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Synthesis, antimalarial activity *in vitro* and docking studies of novel neolignan derivatives

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Running Title: Synthesis and docking studies of new neolignan derivatives with antimalarial activity.

Abstract

The absence of effective vaccines against malaria and the difficulties associated with controlling mosquito vectors has left chemotherapy as the primary control measure against malaria. However, the emergence and spread of parasite resistance to conventional antimalarial drugs result in a worrisome scenario making the search for new drugs a priority. In the present study, the activities of nine neolignan derivatives were evaluated as follows: (i) against blood forms of chloroquine-resistant *Plasmodium falciparum* (clone W2), using the tritiated hypoxanthine incorporation and Anti-HRPII assays; (ii) for cytotoxic activity against cultured human hepatoma cells (HepG2); and, (iii) for intermolecular interaction with the *P. falciparum* cysteine protease of falcipain-2 (F2) by molecular docking. The neolignan derivatives **9** and **10** showed activity against the blood form of the chloroquine-resistant *P. falciparum* clone W2 and were not cytotoxic against cultured human hepatoma cells. A molecular docking study of these two neolignans with FP2 revealed several intermolecular interactions that should guide the design of future analogs.

Introduction

Malaria is the leading public health problem among transmissible diseases in the world. According to the 2015 World Malaria Report, there were around 214 million cases of human malaria with 438.000 reported deaths (1). Despite various attempts not completely successful to produce a vaccine, chemotherapy remains the main control tool against the disease (2). However, multi-resistance of *Plasmodium falciparum* against antimalarial compounds and the lower sensitivity of *P. vivax* to chloroquine have been important obstacles to preventing spread of the disease (3). Many efforts have been made towards identifying novel molecular targets for development of new compounds against malaria. One attractive target is a cysteine-protease of *P. falciparum*, falcipain-2 (FP2), an essential enzyme for parasite development (4). FP2 is particularly suited for the hydrolysis of native hemoglobin in the acidic food vacuole, and several studies reveal that cysteine protease inhibitors such as chalcones block globin hydrolysis by inhibition of FP2 (5, 6), however most chalcones exhibit high toxicity (7, 8)

The neolignans correspond to a kind of lignoids derived from the oxidative homo- or cross-coupling of alylphenols and propenyl phenols (9). They are found in plants from the *Miristicaceae* family (10), and have demonstrated antibacterial (11), anti-*Schistosoma* (12, 13), antifungal (14), trypanosomicidal , anti-*Plasmodium* (15,16) and leishmanicida (17) activities. Neolignan derivatives are active against *Plasmodium* at nanomolar concentrations, and are considered the most active lignoids against malaria, thus promising antimalarial prototypes (15,16, 18).

In an attempt to identify non-toxic FP2 inhibitors retaining good activity, novel neolignan derivatives were synthesized based on their structural similarity with chalcones. Nine neolignan derivatives were tested against chloroquine-resistant *P. falciparum* blood forms (clone W2). The inhibitory drug concentration that eliminates 50% of the parasites was determined using the Anti-HRPII (histidine-rich protein II) and hypoxanthine-tritiated incorporation assays, in parallel with tests of drug cytotoxicity to HepG2 cells (MDL_{50}) to calculate their selectivity indexes (SI). The best compounds were subjected to theoretical docking studies with the FP2.

Methods and Material

Experimental Section

Material

The reactions were monitored by thin-layer chromatography performed on TLC plates with silica gel 60 F254 (Merck®). The spectra data ^{1}H (62.5, 400 and 500 MHz) and ^{13}C (50, 100 and 125 MHz) were obtained by Bruker AC 250/P, Bruker Avance-400 and Varian Inova-500, using $CDCl_3$ and DMSO-d_6 as solvents and TMS as the internal standard (chemical shift δ in ppm). The infrared absorption spectra were obtained by using a spectrophotometer Bomen Model MB Series II, and mass spectra (MS) were evaluated on VG Auto Spec High Resolution Mass Spectrometer (Micromass Company).

Synthesis

Compound preparation (4, 5, 7-10)

A solution of 1.02 equivalent of phenol derivative in anhydrous ethyl methyl ketone (4.5 mL of solvent/mmol of α-bromoketone) and 1.80 equivalent of anhydrous K_2CO_3 was stirred for 10 min at room temperature, and then a solution of α-bromoketone in anhydrous ethyl methyl ketone (1.5 mL of solvent/mmol of α-bromoketone) was added dropwise. The reaction mixture was stirred and refluxed for 12 h. After completion, the mixture was concentrated under vacuum, diluted with H_2O, and extracted with $CHCl_3$ (3x). The organic layer was washed with water, 5% NaOH solution, brine, and dried over Na_2SO_4. The solvent was evaporated to result in a crude product which was purified by crystallization from ethanol.

2-oxo-2-phenylethyl (2E)-3-[4-(2-oxo-2-phenylethoxy)phenyl]prop-2-enoate **4**: This compound was obtained from 0.42 g (2.56 mmol) of 4-hidroxycinnamic acid, 0.50 g (2.51 mmol) of phenacyl bromide and 0.625 g (4.52 mmol) of K_2CO_3. The product obtained was a colorless crystalline solid (0.160 g). Yield 32%. M.P. 145-147 ºC. ^{1}H NMR (400 MHz, DMSO) δ: 7.99 (d, *J*=7.4 Hz, 2H), 8.04 (d, *J* =7.4 Hz, 2H), 7.70 (d, *J* = 16.0 Hz, 1H), 7.60-7.73 (m, 4H), 7.59-7.55 (td, *J* =7.4 Hz and *J*=2.7 Hz, 4H), 7.04 (d, *J* = 9.0 Hz, 2H), 6.64 (d, *J* =16.0 Hz, 1H), 5.68 (s, 2H), 5.60 (s, 2H). ^{13}C NMR (100 MHz, DMSO) δ: 194.2, 193.0, 165.9, 160.0, 145.1, 134.3, 134.0, 130.2, 128.9, 128.9, 127.9, 127.8, 126.9, 115.1, 114.8, 70.2, 66.4. HRMS (ESI): calcd for $C_{25}H_{21}O_5^+$ $[M+H]^+$ 401.1384, found 401,1389. IR (KBr, cm^{-1}): 1721 (C=O), 1706 (C=O).

2-oxo-2-phenylethyl 4-(2-oxo-2-phenylethoxy)benzoate **5**: This compound was obtained from 0.35 g (2.56 mmol) of 4-hydroxybenzoic acid, 0.50 g (2.51 mmol) of phenacyl bromide and 0.62 g (4.52 mmol) of K_2CO_3. The product obtained was a white crystalline solid (0.16 g). Yield 34%. M.P. 146-148 ºC. ^{1}H NMR (400 MHz, DMSO) δ: 8.07 (d, *J* = 9.0 Hz, 2H), 7.98 (d, *J* = 8.5 Hz, 2H), 7.94 (d, *J* = 8.5 Hz, 2H), 7.46-7.52 (m, 4H), 6.97 (d, *J* = 9.0 Hz, 2H), 5.52 (s, 2H), 5,50 (s, 2H). ^{13}C NMR (100 MHz, DMSO) δ: 193.6, 192.3, 165.5, 162.0, 134.4, 134.3, 134.1, 133.8, 132.1, 128.9, 128.9, 128.1, 127.8, 122.7, 114.5, 70.5, 66.3. HRMS (ESI):

calcd for $C_{23}H_{18}NaO_5^+$ $[M+Na]^+$ 397.1046, found 397.1089. IR (KBr, cm^{-1}): 1716 (C=O), 1698 (C=O).

2-oxo-2-phenylethyl (2E)-3-(1,3-benzodioxol-5-yl)prop-2-enoate **7**: This compound was obtained from 0.50 g (2.56 mmol) of 3,4-(methylenedioxy)cinnamic acid, 0.50 g (2.51 mmol) of phenacyl bromide and 0.62 g (4.52 mmol) of K_2CO_3.The product obtained was a colorless crystalline solid (0.54 g). Yield 69%. M.P. 144-146 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.46 (s, 2H), 6.01 (s, 2H), 6.42 (d, J = 16.0 Hz, 1H), 6.81 (d, J = 7.9 Hz, 1H), 7.01-7.05 (m, 2H), 7.49 (t, J = 7.9 Hz, 2H), 7.61 (t, J = 7.3 Hz, 1H), 7.82 (d, J = 16.0 Hz,1H), 7.95 (d, J = 7.3 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 66.0, 101.6, 106.6, 108.5, 114.8, 124.7, 127.8, 128.7, 128.8, 129.2, 133.8, 134.3, 145.8, 148.3, 149.8, 166.4, 192.4. HRMS (ESI): calcd for $C_{18}H_{14}KO_5^+$ $[M+K]^+$ 349.0473, found 349.0478. IR (KBr, cm^{-1}): 1715 (C=O), 1694 (C=O).

2-(4-chlorophenyl)-2-oxoethyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **8**: This compound was obtained from 1.04 g (4.36 mmol) of 3,4,5-trimethoxycinnamic acid, 1.0 g (4.28 mmol) of 2-bromo-4'-chloroacetophenone and 1.1 g (7.74 mmol) of K_2CO_3. The product obtained was a colorless crystalline solid (0.75 g). Yield 45%. M.P. 117-119 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.90 (d, J = 8.0 Hz, 2H), 7.71 (d, J = 16.0 Hz, 1H), 7.48-7.46 (m, 2H), 6.78 (s, 2H), 6.50 (d, J = 16.0 Hz, 1H), 5.44 (s, 2H), 3.89 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 191.2, 166.1, 153.3, 146.2, 140.3, 140.3, 132.5, 129.6, 129.1, 115.9, 105.4, 65.8, 60.9, 56.1.HRMS (ESI): calcd for $C_{20}H_{20}ClO_6^+$ $[M+H]^+$ 391.0943, found 391.0948. IR (KBr, cm^{-1}): 1714 (C=O), 1629 (C=O).

Methyl-(2E)-3-[3-methoxy-4-(2-oxo-2-phenylethoxy)phenyl]prop-2-enoate **9**: This compound was obtained from 0.77 g (3.71 mmol) of methylferulate, 0.70 g (0.35 mmol) of 8-bromoacetophenone and 0.87 g (6.33 mmol) of K_2CO_3. The product obtained was a brown crystalline solid (0.37 g). Yield 32%. M.P. 105-107 ºC. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.00 (d, J = 8.0 Hz, 2H), 7.63-7.59 (m, 2H), 7.50 (t, J_1 = 8.0 Hz, 2H), 7.07 (s, 1H), 7.02 (d, J_1 = 8.0 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.31 (d, J = 16.0 Hz, 1H), 5.41 (s, 2H), 3.92 (s, 3H), 3.79 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 193.7, 167.5, 149.5, 149.3, 144.5, 134.2, 133.9, 128.8, 128.5, 127.9, 122.0, 115.9, 113.6, 110.4, 71.3, 55.9, 51.6. HRMS (ESI): calcd for $C_{19}H_{19}O_5^+$ $[M+H]^+$ 327.1227, found 327.1233. IR (KBr, cm^{-1}): 1721.4 (C=O), 1689.7 (C=O).

1-(4-methoxyphenyl)-1-oxopropan-2-yl-(2E)-3-(4-{[1-(4-methoxyphenyl)-1-oxopropan-2-yl]oxy}phenyl)prop-2-enoate **10**: This compound was obtained from 0.18 g (1.05 mmol) of 4-hydroxycinnamic acid, 0.25 g (1.03 mmol) of 4-methoxy-8-bromopropiophenone and 0.27 g (1.98 mmol) of K_2CO_3. The product obtained was a white crystalline solid (0.18 g). Yield

71%. M.P. 142-144 ºC. ^{1}H NMR (400 MHz, DMSO) δ: 8.12 (*d*, *J* = 8.8 Hz, 2H), 8.05 (d, *J* = 8.8 Hz, 2H), 7.70 (d, *J* = 8.8 Hz, 2H), 7.64 (d, *J* = 16.0 Hz, 1H), 7.19-7.08 (m, 4H), 6.94 (d, *J* = 8.8 Hz, 2H), 6.61 (d, *J* = 16.0 Hz, 1H), 6.16-6.06 (m, 2H), 3.91 (s, 3H), 3.90 (s, 3H), 1.59 (d, *J* = 7.0 Hz, 3H), 1.52 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO) δ: 196.1, 195.1, 165.8, 163.8, 163.6, 159.2, 144.9, 130.9, 130.8, 130.3, 126.9, 126.7, 126.7, 115.3, 115.0, 114.3, 114.2, 74.3, 71.2, 55.7, 18.5, 17.2. ^{13}C NMR – (63 MHz, DMSO) δ: 196.1, 195.1, 165.8, 163.8, 163.6, 159.2, 144.9, 131.0, 130.8, 130.3, 126.9, 126.7, 126.7, 115.3, 115.0, 114.3, 114.3 , 74.3, 71.2, 55.7, 55.6, 18.5, 17.3. HRMS (ESI): calcd for $C_{29}H_{29}O_7^+$ $[M+H]^+$ 489.1941, found 489.1942. IR (KBr, cm^{-1}): 1712.6 (C=O), 1699 (C=O), 1685.5 (C=O).

Continuous *in vitro* culture of *P. falciparum* blood forms

The chloroquine-resistant and mefloquine-sensitive W2 clone of *P. falciparum* was cultivated as described by Trager and Jensen (19), with some modifications. The antiplasmodial activity of neolignan derivatives was evaluated using tritiated hypoxanthine and anti-HRPII (Histidine Rich Protein) assays. At least, two assays for each technique were performed. Each assay was performed in triplicates and chloroquine (as a reference drug) was used in each experiment as a control antimalarial.

Briefly, a 2% hematocrit of human red blood cells type A+ suspended in RPMI 1640 medium supplemented with 10% of Human inactivated-sera type A+ was maintained in culture plates at 37 °C in a gas mixture (5% CO_2, 5% O_2 and 90% N_2 atmosphere). The test of hipoxantine incorporation described previously (20) was used to evaluate drug activity, with some modifications. Briefly, before the test, the blood cultures were kept for at least three days in medium without hypoxanthine. The test was performed after sorbitol-synchronization with ring stages (21); then adjusted for 1% both parasitemia and hematocrit. The parasite suspension was placed in a plate containing the test compounds and incubated in culture conditions for 24 h when ^{3}H-hypoxanthine was added, (0.5 μCi) (PerkinElmer, MA, EUA) to each well. Plates were incubated at 37°C for 18 h and then frozen for at least 24 h at -20°C and thawed to allow cell lysis. The plates were harvested [Tomtec 96-Harvester (Tomtec Inc., Handem, CT, USA)] in glass-fiber filters (WallacOu, Turku, Finland), which were placed in sample bags (Wallac) and subsequently immersed in scintillation fluid (Optiphase super mix, Wallac). The [^{3}H] uptake was measured in a 1450 Microbeta reader (Wallac).

An anti-HRPII immunoenzymatic assay was performed as described (22), using 0.05% parasitemia and 1.5% hematocrit. The compounds were incubated at 37º C with parasite cultures for 72h. The culture was frozen and thawed for cell lysis to occur and release the HRPII protein present in live parasites. Parasite growth was evaluated by specific interactions between the histidine- and alanine-rich parasite protein (HRPII) and commercially available monoclonal antibodies (MPFM ICLLAB-55A® and MPFG55P ICLLAB®, USA), which can be measured at 450 nm using SpectraMax340PC384 (Molecular Devices).

The anti-*P. falciparum* activity of neolignans was evaluated using curve-fitting software (Microcal Origin Software 5.0, Inc.) by comparing parasite growth in relation to drug-free control cultures, considered as 100% growth, and drug test-cultures. A sigmoidal dose-response curve was generated and the half-maximal drug inhibitory concentration (IC_{50}) of the parasite growth was determined. A molecule was considered active when the IC_{50} value was lower than 30 μM.

Cytotoxicity Assays and determination of drug selectivity indexes

The *in vitro* cytotoxicity assays were performed with cells derived from the hepatoma cell line HepG2A16 (ATTC, Manassas, VA, USA), cultivated in 75 cm^2 sterile culture flasks (Nunc) at 37°C in a 5% CO_2 atmosphere. The cells were maintained in RPMI 1640 culture medium (Sigma-Aldrich Co., St. Louis, MO) supplemented with 40 mg/L gentamicin and 10% fetal bovine serum (FBS) (Invitrogen, Life Technologies, Carlsbad, CA, USA). When cell confluence reached 80%, they were trypsinized (0.25% trypsin-EDTA) (Gibco, Life Technologies, Carlsbad, CA, USA), washed, counted, diluted in complete medium at 10^4 cells/well, and placed in a flat-bottomed 96-well plate (Corning, Santa Clara, CA, EUA), then incubated for 18 h at 37 °C to allow cell adhesion. The test and control compounds were added to the plates at various concentrations (up to 1.000 μg/mL) and incubated for another 24 h. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] at 5 mg/mL in water, was added to each well, followed by another 3 h of incubation at 37 ºC (23, 24). The supernatant was removed, and 100 μL of DMSO were added to each well, the reactions were read in a spectrophotometer (Spectra Max $340PC^{384}$, Molecular Devices) with a 570nm filter, and a 630 nm filter for background. The cell viability was expressed as the percentage of the absorbance compared to untreated cells, subtracted from the appropriate background. The minimum lethal dose (MLD_{50}) of the test compounds was determined as previously described (25); each test was performed in duplicates. The selectivity index (SI), or therapeutic activity, was calculated only for the active compounds, as the ratio between cytotoxicity and *P. falciparum* activity (MLD_{50}/IC_{50}), as described (20).

Molecular Docking

All structures were subjected to the geometry optimization using the hybrid density functional (B3LYP) (26, 27) and a basis set 6-31G* using the Gaussian03 package (28). The 3D structure of enzyme FP2 complexed with the **E64** (*L*-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane) were obtained from the Protein Data Bank (PDB accession code 3BPF). All compounds (neolignan derivatives and inhibitor **E64**) were submitted to docking studies using Molegro Virtual Docker (MVD) software. The enzyme cavity was detected with an active site radius of 6.0 Å from the side chain sulfur atom of residue Cys42, according to the literature (29). Docked results were visually inspected to ensure an acceptable drug/enzyme interaction was present. The following parameters were used for the guided differential evolution algorithm: population size = 50, crossover rate = 0.9, scaling factor = 0.5 and max evaluations = 100000. The algorithm used in the docking studies was MolDock Score, an adaptation of the Differential Evolution (DE) algorithm. The results of the molecular docking for neolignan derivatives were compared with the experimental data (29).

Analysis of the molecular electrostatic potential

The maps of electrostatic density can be indicators of nucleophilic and electrophilic centers which control the strength of the connections, and of the unbound interactions and molecular reactivity. With the Adaptive Poisson-Boltzmann Solver (APBS) methodology (30) it was possible to construct around the structure of the FP2 enzyme (**Figure 1**). The three-dimensional surfaces of the molecular electrostatic potential (MEP) were generated using the software Chimera and APBS (31), with an ionic strength of 12 mM. Mapping of the electrostatic potential onto the molecular surface of the protein was performed with a potential range from −8 eV to 8 eV.

Results

Chemistry

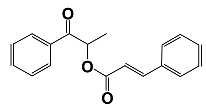
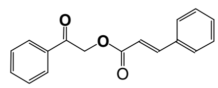
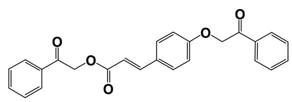
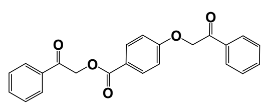
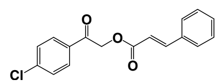
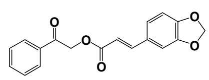
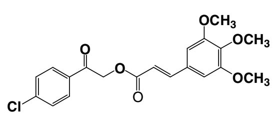
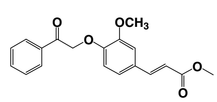
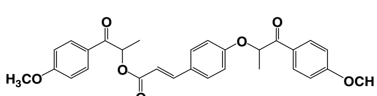
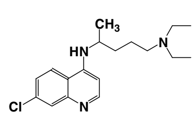
Compounds **2** (32), **3** (33) and **6** (34) were synthesized as previously described. Compounds **7** and **8** were commercial, but their synthesis and spectral data are not available in the literature, therefore we described these in the experimental section. Neolignans derivatives **4**, **5**, **9** and **10** are new compounds and were synthesized as shown in **scheme 1**.

The commercially available aromatic ketones were converted to the corresponding α-bromoketones **1a–c** (**Scheme 1**, step *i*) in one-step according to the methodology (35) and used without purification due to their strong tear-gas properties. All coupling reactions between α-bromoketones and phenoxy or carboxy nucleophiles generated *in situ* with anhydrous K_2CO_3 were performed in reflux of butanone as solvent. Compounds **7** (69% yield) and **8** (45% yield) were synthesized through the reaction of the appropriate α-bromoketone with cinnnamic acid, 3,4-(methylenedioxy)cinnamic acid and 3,4,5-trimethoxycinnamic acid, respectively (Scheme 1, step *iii*). Compound **9** was obtained via reaction of phenacyl bromide with methyl ferulate in 32% yield (**Scheme 1**, step *ii*). Treatment of 4-hydroxycinnamic acid and 4-hydroxybenzoic acid with anhydrous K_2CO_3 (**Scheme 1**, step *iv*) led to the formation of bi-nucleophiles (carboxy and phenoxy) *in situ*. Nucleophilic reactions of bi-nucleophile generated from 4-hidroxycinnamic acid with phenacyl bromide and 4-methoxy-8-bromopropiophenone afforded, respectively, compounds **4** (32% yield) and **10** (71% yield). Nucleophilic reactions of bi-nucleophile generated from 4-hydroxybenzoic acid with phenacyl bromide resulted in compound **5** at 34% yield (Scheme 1, step *v*). The reactions were monitored using TLC, and the products were purified by crystallization from ethanol. All compounds were fully characterized using IR, ^{1}H and ^{13}C NMR and EIMS, where their spectra were consistent with the assigned structures.

Pharmacology

Among the nine neolignan derivatives evaluated *in vitro* against the W2 *P. falciparum* resistant clone, only derivatives **9** and **10** exhibited IC_{50} of 26.6 and 12.1μM, respectively, by ^{3}H-hypoxanthine incorporation assay (**Table 1**).

Table 1. Cytotoxicity assay (MLD_{50}) against human hepatoma cells (line HepG2), *in vitro* activity against *P. falciparum* based on IC_{50} values and selectivity index (SI) of the neolignan derivatives.

Compound	MLD_{50} $\bar{x} \pm$ SD (µM)	IC_{50} $\bar{x} \pm$ SD(µM) ^{3}H-hypoxantine	Anti-HRPII	*SI
 **(2)**	> 4498.0	108.0 ± 80.5	130.4 ± 9.7	-
 **(3)**	> 3755.3	> 187.8	> 187.8	-
 **(4)**	> 2497.4	> 124.9	> 124.9	-
 **(5)**	> 2671.0	> 133.6	> 133.6	-
 **(6)**	> 3325.1	> 166.3	> 166.3	-
 **(7)**	> 3222.7	> 161.1	> 161.1	-
 **(8)**	> 2558.8	> 127.9	> 127.9	-
 **(9)**	>3064.3	26.6 ± 18.8	39.5 ± 11.2	>115.2
 **(10)**	>1545.5	12.1 ± 0.0	NT	>128.8
 **Chloroquine**	330 ± 28	0.12 ± 0.01	0.18 ± 0.37	2750

*SI = MLD_{50}/ IC_{50} obtained by [^{3}H]hypoxantine method which is a gold-test among semi-automated techniques. MLD, minimum lethal dose. IC_{50}, half-maximal drug inhibitory concentration. SD, standard deviation. NT, not tested.

Cytotoxic Activity and Selectivity Index

The nine neolignans tested showed MLDs above 1,500 μM, thus indicating that they are non-toxic to HepG2 cells (**Table 1**). The active derivate **10** (MLD_{50} above 1,545.5 μM) and **9**, (MLD_{50} above 3,064.3 μM) showed selectivity indexes above 128.8 and 115.2, respectively.

Molecular Docking

Co-crystallized ligand (**E64**) was re-docked to their target protein (FP2) to validate the docking protocol. When compared with the crystal structure, the docked conformation of **E64** gave RMSD of 0.15Å and had a MolDock score of -133.2 $kcal.mol^{-1}$, shown in **Figure 2**. These results illustrate a good adjustment between the theoretical and experimental data (29).

Docked conformations of derivatives **9** and **10** were ranked based on their MolDock score and the best conformation (**Table 2**). The RMSD value obtained for the two complexes was 0.19 Å and 0.12 Å, and the score values -102.2 and -111.7 $kcal.mol^{-1}$, respectively. These values are relatively close to the score value of inhibitor **E64** (-133.2 $kcal.mol^{-1}$). Interestingly, the observed activities against clone W2 were 26.6 μM, 12.1 μM and 3.0 μM, for **9**, **10** and **E64**, respectively.

Table 2. Molecular docking studies for **E64** and neolignan derivatives.

Compound	MolDock Score (kcal.mol^{-1})	Hbond score (kcal.mol^{-1})	Number of H-Bonds
2	- 82.3	-2.2	2
3	-77.9	-2.0	2
4	-90.1	0	0
5	-90.5	-0.4	1
6	-80.4	-2.3	2
7	-91.5	-3.2	2
8	-82.6	-1.9	1
9	-102.2	-4.1	1
10	-111.7	-7.3	4
E64*	-133.2	-8.5	4

* Experimental value obtained by Kerr and Lee et al. (27).

In this study, the carbonyl group (*L-trans-Epoxysuccinyl*) and the amide group (*leucylamido*) of **E64** inhibitor were located near the catalytic cysteine residue (Cys42), tryptophan (Trp43) and glycine (Gly83) of the FP2. In addition, the oxyanion hole located in the S2 cavity was occupied by the carbonyl group (*L-trans-Epoxysuccinyl*), which interacts with the thiol group of Cys42 at a distance of 2.5 Å. This carbonyl group also interacts through hydrogen bonds with the aromatic ring of Trp43 at 2.9 Å. For the amide group (*leucylamido*), two interactions with amine and carboxyl group of Gly83 at 3.2 Å and 2.9 Å, respectively, were observed (**Figure 3 A**).

Figure 3B shows the predicted binding site of compound **10** in the F2 catalytic site suggested by molecular docking. In this complex (FP2-10), the ester group of derivate **10** also occupies the oxyanion hole of the cavity S2, interacting with amine and thiol groups of Cys42 at bond lengths of 3.0 Å and 3.1 Å, respectively, same as for the **E64** inhibitor. Moreover, a series of hydrogen bonds can be observed between methoxy groups of derivate **10** and the residues of His174, Ser149 and Ile85 situated within cavities S1' and S2, similar to previous reports (36, 37), being the bond length 3.1 Å, 2.9 Å and 2.9 Å respectively (**Figure 3B**). Lastly, derivate **9** showed only one interaction at a distance of 2.9 Å with residue Ile85 (**Figure 3C**) evidencing the low potency against FP2 as well as its IC_{50} value.

Discussion

The neolignans constitute a class of chemical substances with described biological actions against leishmaniasis, cancer and other diseases (12-14, 15, 38). Some neolignans and their derivatives have shown antimalarial activity at low concentration (39, 40). Derivate **9** in this study exhibited *in vitro* activity, with IC_{50} of 12.1 µM, similar to other neolignan derivatives such as Virolongin A (IC_{50} of 14.9 µM) against chloroquine-resistant *P. falciparum* (clone Dd2) (36). These derivatives have a similar structure to compound **10** with the presence of three benzene nuclei. Furthermore, the presence of methoxyl and the absence of chlorine as substituents in the neolignan derivatives is important for good antimalarial activity (37, 40, 41). In previous work (18) where active lignans were evaluated against a chloroquine-resistant *P. falciparum* strain, the importance of the methoxy substituent for good antiparasitic activity was demonstrated. Most lignoids were not cytotoxic (MLD_{50}> 1,500 µM), and are therefore good new antimalarial candidates as suggested previously (17). The selectivity index was above 100 µM for two new compounds tested, the neolignan derivatives **9** and **10**.

The active site of FP2 can be divided into four cavities denominated S1, S1', S2 and S3 (**Figure 1**), and is located in a cleft between the two structurally distinct domains, with catalytic triad of Cys42, Asn173 and His174 (29). Derivate **10** is a potential inhibitor of FP2, and as can be seen in Figure 1, this derivative effectively occupied cavities S1 and S2. Furthermore, it establishes several interactions with catalytic FP2 residues localized in cavity S2 (**Figure 3**), commonly observed among cysteine protease inhibitors (42). The S2 site is a predominantly hydrophobic region where residues were shown to be preferentially linked to groups of the substrate (43), but can also interact with hydrophilic groups. This fact explains the interactions of the methoxy groups of derivates **9** and **10** in this cavity (44).

Conclusions

Nine neolignans tested were not toxic to HepG2 cells exhibiting minimal lethal doses (MLD_{50}) greater than 1,500 μM. Only derivatives **9** and **10** showed activity against chloroquine-resistant *P. falciparum* clone W2. Interactions and score values obtained by molecular docking techniques for complexes formed with neolignans and FP2 were satisfactory when compared to the crystallographic E64-FP2 complex. These results show that neolignans are promising prototypes to be considered in the search of potent new derivatives against malaria parasites, as well as less toxic products for safe therapy.

Author contributions

CNA and CCFM designed the theoretical study and analyzed the data. GANP performed the *in vivo* and *in vitro* experiments under the supervision of CTDR and AUK. GCS, LSS and LESB designed and performed the synthesis of compounds. Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support. The manuscript was written and revised by all authors.

Conflict of interest

The authors have declared no conflict of interest.

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Supplementary Information

Supplementary data related to this article include representative ^{1}H and ^{13}C NMR and HRMS spectra of new compounds.

Figure Legends

Figure 1. Electrostatic density map around of 3D structures of FP2 with **E64** (pink) and **10** (yellow) ligands.

Figure 2. Superposition of the docked (blue) and crystal structure (pink) conformations of **E64** in the active site of FP2.

Figure 3. Hydrogen bonds formed by the **E64** (pink) and derivatives **10** (yellow) and **9** (orange) with the active site residues of the Falcipaína-2 (green).

Supplementary Figure Legends

Figure S1: ^{1}H NMR (400 MHz, DMSO) spectrum of compound **4**

Figure S2: ^{13}C NMR (100 MHz, DMSO) spectrum of compound **4**

Figure S3: HRMS spectrum of compound **4**

Figure S4: ^{1}H NMR (400 MHz, DMSO) spectrum of compound **5**

Figure S5: ^{13}C NMR (100 MHz, DMSO) spectrum of compound **5**

Figure S6: HRMS spectrum of compound **5**

Figure S7: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound **7**

Figure S8: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of compound **7**

Figure S9: HRMS spectrum of compound **7**

Figure S10: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound **8**

Figure S11: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of compound **8**

Figure S12: HRMS spectrum of compound **8**

Figure S13: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of compound **9**

Figure S14: ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of compound **9**

Figure S15: HRMS spectrum of compound **9**

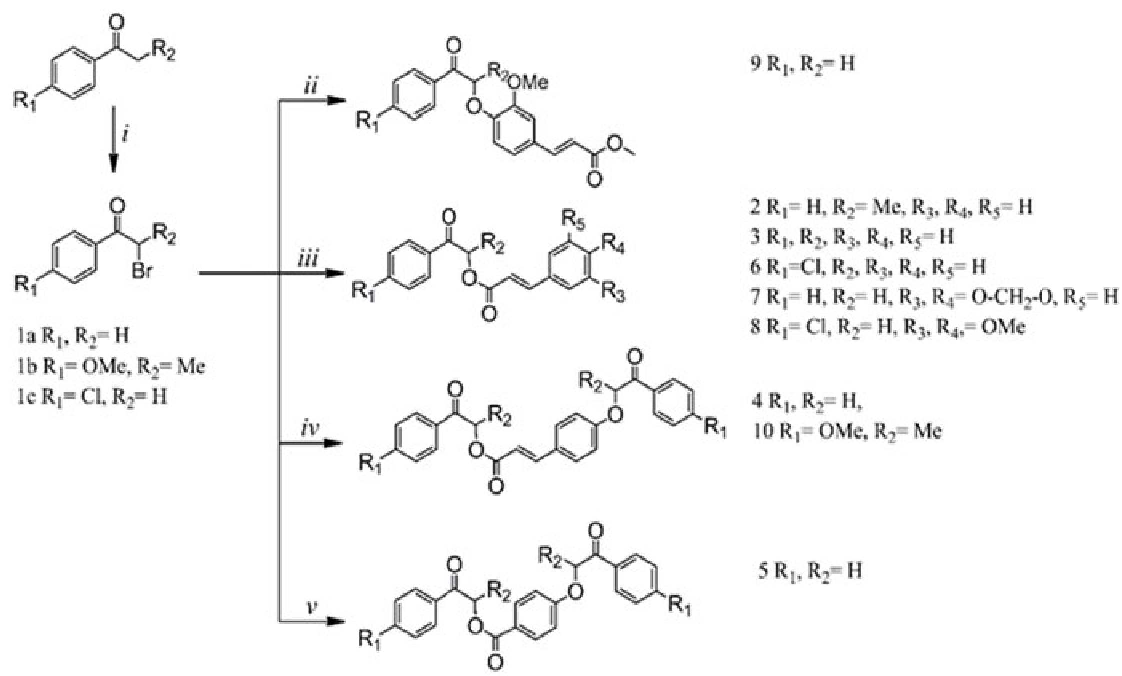
Figure S16: ^{1}H NMR (400 MHz, DMSO) spectrum of compound **10**

Figure S17: ^{13}C NMR (63 MHz, DMSO) spectrum of compound **10**

Figure S18: HRMS spectrum of compound **10**

Scheme Legend

Schemes 1. Synthesis of neolignan 8.O.4' derivatives. Reagents and conditions:(*i*) $CHCl_3$, Br_2, 0.5 h, reflux; (*ii*) Methyl ferulate; K_2CO_3, butanone, 12 h, reflux (*iii*) Cinnamic acid or derivatives, K_2CO_3, butanone, 12 h, reflux. (*iv*) 4-Hydroxycinnamic acid, K_2CO_3, butanone, 12 h, reflux. (*v*) 4-Hydroxybenzoic acid, K_2CO_3, butanone, 12 h, reflux.


i
ii
iii
iv
v
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1b R1= OMe, R2= Me
1c R1= Cl, R2= H
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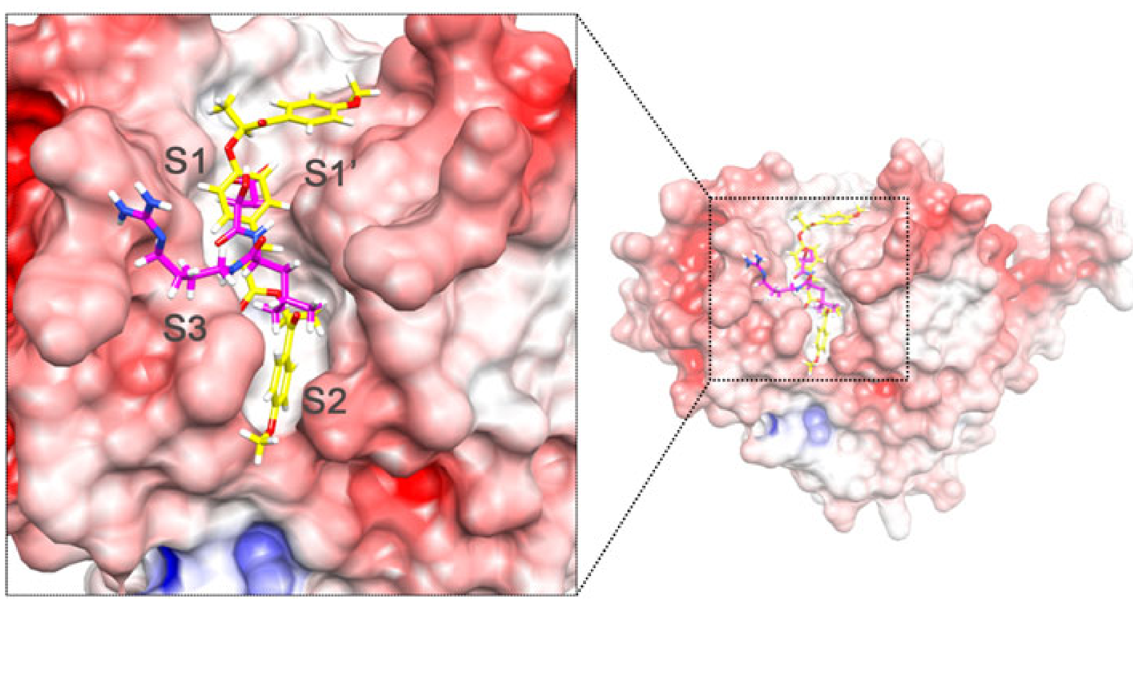
2 R1= H, R2= Me, R3, R4, R5= H
3 R1, R2, R3, R4, R5= H
6 R1=Cl, R2, R3, R4, R5= H
7 R1= H, R2= H, R3, R4= O-CH2-O, R5= H
8 R1= Cl, R2= H, R3, R4,= OMe
4 R1, R2= H,
10 R1= OMe, R2= Me
5 R1, R2= H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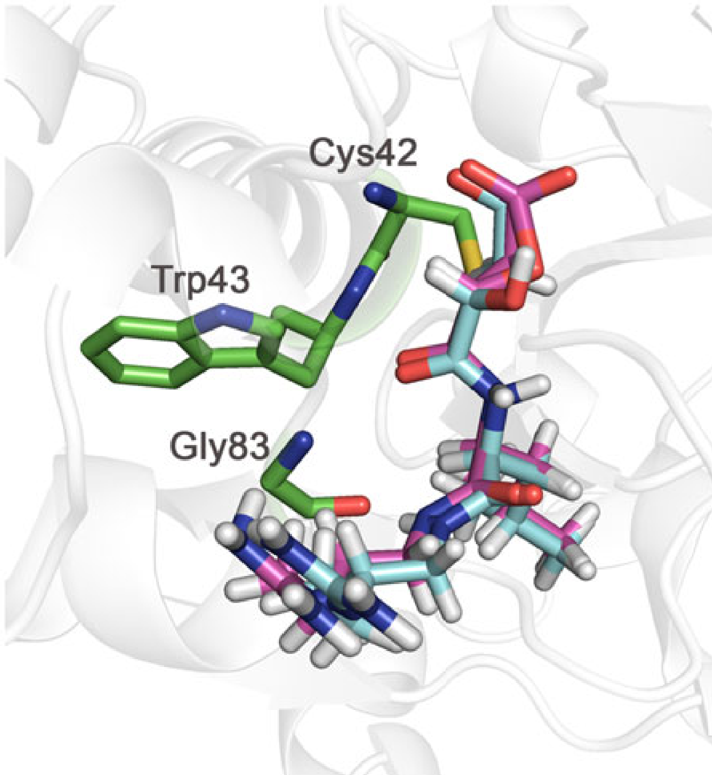
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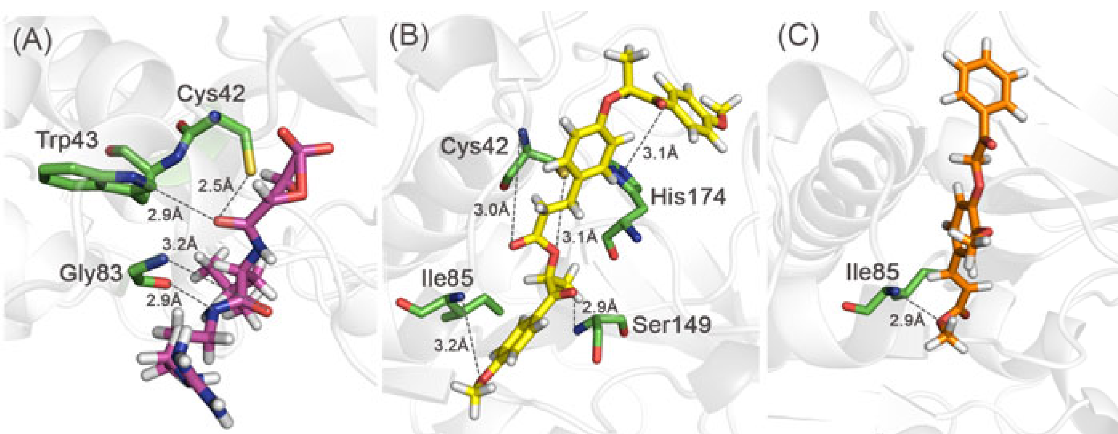
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Trp43
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2.9Å
3.2Å
Gly83
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(B)
Cys42
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His174
3.0Å
3.1Å
Ile85
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Ser149
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(C)
Ile85
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