A new hybrid: Artesunate-Tumacona B

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Nuevo híbrido Artesunato-Tumacona B

Nou híbrid Artesunat-Tumacona B

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SUMMARY

In recent years, the emergence of Plasmodium strains resistant to artemisinin derivatives, such as the commercial antimalarial Artesunate, has been detected. For this reason, in the search for new strategies to malaria control, we used the antiplasmodial activity of natural products from plant *Solanum nudum*, such as Tumacona B (SN2), to synthesize a new hybrid Artesunate-Tumacona B. The antiplasmodial activity and cytotoxicity of this hybrid was evaluated *in vitro*. We found a potent activity with IC_{50} = 0.0044 µM in the strain 3D7 (chloroquine sensitive) and IC_{50} = 0.0059 µM for the strain FCR3 (chloroquine resistant) and low cytotoxicity in HepG2 human liver cells with a CC_{50} = 12.6 µM. This makes the hybrid a new and promising compound.

Keywords: Hybrid; antimalarial; sterols; artemisinins; *Plasmodium falciparum*.

RESUMEN

Se ha detectado en los últimos años la aparición de cepas de Plasmodium resistentes a derivados de artemisininas como el antimalárico comercial Artesunato, es por esto, que en la búsqueda de nuevas estrategias para el control de la malaria se aprovechó la actividad antiplasmodial de productos naturales obtenidos de la planta *Solanum nudum* , como la Tumacona B (SN2), para sintetizar un nuevo híbrido Artesunato–Tumacona B. A este híbrido se le evaluó *in vitro* su actividad antiplasmodial y citotoxicidad. Se encontró una potente actividad con IC_{50} = 0.0044µM en la cepa 3D7 (Sensible a cloroquina) e IC_{50} = 0.0059µM para la cepa FCR3 (Resistente a cloroquina) y baja citotoxicidad en células hepáticas humanas HepG2 con CC_{50} =12.6µM convirtiéndose en un nuevo y promisorio compuesto.

Palabras clave: Híbrido; antimalárico; esteroles; artemisininas; *Plasmodium falciparum.*

RESUM

S'ha detectat en els darrers anys l'aparició de soques de Plasmòdium resistents a derivats d'artemisinines com l'antimalàric comercial Artesunat, és per aquest a rao que a la recerca de noves estrategias pel control de la malaria es va aprofitat l'activitat antiplasmodial de productes naturals obtinguts de la planta *Solanum nudum*, com la Tumacona B (SN2), per sintetitzar un nou híbrid Artesunat–Tumacona B. L'activitat antiplasmodial i la citotoxicitat d'aquest híbrid es va avaluar in vitro. Es va trovar una potent activitat amb IC_{50} = 0.0044µM a la soca 3D7 (resistent a la cloroquina) i IC_{50} = 0.0059µM per a la soca FCR3 (resistent a la cloroquina) i baixa citotoxicitat en cèl.lules hepàtiques humanes HepG2 amb CC_{50} =12.6µM. Convertint l'híbrid en un nou i prometedor compost.

Paraules clau: Híbrid, antimalárico; esterols; artemisinina; *Plasmòdium falciparum.*

HIGHLIGHTS

- We synthesized a new hybrid Artesunate-Tumacona B with *in vitro* antiplasmodial activity in sensitive (3D7) and resistant (FCR3) *P. falciparum* strains.
- A new derivative of Tumacona B (SN2-YAM) with *in vitro* antiplasmodial activity in *P. falciparum* strains was obtained.
- The tested hybrid compounds were more active than their corresponding combinations in chloroquine-sensitive (3D7) and chloroquine-resistant (FCR3) strains of *P. falciparum.*
- The Artesunate-Tumacona B hybrid was 23.4 times more active than chloroquine in a resistant (FCR3) strain of *P. falciparum*.

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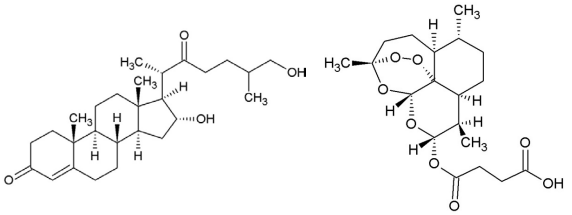
INTRODUCTION

Malaria is a potentially fatal disease caused by parasites of the genus *Plasmodium*, which are transmitted to humans by the bite of infected Anopheles mosquitoes. The species of *Plasmodium* that cause the largest number of cases in the world are *P. vivax* and *P. falciparum*, the latter being the most deadly[1].

The World Health Organization estimates that there were about 214 million cases of the disease worldwide in 2015 that killed 438.000 people[1]. One of the reasons of these figures is the difficulty in controlling the emergence and spread of strains of *Plasmodium Spp* resistant to antimalarial therapy. In the case of *P. falciparum* malaria, combined therapy based in artemisinin derivatives has been used in an attempt to overcome this obstacle. However, in recent years, resistance to this treatment option has been detected and resistance of *P. falciparum* to artemisinin-type drugs in regions such as Cambodia, Myanmar, Thailand and Vietnam, with great potential for global spread, has been reported[2, 3, 4, 5, 6.] The problem of resistance, in addition to the absence of an effective vaccine for the control of the disease, require the search for new treatments.

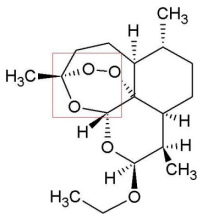
Currently, several studies have shown that molecules composed of two chemically linked structures, with two different pharmacophores and dual function, have become an important alternative for the treatment of diseases that share the problem of resistance. These molecules are called hybrids and potentiate the activity compared to the molecules used separately, in Plasmodium strains resistant to them[7], as is the case with ferroquine[8] and some trioxaquine derivatives[9].

In this work, we synthesized a hybrid by covalently binding Artesunate (artemisinin derivative) with Tumacona B (SN2) (Figure 1), a steroid isolated from plant *S. nudum* (HUA, Voucher 179201-Catalog of Colombian Flora). This steroid has antiplasmodial activity (IC_{50} 19,55 µM) in strain 7G8 CQ-resistant[10] and its structure includes reactive hydroxyls moieties[11,12], which allows the formation of a hybrid. Additionally, its A cycle has an α, β-unsaturated carbonyl group which has been related by studies of structure-activity relationship (SAR) with the promising antiplasmodial activity of Diosgenone[13], a natural compound also found in *S. nudum*. In our case, this α, β-unsaturated carbonyl group fulfills the role one of the two pharmacophores for the hybrid's formation. (See Figure 4)

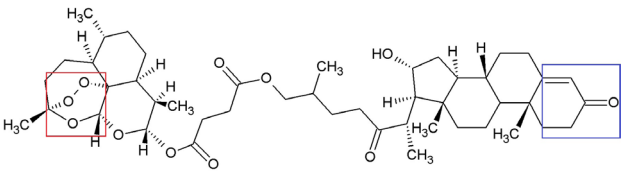


Compound 1. Artesunate (1) *Compound 2. Tumacona B (SN2) (2)*

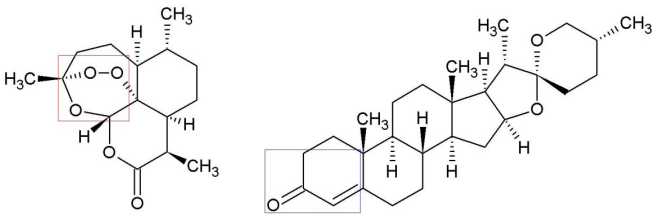
Figure 1. *Chemical structures of molecular entities that formed the hybrid Artesunate-Tumacona B (3)*



Compound 3. Arteether



Compound 4. Hybrid (ART-SN2) (3)



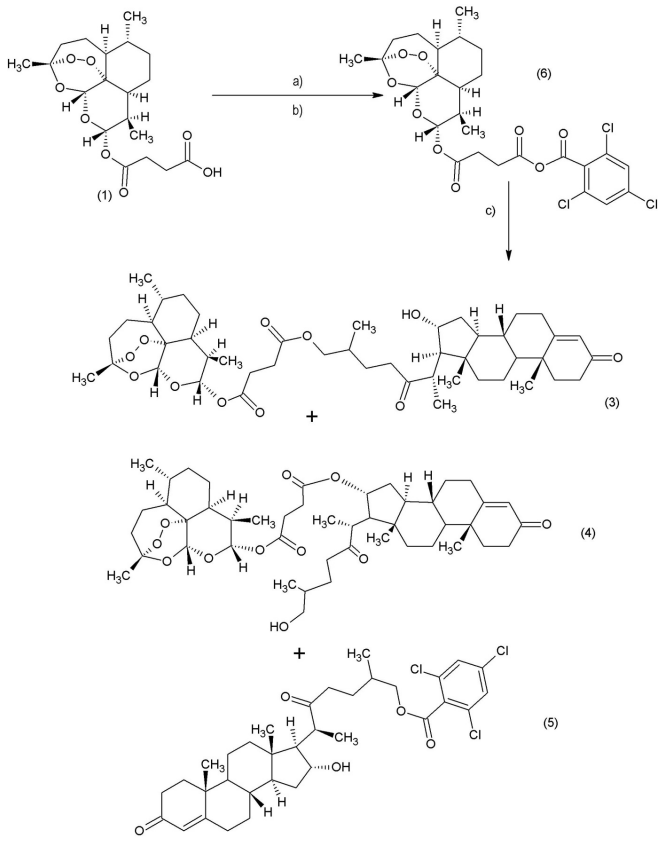
Compound 5. Artemisinin *Compound 6. Diosgenone*

Figure 2. *Hybrid design based on antiplasmodial pharmacophores found in artemisinin (1, 2, 4-trioxanes) and sterols (α,β-unsaturated system in A ring).*

RESULTS AND DISCUSSION CHEMISTRY

Synthesis of the hybrid Artesunate-Tumacona B (ART-SN2) 3 was developed in one step as shown in scheme 1, where the starting materials were Artesunate (Sigma®) and pure Tumacona B (2) extracted from the plant *S. nudum*. Esterification between the carboxyl group of Artesunate and the two hydroxyl groups of Tumacona B was set in order to obtain two different hybrids (compounds 3 and 4) in which pharmacophores are held. To do this, the Yamaguchi-type esterification procedure was followed[14] where compound 2, 4, 6- trichlorobenzoyl chloride, known as Yamaguchi reagent, was coupled to Artesunate (1), which facilitated the primary alcohol nucleophilic attack (the most exposed and more reactive) of natural compound Tumacona B (2) for the formation of an ester-type hydrolyzable connector.

Compound 3 is hydrolyzable, so it becomes a dual prodrug and its individual molecules, after metabolized, could be considered as the active substances. The expected compound 4 was not obtained, possibly due to steric hindrance in the region of the secondary hydroxyl of Tumacona B or the low reactivity this hydroxyl has with respect to the primary hydroxyl. However, we obtained compound 5, as an unexpected product resulting from the nucleophilic attack of the primary alcohol of Tumacona B to the Yamaguchi reagent acyl chloride, which is found in excess in the reaction mixture.



Scheme 1. *Synthesis of Artesunate - Tumacona B hybrid 3. a.) THF at 0°C b) TEA, 2,4,6- trichlorobenzoyl chloride, rt, 2 h. (c) Tumacona B (2), DCM, DMAP, 24 h, rt.*

Biological Assays

In vitro antiplasmodial assays were performed to hybrid 3 (SN2-ART and derivative 5 (SN2-YAM) in 3D7 (chloroquine-sensitive) and FCR3 (chloroquine-resistant) *P. falciparum* strains following the protocol adapted according to the methodologies described by Bravo and collaborators [15], and Desjardins and collaborators[16]. The same procedure was used to perform antiplasmodial assays a to molecules Tumacona B, Artesunate and its combination. In addition Mosmann method[17] was followed to determine the cytotoxicity of compounds and the HepG2 cell line was employed.

In Table 1, we can observe that hybrid **3** (ART-SN2) showed an IC_{50} of 0.0059 µM for the FCR3 strain. For individual molecules in this same strain, an IC_{50} of 0.0025 µM for Artesunate (**1**) and 60.6 µM for Tumacona B (SN2) was obtained. The hybrid **3** antiplasmodial activity is in the same order of magnitude as the potent antimalarial Artesunate and is much better than the natural starting compound Tumacona B (**2**). It is important to note that the IC_{50} of hybrid **3**, is approximately 23.4 times more potent than the one shown by chloroquine in resistant strains. Hybrid **3** was less toxic (12.6 µM) than Artesunate (**1**) (8.5 µM), exhibiting a SI of 2135.6, a high value that indicates its high selectivity for erythrocytes parasitized with *P. falciparum*. This allows us to state that this compound has very low cytotoxicity.

Table 1. *In vitro antiplasmodial activity and cytotoxicity of molecules obtained and of starting compounds.*

Compound	[a]IC_{50} ± SD (µM)		[b]CC_{50} ± SD (µM)	[c]SI
	FCR3	3D7	HepG2	
Artesunate (1)	0.0025±0.0007	0.0020±0.0002	8.5± 4.1	3400
Tumacona B (2)	60.6±11.7	53.9±9,8	464.4± 20,0	7.7
ART-SN2 (3)	0.0059±0.0011	0.0044±0.0006	12.60± 2.03	2135.6
ART-SN2 (4)	--	--	--	--
SN2-YAM (5)	0.0445±0.0289	0.0410±0.0021	3.87± 0.21	87.0
Cloroquine	0.138±0.016	0.0111±0.0018	NA	NA

[a] IC_{50} (µM): Concentration corresponding to the 50% inhibition of parasite growth; [b] CC_{50} (µM): Concentration corresponding to the 50% cytotoxic dose in HepG2 cells; [c] Selectivity Index (SI) : HepG2 CC_{50} / FCR3 IC_{50}; Antiplasmodial assay control. SD: standard deviation. FCR3: cloroquine resistant strain, 3D7: cloroquine sensitive strain.

Ester 5 (SN2-YAM) formed between SN2 and the Yamaguchi reagent presented an IC_{50} of 0.0445 µM in FCR3, and 0.041 µM in 3D7, showing that the derivatization with the chlorinated compound dramatically increased the antiplasmodial activity with respect to SN2 (IC_{50}: 60.6 µM FCR3, 53.9 µM 3D7), resulting more potent than chloroquine. This IC_{50} exhibited by the derivative was 3 times higher than that of chloroquine. Despite the fact that compound 5 presented a higher cytotoxicity with a 3.87 µM CC_{50} in HepG2, the SI was 87, which tells us that this is a selective compound towards the red blood cells parasitized with *P. falciparum*; that is, it shows very low cytotoxicity.

The results from antiplasmodial activity in **table 1** indicate that the activity of hybrid 3 is greater than that of natural compound SN2; i.e., the covalent binding with Artesunate (1) increases its individual antiplasmodial activity and favors the formation of a new artemisinin type derivative with an IC_{50} value of the same order as the Artesunate IC_{50}. This result may become relevant in studies related to malaria episodes caused by parasites of *P.falciparum* artemisinin-resistant strains, since the hybrid could be a good alternative due to its potent activity, comparable to the Artemisinin derivatives currently used[18].

The antiplasmodial activity of combinations between the starting molecules can be calculated from the FICs (fractional inhibitory concentrations); this index allows us to know the effect of the interaction between two molecules. In table 2, FICs for strains 3D7 and FCR3 were indifferent (3D7 FIC: 1.8; FCR3 FIC: 1.58), indicating that the molecules in combination do not exert any effect (synergistic or antagonistic) on each other, since it is established that a value between 0.51 to 4 is considered indifferent[19]. However, a molecule with an IC_{50} in the same order as Artesunate is obtained when they are chemically linked.

Table 2. *Antiplasmodial activity of combinations of starting molecules.*

Strain	Compound and Combinations	IC_{50} (µM)	[a]SD	[b]FICs
3D7	[c]ART	0.0039	0.0011	1.8
	ART+[e]IC_{10} SN2	0.0037	0.0006	
	[d]SN2	29.85	0.01	
	SN2+IC_{10} ART	28.39	0.46	
FCR3	ART	0.0066	0.0013	1.58
	ART+IC_{10} SN2	0.0058	0.0012	
	SN2	50.35	6.89	
	SN2+IC_{10} ART	35.35	2.74	

[a]SD: Standard Deviation; [b]FICs: Fractional inhibitory concentrations; [c]ART: Artesunate; [d]SN2: Tumacona B ; [e]IC_{10}: Inhibitory concentration of 10% parasite growth.
IC_{10} SN2: 7.250 mM; IC_{10} ART: 2.0x10^{-3} mM

CONCLUSIONS

We obtained a promising Artesunate-Tumacona B hybrid (3) (ART- SN2) that exhibits potent antiplasmodial activity *in vitro* in chloroquine-sensitive and chloroquine -resistant strains, and also has a low cytotoxicity. This antiplasmodial activity was 23.4 times more potent than chloroquine for the resistant strain. The best therapeutic option from the starting compounds is the formation of a hybrid because they do not potentiate between each other when are tested as a combination. (3D7 FIC: 1.8; FCR3 FIC: 1.58).

Although the hybrid displays the Artesunate pharmacophore, this is a new molecule and it is possible to think that it has antiplasmodial effect in strains resistant to artemisinin, which could represent an alternative for artemisinin combination therapies.

The derivative SN2-YAM (5), obtained from the esterification reaction with the coupling reagent, showed greater activity and selectivity than the starting natural compound SN2; that is, a new compound was synthesized with interesting physicochemical and biological characteristics.

The two compounds, obtained synthetically, are new and were analyzed by nuclear magnetic resonance and mass analysis to verify their structure and molecular composition.

The results obtained in this study confirm the importance of hybrids as a therapeutic alternative for the treatment of malaria and could be useful to guide future efforts to discover new antiplasmodial compounds of this type.

EXPERIMENTAL METHODS

General information

We used Artesunate, 2, 4, 6-trichlorobenzoyl chloride (Yamaguchi reagent), triethylamine (TEA), 4-dimethylaminopyridine (DMAP) and sodium borohydride (SBH) from Sigma®; and tetrahydrofuran (THF) and Dichloromethane from Merck®. Methanol, hexane and ethyl acetate were commercial grade. The progress of the reactions was monitored by thin layer chromatography (TLC), silica gel 60 F254 (Merck®). The plates were developed in a mix of acetic acid, sulfuric acid and water, as well as sublimated iodine and ultraviolet light. Chromatographic columns were made using a matrix of silica gel 60 (0.063-0.200 mm) Merck®. The IR spectrum was obtained in a 100 Spectrum (Perkin Elmer®) and the software used to interpret the results was the Spectrum v5.3.1. We utilized a Bruker® spectrometer for the spectroscopic analysis of ^{1}H-NMR and ^{13}C-NMR, 300 MHz for ^{1}H and 75 MHz for ^{13}C, using trimethylsilane as internal standard, and deuterated methanol (DME) and deuterated chloroform ($DCCl_3$) from Merck® as solvents. The mass spectrum of hybrid 3 was obtained in a 6300 LC/MSD Trap SL mass spectrometer (Agilent Technologies®).

Procedure for the synthesis of the SN2-ART hybrid (3)

Hybrid 3 was synthesized from an esterification reaction via Yamaguchi. The following methodology was developed: 89.2 mg (0.23 mmol) of Artesunate 1 were diluted with 2.0 mL of THF, 0°C, then 57.5 µL of TEA and 54.4 µL of Yamaguchi reagent (0.34 mmol) were added, then stirred in inert atmosphere and room temperature for two hours. Subsequently, 100 mg (0.23 mmol) of Tumacona B 2 and 44.25 mg (0.36 mmol) of DMAP were dissolved with 3.0 mL of DCM, which were slowly added to the solution and allowed to react for 24 hours (scheme 1). The reaction was stopped adding to the mixture a solution of distilled water saturated with ammonium chloride.

Two products were detected by TLC in the reaction mixture. Subsequently, a liquid–liquid extraction was performed using three washes of 15 mL of dichloromethane. Then the organic phase was separated and concentrated to dryness and transferred to a chromatographic column where separation and purification of compounds was obtained by gradient elution (hexane: ethyl acetate). Two white solids were obtained and identified after characterization as pure compounds **3** and **5**. These are new compounds, obtained synthetically. Both the Artesunate-Tumacona B hybrid 3 and compound 5, a chlorinated derivative of Tumacona B, were analyzed by nuclear magnetic resonance and mass analysis to verify their structure and molecular composition.

Compound **3**, white solid, showed a molecular ion [M+Na^+] at m/z 819.6 (calculated for $C_{46}H_{68}O_{11}Na$) in the spectrum of ESI-API-MS, corresponding to the molecular formula $C_{46}H_{68}O_{11}$. Melting point (°C): 176.34. IR (KBr, cm^{-1}): 1735 (COOR), 1671 (C=O), 1014 (C-O), 3492 (C-OH). The ^{1}H NMR spectrum (300 MHz, DME) showed characteristic singlets attributed to three methyl groups δ 0.826 (s, 3H), 1.245 (s, 3H), 1.368 (s, 3H), two doublets at δ 0.87 (d, 3H, J: 9Hz), 1.189 (d, 3H, J:6 Hz), one triplet δ 0.964 (t br, 6H, J: 9Hz, 3 Hz) and signal for one olefinic proton at δ 5.530 (s, 1H); also one doublet is observed at δ 5.772 (d, 1H, J: 9 Hz) and a markedly displaced singlet at δ 5.730 (s,1H), belonging to the structural entity of Ar-

tesunate and aliphatic protons between δ 1,0 y 2,4, typical of a steroid skeleton. The ^{13}C NMR spectrum (75MHz, DME) showed 46 signals, including the four carbonyls, one for α, β-unsaturated ketone at δ 20.8, another for ketone at δ 216.13 and signals for two esters at δ 172.5 and δ 171.43. This demonstrates the covalent binding between Artesunate and the natural molecule Tumacona B (SN2). The rest of the chemical shifts for carbon were δ: 173.57, 122.80, 103.73, 92.12, 91.14, 79.90, 75.25, 68.66, 62.35, 53.77, 52.70, 51.57, 47.87, 45.18, 43.77, 39.45, 38.54, 37.91, 36.90, 35.87, 35.67, 35.26, 34.85, 33.91, 33.28, 32.44, 31.97, 31.84, 31.66, 28.67, 28.35, 26.67, 24.49, 24.40, 21.46, 20.33, 19.18, 16.23, 15.89, 15.51, 12.41, 11.02. Compound **3** was obtained in a 47% yield.

Compound **5** is a white solid with a molecular formula $C_{34}H_{43}Cl_3O_5$, established by ESI-API-MS, showing a molecular ion [M+Na$^+$] at m/z 659.3 (calculated for $C_{34}H_{43}Cl_3O_5Na$). Melting point (°C): 172.5. IR (KBr, cm^{-1}): 1736 (COOR), 1670 (C=O), 1578 (C=C), 3422 (C-OH). The ^1H NMR spectrum (300 MHz, $CDCl_3$) showed the characteristic singlets of the steroidal part attributed to methyl groups in δ 0.78 (s, 3H) and δ 1.21 (s, 3H), as well as δ 5.63 corresponding to the olefinic proton of the α, β unsaturated system and one singlet at δ 7.39 corresponding to the two aromatic protons (s, 2H). One doublet is observed at δ 1.05 (d, 4H, J: 6Hz). One triplet can also be observed in δ 3.80 (t br, 1H, J: 3Hz) and one multiplet δ 4.27 (m, 2H, J: 4.27). There are also aliphatic protons between δ 1.38 and 3.32 typical of the steroidal skeleton. The ^{13}C NMR spectrum (75MHz, $CDCl_3$) showed 34 signals in total, including 3 carbonyls, one for α, β-unsaturated ketone at δ 199.4 and one ester type in the region δ 164.2, signal that suggests the binding between the entity of Artesunate and the Yamaguchi reagent (2,4,6-trichlorobenzoyl chloride). The olefinic carbon was also observed at δ 171.0 and the hydroxylated secondary carbon, belonging to the structural entity of Tumacona B, at δ 76.6. The other chemical shifts for carbon were δ: 136.18, 132.57 (2C), 128.08 (2C), 124.0, 70.78, 62.58, 53.48, 52.42, 48.63, 44.09, 30.72, 38.77, 38.54, 35.62, 35.54, 34.97, 33.95, 32.79, 32.08, 31.85, 27.09, 20.71, 17.33, 17.11, 17.06, 16.81, 13.75. Compound **5** was obtained in an 18% yield.

Biological Assays

***In vitro* antiplasmodial assays** compounds **1**, **2**, **3**, **5**, were subjected to *in vitro* antiplasmodial activity assays in *P. falciparum* strains 3D7 (sensitive to chloroquine) and FCR3 (resistant to chloroquine) at 1% parasitemia and 2% hematocrit, following the protocol described by Bravo and collaborators in 1999 and Desjardins and collaborators in 1979. The following concentrations were evaluated: **1** (0.00098 to 0.065 μM), **2** (3.62 to 232 μM) **3** (0.00047 to 0.031 μM) and **5** (0.0061 to 0.39μM), all dissolved in pure DMSO. Chloroquine was used as a positive control from 0.15 to 0.00234 μM for the sensitive strain and 2.0 to 0.31 μM for the resistant strain, all dissolved in distilled water. All experiments were performed in triplicate, independently, and the final concentration of the DMSO was always less than 0.1%. Antiplasmodial activity was done using radiolabeled hypoxanthine as follows: 96-well flat-bottom plates, containing the concentrations to be evaluated, were treated with 200uL of parasitized erythrocytes (1% parasitemia and 2% hematocrit) and 1 μC/mL of ^3H-hypoxanthine (MP Biomedicals, Santa Ana). The plates were incubated for 48 hours at 37°C in a 5% O_2, 5% CO_2 atmosphere (balanced nitrogen). DNA of parasites was collected with the help of a semi-automatic harvester and radioactivity in μCi was measured on a scintillation counter (Merck®). An experiment was considered valid when reading of untreated control (culture media) wells was superior to 2000 cpm. Results were expressed as the average of the 50% inhibition concentration. Moreover, an IC_{50}, for each compound, less than 1 μM[20] was considered a promising activity or hit molecules, good from 1 to 20μM[21], moderate 20 to 100μM[21], low activity 100 to 200 μM[21] and not active when greater than 200 μM[21].

To determine the type of drug interaction between the Tumacona B (**2**) and Artesunate (**1**), we calculated the combination IC_{50} with a fixed concentration of Artesunate in the case of Tumacona B (SN2), and a fixed concentration of Tumacona B (SN2) in the case of Artesunate. The fixed concentration used was the IC_{10} of the maximum antiplasmodial effect.

The combination IC_{50} values were employed to calculate the fractional inhibitory concentrations (FICs) using equation 1.

$$FIC = \frac{In\ combination\ Drug\ A\ IC50}{Single\ drug\ A\ IC50} + \frac{In\ combination\ Drug\ B\ IC50}{Single\ drug\ B\ IC50} \quad (1)$$

FICs: If the combination IC_{50} is less than that obtained with the single molecule, it is considered a synergistic effect (FIC < 0.5); if it is greater, anantagonistic (FIC > 4) or an indifferent effect when the FIC is (0.51 < FIC < 4).

Cytotoxicity assays

Mosmann method[17] was followed to determine the cytotoxicity of compounds. The HepG2 cell line was employed and cultivated in 96-well flat-bottom plates at a concentration of $2x10^5$ cells/well in 100 μL of DMEM/F12 medium supplemented with 10% fetal bovine serum. The cells were incubated at 37°C with 5% CO_2 for 30 hours to allow monolayer formation. After that, 100 μL of each compound was added to each well in serial concentrations as follows: 520.26 to 8.13μM for Artesunate (**1**), 464.4 to 7.257μM for Tumacona B (2), 250.93 to 3.92μM for compound 3 and 313.45 to 2.82μM for compound 5, and were solubilized in pure dimethyl sulfoxide. The plates were incubated for 48 hours at 37°C. Afterwards, the MTT reagent was added and the absorbance of each well read at 595nm. The toxicity of Artesunate (1) in combination with Tumacona B (**2**) was evaluated using the CC_{10} of Artesunate (1) and different concentrations of Tumacona B (2) (464.4 to 7.257 μM). DMEM/F12 medium with 10% fetal bovine serum was employed as control of cell growth. The Tumacona B (2) and 2,4,6-trichlorobenzoyl chloride compounds could not be assessed in combination because chloride decomposed immediately after it was added to the assay culture medium due to easy hydrolysis of acid chloride.

The toxicity of a molecule was considered according to the following ranges: CC_{50} <1.0 μg/mL (highly cytotoxic), 1.0-10.0 μg/mL (moderately cytotoxic), 10.0 -30.0 μg/mL (medium toxicity) and >30 μg/mL (non-toxic)[22].

Regarding the selectivity index for classifying toxic

molecules, which is obtained from the ratio between the 50% toxic concentration (CC_{50}) and the 50% antiplasmodial concentration (IC_{50}), it is considered a medium toxicity when this index is less than 10[22].

Statistical analysis. A non-linear regression analysis was performed to estimate the antiplasmodial activity. These results correspond to means plus or minus standard deviations, which were obtained from three independent experiments. Statistical analyses were obtained in the Prism 5.0 (GraphPad) program.

A non-linear regression analysis was performed to estimate the cytotoxicity. The results correspond to means plus or minus standard deviations, obtained from two independent experiments. Statistical analyses were obtained in the Prism 5.0 (GraphPad) program.

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