, the number of hydrogen bonds donors (<5) and hydrogen bonds acceptors (<10) as well as the molecular mass (<500 D) were also respected, suggesting that these molecules may have adequate bioavailability when orally administered.

3.2.2. Toxicity of menadione derivatives to the Vero cell line

Menadione derivatives and isoniazid were tested by the MTT reduction assay in the Vero cell line at 5, 10, 25 and 50 μM . Among evaluated derivatives, **8c** showed the lowest CC₅₀ value (7.7 μM) and consequently a low SI (selective index) value (Vero/H37Rv 0.9). Derivatives **8a** and **8f** showed CC₅₀ above the highest dose (>50 μM), which pointed to SI values (Vero/H37Rv) above 23.8 and 6.2, respectively, as reported in Table 2. Isoniazid showed an SI (>22.7) very similar to **8a**, which represents a starting point for advanced studies of this derivative, given the correlation between toxicity in Vero cells, isolated renal tissue and nephrotoxicity in rats [57].

4. Conclusions

In summary, we report in this paper a new series of selenium-

Table 2

CLogP values, *in vitro* activity against three *M. tuberculosis* strains (H37Rv, CDCT-16 and CDCT-27), CC₅₀ values for Vero cell line, and calculated selectivity index (SI) values for the selenium-containing naphthoquinones.

Compounds	miLogP	MIC (μM)			CC ₅₀ Vero (μM)	SI		
		H37Rv	CDCT-16	CDCT- 27		H37Rv	CDCT-16	CDCT- 27
8a	4.39	2.1	0.8	0.8	>50	>23.8	>62.5	>62.5
8b	4.89	>10	>10	>10	—	—	—	—
8c	4.89	8.1	3.1	3.1	7.7	0.9	2.5	2.5
8d	5.42	>10	>10	>10	—	—	—	—
8e	4.31	>10	>10	>10	—	—	—	—
8f	4.53	8.0	0.8	3.1	>50	>6.2	>62.5	>16.1
8g	5.10	>10	>10	>10	—	—	—	—
8h	6.48	>10	>10	>10	—	—	—	—
8i	6.88	>10	>10	>10	—	—	—	—
Isoniazid	−0.67	2.2	100	25	>50	>22.7	—	—

^aClogP calculated by ChemBioDraw Ultra version 13.0.0.3015.

containing naphthoquinones and the evaluation of their biological activity against *M. tuberculosis* H37Rv and MDR clinical isolates (CDCT-16 and CDCT-27) that were isolated from patients undergoing clinical treatments. The compounds were designed as hybrids by combining two pharmacophoric groups (naphthoquinone and selenoester) known to have similar biological activities. The hybrid compounds were synthesized in adequate yields and showed good profiles of anti-tubercular activity against multidrug-resistant strains of *M. tuberculosis*. A convenient synthesis of compounds, as well as in biological activity and toxicity, suggest the continuation of this study for *in vivo* tests, to overcome the phenomenon of resistance to multiple drugs of TB, and in the synthesis of new hybrid selenoesters with menadione that may have better profiles of anti-tubercular activity with low toxicity.

Considering the promising results obtained in terms of synthesis, yields, biological activity and toxicity, this work suggests the potential application of this series of selenoesters derived from menadione in subsequent biological and structure-activity studies (SAR). Although the mechanism of action of the synthesized and tested compounds is still not known, these represent interesting starting points for the development of new antimycobacterial agents, in particular for overcoming the phenomenon of resistance to multiple TB drugs. The next steps to understand the mechanism of action and to determine the molecular target(s) of the selenium-containing naphthoquinones will include the selection of spontaneous mutants, the whole-genome sequencing of the obtained mutants, and the use of genetic approaches to validate the gene(s) involved in the mechanism of action and resistance. Besides, considering that ebselen is known to bind and inhibit the antigen 85 (Ag85) complex activity, which is involved in the biosynthesis of trehalose dimycolate and mycolylarabinogalactan, both essential components of the mycomembrane [28], attempts will be made to evaluate the capacity of our compounds to inhibit *in vitro* the activity of enzymes of this complex and the synthesis of different mycolates by growing cultures of *M. tuberculosis*.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2020.112859>.

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