Quinonoid and phenazine compounds: Synthesis and evaluation against H37Rv, rifampicin and isoniazid-resistance strains of Mycobacterium tuberculosis


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Abstract

Several quinonoid and phenazine compounds were synthesized in moderate to high yields and showed activity against H37Rv, rifampicin and isoniazid-resistance strains of Mycobacterium tuberculosis. The cytotoxicity of the compounds were evaluated against human peripheral blood mononuclear cells (PBMC) and these substances emerge as promising antitubercular prototypes.

1. Introduction

Tuberculosis (TB) is a very aggressive elderly lung disease caused by Mycobacterium tuberculosis [1] and still represents nowadays a cause of global death [2]. According to WHO, 9.27 million new cases of TB occurred in 2007 and about these cases, estimates that, 1.37 million (14.8%) were HIV-positive [3]. The emergence of the M. tuberculosis in the immunocompromised population represents a problem that suggest continuing search of new compounds against this pathogen [4]. Moreover, in the absence of an effective vaccine, treatment is the main tool for control ling the dissemination of TB, but the length of treatment (usually 6 months) makes it difficult for patients to comply with treatment [5].

Clofazimine, an important phenazine compound, was originally reported in 1957 as a potent antituberculosis agent and has been used as prototype to developed new drugs with antimicrobial activities [6]. Phenazine-1-carboxylic acids, also known as tubermcins present potent activity against M. tuberculosis, and represent a class of phenazines used to obtain new actives substances by chemical modifications (Fig. 1).

Quinonoid compounds have been described as important structures with pharmacological diversity [7]. Recently, our research group has investigated the antitumor[8,9], trypanocidal [10,11] and antibacterial [12] activity of a large number of substances obtained from lapachol (1) as part of our study to search new heterocyclic compounds [13] with potential activity against neglected diseases. In this context, some of us have synthesized and evaluated a group of the macrolactones and phenazine compounds identifying new actives substances against M. tuberculosis [14].

Following our program to developed new compounds with potent antinycobacterial activities, herein, we present the synthesis and evaluation of quinones and new phenazine compounds against M. tuberculosis pan-susceptible and resistants strains.
2. Chemistry

Lapachol (1) was extracted from the heartwood of Tabebuia sp. (Tecoma) and purified by a series of recrystallizations [15]. From this quinone, nor-lapachol (11) was obtained by Hooker oxidation method [16].

Initially, from lapachol (1) the phenazine 2 was prepared in two steps as previously described by us [17]. Lapachol was reduced by catalytic reduction with Pd/C as catalyst and the substances 3 and 4 were prepared [18] and used to synthesize the phenazine compounds 5 and 7 in good yields and the ether derivatives 6 and 8, respectively. Finally, the ether phenazines 9 and 10 were obtained from the substances 6 and 8, as yellow solids (Scheme 2).

Nor-lapachol (11) was submitted to reaction with ortho-phenylenediamine in acetic acid to obtain the phenazine 12 and from the reaction of the catalytic reduction the substances 13 and 14 were also obtained from this naphthoquinone. In order, to obtain new phenazine compounds 15 and 18 from 13 and 14 the same reaction for form the phenazinic ring was employed (Scheme 2).

The Hooker’s reaction with PbO2 [19] with minor modifications [13,20] was used to produce the ether derivatives 16 and 17 from the reduced quinones 13 and 14 and subsequently these substances were used to obtain the ether phenazine derivatives 19 and 20 with the side chain and aromatic portions reduced (Scheme 2).

From C-allyl lawsone (21) [21] was obtained the reduced products 22 and 25 and the ether derivative 24. The phenazinic compounds 23 and 26 were obtained from C-allyl lawson and reduced quinone 25, respectively. From ether quinones 24 and 27 the ether phenazine compounds 28, 30 and 31 were synthesized using the reaction with the diamine in acetic acid (Scheme 3).

All the compounds were purified by silica gel column chromatography and in the case of the ether phenazines the recrystallization were preferred to avoid the cleavage of the C–O–C bonds as previously described by us [18] and in general, the substances were obtained in excellent yields.

The structures of the compounds were confirmed by techniques such as 1H and 13C NMR, IR, high-resolution (electrospray ionization) mass spectra. For the compounds 12 and 23 the 13C NMR spectra were not obtained due the low solubility in different solvents in appropriate concentration to get these spectra. The substance 23 was obtained in your crystalline form and the structure was solved by crystallographic methods.

Ortep-3 diagram of the molecule is shown in Fig. 2, and Table 1 lists main crystallographic parameters. In the unit cell there are four independent molecules not related by symmetry and for the sake of clarity in Fig. 2 we are showing only one of four molecules. Bond lengths and angles are available in supporting information and are in good agreement with the expected values reported in the literature [22]. The dihedral angle formed between the least squares plane passing through the atoms of benzophenazine ring (A–B) and that plane passing through the atoms of benzene ring (D) is 2.3 (3)°, therefore, all rings of the molecule are almost planar as showing in Fig. 3a. The torsion angle between atoms C6–C13–C14–C15 is 47.5 (2)°. The packing of the crystal structure is show in Fig. 3b.

3. Results and discussion

The minimum inhibitory concentrations (MICs) for all the phenazine compounds and precursor quinones were evaluated against M. tuberculosis H37Rv, M. tuberculosis rifampicin resistance (ATCC 35338) and M. tuberculosis isoniazid resistance (ATCC 35822) (Table 2). The toxicity of compounds toward a normal proliferating cell was investigated using the Alamar Blue assay performed with peripheral blood mononuclear cells (PBMC) after 72 h of drug exposure. The compounds were classified by us according to their activity against M. tuberculosis as highly active (MIC ≤ 3 μg/mL), lowly active (3 μg/mL < MIC < 100 μg/mL), or inactive (MIC > 100 μg/mL).

Lapachol (1), an important naphthoquinone precursor presents lowly active against H37Rv and ATCC 35338 strains and for ATCC 35822 strains the substance was inactive.

Compounds 2, 9, 10, 15, 18, 19, 26, 28, 30 and 31 were considered inactive when MIC > 100 μg/mL for H37Rv, ATCC 35338 and ATCC 35822 strains.

The substances 3, 4, 12, 13, 14, 20, 21, 22, 23 and 25 were lowly active (12.5 μg/mL < MIC < 100 μg/mL for H37Rv, ATCC 35338 and ATCC 35822 strains). With MIC value of the 25, 12.5 and 25 μg/mL for the M. tuberculosis H37Rv, M. tuberculosis rifampicin resistance (ATCC 35338) and M. tuberculosis isoniazid resistance (ATCC 35822) the reduced quinones 4 and 14 are important prototypes for new derivation for the achievement of compounds more actives.

The substance 7 was considered highly active [MIC ≤ 3 μg/mL (9.75 μM)] against M. tuberculosis H37Rv. For ATCC 35822 strain the compound was less active with MIC value of 12.5 μg/mL and for ATCC 35822 strain was inactive, this could be related to the molecular basis of resistance of these strains. This compound also was not cytotoxic against normal cells [IC50 > 25 μg/mL (>78.02 μM)] and represents an important prototype for development of new drugs against TB.

Nor-lapachol (11) showed promising activity against ATCC 35338 strains MIC [3.12 μg/mL (13.7 μM)] but lowly active for H37Rv and not active for ATCC 35822 strains (MIC = 100 μg/mL). As previously described nor-lapachol (11) was not cytotoxic against normal cells (IC50 > 25 μg/mL [>109.53 μM]) and this substance appears as excellent prototype against resistant to rifampicin strains.

4. Conclusions

Some phenazine compounds were synthesized and evaluated against M. tuberculosis and the compounds 7 and 11 presented important activity and could be considered new antimycobacterial prototypes. The substances were inactive against human peripheral blood mononuclear cells (PBMC) in a concentrate of 25 μg/mL and present great selectivity against the agent that cause TB.

5. Experimental

5.1. Chemistry

Melting points were obtained on Thomas Hoover and are uncorrected. Analytical grade solvents were used. Column chromatography was performed on silica gel (Acros Organics 0.035–0.070 mm, pore diameter ca 6 nm). Infrared spectra were
recorded on an FTIR Spectrometer IR Prestige-21 – Shimadzu. $^1$H and $^{13}$C NMR were recorded at room temperature using a VNMRSYS-500, Varian MR 400 instrument. Varian Mercury Plus 300 instrument and Bruker AVANCE DRX200, in the solvents indicated, with TMS as internal standard. Chemical shifts ($\delta$) are given in ppm and coupling constants ($J$) in Hertz. High resolution mass spectra (electrospray ionization) were obtained using a MicroTOF Ic – Bruker Daltonics. In some cases the spectra were obtained using a gas chromatograph mass spectrometer GCMS-QP2010 PLUS Shimadzu with column DB-5MS and GCMS-QP5000 (70 eV). The fragments were described as a relation between atomic mass units and the charge ($m/z$) and the relative abundance

Scheme 1. Obtention of phenazine compounds 2, 5, 7, 9 and 10. Reagents and conditions: (i) THF, 10 mol% Pd/C, 30 psi of H$_2$, 15 min; (ii) AcOH, 10 mol% Pd/C, 55 psi of H$_2$, 6 h; (iii) AcOH, PbO$_2$.

Scheme 2. Obtention of phenazine compounds 12, 15, 18, 19 and 20. Reagents and conditions: (i) THF, 10 mol% Pd/C, 30 psi of H$_2$, 15 min; (ii) AcOH, 10 mol% Pd/C, 55 psi of H$_2$, 6 h; (iii) AcOH, PbO$_2$. 
in percentage of the base peak intensity. All the compounds were nominated using the program CS ChemDraw Ultra version 10.0.

5.2. General procedures to prepare the phenazine compounds

The respective quinone (1.0 mmol), 7.9 mmol of crystalline sodium acetate, 2.3 mmol of ortho-phenylenediamine and 5 mL of glacial acetic acid were heated on steam-bath for a short time and monitored by silica gel TLC (eluted with hexane and ethyl acetate and dichloromethane 8:1:1). After this time, the solution was poured on ice and filtered under vacuum. The phenazine compounds obtained from lapachol (1), nor-lapachol (11), C-allyl lawsone (21) and the reduced derivatives were purified by column chromatography eluted with an increasing polarity gradient mixture of hexane and ethyl acetate.

5.2.1. 6-Isopentylbenzo[a]phenazin-5(7H)-one (5)

The reaction of 2-hydroxy-3-isopentylnaphthalene-1,4-dione (3), (244 mg, 1 mmol), 7.9 mmol of crystalline sodium acetate, 2.3 mmol of ortho-phenylenediamine in 5 mL of glacial acetic acid yielded product 5, (287 mg, 0.9 mmol, 90% yield) as a red solid; mp 79–80 °C; IR (KBr) 3466, 3352, 3057, 2953, 2918, 2868, 2851, 1661, 1647, 1583, 1580, 1545, 1530, 1499, 1468, 1458, 1435, 1413, 1381, 1362, 1346, 1335, 1312, 1277, 1246, 1220, 1111, 1040, 1030, 1015, 964, 934, 770, 756, 723 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 9.49–9.23 (m, 1H), 8.38–8.05 (m, 3H), 7.86–7.71 (m, 4H), 3.46–3.18 (m, 2H), 1.83–1.72 (m, 1H), 1.66–1.59 (m, 2H), 1.07 (s, 3H), 0.97 (s, 3H), 0.83 (s, 3H), 0.75 (s, 3H).
Table 1
Crystal data and structure refinement.

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5.2.2. 6-Isopentyl-1,2,3,4-tetrahydrobenzo[a]phenazin-6-yloxy)naphthalene-1,4-dione 9

The reaction of 6-phenylenediamine in 5 mL of glacial acetic acid yielded product 9, (340 mg, 0.6 mmol, 61% yield) as an yellow solid; mp 160–162 ºC; IR (KBr) 3070, 2953, 2930, 2870, 2855, 1699, 1655, 1547, 1574, 1490, 1460, 1433, 1287, 1267, 1211, 1202, 1157, 1107, 1016, 1078, 1050, 1016, 954, 594, 734, 718 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.95 (d, 1H), 8.24 (d, 1H), 8.15 (d, 1H), 7.99–7.86 (m, 3H), 7.76–7.63 (m, 3H), 7.52 (t, 1H), 7.43–7.38 (d, 1H), 7.34 (t, 1H), 2.99–2.91 (t, 2H), 2.27–2.16 (m, 1H), 2.14–2.03 (m, 1H), 1.90–1.79 (m, 2H), 1.51–1.64 (m, 1H), 1.49–1.35 (m, 2H), 1.32–1.23 (m, 1H), 1.15–1.06 (dd, 6H), 0.76 (dd, 6H); ¹³C NMR (100 MHz, CDCl₃) δ 192.8, 184.1, 181.1, 153.6, 152.5, 145.6, 144.1, 141.3, 131.5, 134.6, 133.6, 133.5, 132.4, 131.7, 131.4, 130.6, 130.0, 129.9, 129.3, 129.1, 127.7, 126.9, 125.7, 88.1, 40.4, 37.0, 31.8, 28.8, 28.1, 22.7, 22.6, 22.4, 22.2, 22.2; El/HRMS (m/z) [M + H]+ 559.2604. Calcld for [C_{38}H_{34}N_{2}O_{4}]⁺: 559.2596.

5.2.4. 6-Isopentyl-1,2,3,4,5,6-hexahydrobenzo[a]phenazin-6-yloxy)naphthalene-1,4-dione 10

The reaction of 6-phenylenediamine in 5 mL of glacial acetic acid yielded product 10, (226 mg, 0.4 mmol, 40% yield) as an yellow solid; mp 105–107 ºC; IR (KBr) 3040, 2953, 2985, 1680, 1643, 1605, 1500, 1470, 1460, 1449, 1429, 1412, 1287, 1267, 1210, 1150, 1105, 1070, 946, 840, 766 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.80–8.74 (m, 4H), 7.64–7.68 (m, 2H), 3.34–3.23 (m, 1H), 2.98–2.85 (m, 4H), 2.78–2.64 (m, 3H), 2.49–2.39 (m, 1H), 2.35–2.28 (m, 2H), 2.21–2.05 (m, 1H), 2.00–1.66 (m, 8H), 1.59–1.30 (m, 8H), 1.22–1.11 (m, 1H), 1.03 (dd, 6H), 0.79 (dd, 6H); ¹³C NMR (100 MHz, CDCl₃) δ 194.9, 187.6, 183.5, 154.4, 150.0, 147.6, 145.8, 141.3, 140.2, 139.0, 138.0, 129.9, 129.5, 129.4, 128.9, 110.8, 87.3, 40.9, 37.0, 32.0, 28.7, 21.8, 24.9, 23.3, 22.7, 22.6, 22.4, 22.2, 22.2; El/HRMS (m/z) [M + H]+ 567.3215. Calcld for [C_{41}H_{36}N_{2}O_{4}]⁺: 567.32231.

Fig. 3. (a): A view of the molecule parallel to planes of the rings and (b): The packing of the molecules, viewed down the c axis.
6.5. 6-Isobutylbenz[a]phenazin-5(7H)-one 15

The reaction of 2-hydroxy-3-isobutylnaphthalene-1,4-dione (13), (230 mg, 1 mmol), 7.9 mmol of crystalline sodium acetate, 2.3 mmol of ortho-phenylenediamine in 5 mL of glacial acetic acid yielded product 15, (263 mg, 0.88 mmol, 87% yield) as an red solid; mp 120–124 °C; IR (KBr) 3449, 3374, 3100, 2951, 2920, 2866, 1655, 1643, 1624, 1591, 1460, 1369, 1350, 1311, 1279, 1236, 1179, 1169, 1040, 1020, 970, 910, 780, 760, 727 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 9.43–9.37 (1H, m, H-1), 8.43–8.21 (3H, m, H-2'/H-3'; 1H, s, H-7), 2.19 (s, 3H, H-8); 13C NMR (100 MHz, CDCl₃) δ 153.4, 142.9, 142.1, 140.5, 139.8, 139.7, 139.4, 137.1, 135.8, 134.2, 132.0, 129.6, 129.3, 129.0, 128.1, 127.9, 126.2, 125.6, 125.4, 123.4, 122.8, 115.7, 32.0, 28.2, 22.5; MS [70 eV, m/z (%)]: 300 (33), 285 (100), 268 (44), 255 (23), 249 (22), 235 (22), 219 (16), 205 (14), 193 (13), 181 (4), 167 (3), 141 (13), 135 (11), 125 (11), 123 (11), 105 (11), 77 (20), 65 (8), 43 (38), 41 (32).

5.2.6. 6-Isobutylbenz[a]phenazin-5(7H)-one 18

The reaction of 2-hydroxy-3-isobutylnaphthalene-1,4-dione (13), (230 mg, 1 mmol), 7.9 mmol of crystalline sodium acetate, 2.3 mmol of ortho-phenylenediamine in 5 mL of glacial acetic acid yielded product 18, (294 mg, 0.98 mmol, 96% yield) as an yellow solid; mp 131–133 °C; IR (KBr) 3399, 3057, 2949, 2930, 2864, 2837, 2525, 1624, 1603, 1524, 1458, 1431, 1381, 1364, 1335, 1231, 1200, 1175, 1152, 1134, 1111, 1028, 945, 840, 756 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.43–8.21 (3H, m, H-2'/H-3'; 1H, s, H-7), 2.19 (s, 3H, H-8); 13C NMR (100 MHz, CDCl₃) δ 153.4, 142.9, 142.1, 140.5, 139.8, 139.7, 139.4, 137.1, 135.8, 134.2, 132.0, 129.6, 129.3, 129.0, 128.1, 127.9, 126.2, 125.6, 125.4, 123.4, 122.8, 115.7, 32.0, 28.2, 22.5; MS [70 eV, m/z (%)]: 300 (33), 285 (100), 268 (44), 255 (23), 249 (22), 235 (22), 219 (16), 205 (14), 193 (13), 181 (4), 167 (3), 141 (13), 135 (11), 125 (11), 123 (11), 105 (11), 77 (20), 65 (8), 43 (38), 41 (32).

5.2.7. 6-Isobutyl-1,2,3,4-tetrahydrobenz[a]phenazin-5(7H)-one 18

The reaction of 2-hydroxy-3-isobutyl-5,6,7,8-tetrahydronaphthalene-1,4-dione (18), (234 mg, 1 mmol), 7.9 mmol of crystalline sodium acetate, 2.3 mmol of ortho-phenylenediamine in 5 mL of glacial acetic acid yielded product 18, (294 mg, 0.98 mmol, 96% yield) as an yellow solid; mp 131–133 °C; IR (KBr) 3399, 3057, 2949, 2930, 2864, 2837, 2525, 1624, 1603, 1524, 1458, 1431, 1364, 1335, 1231, 1200, 1175, 1152, 1134, 1111, 1028, 945, 840, 756 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.43–8.21 (3H, m, H-2'/H-3'; 1H, s, H-7), 2.19 (s, 3H, H-8); 13C NMR (100 MHz, CDCl₃) δ 153.4, 142.9, 142.1, 140.5, 139.8, 139.7, 139.4, 137.1, 135.8, 134.2, 132.0, 129.6, 129.3, 129.0, 128.1, 127.9, 126.2, 125.6, 125.4, 123.4, 122.8, 115.7, 32.0, 28.2, 22.5; MS [70 eV, m/z (%)]: 300 (33), 285 (100), 268 (44), 255 (23), 249 (22), 235 (22), 219 (16), 205 (14), 193 (13), 181 (4), 167 (3), 141 (13), 135 (11), 125 (11), 123 (11), 105 (11), 77 (20), 65 (8), 43 (38), 41 (32).
5.2.11. 6-Propyl-1,2,3,4-tetrahydrobenzo[a]phenazin-5(7H)-one 7), 233 (10), 217 (4), 164 (1), 142 (16), 142 (16), 128 (21), 121 (20), sodium acetate, 2.3 mmol of 4.57 (m, 2H); MS [70 eV, (m, 2H), 3.31
8.43
NMR (400 MHz, CDCl3)
1385, 1364, 1346, 1317, 1250, 1229, 770 cm
4.99 (d, 1H), 4.90 (d, 1H), 3.72 (d, 2H), 3.03 (dd, 1H), 2.88 (dd, 1H);
3080, 2940, 2932, 2872, 1676, 1643, 1603, 1480, 1456, 1420, 1317, 1265, 1247, 1243, 1215, 1189, 1173, 1136, 1105, 1034, 1020, 949, 758 cm−1; 1H NMR (400 MHz, CDCl3) δ 8.22—8.08 (m, 2H), 7.80—7.62 (m, 2H), 5.79—5.28 (m, 7H). 3.45—3.34 (m, 2H), 3.31—3.18 (m, 2H), 2.91—2.82 (m, 2H), 2.00—1.90 (m, 4H), 1.81—1.67 (m, 2H), 1.05 (t, 3H); 13C NMR (50 MHz, DMSO-d6) δ 157.8, 149.1, 147.1, 142.5, 141.8, 135.7, 134.4, 130.8, 130.4, 130.3, 129.9, 118.2, 66.8, 66.2, 54.8, 24.2, 23.7, 23.4, 15.8; MS [70 eV, m/z (\%)]; 292 (74), 277 (81), 264 (100); 249 (18), 237 (38), 219 (26), 205 (18), 193 (12), 181 (9), 167 (5), 140 (6), 132 (13), 122 (19), 109 (46), 97 (10), 77 (36), 65 (14), 43 (32).
5.2.12. 2- Allyl -3- (6- isobutyl -5- oxo- 1,2,3,4,5,6- hexahydrobenzo[a]phenazin-6- yloxy)-3-propyl-5,6,7,8-tetrahydronaphthalene-1,4-dione 28
The reaction of 2-allyl-3-(6-isobutyl-5-oxo-1,2,3,4,5,6-hexahydropbenzo[a]phenazin-6-yloxy)-3-propyl-5,6,7,8-tetrahydronaphthalene-1,4-dione 28 (245 mg, 0.7 mmol, 79% yield) as an yellow solid; mp 134
5.3. X-ray analysis
X-ray diffraction data collection were performed on an Enraf-Nonius Kappa-CCD diffractometer (95 mm CCD camera on k-goniostat) using graphite monochromated MoKα radiation (0.71073 Å), at room temperature. Data collection were carried out using the COLLECT software [23] up to 50° in 2θ. Final unit cell parameters were based on 9578 reflections. Integration and scaling of the reflections, correction for Lorentz and polarization effects were performed with the HKL DENZO-Scalepack system of programs [24]. The structure of the compound was solved by direct methods with SHELXS-97 [25]. The models were refined by full-matrix least squares on F2 using SHELXL-97 [26]. The program ORTEP-3 [27] was used for graphic representation and the program WINGX [28] to prepare materials for publication. All H atoms were located by geometric considerations placed (C—H = 0.93—0.97 Å; N—H = 0.86 Å) and refined as riding with Uiso(H) = 1.2Ueq.
Crystallographic data for compound 23 have been deposited with the Cambridge Crystallographic Data Center as Supplementary Publication No. CCDC 823816. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: +44 1223 36 033 or e-mail: deposit@ccdc.cam.ac.uk).
6. Biological assay in vitro

6.1. Strains and minimum inhibitory concentration (MIC) determination

The experiment was carried out using M. tuberculosis H37Rv (ATCC27294) – a pan-susceptible strain – rifampicin-resistant strain (ATCC35338) with a His-526-Tir mutation in the rpoB gene and isoniazid-resistant strain (ATCC35822) with mutation in the gene katG Ser-315-Tir of M. tuberculosis. The strains were grown in the Ogawa Kudoh medium for 14 days at 37 °C. The determination of antimicrobial activity was performed using the REMA (Resazurin Microtitre Assay), as previously described [29] In brief, the bacterial suspensions were homogenized by vortex agitation, and the turbidity was adjusted according to the McFarland scale 1.0 (3.2 × 10^8 colony-forming units/mL). The inoculums was prepared by diluting the bacterial suspension 1:20 in Middlebrook 7H9 OADC medium (4.7 g Middlebrook 7H9 base; Difco, Becton Dickinson).

The MIC assay was performed a 96-well microplates, with concentrations of 100 μg/mL in the first well, then serially decreased to 3.12 μg/mL in the last well. Each 96-well microplate was incubated for 7 days at 37 °C. Following the initial incubation period, 30 μL of resazurin was added into each well and then incubated for an additional two days at 37 °C. The cell viability observation was done based on the oxi-reduction of the resazurin by noting the change of color when cellular growth was occurring.

6.2. Inhibition of PBMC proliferation — Alamar Blue assay

To investigate the selectivity of compounds toward a normal proliferating cell, the Alamar blue assay was performed with peripheral blood mononuclear cells (PBMC) after 72 h of drug exposure. After 24 h, compounds (0.048–25 μg/mL) dissolved in DMSO (0.1%) were added to each well and incubated for 72 h. Doxorubicin (0.01–1.06 μM) was used as positive control. Twenty-four h before the end of the incubation, 10 μL of stock solution (0.312 mg/mL) of the Alamar Blue (Resazurin, Sigma–Aldrich Co) was added to each well. The absorbance was measured using a multplate reader (DTX 880 Multimode Detector, Beckman Coulter®) and the drug effect was quantified as the percentage of control absorbance at 570 nm and 595 nm. The absorbance of Alamar Blue in culture medium is measured at a higher wavelength and a lower wavelength. The absorbance of the medium is also measured at the higher and lower wavelengths. The absorbance of the medium alone is subtracted from the absorbance of medium plus Alamar Blue at the higher wavelength. This value is called AOHW. The absorbance of the medium alone is subtracted from the absorbance of medium plus Alamar Blue at the lower wavelength. This value is called AOWL. A correction factor, R0, can be calculated from AOHW and AOWL, where R0 = AOWL/AOHW. The percent Alamar Blue reduced is then expressed as follows: % reduced = AOWL – (AHW × R0) × 100.

Conflict of interest

Authors declare no conflict of interest.

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Appendix. Supplementary material

Supplementary material related to this article can be found online at doi:10.1016/j.ejmech.2011.07.026.


