Synthesis of a new class of naphthoquinone glycoconjugates and evaluation of their potential as antitumoral agents†

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A novel series of carbohydrate-based naphthoquinones was synthesized and evaluated for cytotoxicity against different cancer cell lines. The compounds derived from 5-hydroxy-1,4-naphthoquinone (juglone) showed better cytotoxicity profiles against HCT-116, A-549 and MDA-MB 435 human cancer cells than the parent compound. The results suggest that the hydroxyl group on the aromatic ring increased the pro-oxidant activity of these new naphthoquinone derivatives. Furthermore, two derivatives were found to be more active against melanoma cells (MDA-MB435) than the clinically useful anticancer agent doxorubicin, and none of the compounds caused mouse erythrocyte lysis.

1 Introduction

Natural glycoconjugated compounds, 1–7 (Fig. 1), are useful drugs that are commonly used clinically in the treatment of cancer. The importance of the carbohydrate residues in these structures has been well established: these moieties are important for solubilization and also decrease the toxicity and improve the pharmacokinetics of the compounds. Indeed, several cases in the literature show the benefit to biological activity of a carbohydrate moiety in the structure of a molecule. For example, the sugar moieties in antibiotic anthracycline derivatives 1–3 (Fig. 1) participate in the molecular recognition of the main cellular targets, DNA and topoisomerase II, forming a DNA-drug-topoisomerase II ternary complex in which the enzyme is covalently linked to a broken DNA strand, which is critical for the induction of apoptosis and cell death. 6, 7

Other mechanisms can be involved in quinone toxicity of such redox cycles, which result in the production of semi-quinone radicals and reactive oxygen species (ROS). These events lead to the depletion of glutathione in cells as well as damage to other intracellular components. 3

The importance of exploring the synthesis of sugar-conjugated compounds to increase the selectivity of drug uptake is based on the Warburg effect, 4 which is related to the large consumption of glucose by cancerous cells compared to normal cells due to the high rate of aerobic glycolysis in the former. 4

Recently, our group described the synthesis and pharmacological evaluation of a series of carbohydrate-based quinones. In this study, 5 compounds 8a and 8b (Fig. 2) (IC50 below 3.0 M) were found to exhibit significant activity against a melanoma cell line (MDA-MB 435).

Quinones containing heterocyclic aromatic rings in their structures have been extensively explored by many research groups. 8, 9 Structure–activity relationship studies have revealed that the number and position of nitrogen (N) atoms in the five- or six-membered heterocyclic ring are critical factors for enhancing antitumor activity. 9, 10

Among nitrogen-containing heterocycles, 1,2,3-triazole derivatives exhibit a broad spectrum of biological properties 11–15 and may serve as scaffold structures for the development of new pharmaceutical compounds. This heterocyclic system is resistant to metabolic degradation and can interact with biological targets through hydrogen bonds and dipole interactions, properties that may be responsible for the enhanced biological activities of many synthetically versatile molecules. 16

To extend our investigation into the synthesis and biological evaluation of quinone glycoconjugate compounds 16 and 1,2,3-triazole derivatives, 11–15 we prepared a new series of naphthotriazoles, 9a–c (Scheme 1), and assessed their activity against three types of cancer cells: human colorectal carcinoma...
The synthesis of derivatives $\text{17} - 10a - c$, which possess amino-carbohydrate chains at the C-2 position of the quinone moiety (Scheme 1), was also performed to verify the possible pharmacophoric effects of the 1,2,3-triazole moiety fused to naphthoquinone.

Naturally occurring hydroxy naphthoquinone (juglone, 12) exhibits a toxicological effect through its ability to undergo redox cycling to damage macromolecules, such as DNA, lipids and proteins.$^{18}$ Several studies have shown that the introduction of a hydroxyl group at the carbon of the benzene moiety improves the pro-oxidant properties of naphthoquinone derivatives, stimulating lipid peroxidation and adduct formation with macromolecules.$^{18}$

Based on these reports, we also decided to prepare a new series of hydroxylated naphthoquinones, $11a-c$, exploring cycloaddition between juglone (12) and azide-functionalized carbohydrates $13a-c$ (Scheme 1), and to investigate the influence on biological activity of a hydroxyl group at position 5 of the benzene ring.

The compounds $10a-c$ and $11a-c$ were also evaluated for their cytotoxic activities against cancer cell lines HCT-116, A-549 and MDA-MB435.

Scheme 1  Strategy for the synthesis of naphthoquinone derivatives $9a-c$, $10a-c$ and $11a-c$. 

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Fig. 1  Examples of natural glycoconjugated compounds (1–7) with antitumoral activity.

Fig. 2  Examples of synthetic molecules ($8a$ and $8b$) with antitumoral activities.

(HCT-116), human lung adenocarcinoma (A-549) and human melanoma (MDA-MB435).

The synthesis of derivatives$^{17}$ $10a-c$, which possess amino-carbohydrate chains at the C-2 position of the quinone moiety (Scheme 1), was also performed to verify the possible pharmacophoric effects of the 1,2,3-triazole moiety fused to naphthoquinone.
2 Results and discussion

2.1 Chemistry

Naphthotriazole derivatives 9a–c (Scheme 2) were obtained in moderate yields via a [3 + 2] cycloaddition reaction between glycosyl azides 13a–c and 1,4-naphthoquinone (14) (Scheme 2). The probable mechanism for the formation of these compounds involves an initial 1,3-dipolar cycloaddition reaction between the azide carbohydrates 13a–c and 1,4-benzoquinone (14), producing the corresponding triazoline intermediates 15a–c, which are tautomerized and oxidized to form the desired glycotriazole derivatives 9a–c.

Glycosyl azides 13a–c were prepared from their corresponding commercially available reagents D-ribose, D-xylose and D-galactose using previously described methods for carbohydrate derivatization19,20 (Scheme 3).

The aminonaphthoquinone analogs 10a–c (Scheme 2) were synthesized in moderate yields via ultrasound-accelerated 1,4-cycloaddition between 1,4-naphthoquinone (14) and different aminocarbohydrates (16a–c), according to our previous report.17 Reduction of the azide derivatives 13a–c in the presence of a catalytic amount of 10% Pd/C (Scheme 3) led to the corresponding amines 16a–c.

Juglone 12, prepared in 85% yield via the methodology described by Ferreira and coworkers,21 was reacted with azide compounds 13a and 13b. Although it is possible to form a mixture of the 5- and 8-hydroxy-naphthotriazole regioisomers (Scheme 4) via this thermal 1,3-dipolar cycloaddition reaction, only 5-hydroxy-1-substituted-1H-naphtho[2,3-d][1,2,3]-triazole-4,9-dione derivatives 11a and 11b were isolated, in moderate yields. Furthermore, unexpected products 22a and 22b, possessing a carbohydrate chain at the C-2 position of the quinone ring, were also isolated.

The reaction of compound 13c with juglone (12) gave a crude product which upon TLC analysis showed to be a mixture of two major compounds with retention factors (R_f) very similar. The 1H NMR spectrum of this mixture led to the conclusion that one of the substances corresponded to 11c, the desired compound, and that the other substance was the amine 22c. Attempts to separate the products by chromatography on silica gel column were unsuccessful (Scheme 5).
The structures of the new compounds 9a–c, 11a, 11b, 12, 14, 22a and 22b were established by spectral data: one- and two-dimensional $^1$H and $^{13}$C NMR spectra \([^{1}H, ^{13}C-APT, COSY-^{1}H \times ^{1}H, HSQC and HMBC}\), IR and elemental analysis.

The $^1$H NMR spectra of 11a, 11b, 22a and 22b indicate that only one of the two possible isomers was formed. The regiochemistry of the reaction was investigated based on X-ray crystallographic analysis of substances 11a and 22a (Fig. 3). Fig. 3 shows the ORTEP diagrams of these compounds, and crystal data and refinements are provided in Table 2. The diagram shows that the carbohydrate chains are in the anti-position to the hydroxyl group attached to the quinone ring of 11a and 22a. The carbohydrate moieties of 11a and 22a adopt envelope and twist conformations, respectively.

### 2.2 Biological assay

Compounds 9a–c, 10a–c, 11a, 11b, 12, 14, 22a and 22b were tested *in vitro* against three cancer cell lines and normal cells, human blood peripheral leukocytes, using the MTT assay. Doxorubicin was used as the positive control. Erythrocyte lysis was evaluated at a concentration of 200 μM. The concentrations inducing 50% inhibition of cell growth (IC$_{50}$) are reported in Table 2. The compounds were classified according to their activity as highly active (IC$_{50}$ < 2 μM), moderately active (2 μM < IC$_{50}$ < 10 μM), or inactive (IC$_{50}$ > 10 μM).\(^\text{22}\)

The naphthoquinone derivatives 9a–c, 10a–c, 11a, 11b, 12, 14, 22a and 22b (Table 2) did not exhibit a lytic effect against either erythrocytes or normal human leukocytes.

Among naphthotriazoles 9a–c, only derivative 9b showed significant activity against HCT-116 cells, with an IC$_{50}$ value of 4.85 μM. This result indicates that the presence of the glycol 1,2,3-triazole subunit determines the biological activity assessed. Aminoquinones 10a–c showed no activity against the three cell lines, allowing us to speculate that the presence of the amino group attached to carbon 2 has no effect on the biological activity of the compounds.

Aminoquinone analogs 22a and 22b showed higher cytotoxic activities than compounds 10a and 10b. This can be attributed to the presence of the hydroxyl group connected to the benzene ring in these derivatives, possibly contributing to their pro-oxidant activities.\(^\text{18}\)

In addition, the new glycoconjugate compounds 22a and 22b were also more active than juglone (12). We speculate that cellular permeability of these 5-hydroxy-1,4-naphthoquinonic derivatives could be improved by the presence of the carbohydrate chain at the C-2 position of the quinone moiety. As 22a and 22b were active against different cell lines, it appears that they can bind to different targets in a specific background.

The series of quinones 11a–c displayed greater activity in all tested cancer cell lines than naphthoquinone derivatives 9a and 9b, possibly indicating that these quinones have increased pro-oxidant capacity. One can speculate that the hydroxyl attached to the C5 carbon in these derivatives is responsible for their increased anti-tumor activity.
3 Conclusion

The thermal cycloaddition reaction of glycosyl azides 13a–c with 1,4-naphthoquinone (14) produced the new glycoconjugates 9a–c in moderate yields. Under the same conditions, parent compound 21 afforded the corresponding glycoconjugates, 11a and 11b, in moderate yields as well as the unexpected aminonaphthoquinones 22a and 22b. These carbohydrate-based naphthoquinones, 11a, 11b, 22a and 22b, derived from juglone (12) showed the best cytotoxicity profile against human cell lines HCT-116, A-549 and MDA-MB 435 when compared with the parent compounds, 9a, 9b, 10a and 10b, respectively. These data show the importance of the hydroxyl group to the pro-oxidant action of these naphthoquinone compounds. Indeed, derivatives 11a and 11b were more active than the clinically useful anticancer agent doxorubicin against melanoma cancer cells (MDA-MB435 cells). However, none of the compounds exhibited lytic effects against mouse erythrocytes.

Molecules 11a, 11b, 22a and 22b can be considered promising prototypes for cancer therapy.

4 Experimental

4.1 Materials and measurements

Melting points were determined with a Fisher-Johns – Melting Point Apparatus instrument, and the values are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer FT-IR 1600 spectrophotometer using KBr pellets. NMR spectra were recorded on a Varian Unity Plus 300 MHz or 500 MHz spectrometer using the specified solvents. Chemical shifts (δ) are reported in ppm, and the coupling constants (J) are expressed in Hz. Column chromatography was performed with silica gel flash from Acros. The reactions were routinely monitored by thin layer chromatography (TLC) on silica gel pre-coated F254.

Table 1 X-ray crystallographic data for compounds 11a and 22a

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<tr>
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<th>11a</th>
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<tr>
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<td>M_r (g mol⁻¹)</td>
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<td>375.37</td>
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<td>[0.43 × 0.33 x]</td>
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<td>Yellow, prism</td>
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<td>Monoclinic, P2₁</td>
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<td>150</td>
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<td>11.9174 (4), 12.2331 (5)</td>
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<td>94.745 (4)</td>
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<td>Mo Kα</td>
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<td>0.11</td>
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<tr>
<td>No. of measured, independent and observed [I &gt; 2σ(I)] reflections</td>
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<td>9555, 3667, 3075</td>
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Table 2 Cytotoxic activity expressed as the IC₅₀ (µM) of the compounds against different cell lines

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<th>9b</th>
<th>9c</th>
<th>10a</th>
<th>10b</th>
<th>10c</th>
<th>11a</th>
<th>11b</th>
<th>12</th>
<th>14</th>
<th>22a</th>
<th>22b</th>
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</tbody>
</table>

² Data are presented as IC₅₀ values (µM), and 95% confidence intervals were obtained by nonlinear regression for all cell lines from three independent experiments.³ Doxorubicin was used as a positive control. Only compounds with an IC₅₀ value lower than 5 mg mL⁻¹ for at least one cell line were considered active.⁴ Concentration of compound that induced erythrocyte lysis.⁵ NT – not tested.
Merck plates. Microanalyses were performed using a Perkin-Elmer Model 2400 instrument, and all values were within ±0.4% of the calculated compositions.

4.2 General procedure for the preparation of new naphthotriazoles 9a-c

The glycosyl azides 13a-c (1.0 mmol) and 1,4-naphthoquinone (14) (0.158 mg, 1.0 mmol) were dissolved in toluene and heated at 100 °C for 24 hours. After completion of the reaction, the mixture was cooled, and the solvent was removed. The residual solid product was purified by column chromatography on silica gel and eluted with an increasing polarity gradient mixture of hexane and ethyl acetate (100% to 50%) to afford compounds 9a-c.

(Methyl-5′-deoxy-2′,3′-O-isopropylidene-β-D-ribofuranosid-5′-yl)-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (9a). The 1,3-dipolar cycloaddition reaction between naphthoquinone 12 and glycosyl azide 13a yielded glyconaphthotriazole 9a (121.0 mg, 63%) as an orange solid: mp 225–227 °C; IR (KBr) ν (cm⁻¹): 1588 and 1686 (C=O). ¹H NMR (500.00 MHz, CDCl₃) δ: 1.30 (s, 3H, CH₃), 1.44 (s, 3H, CH₃), 3.41 (s, 3H, OCH₃), 4.72–4.73 (m, 1H, H-4), 4.74 (d, 1H, J = 6.0 Hz, H-2), 4.86 (d, 1H, J = 6.0 Hz, H-3), 5.01 (dd, 2H, J = 7.5 and 1.0 Hz, H-5a and H-5b), 5.02 (s, 1H, H-1), 7.82 (td, 1H, J = 7.5 and 1.5 Hz, H-7), 7.86 (dd, 1H, J = 7.5 and 1.5 Hz, H-6), 8.24 (dd, 1H, J = 7.5 and 1.5 Hz, H-8), 8.34 (dd, 1H, J = 7.5 and 1.5 Hz, H-5) ppm. ¹³C NMR (125.00 MHz, CDCl₃) δ: 25.1 (CH₃), 26.5 (CH₃), 53.2 (C-5’), 55.9 (OCH₃), 81.8 (C-3’), 84.6 (C-4’), 85.3 (C-2’), 110.4 (C-1’), 113.1 (–OOC–), 127.5 (C-8), 128.5 (C-5), 132.8 (C-8a), 133.5 (C-4a), 133.6 (C-9a), 134.5 (C-7), 135.4 (C-14), 145.7 (C-3a), 175.6 (C-9), 176.7 (C-4) ppm. Anal. calc. for C₁₉H₁₉N₃O₆: C, 59.32; H, 5.63; N, 9.25%.

4.3 General procedure for the preparation of new naphthoquinone derivatives 11a, 11b, 22a and 22b

The glycosyl azides 13a and 13b (1.0 mmol) and juglone 12 (0.174 mg, 1.0 mmol) were dissolved in toluene and heated at 100 °C for 24 hours. The reaction mixture was concentrated under reduced pressure and the resulting residue was purified by column chromatography using silica gel and eluted with an increasing polarity gradient mixture of hexane and ethyl acetate (100% to 70%) to give the desired compounds 11a and 11b and the unexpected aminoquinones 22a and 22b.

5-Hydroxy-(methyl-5′-deoxy-2′,3′-O-isopropylidene-β-D-ribofuranosid-5′-yl)-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (11a) and 5-hydroxy-(methyl-5′-deoxy-2′,3′-O-isopropylidene-β-D-ribofuranosid-5′-yl)-amino-1,4-naphthoquinone (22a). The 1,3-dipolar cycloaddition reaction between juglone 12 and glycosyl azide 13a gave glyconaphthotriazole 11a (136.0 mg, 68%) and amino compound 22a (5.6 mg, 3%).

The compound 11a was obtained as a yellow solid: mp 144–147 °C; IR (KBr) ν (cm⁻¹): 3434 (OH), 1649 (C=O), 1449 (C=C). ¹H NMR (500.00 MHz, CDCl₃) δ: 1.31 (s, 3H, CH₃), 1.45 (s, 3H, CH₃), 3.41 (s, 3H, OCH₃), 4.70–4.72 (m, 1H, H-4), 4.72 (d, 1H, J = 6.0 Hz, H-2), 4.85 (d, 1H, J = 6.0 Hz, H-3), 4.98 (dd, 2H, J = 7.5 and 2.5 Hz, H-5a and H-5b), 5.02 (s, 1H, H-1), 7.38 (dd, 1H, J = 8.5 and 1.0 Hz, H-6), 7.68 (dd, 1H, J = 8.5 and 7.5 Hz, H-7), 7.80 (dd, 1H, J = 7.5 and 1.0 Hz, H-8), 12.31 (s, 1H, OH) ppm. ¹³C NMR (125.00 MHz, CDCl₃) δ: 25.1 (CH₃), 26.5 (CH₃), 53.3 (C-5’), 56.0 (OCH₃), 81.8 (C-4’), 85.3 (C-2’), 110.4 (C-1’), 113.1 (–OOC–), 115.5 (C-4a), 120.8 (C-8), 126.9 (C-6), 133.2 (C-8a), 133.9 (C-9a), 136.9 (C-7), 145.5 (C-3a), 163.6 (C-9), 174.8 (C-9), 182.7 (C-4) ppm. Anal. calc. for C₁₉H₁₉N₃O₅·C₂H₂O: C, 58.86; H, 4.77; N, 10.47. Found: C, 58.04; H, 5.25; N, 9.05%.

The compound 22a was obtained as a red solid: mp 128–130 °C; IR (KBr) ν (cm⁻¹): 3401 (OH), 3263 (N=H), 1623, 1592 (C=O), 1468 (C=C). ¹H NMR (500.00 MHz, CDCl₃) δ: 1.32 (s, 3H, CH₃), 1.50 (s, 3H, CH₃), 3.29–3.34 (m, 2H, H-5a and H-5b), 3.41 (s, 3H, OCH₃), 4.49–4.51 (m, 1H, H-4), 4.63 (dd, 1H, J = 6.0 and 1.0 Hz, H-3), 4.66 (dd, 1H, J = 6.0 Hz, H-2), 5.05 (s, 1H, H-1), 5.64 (s, 1H, H-3), 6.66–6.68 (m, 1H, H=), 7.25 (dd, 1H, J = 8.5 and 1.0 Hz, H-6).
Hz, H-6), 7.47 (dd, 1H, J = 8.5 and 7.5 Hz, H-7), 7.59 (dd, 1H, J = 7.5 and 1.0 Hz, H-8), 12.98 [s, 1H, OH] ppm. 13C/APT NMR (125.0 MHz, CDCl3) δ ppm: 25.1 (CH3), 26.6 (CH3), 45.6 (C-5'), 55.7 (OH), 82.3 (C-3), 84.5 (C-2'), 85.4 (C-2'), 100.5 (C-3), 109.8 (C-1'), 111.3 (-OOC-), 115.0 (C-4a), 119.2 (C-8), 126.1 (C-6), 130.7 (C-8a), 134.2 (C-7), 149.0 (C-2), 161.3 (C-5), 181.1 (C-1), 189.2 (C-4) ppm. Anal. calc. for C15H23NO2: C, 60.79; H, 5.64; N, 3.73. Found: C, 61.61; H, 6.00; N, 3.21%.

5-Hydroxy-1-(6-deoxy-1',2',3',4'-di-O-isopropylidene-D-galactopyranos-6-yl)-1H-nafto[2,3-d][1,3,4-triazole-4,9-dione (11b) and 5-hydroxy-2-(6-deoxy-1,2',3',4'-di-O-isopropylidene-D-galactopyranos-6-yl)-amino-1,4-naphthoquinone (22b). The 1,3-dipolar cycloaddition reaction between juglone 12 and glycosyl azide 13b gave glyconaphthotriazone 11b (64.0 mg, 28%) and amino compound 22b (4.3 mg, 2%).

The compound 11b was obtained as a yellow solid: mp 160-162 °C; IR (KBr) ν (cm⁻¹): 3416 (OH), 1654 (C=O), 1453 (C=C). 1H NMR (300.00 MHz, CDCl3) δ: 1.23 [s, 3H, CH3], 1.36 [s, 3H, CH3], 1.38 [s, 3H, CH3], 1.57 [s, 3H, CH3], 1.83 [s, 3H, CH3], 3.64-3.68 (m, 2H, H-6a and H-6b), 4.00-4.05 (m, 1H, H-5'), 4.25 (dd, 1H, J = 7.8 and 1.8 Hz, H-4'), 4.35 (dd, 1H, J = 5.1 and 2.4 Hz, H-2'), 4.65 (dd, 1H, J = 7.8 and 2.4 Hz, H-3'), 5.55 (d, 1H, J = 5.1 Hz, H-1'), 6.22 (s, 1H, H-3), 6.50-6.56 (m, 1H, H-7), 7.32 (dd, 1H, J = 8.4 and 2.4 Hz, H-6), 7.46 (dd, 1H, J = 8.4 and 7.5 Hz, H-7), 7.59 (dd, 1H, J = 7.5 and 1.2 Hz, H-8), 13.05 [s, 1H, OH] ppm. 13C/APT NMR (75.0 MHz, CDCl3) δ: 24.5 (CH3), 25.0 (CH3), 26.1 (CH3), 26.2 (CH3), 43.3 (C-6'), 64.9 (C-5'), 70.5 (C-3'), 70.6 (C-4'), 71.9 (C-2'), 96.5 (C-1'), 99.9 (C-3), 109.0 (-OOC-), 110.0 (-OOC-), 115.1 (C-4a), 119.2 (C-8), 125.9 (C-6), 130.7 (C-8a), 134.0 (C-7), 149.1 (C-2'), 161, 1 (C-5), 189.1 (C-4) ppm. Anal. calc. for C15H23NO2: C, 61.25; H, 5.84; N, 3.25. Found: C, 61.97; H, 6.18; N, 3.31%.

5 X-ray determination

X-ray diffraction was performed using an Oxford Diffraction Xcalibur Atlas Gemini ultra Diffractometer at 150 K with Mo-Kα radiation. The collection and reduction of the data were performed using the CrysAlisPro software. The structures (Table 1) were solved by direct methods and refined using full-matrix least squares on F² with the SHELX-97 software package. The CCDC reference numbers for 11a and 22a are 1055525 and 1055524, respectively.

6 Biology

6.1 Cytotoxicity against cancer cell lines

The compounds (0.15–20 μM) were tested for cytotoxic activity against cancer cell lines HCT-116 (human colorectal carcinoma, ATCC # CCL-247), A-549 (human lung adenocarcinoma, ATCC # CCL-185) and MDA-MB-435 (human melanoma, ATCC # HTB-129) as well as against freshly prepared human blood leucocytes and erythrocytes. All cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U mL⁻¹ penicillin, and 100 mg mL⁻¹ streptomycin at 37 °C in an atmosphere containing 5% CO₂. Each compound was dissolved with DMSO and diluted with the cell culture medium to obtain a concentration of 100 μM, which was then incubated with the cells for 72 hours. The negative control received the same amount of DMSO (0.005% at the highest concentration). Doxorubicin was used as a positive control. Cell viability was determined by the reduction of the yellow dye 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product after 3 hours of incubation, as described by Denizot (1986).

6.2 Cell membrane disruption

Cytotoxicity testing was performed as described previously in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mM CaCl₂. The compounds, which were diluted as mentioned above, were tested at a concentration of 200 μM. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed, and the liberated hemoglobin was measured spectrophotometrically at 540 nm. DMSO was used as a negative control and Triton X-100 (1%) was used as a positive control.

Acknowledgements

This work was supported by the Brazilian agency FAPERJ-PRONEX. Fellowships granted to UFF, by FAPERJ, CAPES, and CNPq-PIBIC are gratefully acknowledged. We would like to thank the LabCrib/UFMG for the use of the X-ray facilities.

References

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