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Preparation and evaluation of a set of *bis*(methoxycarbonylmethylthio) heteroquinones as CDC25B phosphatase inhibitors

Tatiana Besset[a], Emmanuelle Braud[b,*], Rafika Jarray[b], Christiane Garbay[b], Stéphanie Kolb[b], Pierre-Marc Léo[a] and Christophe Morin[a,*]

[a] *Département de Chimie Moléculaire (CNRS, UMR 5250, ICMG FR-2607), Université Joseph Fourier, 301 Rue de la chimie, F-38402 Grenoble Cedex, France*
[b] *Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, CNRS UMR 8601, Université Paris Descartes, 45 Rue des Saints Pères, F-75006 Paris, France*

**Corresponding author at: Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, CNRS UMR 8601, Université Paris Descartes, 45 Rue des Saints Pères, F-75006 Paris, France. Tel.: +33.142864085; fax: +33.142864082. E-mail address: emmanuelle.braud@parisdescartes.fr (E. Braud), christophe.morin@ujf-grenoble.fr (C. Morin).*

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ABSTRACT

A set of new heteroquinone derivatives bearing two methoxycarbonylmethylthio groups on the benzoquinone ring were synthesized and evaluated for CDC25B phosphatase inhibitory activity. All compounds inhibited the enzyme with IC_{50} values in the micromolar range regardless of the size and heteroatoms constituting the heterocycle fused to the quinone ring. Moreover, these quinonoid-based compounds showed moderate antiproliferative activity toward two cancer cell lines (HeLa and MiaPaca-2). These results provide additional data for CDC25 inhibition by quinone-type derivatives and highlight the importance of substituents on the quinonic moiety.

1. Introduction

Cell signaling is regulated by covalent and reversible phosphorylation and dephosphorylation reactions, which are controlled by protein kinases and phosphatases. These signaling pathways are responsible for essential cell events such as growth, division or death, their deregulation leading to the apparition of pathologies such as cancers. Cell components involved in cell cycle regulation and in the checkpoint machinery are considered as promising targets in therapeutic and especially for cancer treatment. Indeed, inhibiting cell cycle progression will limit cell proliferation. Among possible targets, cyclin-dependent kinases (CDK) are currently widely studied. Among CDK regulators, CDC25 phosphatases have received increasing interest over the past years. These enzymes play a central role in cell cycle control in eukaryotes and in the checkpoint response to DNA damage [1-3]. Up-regulation of CDC25 has been observed in a wide variety of human cancers [4,5] and the phosphatases are also involved in oncogenic transformation [6].

Consequently, many efforts have been devoted to search for CDC25 inhibitors. The most potent are currently quinonoid-based structures, which have been extensively studied [7]. Compounds **1** and **2** are lead compounds with IC_{50} values of 5 µM and 125 nM, respectively (Figure 1) [8,9]. They led to the preparation of a number of quinonic derivatives including heteroquinones [10] based on quinoline [11-17], quinazoline [11], phtalazine [11], quinoxaline [16], isoquinoline [11,12,18], isoindole [19], pyrazole [19], isoxazole [19], isothiazole [19] and imidazole [14,19] scaffolds. Since sub-micromolar activities and encouraging selectivities were observed with benzothiazolequinone, **11**, and benzoxazolequinone, **12**, [19] (Figure 1), these two pharmacophores were assembled into a *bis*-quinone moiety, which resulted in a *ca.* 10-fold increase in CDC25 phosphatase inhibition. In addition, this compound was demonstrated to inhibit the growth of human tumor xenografted on mice [20].

We previously reported the synthesis and the inhibitory activity of naphthoquinones and quinolinequinones, **3**-**6**, bearing two identical alkylthio substituents on the quinone ring, these inhibitors displaying IC_{50} values in the micromolar range (Figure 1) [17,21]. Searching for a potent pharmacophore for CDC25 inhibition, Lazo and colleagues identified the bis-substituted naphthoquinone, **2**, (IC_{50} = 125 nM) as well as quinoleine, isoquinoleine and imidazole derivatives, **7**-**10**, as micromolar CDC25 inhibitors [12].

In order to evaluate the effects of five-membered heterocycles fused to the quinone ring, Lavergne *et al.* synthesized and evaluated a set of heterocyclic quinones [19]. They observed that the nature of the heterocycle could modulate the activity of the quinonic derivatives mono-substituted with a *N*,*N*-dimethylethylenediamino group on the quinone ring. In this study, the most potent inhibitors **11** and **12** displayed IC_{50} values in the hundred nanomolar range [19].

In order to investigate the influence, on CDC25 phosphatase activity, of five and six-membered heterocycles fused to the 1,4-dione pharmacophore substituted with two alkylthio groups, we designed, synthesized and evaluated a set of nine heteroquinones bearing two methoxycarbonyl methylthio substituents on the quinone core.

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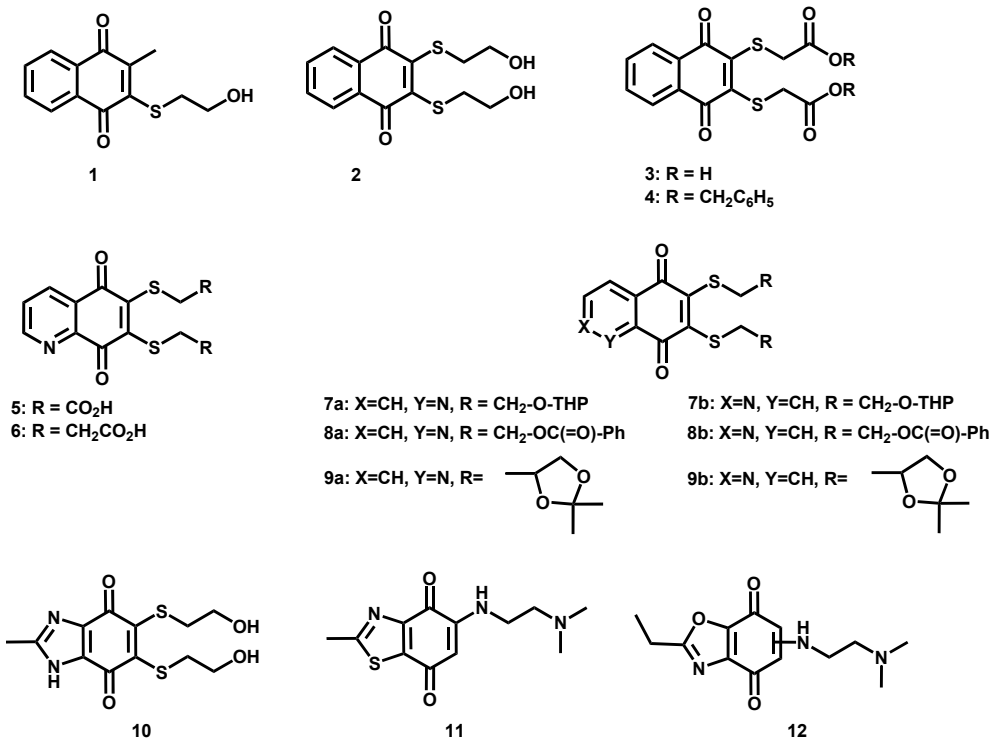


Figure 1. Inhibitors of CDC25 phosphatases.

2. Experimental

2.1. Instrumentation

Melting points were determined on a Büchi B-545 apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance 300 or Avance III 400 spectrometers. IR spectra were recorded on a Nicolet Magna IR-550 FT-IR spectrometer using KBr pellets or an ATR module. Mass spectra were recorded on a Bruker Daltonics Esquire 3000+ or a Thermo Fischer Scientific Polaris Q ion-trap spectrometer. High resolution mass spectra were obtained using a Thermoquest Orbitrap apparatus. Elemental analyses (Service d'Analyse du Département de Chimie Moléculaire de Grenoble, France) were performed using a CHN analyzer built by the Service Central d'Analyses du CNRS (Vernaison, France).

2.2. Synthesis

2.2.1. Preparation of compounds 14-17 and 21-26

2.2.1.1. 2,3-Bis(1-methoxycarbonylmethylthio)-1,4-naphthoquinone **(14)**

To a mixture of 2,3-dichloro-1,4-naphthoquinone (50 mg, 0.22 mmol) and pyridine (50 µL, 0.62 mmol) in ethanol (1 mL) at 0 °C methyl thioglycolate (0.484 mmol, 43 µL) was added. The mixture was stirred at 4 °C for 68 hours then diluted with ethanol. After filtration and evaporation of the volatiles, the residue was recrystallized from hot acetone to give pure **14** as dark violet needles. Yield: 43%. M.p.: 113 °C. FT-IR (film): 2939, 1738 ν(C=O), 1650 ν(C=O), 1583 ν(Aromatic C=C), 1435, 1276, 1164, 1140 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 8.03 (m, 2H, Ar-H), 7.68 (m, 2H, Ar-H), 4.02 (s, 4H, 2×CH_2), 3.70 (s, 6H, 2×CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 178.8 (2C=O), 169.3 (2C=O), 146.2 (2C), 133.7 (2C), 132.8 (2C), 127.0 (2C), 52.7 (2C), 35.5 (2C). MS (ESI$^+$, *m/z*): 367 [(M+H)$^+$], 389 [(M+Na)$^+$]. Anal. calcd. for $C_{16}H_{14}O_6S_2$: C, 52.45; H, 3.86. Found: C, 52.71; H, 4.23.

2.2.1.2. 6,7-Bis(1-methoxycarbonylmethylthio)-5,8-quinolinequinone **(15)**

Under argon, to a solution of methyl thioglycolate (98 µL, 0.97 mmol) in THF (1 mL) was added triethylamine (135 µL, 0.97 mmol) and after 15 min 6,7-dichloroquinoline-5,8-dione (100 mg, 0.44 mmol). After 1 h stirring, diethyl ether (5 mL) was added, which was followed by filtration. The residue obtained after evaporation of the volatiles was purified by column chromatography on silica gel (ethyl acetate) to afford **15** as a red oil. Yield: 51%. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 8.94 (dd, 1H, *J* = 4.5, 1.5 Hz, Ar-H), 8.34 (dd, 1H, *J* = 7.8, 1.5 Hz, Ar-H), 7.62 (dd, 1H, *J* = 7.8, 4.5 Hz, Ar-H), 4.08 (s, 2H, CH_2), 4.00 (s, 2H, CH_2), 3.68 (s, 3H, CH_3), 3.67 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 177.9 (C=O), 177.2 (C=O), 169.0 (C=O), 168.9 (C=O), 154.3 (C), 148.2 (C), 147.8 (C), 144.4 (C), 134.8 (C), 129.7 (C), 127.4 (C), 52.7 (C), 52.6 (C), 35.3 (C), 35.2 (C). MS (ESI$^+$, *m/z*): 368 [(M+H)$^+$], 390 [(M+Na)$^+$]. HRMS (ESI$^+$) calcd. for $C_{15}H_{14}NO_6S_2$ [(M+H)$^+$]: 368.02571. Found: 368.02591.

2.2.1.3. 6,7-Bis(1-methoxycarbonylmethylthio)-5,8-phthalazinequinone **(16)**

A solution of methyl thioglycolate (26 µL, 0.28 mmol) and triethylamine (40 µL, 0.28 mmol) in THF (0.7 mL) was stirred at room temperature during 15 min before addition of 6,7-dichloro-5,8-phtalazinequinone (30 mg, 0.13 mmol). After 2 h stirring, CH_2Cl_2 and water were added and the aqueous layer was extracted (x3) with CH_2Cl_2. The combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure to afford 6,7-*bis*(methoxycarbonyl methylthio)-5,8-dihydroxyphthalazine (37 mg) as a brown oil (^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 9.82 (s, 2H, Ar-H), 3.67 (s, 4H, 2×CH_2), 3.65 (s, 6H, 2×CH_3). IR (film, ν, cm^{-1}): 3383, 2953,

2924, 1737, 1435, 1291, 1197, 1162, 1127, 1009. MS (ESI+, m/z): 371 [(M+H)+]) which was oxidized without purification. Thus 11 mg (0.030 mmol) of it was stirred in acetonitrile:water (1:1) (200 µL) to which was added dropwise a solution of 41 mg of cerium ammonium nitrate (0.075 mmol) in acetonitrile:water (20:1) (320 µL). The mixture was then stirred at room temperature for 30 min, before being diluted with an ice-water slurry (5 mL) and CH_2Cl_2 (5 mL). After separation of the layers, the aqueous phase was extracted with CH_2Cl_2 (3x5 mL), the organic layers were combined and washed with brine (5 mL), dried over Na_2SO_4 and evaporated under reduced pressure. Preparative TLC (Et_2O) gave **16** as a red oil. Yield: 10%. FT-IR (film, cm^{-1}): 3054, 2986, 2955, 2925, 1738 ν(C=O), 1666 ν(C=O), 1461, 1438, 1265, 1158, 896. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 9.75 (s, 2H, Ar-H), 4.08 (s, 4H, 2×CH_2), 3.72 (s, 6H, 2×CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 177.5 (2C=O), 168.9 (2C=O), 146.3 (2C), 146.1 (2C), 125.4 (2C), 52.9 (2C), 35.1 (2C). MS (ESI+, *m/z*): 369 [(M+H)+]. HRMS (ESI+) calcd. for $C_{14}H_{13}N_2O_6S_2$ (M+H)+: 369.02095, found: 369.02098.

2.2.1.4. 2-Methyl-5,6-bis(1-methoxycarbonylmethylthio)-4,7-benzoxazolequinone (17)

To a solution of **21** (80 mg, 0.34 mmol) in ethanol (1.7 mL) was added pyridine (80 µL, 0.99 mmol) and the reaction mixture was cooled to 0 °C before methyl thioglycolate (70 µL, 0.76 mmol) was added. Then, the reaction mixture was stirred at room temperature for 18 h before being diluted with CH_2Cl_2 (5 mL) and water (5 mL) and the aqueous layer was extracted with CH_2Cl_2 (3x5 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure to afford a burgundy-red oil (25 mg) which is a mixture of **17** and 5,6-*bis*(methoxycarbonylmethylthio)-4,7-dihydroxy-2-methyl-benzo[*d*]oxazole (^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 8.62 (br s, 2H, OH), 4.05 (s, 4H, 2×CH_2), 3.73 (s, 6H, 2×CH_3), 2.66 (s, 3H, CH_3)). This mixture was diluted with $CHCl_3$:EtOH (1:1) (220 µL) and cooled to 0 °C before the addition of a solution of $FeCl_3.6H_2O$ (122 mg, 0.45 mmol) in water (110 µL). After stirring for 30 min at room temperature, CH_2Cl_2 (5 mL) and water (5 mL) were added. The aqueous layer was extracted with CH_2Cl_2 (3 x 5 mL) and the combined organic layers were washed with brine (5 mL), dried over Na_2SO_4 and the volatiles were removed under reduced pressure. The dark purple oil thus obtained was purified by column chromatography on silica gel (Et_2O:pentane, 1:1) to afford **17** as a dark red oil. Yield: 79 %. FT-IR (KBr, cm^{-1}): 3054, 2987, 1739 ν(C=O), 1678 ν(C=O), 1421, 1265, 896. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 4.05 (s, 2H, CH_2), 3.99 (s, 2H, CH_2), 3.73 (s, 3H, CH_3), 3.72 (s, 3H, CH_3), 2.55 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 175.3 (C=O), 169.0 (C=O), 168.9 (C=O), 168.5 (C=O), 163.8 (C), 158.0 (C), 147.6 (C), 141.9 (C), 119.6 (C), 52.8 (C), 52.8 (C), 35.8 (C), 35.2 (C), 10.6 (C). MS (ESI+, *m/z*): 372 [(M+H)+], 394 [(M+Na)+]. HRMS (ESI+) calcd. for $C_{14}H_{14}NO_7S_2$ (M+H)+: 372.02062. Found: 372.02112.

2.2.1.5. 5,6-Dichloro-2-methyl-4,7-benzo[d]oxazolequinone (21)

Sodium chlorate (1.06 g, 10.0 mmol) was added in portions over a period of 30 min to a stirred solution of 4-hydroxy-2-methylbenzoxazole (200 mg, 1.34 mmol) in conc. HCl (8 mL) at 65 °C. After 3 h stirring at this temperature, the reaction mixture was cooled to room temperature before dilution with water (20 mL) and CH_2Cl_2 (20 mL). The layers were separated and the aqueous phase was extracted with CH_2Cl_2 (3x10 mL). The organic layers were combined, washed with brine (10 mL), dried over Na_2SO_4 and evaporated under reduced pressure to afford an orange solid which was recrystallized from CH_2Cl_2/MeOH to give **21** as a yellow solid. Yield: 25%. M.p.: 197-199 °C. FT-IR (KBr, cm^{-1}): 3387, 1688 ν(C=O), 1616 ν(C=O), 1543, 1174, 812. ^1H NMR (400 MHz, CD_3OD, δ, ppm): 2.58 (s, 3H, CH_3). ^{13}C NMR (100 MHz, Acetone, δ, ppm): 173.4 (C=O), 167.7 (C=O), 165.6 (C), 159.9 (C), 144.2 (C), 142.4 (C), 120.6 (C), 11.4 (C). MS (ESI+, *m/z*): 233 [(M+H)+]. Anal. calcd. for $C_8H_3Cl_2NO_3$: C, 41.42; H, 1.31; N, 6.04. Found: C, 41.29; H, 1.54; N, 5.67%.

2.2.1.6. 5,6-Bis(1-methoxycarbonylmethylthio)-4,7-benzo[b]thiophenequinone (22)

A solution of benzo[*b*]thiophene-4,7-quinone (49 mg, 0.30 mmol) in 95% EtOH (6 mL) was added to a solution of methyl thioglycolate (56 µL, 0.62 mmol) in 95% EtOH (6 mL) The resulting solution was refluxed for 1 h and after cooling, it was diluted with water (10 mL) and CH_2Cl_2 (10 mL). The layers were separated and the aqueous phase was extracted with CH_2Cl_2 (3x10 mL). The combined organic layers were washed with brine (10 mL), dried over Na_2SO_4 and evaporated under reduced pressure to afford light yellow oil (70 mg). This oil was diluted with acetonitrile:water (1:1) (1.4 mL) before the dropwise addition of a solution of cerium ammonium nitrate (321 mg, 0.58 mmol) in 20:1 of acetonitrile:water (2.7 mL). The mixture was stirred at room temperature for 30 min before dilution with an ice/water slurry (5 mL) and CH_2Cl_2 (5 mL). The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (3x5 mL). The combined organic layers were washed with brine (5 mL), dried over Na_2SO_4 and evaporated under reduced pressure to afford a red solid (60 mg). This material was taken up in 95% EtOH (4.5 mL) and added to a solution of methyl thioglycolate (42 µL, 0.47 mmol) in 95% EtOH (4.5 mL). The resulting solution was then refluxed for 140 min and after cooling, was diluted with water (10 mL) and CH_2Cl_2 (10 mL). The layers were separated and the aqueous phase was extracted with CH_2Cl_2 (3x10 mL). The organic layers were combined, washed with brine (10 mL), dried over Na_2SO_4 and evaporated under reduced pressure to afford a brown oil (91 mg). Once this oil was taken up in acetonitrile:water (1:1) (1.4 mL) a solution of ceric ammonium nitrate (333 mg, 0.61 mmol) in acetonitrile:water (20:1) (2.7 mL) was added dropwise. After stirring for 30 minutes, an ice/water slurry (5 mL) and CH_2Cl_2 (5 mL) were added. The aqueous layer was then extracted with CH_2Cl_2 (3x5 mL) and the combined organic layers were washed with brine (5 mL), dried over Na_2SO_4 and evaporated under reduced pressure to afford a dark purple oil that was purified by column chromatography (CH_2Cl_2) over silica gel to give **22** as a purple oil. Yield: 40 %. FT-IR (film, cm^{-1}): 3054, 2987, 2955, 1736 ν(C=O), 1663 ν(C=O), 1437, 1265, 1147. ^1H NMR (300 MHz, $CDCl_3$, δ, ppm): 7.62 (d, 1H, *J* = 4.8 Hz, Ar-H), 7.48 (d, 1H, *J* = 4.8 Hz, Ar-H), 4.00 (s, 2H, CH_2), 3.99 (s, 2H, CH_2), 3.71 (br s, 6H, 2×CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 174.9 (C=O), 173.5 (C=O), 169.2 (C=O), 169.1 (C=O), 145.1 (C), 144.8 (C), 143.3 (C), 141.6 (C), 133.7 (C), 126.7 (C), 52.7 (C), 52.6 (C), 35.7 (C), 35.6 (C). MS (ESI+, *m/z*): 372 [(M+H)+], 394 [(M+Na)+]. HRMS (ESI+) calcd. for $C_{14}H_{12}O_6S_3Na$ (M+Na)+: 394.96882. Found: 394.96943.

2.2.1.7. 2-Methyl-5,6-bis(1-methoxycarbonylmethylthio)-4,7-benzo[d]thiazolequinone (23)

Compound **23** was obtained as a purple oil from 2-methyl-4,7-benzo[*d*]thiazolequinone, using the procedure described for the preparation of **22**. Yield: 46 %. FT-IR (film, cm^{-1}): 3053, 2986, 1739 ν(C=O), 1671 ν(C=O), 1422, 1265. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 4.04 (s, 2H, CH_2), 3.97 (s, 2H, CH_2), 3.71 (s, 3H, CH_3), 3.70 (s, 3H, CH_3), 2.81 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 173.7 (C=O), 173.4 (C=O), 173.3 (C=O), 169.1 (C), 169.0 (C), 153.2 (C), 145.3 (C), 143.5 (C), 140.0 (C), 52.8 (C), 52.7 (C), 35.7 (C), 35.6 (C), 20.0 (C). MS (ESI+, *m/z*): 410 [(M+Na)+]. HRMS (ESI+) calcd. for $C_{14}H_{14}NO_6S_3$ (M+H)+: 387.99778, found: 387.99872.

2.2.1.8. 6,7-Bis(1-methoxycarbonylmethylthio)-5,8-quinazolinequinone (24)

Compound **24** was obtained from 5,8-quinazolequinone using the procedure described for the preparation of **22**. Yield: 31 %. FT-IR (film, cm^{-1}): 2996, 2954, 1737 ν(C=O), 1666 ν(C=O), 1566 ν(Aromatic, C=C), 1435, 1280. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 9.58 (s, 1H, Ar-H), 9.42 (s, 1H, Ar-H), 4.09 (s, 2H, CH_2), 4.07 (s, 2H, CH_2), 3.70 (s, 3H, CH_3) 3,68 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 176.8 (C=O), 176.4 (C=O), 168.9 (C=O), 168.8 (C=O), 162.5 (C), 157.2 (C), 153.5 (C), 147.3 (C), 145.5 (C), 125.3 (C), 52.9 (C), 52.8 (C), 35.2 (C), 35.1 (C). MS (ESI^+, *m/z*): 369 [$(M+H)^+$], 391 [$(M+Na)^+$]. HRMS (ESI^+) calcd. for $C_{14}H_{13}N_2O_6S_2$ $(M+H)^+$: 369.02095. Found: 369.02101.

2.2.1.9. 6,7-Bis(1-methoxycarbonylmethylthio)-5,8-quinoxalinequinone (25)

Methyl thioglycolate (25 µL, 0.275 mmol) and triethylamine (38 µL, 0.275 mmol) were mixed in THF (620 µL) at room temperature and stirred for 15 min before 5,8-quinoxalinequinone (20 mg, 0.125 mmol) was added. After stirring for 12h, the volatiles were evaporated under reduced pressure and the residue was dissolved in water and then extracted with CH_2Cl_2 (3x10 mL). The combined organic layers were washed with brine (15 mL), dried over Na_2SO_4 and evaporated under reduced pressure to afford a yellow solid which was purified by column chromatography on silica gel (Et_2O:MeOH, 94:6) and crystallized from hot ethanol to afford 5,8-dihydroxy-6,7-*bis*(1-methoxycarbonylmethylthio)quinoxaline (17 mg, 37 %) as a yellow solid. M.p.: 129-130 °C. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 8.83 (s, 2H, Ar-H), 8.01 (s, 2H, OH), 3.86 (s, 4H, 2×CH_2), 3.68 (s, 6H, 2×CH_3). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 170.4 (2C=O), 147.3 (C), 144.3 (C), 133.6 (C), 119.3 (C), 52.7 (C), 36.7 (C). IR (KBr, ν, cm^{-1}): 1720, 1439, 1400, 1278, 1154, 1121, 1100. HRMS (ESI^+) calcd. for $C_{14}H_{14}N_2O_6S_2Na$ $(M+Na)^+$: 393.01855, Found: 393.01857. This material (17 mg, 0.046 mmol) was taken-up in acetonitrile:water (1:1) (500 µL), to which was added dropwise under stirring a solution of cerium ammonium nitrate (55 mg, 0.101 mmol) in acetonitrile:water (20:1) (950 µL). The mixture was then stirred at room temperature for 30 min before being diluted with a ice/water slurry (5 mL) and CH_2Cl_2 (5 mL). The aqueous phase was extracted with CH_2Cl_2 (4x5 mL) and the combined organic layers were washed with brine (5 mL), and dried over Na_2SO_4. Evaporation of the volatiles under reduced pressure afforded a dark purple oil which was purified by column chromatography on silica gel (CH_2Cl_2 then CH_2Cl_2:MeOH, 95:5) to give **25** as an orange oil. Yield: 24%. FT-IR (film, cm^{-1}): 3003, 2954, 2924, 2851, 1731 ν(C=O), 1667 ν(C=O), 1530 ν(Aromatic C=C), 1485, 1435, 1286, 1191, 1004. ^1H NMR (300 MHz, $CDCl_3$, δ, ppm): 8.69 (s, 2H, Ar-H), 4.12 (s, 4H, 2×CH_2), 3.70 (s, 6H, 2×CH_3). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 176.7 (2C=O), 169.0 (2C=O), 148.6 (C), 146.6 (C), 144.6 (C), 52.8 (C), 35.2 (C). MS (ESI^+, *m/z*): 369 [$(M+H)^+$], 391 [$(M+Na)^+$]. Anal. calcd. for $C_{14}H_{12}N_2O_6S_2$: C, 45.65; H, 3.28; N, 7.60. Found: C, 45.89; H, 3.22; N, 7.23%.

2.2.1.10. 5,6-Bis(1-methoxycarbonylmethylthio)-4,7-indolequinone (26)

Methyl thioglycolate (94 µL, 1.05 mmol) and triethylamine (150 µL, 1.0 mmol) were added to THF (4.8 mL) and the mixture was stirred at room temperature for 15 min before the addition of 4,7-indolequinone (70 mg, 0.476 mmol). The reaction mixture was stirred for 16 h, then was diluted with water (5 mL) and CH_2Cl_2 (5 mL). The aqueous layer was extracted with CH_2Cl_2 (3x10 mL) and the combined organic layers were washed with brine (15 mL), dried over Na_2SO_4 and evaporated under reduced pressure. Purification of the crude product was achieved by column chromatography on silica gel (CH_2Cl_2:AcOEt, 94:6) to give **26** as a purple oil. Yield: 36%. FT-IR (KBr, cm^{-1}): 3300 ν(N-H), 2953, 2926, 1737 ν(C=O), 1649 ν(C=O), 1435, 1381, 1292, 1194, 1157, 1124, 1008. ^1H NMR (300 MHz, $CDCl_3$, δ, ppm): 10.28 (br s, 1H, NH), 7.01 (d, 1H, *J*=2.7 Hz, Ar-H), 6.55 (d, 1H, *J* = 2.7 Hz, Ar-H), 4.01 (s, 2H, CH_2), 3.94 (s, 2H, CH_2), 3.73 (s, 3H, CH_3), 3.72 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 176.6 (C=O), 171.2 (C=O), 169.8 (C=O), 169.6 (C=O), 146.8 (C), 141.5 (C), 130.8 (C), 126.2 (C), 126.0 (C), 109.4 (C), 52.7 (C), 52.6 (C), 36.1 (C), 35.8 (C). MS (ESI^+, *m/z*): 356 [$(M+H)^+$], 378 [$(M+Na)^+$]. HRMS (ESI^+) calcd. for $C_{14}H_{14}NO_6S_2$ $(M+H)^+$: 356.02571. Found: 356.02617. Anal. Calcd. for $C_{14}H_{13}NO_6S_2$: C, 47.31; H, 3.69; N, 3.94. Found: C, 47.89; H, 3.85; N, 3.76%.

2.2.2. In vitro enzymatic assay

The activity of the MBP-CDC25B [22] recombinant enzyme was monitored using fluorescein diphosphate. The assay was performed in 96-well plates in a final volume of 200 µL. MBP-CDC25B3 was diluted in assay buffer [30 mM Tris-HCl (pH = 8.2), 75 mM NaCl, 0.67 mM EDTA, 0.033% BSA, 1 mM DTT] so that the final concentration was 90 ng/well. The reaction was initiated by addition of 25 µM of fluorescein diphosphate followed by immediate measurement of fluorescein phosphate emission with a Fluoroskan Ascent (Lab Systems; excitation filter: 485 nm, emission filter: 530 nm). For each compound, the drug concentration required for 50% inhibition (IC_{50}) was determined from a sigmoidal dose-response curve using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Results are expressed as the mean of at least two independent experiments with three determinations per tested concentration and per experiment.

2.2.3. Cell proliferation assay

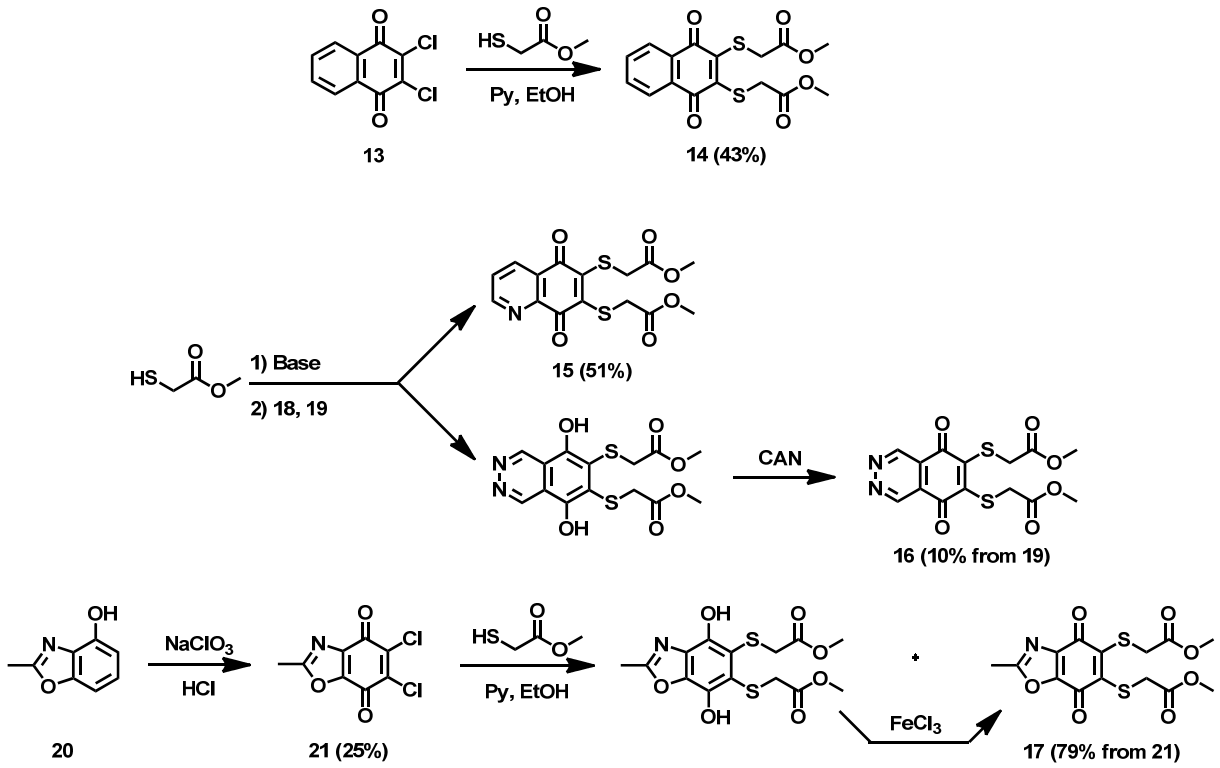
The inhibition of cell proliferation was determined using a colorimetric assay based on the cleavage of the WST-1 tetrazolium salt by mitochondrial dehydrogenases in viable cells, leading to formazan formation. At day 0, HeLa and MiaPaCa-2 cells were plated at 5500 and 5000 cells/well, respectively in 96-well culture plates with 95 µL of medium/well. At day 1, cells were treated for 48 h with 5 µL of increasing concentrations of drug (dissolved in DMSO, to a final concentration < 0.05%). At day 3, after addition of 10 µL of WST-1 per well, cells were incubated 2 hours at 37 °C in a humidified atmosphere of 5% CO_2. Absorbance was then measured at 490 nm with a Bio-Rad microplate reader. The results are expressed as the mean of three independent experiments with three determinations per tested concentration and per experiment. The IC_{50} value were determined from a sigmoidal dose-response using GraphPad Prism (GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. Chemistry

Parent compound *bis*(methoxycarbonylmethylthio) naphthaquinone, **14**, was prepared from 6,7-dichloro-naphthoquinone, **13**, by nucleophilic attack of methyl mercaptoacetate [23].

Compounds **15** and **16** were obtained from 6,7-dichloro-5,8-quinolinequinone, **18**, [24] and 6,7-dichloro-5,8-phthalazinequinone, **19**, [24], respectively, using 'reverse addition' conditions (i.e. addition of the dichloroheteroquinone to a solution of sodium methoxy carbonylmethyl-thiolate). Derivative **17** was synthesized from 5,6-dichloro-2-methyl-4,7-benzoxazolequinone, **21**, prepared by oxidative halogenation of 7-hydroxy-2-methylbenzoxazole, **20**, [25].



Scheme 1

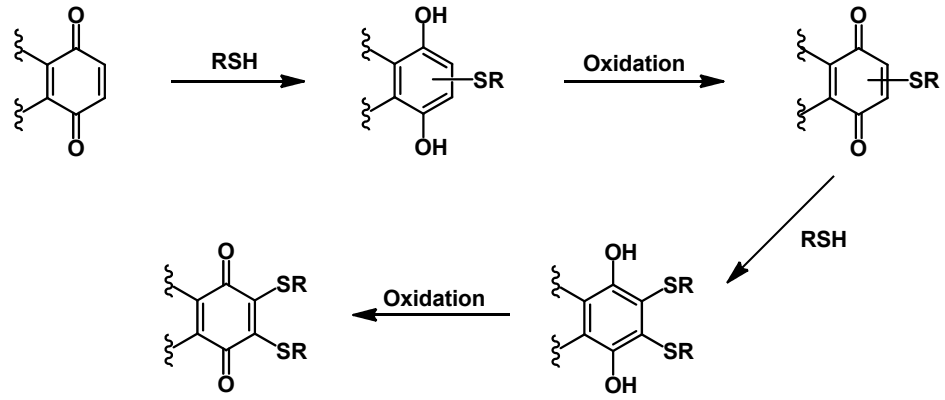


Figure 2. Addition of a thiol onto heteroquinones (regioisomers are not highlighted).

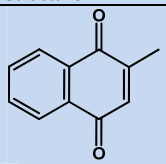
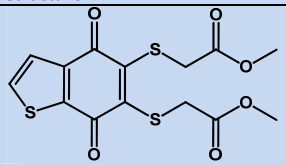
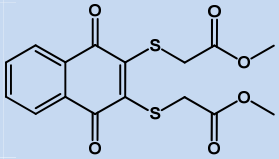
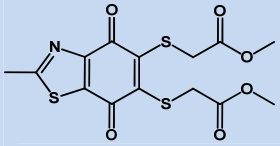
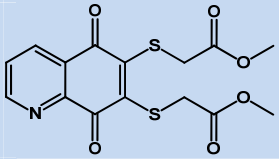
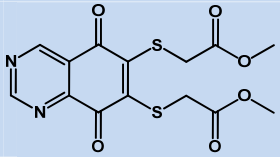
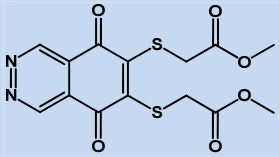
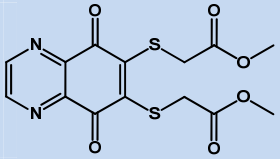
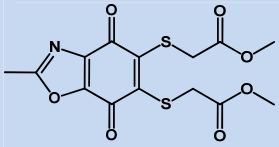
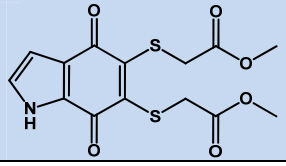
Since *bis*(methoxycarbonylmethyl-thio)hydroquinones are the primary products obtained from **19** or **21**, an oxidation step was required which was carried out conventionally ($FeCl_3$ or CAN) (Scheme 1).

When thiols react with unsubstituted quinones, three intermediates are met *en route* to get a disubstituted quinone (Scheme 2). These can be observed spectroscopically but do not need to be isolated as a sequential addition/oxidation/addition/oxidation process can be carried out up to the final product, as it has been previously exemplified for the preparation of *bis*(thio) derivatives of quinocarcin [26].

Accordingly, reacting benzo[*b*]thiophene-4,7-quinone [27,28], 2-methyl-4,7-benzo[*d*]thiazolequinone [29-32] and 5,8-quinazolinequinone [33,34] with methyl mercaptoacetate, followed by oxidation (CAN) in a stepwise and duplicated manner (see experimental section), afforded *bis*-substituted heteroquinones, **22**-**24**, (Figure 2). Concerning the 5,8-quinoxalinequinone scaffold [24,35,36], it yielded a *bis*-(thio)hydroquinone in a single step which was followed by oxidation to get **25**. Moreover, in the case of indole-4,7-dione [37,38], the *bis*-substituted quinone, **26**, could be obtained directly, *i.e.* without the need for an external oxidizing agent, which presumably arises from relative redox properties of intermediate species within the reaction system.

Although *bis*(methoxycarbonylmethylthio)-heteroquinones, **14**-**17** and **22**-**26** have been isolated in fair-to-low (but unoptimized) yields (Figure 3), they have been obtained in pure state, which has allowed their evaluation on CDC25 phosphatase enzymatic activity.

Table 1. Inhibitory activities of heteroquinones **14**-**17** and **22**-**26** toward CDC25.

Compound	Structure	IC_{50}±SEM (μM)	Compound	Structure	IC_{50}±SEM (μM)
Menadione		2.20±0.60	22		1.30±0.03
14		1.17±0.04	23		3.81±0.06
15		1.63±0.21	24		1.83±0.28
16		3.38±0.17	25		5.40±1.81
17		5.70±0.47	26		4.49±1.45

[a]The IC_{50} values were calculated from three independent experiments.

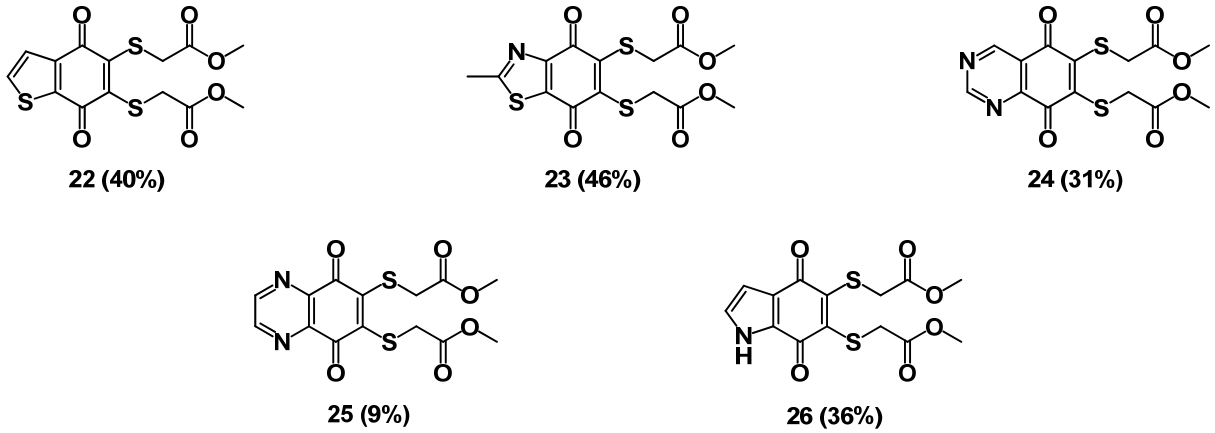


Figure 3. Structures of quinones, **22**-**26**.

3.2. Enzymatic activity studies

The nine newly synthesized heteroquinones, **14**-**17** and **22**-**26** were examined for their inhibitory activity against the recombinant protein MBP-CDC25B using fluorescein-3,6-diphosphate (FDP) as the artificial substrate. The fluorescent emission resulting from dephosphorylation was monitored during 30 min at 30 °C in the presence of increasing concentrations of inhibitors [21]. Menadione was used as a reference. Results are reported in Table 1.

All tested quinones show comparable inhibitory activities with IC_{50} values ranging from 1.17 to 5.70 μM, naphthoquinone being the most potent pharmacophore for this set of heteroquinones. With regard to the six-membered heterocyclic derivatives, **14**-**16**, and **24**-**25**, increasing the number of nitrogen atoms on the heterocycle led to a slight decrease of activity, quinoxalinequinone, **25**, being the less potent inhibitor.

The parent compound **14** with two methyl ester groups is 10-fold less potent than the *bis*(2-hydroxyethylthio) analogue NSC95397 [12] for which an IC_{50} of 125 nM was reported though we previously measured for this compound an IC_{50} value of 3.8 μM under our conditions [21]. On the other hand, **14** displayed similar activity when compared with the *bis*-carboxylic acid **3** and the corresponding benzyl di-ester **4** [21].

Table 2. In vitro growth inhibition of HeLa and MiaPaCa-2 cell lines.

Compound	HeLa % of growth inhibition		MiaPaCa-2 % of growth inhibition	
	100 µM	10 µM	100 µM	10 µM
Menadione	IC_{50} = 37.5±3.5 µM		IC_{50} = 33 µM	
15	79±0.9	13±0.9	77±2.1	24±15.4
16	60±2.1	21.0±1.5	74±0.7	10±3.7
17	62±3.1	6.0±4.1	75±1.2	0
22	IC_{50} = 26.9±6.3 µM		IC_{50} = 29.0± µM	
23	IC_{50} = 34.5±4.9 µM		78±1.7	27
24	47.0±5.5	22.0±3.0	71±0.2	4±3.6
25	0	0	24±4.6	6±4.8
26	82.0±0.9	8.0±6.2	75±2.0	5±5.0

In contrast, the bis-ester quinolinequinone **15** (IC_{50} = 1.63 µM) is more than 5-fold more potent than its bis-carboxylic acid counterpart (IC_{50} = 11.2 µM for **5**) [17].

Within the five-membered heterocycle sub-set, benzoxazolequinone, **17**, and benzothiazolequinone, **23**, bearing a methyl group on the heterocyclic moiety did not lead to the expected increase of activity when compared with **11** and **12** [19]. Extending the scope of the study to scaffolds such as benzothiophenequinone, **22**, and indolequinone, **26**, which had not been previously considered for CDC25 inhibition did not bring any improvement.

Finally, when comparing both sub-families, results indicate that the six-membered heterocyclic derivatives are slightly more active than the five-membered heterocyclic set, benzothiophenequinone, **22**, being an exception.

3.3. Antiproliferative activity

The cytotoxicity of **8** quinonoid-based CDC25B inhibitors was evaluated on the human cancer cell lines HeLa (cervix) and MiaPaCa-2 (pancreatic adenocarcinoma) using a WST-1 colorimetric cleavage assay. The percentage of growth inhibition was determined at 100 µM and 10 µM for all compounds and the IC_{50} values were measured for the most efficient heterocyclic quinones only. Menadione was also tested and inhibited HeLa and MiaPaCa-2 cell lines with comparable IC_{50} of 37.5 and 33 µM, respectively (Table 2).

All compounds showed cytotoxic activity toward both cell lines at 100 µM with percentages of growth inhibition above 60% except derivative, **25**, which was totally inactive on HeLa cells and displayed a percentage of 24% inhibition at 100 µM toward the pancreatic cell line. These series of quinone is less potent on HeLa cells than compounds **1** and **2** for which we previously reported IC_{50} values of 8.8 and 10.8 µM, respectively [21]. Benzothiophenequinone, **22**, which also showed one of the highest anti-phosphatase activity was the most cytotoxic quinone with activities comparable to that of menadione.

4. Conclusion

Quinonoid-based compounds are currently the most potent CDC25 phosphatase inhibitors. In the present study, we examined the effect on CDC25B activity of heterocycles fused to the 1,4-dione pharmacophore substituted with two thiol groups. For this limited series of *bis*-substituted heteroquinones, important modifications of the heterocyclic ring are well tolerated since all derivatives show similar micromolar activities. Unfortunately, we were not able to increase the affinity of the quinonic compounds by modulating the heterocyclic part as expected. These results underline the role of substitution of the quinone ring, which is essential for potent CDC25 inhibition [12,19]. Thus, for this set of quinonic derivatives, the substitution pattern appears to be responsible for the moderate inhibitory activity level and probably explains the absence of significant differences in activities between these heteroquinones. Therefore, it could be valuable to prepare and evaluate on CDC25 phosphatase activity heteroquinones bearing two *N*,*N*-dimethylethylenediamino or 2-hydroxyethylthio groups on the quinone ring.

Acknowledgements

Martine Fayolle is thanked for the preparation of 4-hydroxy-2-methylbenzoxazole (**20**) and Tatiana Besset is grateful to the 'Ministère de l'Education Nationale, de la Recherche et de la Technologie' (MENRT) for a doctoral fellowship. MBP-CDC25B3 was provided by Bernard Ducommun.

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