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Article

Hydrazone Derivatives Enhance Antileishmanial Activity of Thiochroman-4-ones

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Abstract: Cutaneous Leishmaniasis (CL) is a neglected tropical disease, which causes severe skin lesions. Due to the lack of effective vaccines, treatment can be complex and prolonged, high toxicity, side effects and high cost, there is an urgent need to develop alternatives for the treatment for CL that may have different mechanisms of action. In our effort to search for new promising hits against *Leishmania* parasites we prepared 18 acyl hydrazone derivatives of thiochroman-4-ones. Compounds were evaluated for their *in vitro* antileishmanial activity against intracellular amastigotes form of *Leishmania panamensis* and cytotoxic activity against human monocytes (U-937 ATCC CRL-1593.2); our results show that derivatization with acyl hydrazones significantly enhance the antileishmanial activity, among the compounds tested semicarbazone (**19**) and thiosemicarbazone (**20**) derivatives of thioflavanone display the highest antileishmanial activities with EC_{50} values of 5.4 and 5.1 μM both with low cytotoxicities, 100.2 a 50.1 μM resulting in high selectivity index (SI).

Keywords: *Leishmania*; thiochroman-4-ones; acyl hydrazone; cytotoxicity

1. Introduction

Leihsmaniasis is a parasitic disease caused by some species of protozoans of the genus *Leishmania* [1]. The parasites has complex life cycle including stages in the mammal host and the vector, in the mammal host parasites exist in two forms: as intracellular amastigotes, the replicative form, and as promastigotes, non-replicative forms, the later occurs also in the vector [1–3].

Cutaneous leishmaniasis causes severe skin lesions, mainly in face, arms and feet. Because of the high occurrence and the lack of therapeutic alternatives the World Health Organization, WHO, considered leishmaniasis a neglected tropical disease and encourages countries to search for new antileishmanial drugs with novel mechanism of action [1,4].

In our search for new chemotherapeutic alternatives to fight leishmaniasis we have explored thiochroman compounds, which could be considered a potential privileged scaffold [5,6] because of its great similarity with the chroman compounds which display a broad range of bioactivities [7].

In addition, numerous hydrazones have been found to show interesting biological activities [8,9]; we focused our attention to those bioactivities that deals with parasitic diseases as malaria, leishmaniasis and trypanosomiasis [3,10–17]; the inhibition of proteases [15,18–20] represent the most common mechanism for the hydrazones. Avery *et al.* [15,16] in the search for new drugs against novel targets in *Leishmania* explored the cysteine proteases inhibitors, 241000 compounds were screened and found a total of 24 interesting compounds which showed inhibition of one or more cysteine proteases or antileishmanial activity, 16 out of the 24 interesting compounds possess hydrazone or imine moieties. Semicarbazones and thiosemicarbazones have also been shown to be potent inhibitors of cysteine proteases, specifically of cathepsin L [19–23], the thiosemicarbazone

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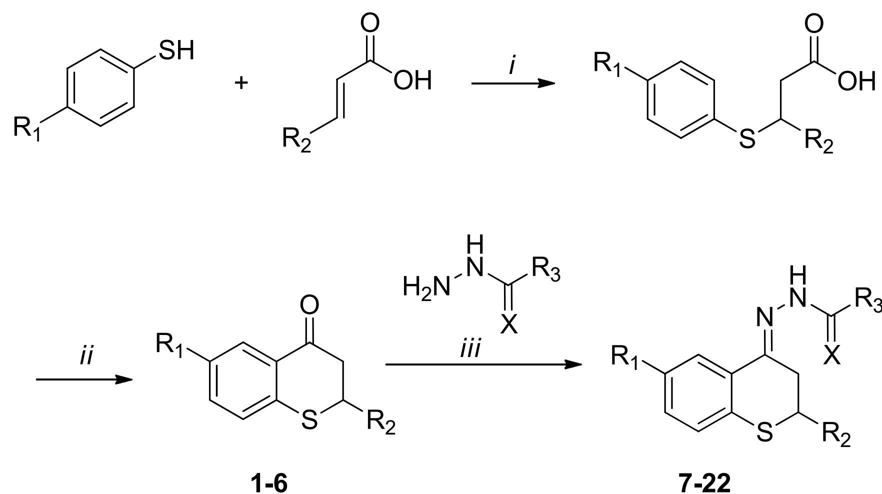
47 derivatives of thiochroman-4ones also showed inhibition of Cathepsin L [20]. Although these
48 compounds inhibited the cysteine proteases there are no reports on their antileishmanial or cytotoxic
49 activities. After considering the many interesting biological activities displayed by compounds
50 bearing the hydrazone moiety and the ability of some thiosemicarbazone derivatives of
51 thiochroman-4ones to inhibit cysteine proteases of protozoan parasites we synthesized a series of
52 analogues in order to screen their antileishmanial and cytotoxic activities.

53 **2. Results**

54 *2.1. Synthesis.*

55 Thiochroman-4-ones were prepared by addition of thiophenol or 4-fluorothiophenol to
56 α,β-unsaturated carboxylic acids (acrylic acid, crotonic acid or cinnamic acid). The resulting
57 carboxylic acids undergo a ring closing reaction upon treatment with sulfuric acid to give the
58 thiochroman-4-ones; in the case of thioflavanone ring closing reaction was carried out with oxalyl
59 chloride followed by tin chloride. Resultant ketones were condensed with acyl hydrazides (benzoic
60 hydrazide, isonicotin hydrazide, semicarbazide or thiosemicarbazide) and afforded the desired acyl
61 hydrazones in moderate to good yields.
62
63



64 (*i*) I_2 or TBAF; (*ii*) H_2SO_4 or CH_3SO_3H or $(COCl)_2$, $SnCl_4$; (*iii*) CH_3COOH.
65

66 *2.2. Antileishmanial and Cytotoxic activities.*
67

68 All synthesized compounds were screened for their *in-vitro* antileishmanial and cytotoxic activities
69 in comparison with amphotericin B as a control with EC_{50} and LC_{50} values of 0.32 μM and 39.6 μM
70 respectively (Table 1).
71 Selectivity was calculated by the ratio of LC_{50}/ EC_{50} and defined as selectivity index, SI.
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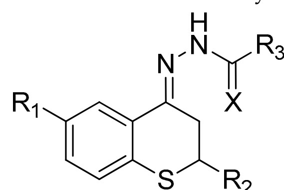
Table 1. In vitro antileishmanial and cytotoxic activities.



Compound	R_1	R_2	R_3	X	EC_{50} (μM)[1]	LC_{50} (μM)[1]	SI[2]
1	H	H	-	-	343.8 ± 75.6	>1000	-
2	F	H	-	-	> 109.9	>706.0 ± 34.6	< 6.5
3	H	CH_3	-	-	444.6 ± 7.3	604.2 ± 86.4	1.4
4	F	CH_3	-	-	422.0 ± 9.2	578.9 ± 59.1	1.4
5	H	C_6H_5	-	-	44.1 ± 0.9	>41.61	> 0.9
6	H	H		O	63.7 ± 9.2	248.3 ± 49.6	3.9
7	H	H	N	O	56.8	> 705.8	> 12.4
8	H	H	—NH_2	O	91.5 ± 33.4	637.2 ± 37.9	7.0
9	H	H	—NH_2	S	55.7 ± 22.1	>842.6	> 15.1
10	F	H		O	37.3 ± 3.3	>665.9	> 17.6
11	F	H	N	O	39.9 ± 5.3	>663.7	> 16.6
12	F	H	—NH_2	O	95.5 ± 6.9	>634.2	> 6.6
13	H	CH_3		O	38.1 ± 16.2	150.1 ± 24.3	3.9
14	H	CH_3	N	O	56.6 ± 0.7	>672.5	> 11.9
15	H	CH_3	—NH_2	O	91.8 ± 13.5	102.1 ± 15.1	1.1
16	F	CH_3		O	43.9 ± 4.5	>636.2	14.5
17	F	CH_3	N	O	98.9 ± 19.3	203.3 ± 25.4	2.1
18	F	CH_3	—NH_2	O	160.7 ± 2.4	31.0 ± 7.0	0.2
19	H	C_6H_5	—NH_2	O	5.4 ± 1.0	100.2 ± 19.8	18.6
20	H	C_6H_5	—NH_2	S	5.1 ± 1.3	50.1 ± 4.1	9.8
21	H	C_6H_5		O	28.5 ± 2.8	528.6 ± 7.0	19.6
22	H	C_6H_5	N	O	16.4 ± 3.6	>556.4	> 33.9
thiosemicarbazide					nt	>1000	-
Amphotericin B	-	-	-	-	0.32 ± 1.04	39.6 ± 8.7	132.0

[1] Results reported as the mean value ± standard deviation of the half-maximum concentration in μM.
[2] Selectivity Index (SI) = LC_{50}/EC_{50}.
[3] Abbreviation: nt, not tested because the inhibition percent at the highest evaluated concentration 50 μM was 0.0.

3. Discussion

Complete data concerning antileishmanial and cytotoxic activities are reported in Table 1. Almost all the thiochroman-4-one compounds (**1-6**) revealed weak antileishmanial activity since none of EC_{50} values was lower than 10 µM. Thioflavanone **6** displayed moderate antileishmanial activity but also high cytotoxic activity resulting in a low selectivity index. In general the antileishmanial activities of hydrazones were higher than those of their ketone precursors; cytotoxic activity for the hydrazones has remained low resulting in a selectivity index higher than those of the thiochroman-4-ones. Incorporation of an aromatic ring at C2 (thioflavanone **6**) resulted in improved antileishmanial activity (compare **19**, **20**, **21**, **21** with **6**, **7**, **8**, **9**), semicarbazone **20** and thiosemicarbazone **21** analogues displayed the highest antileishmanial activity (EC_{50} values of 5.38 and 5.11 µM, respectively). 4-amino benzoic hydrazones (**12**, **15** and **18**) are more basic than other hydrazones; the increased basicity due to the amino group could be related with the lower antileishmanial activity of these compounds.

Although hydrazones have good stability towards hydrolysis [24] it is important to determine whether the increased of antileishmanial activity is due to the hydrazone itself or perhaps, the *in situ* hydrolysis products are responsible enhanced activity; Garces-Eisele and Scior [25] studied the *in vitro* antituberculotic activity of over 200 hydrazone derivatives of isonicotinic acid hydrazide (isoniazid, INH) and found that the derivatives do not improve the activity of isonicotinic acid hydrazide, hydrolysis delivers extra or/and intracelular portions of INH which itself is the active compound nor the hydrazones. In this work the antileishmanial activity of thiosemicarbazide was evaluated and compared with its hydrazones **9** y **20** in both cases the derivative showed better activity than its precursors, in fact thiosemicarbazide itself is inactive against *Leishmania parasites*. In spite of the available information, however, it cannot be ruled out that acyl hydrazone derivatives can act as a prodrug [26,27] which facilitates passage through the membrane of the macrophage, parasitophorous vacuole or to enter the parasite carrying the hydrazide to site of action.

Since several hydrazones have shown to inhibit cysteine proteases the mechanism of action may be related to this therapeutic target, moreover, Song *et al* [20] showed that semicarbazones of some thiochroman-4-ones inhibit Cathepsin L, which is structurally related with the cysteine protease, cruzain present in other trypanosomatyd parasites responsible for the Chagas disease.

4. Materials and Methods

4.1. Chemistry

4.1.1. General

All commercially available reagents and solvents were obtained from commercial suppliers and used without further purification. Commercial thiocroman-4-one was purchased from Sigma Chemical Co. (St. Louis, MO). The reaction progress was monitored with thin layer chromatography on silica gel TLC aluminum sheets ($60F_{254}$, Merck, Darmstadt, Germany). The melting points were determined using a Mel-Temp apparatus (Electrothermal, Staffordshire, UK) and are uncorrected. FTIR spectra were obtained on a Bruker Alpha FTIR spectrometer (Bruker Optic GmbH, Ettlingen, Germany. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using Bruker DRX 300 spectrometer (Bruker Bio-Spin GmbH, Rheinstetten, Germany) operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts were reported relative to internal tetramethylsilane (δ 0.00 ppm) for ^{1}H, and $CDCl_3$ (δ 77.0 ppm) for ^{13}C. HRMS was obtained using Q-ToF quadrupole/orthogonal spectrometry (Waters, Milford, MA, USA) in either negative (reported as $[M - H]^-$) or positive mode (reported as $[M + H]^+$) and Bruker Impact II UHR-Q-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) in positive mode.

134 4.1.2. Synthesis of Thiochroman-4-ones and Thioflavanone.
135
136 *6-fluorothiochroman-4-one* **(2)** To a mixture of acrylic acid (700 µL, 720 mg, 10 mmol) and
137 4-fluorothiophenol (1985 mg, 15 mmol) was added I_2 (20% mol, 760 mg, 3 mmol) and the mixture
138 was stirred at 50 °C for 24 h. After completion of reaction (monitored by TLC), a cold saturated
139 sodium thiosulfate solution (30 mL) was added and extracted with dichloromethane (2 x 25 mL); the
140 combined organic layers were mixed with a saturated solution of sodium bicarbonate and extracted
141 to remove the unreacted starting material. The water layer was acidified with hydrochloric acid
142 (10% aq) and extracted with dichloromethane (3 × 50 mL). The combined organic layers were dried
143 over Na_2SO_4, evaporation of the solvent under reduced pressure afforded 1150 mg (64%) of the
144 desired addition product. The product was cooled down to 0 °C in an ice bath and 3 mL of
145 concentrated sulfuric acid was added and the reaction mixture was allowed to warm to room
146 temperature for 2 h with magnetic stirring. The reaction was quenched with ice and the mixture was
147 extracted with dichloromethane (3 × 50 mL). The combined organic layers were washed once with
148 water, followed by saturated $NaHCO_3$ solution. The combined organic layers were washed with
149 brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by
150 column chromatography over silica gel using hexane:EtOAc (9:1) as eluent to give 570 mg (90%) of
151 pure **2** as a yellow solid. mp: 86–88 °C. ^1H NMR (300 MHz, $CDCl_3$) δ 8.07 (dd, J=8.0, 1.4 Hz, 1H),
152 7.16—7.11 (m, 2H), 3.25—3.23 (m, 2H), 2.99—2.96 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.5, 160.9
153 (d, $J_{C\text{-}F}$ = 245 Hz), 137.8, 132.7, 129.8 (d, $J_{C\text{-}F}$ = 7 Hz), 121.70 (d, $J_{C\text{-}F}$ = 23.0 Hz), 115.6 (d, $J_{C\text{-}F}$ = 22.6 Hz),
154 39.7, 27.1. IR ν: 1659, 1595, 1565. HRMS (ESI) calculated for C_9H_6FOS $[M - H]^-$ 181.0123, found
155 181.0165.
156
157 *2-Methylthiochroman-4-one* **(3).** To a mixture of crotonic acid (860 mg, 10 mmol) and thiophenol (1.650
158 g, 15 mmol) was added I_2 (20 mol %, 255 mg, 1 mmol) and the mixture was stirred at room
159 temperature for 12 h. After completion of the reaction (monitored by TLC), a cold saturated sodium
160 thiosulfate solution (20 mL) was added and extracted with dichloromethane (2 x 50 mL); then,
161 combined organic layers were mixed with a saturated solution of sodium bicarbonate and extracted
162 to remove the unreacted starting material. The water layer was acidified with hydrochloric acid
163 (10% aq) and extracted with dichloromethane (3 × 40 mL). The combined organic layers were dried
164 over Na_2SO_4, evaporation of the solvent under reduced pressure afforded 1.962 g (86%) of the
165 desired addition product. After, 200 mg (1.0 mmol) of this product were cooled down to 0 °C in an
166 ice bath and 3.0 mL of concentrated sulfuric acid was added; the reaction mixture was stirred for 30
167 min, and, after that, the ice bath was removed allowing the reaction mixture to warm to room
168 temperature for another 2 hours under continuous stirring. The reaction was quenched with ice and
169 the mixture was extracted with dichloromethane (3 × 25 mL). The combined organic layers were
170 washed once with water, followed by addition of a saturated $NaHCO_3$ solution. The combined
171 organic layers were dried over Na_2SO_4, and concentrated under reduced pressure. The residue was
172 purified by column chromatography over silica gel using hexane:EtOAc (9:1) as eluent, to give 137
173 mg (75%) of pure **3** as a yellowish oil. ^1H NMR (300 MHz, $CDCl_3$) δ 8.08 (d, J = 8.0 Hz, 1H), 7.38 (t, J =
174 7.5 Hz, 1H), 7.25 (d, J = 7.8 Hz, 1H), 7.16 (t, J = 7.6 Hz, 1H), 3.74–3.53 (m, 1H), 2.98 (dd, J = 17.6, 8.8 Hz,
175 1H), 2.84–2.66 (m, 1H), 1.43 (d, J = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.9, 141.9, 133.7, 130.1,
176 129.1, 127.65, 125.1, 48.0, 36.5, 20.6. IR ν**:** 2964, 1679, 1587. HRMS (ESI) calculated for $C_{10}H_{11}OS$ [M +
177 $H]^+$ 179.0525, found 179.0536.
178
179 *6-fluoro-2-methylthiochroman-4-one* **(4).** To a mixture of crotonic acid (172 mg, 2 mmol) and
180 4-fluorothiophenol (385 mg, 3.0 mmol) was added I_2 (20% mol, 52 mg, 0.2 mmol) and the mixture
181 was stirred at room temperature for 12 h. After completion of reaction (monitored by TLC), a cold
182 saturated sodium thiosulfate solution (20 mL) was added and extracted with dichloromethane (2 x
183 25 mL); the combined organic layers were mixed with a saturated solution of sodium bicarbonate
184 and extracted to remove the unreacted starting material. The water layer was acidified with
185 hydrochloric acid (10% aq) and extracted with dichloromethane (3 × 25 mL). The combined organic

186 layers were dried over Na_2SO_4, evaporation of the solvent under reduced pressure afforded 200 mg
187 (94%) of the addition product. Thus, compounds were cooled down to 0 °C in an ice bath and 2.0 mL
188 of concentrated sulfuric acid was added and the reaction mixture was allowed to warm to room
189 temperature for 2 h with continuous stirring. The reaction was quenched with ice and the mixture
190 was extracted with dichloromethane (3×25 mL). The combined organic layers were washed once
191 with water, followed by saturated $NaHCO_3$ solution. The combined organic layers were washed
192 with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified
193 by column chromatography over silica gel using hexane:EtOAc (9:1) as eluent to give 128 mg (64%)
194 of pure **4** as a yellowish oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84–7.72 (m, 1H), 7.39–6.97 (m, 2H),
195 3.78–3.47 (m, 1H), 3.07–2.95 (m, 1H), 2.80–2.68 (m, 1H), 1.43 (d, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz,
196 $CDCl_3$) δ 194.2, 160.7 (d, J_{C-F} = 246 Hz), 137.6, 132.3, 129.7 (d, J_{C-F} = 7.0 Hz), 121.8 (d, J_{C-F} = 23.1 Hz),
197 115.4 (d, J_{C-F} = 22.8 Hz), 47.9, 37.0, 20.7. IR ν: 2967, 1684, 1602. HRMS (ESI) calculated for $C_{10}H_{10}FOS$
198 $[M + H]^+$ 197.0431, found 197.0443.
199
200 *2-phenylthiochroman-4-one (thioflavanone)* **(5).**
201 Cinnamic acid (297 mg, 2 mmol) and thiophenol (330 mg, 3 mmol) were mixed with 75% aqueous
202 solution of TBAF (140 μL sln, 0.4 mmol) and the mixture was stirred for 4 h at 60 °C. A saturated
203 solution of sodium bicarbonate was added and extracted with dichloromethane (3 x 25 mL) to
204 remove the unreacted starting material. The water layer was acidified with hydrochloric acid (10%
205 aq) and extracted with dichloromethane (3 × 30 mL). The combined organic layers were dried over
206 Na_2SO_4; evaporation of the solvent under reduced pressure gave the crude addition product which
207 was dissolved in anhydrous dichloromethane and placed in an oven-dried round bottomed flask
208 under N_2 in an ice cooling bath. Consequently, oxalyl chloride (365 μL, 3.0 mmol) was added
209 dropwise followed by two drops of DMF and the reaction mixture is left to warm to room
210 temperature. After stirring for 2.5 h, the solution was cooled to –10 °C, and a solution of 1M $SnCl_4$
211 (3.0 mL, 3.0 mmol) in CH_2Cl_2 was added dropwise. The resulting mixture was stirred at 0 °C for 10
212 min and then allowed to warm to room temperature. After stirring at room temperature for 12 h,
213 water (25 mL) was added and extracted with dichloromethane (3 x 25 mL). The combined organic
214 layers dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was
215 purified by column chromatography over silica gel using hexane:EtOAc (2:1) as eluent to give the
216 desired thioflavanone **5**. Yield 215 mg (45%) white solid. mp. 155–157 °C. ^{1}H NMR (300 MHz, $CDCl_3$)
217 δ 8.20 (dd, J = 7.9, 1.2 Hz, 1H), 7.52–7.36 (m, 6H), 7.35–7.30 (m, 1H), 7.30–7.21 (m, 1H), 4.77 (dd, J =
218 12.7, 3.3 Hz, 1H), 3.56-3.07 (m, 2H). IR ν: 1665, 1586, 1556, 1452, 1433. HRMS (ESI) calculated for
219 $C_{15}H_{13}OS$ $[M + H]^+$ 241.0687, found 241.0694.
220
221 4.1.3. General procedure for the preparation of acyl hydrazone derivatives.
222
223 thiochroman-4-ones (0.5 mmol) were dissolved in anhydrous methanol (25 mL). The mixture was
224 heated at reflux and then hydrazide (1.0 mmol, 2 equiv) and glacial acetic acid 60 μL were added.
225 After 12 h at reflux, the resulting precipitate was collected through filtration and washed with
226 methanol. After drying under vacuum, the residue was passed through a small pad of silica gel with
227 ethyl acetate, after evaporation of the solvent acyl hydrazones were obtained as white solids.
228
229 *(E)-N'-(thiochroman-4-ylidene)benzohydrazide* **(6).** Yield 80%, mp: 160-161°C. ^{1}H NMR (300 MHz,
230 DMSO) δ (300MHz, DMSO) 10.79 (s , 1H), 8.20 (br s, 1H), 7.87 (br s, 2H), 7.54 (d,J = 7.8Hz, 1H), 7.52
231 (d, J = 7.8Hz, 2H), 7.24 (m, 2H), 7.16 (br s, 1H), 3.07 (br s 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.5,
232 153.1, 136.8, 134.5, 132.0, 129.9, 128.8, 128.40, 128.39, 121.8, 126.0, 125.7, 25.7, 28.4. HRMS (ESI)
233 calculated for $C_{16}H_{15}N_2OS$ $[M + H]^+$ 283.0900 found 283.0906. IR ν: 2997, 1645, 1597, 1532, 1275, 1136.
234
235 *(E)-N'-(thiochroman-4-ylidene)isonicotinohydrazide* **(7).** Yield 60%, mp: 175-176°C. ^{1}H NMR (300 MHz,
236 $CDCl_3$) δ 9.56 (s, H), 8.81 (d, J = 5.9 Hz, 2H), 7.70 (d, J = 5.9 Hz, 2H), 7.35 – 7.03 (m, 4H), 3.16 – 3.02 (m,
237 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.1, 149.8, 146.8, 140.8, 136.6, 131.0, 129.6, 128.4, 127.6, 125.9,

238 123.7, 27.6, 25.8. HRMS (ESI) calculated for $C_{15}H_{14}N_3OS$ [M + H]$^+$ 284.0852 found 284.0864. IR ν:
239 2924, 1637, 1597, 1530, 761.
240
241 *(E)-2-(thiochroman-4-ylidene)hydrazinecarboxamide* **(8)** Yield 79%, mp:214-216°C. ^{1}H NMR (300 MHz,
242 DMSO) δ 9.34 (s, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 7.24 – 7.03 (m, 3H), 6.56 (s, 2H), 3.15 – 2.90 (m, 2H), 2.91
243 – 2.72 (m, 2H). ^{13}C NMR (75 MHz, DMSO) δ 157.7, 142.4, 135.5, 132.2, 128.9, 128.3, 127.1, 125.8, 28.2,
244 25.7. HRMS (ESI) calculated for $C_{10}H_{12}N_3OS$ [M + H]$^+$ 222.0696 found 222.0703. IR ν: 3456, 3186,
245 1697, 1584, 1430, 1248, 1201.
246
247 *(E)-2-(thiochroman-4-ylidene)hydrazinecarbothioamide* **(9).** Yield 55%, mp: 228-230°C. ^{1}H NMR (300
248 MHz, DMSO) δ 10.21 (s, 1H), 8.32 (d, *J* = 8.0 Hz, 2H), 8.00 (s, 1H), 7.27 – 7.19 (m, 2H), 7.12 (ddd, *J* =
249 8.3, 6.1, 2.5 Hz, 1H), 3.01 (s, 4H).^{13}C NMR (75 MHz, DMSO) δ 179.3, 145.8, 136.6, 131.4, 129.7, 128.3,
250 127.8, 125.7, 28.4, 25.5. HRMS (ESI) calculated for $C_{10}H_{12}N_3S_2$ [M + H]$^+$ 238.0467 found 238.0482. IR ν:
251 3410, 3126, 1596, 1468, 1285.
252
253 *(E)-N'-(6-fluorothiochroman-4-ylidene)benzohydrazide* **(10).** Yield 76%, mp:168°C. ^{1}H NMR (300 MHz,
254 DMSO) δ 10.91 (s, 1H), 8.02 – 7.77 (m, 3H), 7.58 (dd, *J* = 15.3, 8.6 Hz, 1H), 7.52 (t, *J* = 7.3 Hz, 2H), 7.33
255 (dd, *J* = 8.9, 5.2 Hz, 1H), 7.18 (t, *J* = 8.0 Hz, 1H), 3.10 (br s, *J* = 24.2 Hz, 4H). ^{13}C NMR (75 MHz, DMSO)
256 δ 164.2, 159.9 (d, *J* = 241.4 Hz), 151.0, 133.9, 133.2 (d, *J* = 7.3 Hz), 131.9, 131.7, 130.0 (d, *J* = 7.7 Hz), 128.4
257 (s), 128.1, 117.1 (d, *J* = 18.7 Hz), 112.5 (d, *J* = 24.1 Hz), 27.6, 25.21. HRMS (ESI) calculated for
258 $C_{16}H_{14}FN_2OS$ [M + H]$^+$ 301.0805 found 301.0820. IR ν: 1666, 1643, 1537, 1282, 1138.
259
260 *(E)-N'-(6-fluorothiochroman-4-ylidene)isonicotinohydrazide* **(11).** Yield 53%, mp: 182-183°C. ^{1}H NMR
261 (300 MHz, DMSO) δ 11.14 (s, 1H), 8.77 (br s, 2H), 7.89 (d, *J* = 10.5 Hz, 1H), 7.81 (br s, 2H), 7.33 (dd, *J* =
262 12.1, 6.3 Hz, 1H), 7.21 (t, *J* = 9.5 Hz, 1H), 3.10 (br s, 4H). ^{13}C NMR (75 MHz, DMSO) δ 162.8, 159.9 (d, *J*
263 = 242.6 Hz), 152.4, 150.2, 140.9, 132.9, 132.3, 130.1 (d, *J* = 7.6 Hz), 122.0, 117.5 (d, *J* = 22.8 Hz), 112.7 (d, *J*
264 = 23.7 Hz), 27.8, 25.2. HRMS (ESI) calculated for $C_{15}H_{13}FN_3OS$ [M + H]$^+$ 302.0758 found 302.0773. IR ν:
265 3461, 3100, 2920, 1666, 1539, 1281.
266
267 *(E)-4-amino-N'-(6-fluorothiochroman-4-ylidene)benzohydrazide* **(12).** Yield 82%, mp: 223-224°C. ^{1}H NMR
268 (300 MHz, DMSO) δ 10.40 (s, 1H), 7.84 (d, *J* = 10.8 Hz, 1H), 7.64 (d, *J* = 8.5 Hz, 2H), 7.30 (dd, *J* = 8.7, 5.6
269 Hz, 1H), 7.15 (td, *J* = 8.4, 2.9 Hz, 1H), 6.60 (d, *J* = 8.6 Hz, 2H), 5.75 (d, *J* = 17.4 Hz, 2H), 3.07 (s, 4H). ^{13}C
270 NMR (75 MHz, DMSO) δ 164.7, 160.1 (d, *J* = 241.1 Hz), 152.5, 148.5, 133.6 (d, *J* = 7.2 Hz), 131.7 (d, *J* =
271 2.5 Hz), 130.1 (d, *J* = 7.5 Hz), 130.0, 119.7, 116.8 (d, *J* = 22.5 Hz), 112.7, 112.5 (d, *J* = 22.3 Hz), 27.5, 25.4.
272 HRMS (ESI) calculated for $C_{16}H_{15}FN_3OS$ [M + H]$^+$ 316.0914 found 316.0933. IR ν: 3480, 3332, 1647,
273 1634, 1614, 1594, 1533, 1507, 1267, 1148.
274
275 *(E)-N'-(2-methylthiochroman-4-ylidene)benzohydrazide* **(13).** Yield 79%, mp: 205-206°C. ^{1}H NMR (300
276 MHz, DMSO) δ 10.86 (s, *J* = 46.9 Hz, 1H), 8.18 (br s, 1H), 7.88 (br s, 2H), 7.64 – 7.45 (m, *J* = 14.3, 6.8 Hz,
277 3H), 7.34 – 7.14 (m, 3H), 3.58 – 3.32 (m, 2H), 2.70 (dd, *J* = 17.1, 10.8 Hz, 1H), 1.36 (d, *J* = 6.7 Hz, 3H). ^{13}C
278 NMR (75 MHz, DMSO) δ 164.0, 152.7, 135.8, 134.0, 131.6, 130.8, 129.8, 128.3, 128.0, 127.8, 126.7, 125.2,
279 35.6, 34.8, 20.3. HRMS (ESI) calculated for $C_{17}H_{17}N_2OS$ [M + H]$^+$ 297.1056 found 297.1067. IR ν:
280 3217,1665, 1527, 1276, 1132.
281
282 *(E)-N'-(2-methylthiochroman-4-ylidene)isonicotinohydrazide* **(14).** Yield 65%, mp: 194-195°C. ^{1}H NMR
283 (300 MHz, $CDCl_3$) δ 9.57 (s, 1H), 8.90-8.75 (m,2H), 7.85-7.71 (m, 2H), 7.05 – 7.40 (m, 4H), 3.57 – 3.36
284 (m, 1H), 3.21 (dd, *J* = 16.4, 3.0 Hz, 1H), 2.66 (dd, *J* = 16.4, 11.0 Hz, 1H), 1.50 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR
285 (75 MHz, DMSO) δ 162.6, 154.1, 150.2, 141.0, 136.1, 130.5, 130.1, 127.8, 126.8, 125.2, 122.0, 35.7, 34.7,
286 20.3. HRMS (ESI) calculated for $C_{16}H_{16}N_3OS$ [M + H]$^+$ 298.1009 found 298.1024. IR ν: 3174, 2962,
287 1650, 1600, 1529, 1281.
288

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289 *(E)-4-amino-N′-(2-methylthiochroman-4-ylidene)benzohydrazide* **(15).** Yield 60%, mp: 168-169°C. ^{1}H
290 NMR (300 MHz, DMSO) δ 10.35 (s, 1H), 8.12 (d, *J* = 7.8 Hz, 1H), 7.65 (d, *J* = 8.5 Hz, 2H), 7.38 – 6.97 (m,
291 3H), 6.60 (d, *J* = 8.6 Hz, 2H), 5.74 (d, *J* = 9.2 Hz, 2H), 3.65 – 3.11 (m, 2H), 2.67 (dd, *J* = 16.8, 10.4 Hz, 1H),
292 1.35 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, DMSO) δ 164.3, 152.4, 150.5, 135.5, 131.2, 130.1, 129.6,
293 127.9, 126.6, 125.3, 119.9, 112.7, 35.4, 34.9, 20.5. HRMS (ESI) calculated for $C_{17}H_{18}N_3OS$ $[M + H]^+$
294 312.1165 found 312.1187. IR ν: 3473, 3360, 2921, 1634, 1613, 1612, 1372.
295
296 *(E)-N′-(6-fluoro-2-methylthiochroman-4-ylidene)benzohydrazide* **(16).** Yield 52%, mp: 206-207°C. ^{1}H NMR
297 (300 MHz, DMSO) δ 10.92 (s, 1H), 8.03 – 7.75 (m, 3H), 7.65 – 7.46 (m, 3H), 7.30 (dd, *J* = 8.8, 5.4 Hz, 1H),
298 7.25 – 7.15 (m, 1H), 3.50 – 3.27 (m, 2H), 2.69 (dd, *J* = 17.1, 10.9 Hz, 1H), 1.35 (d, *J* = 6.6 Hz, 3H). . ^{13}C
299 NMR (75 MHz, DMSO) δ 164.2, 159.9 (d, *J* = 241.4 Hz), 151.1, 133.9, 132.7 (d, *J* = 7.3 Hz), 131.7, 131.3,
300 129.8 (d, *J* = 7.7 Hz), 128.3, 128.1, 117.3 (d, *J* = 23.0 Hz), 112.3 (d, *J* = 24.3 Hz), 35.3, 34.8, 20.2. HRMS
301 (ESI) calculated for $C_{17}H_{16}FN_2OS$ $[M + H]^+$ 315.0962 found 315.0977. IR ν: 3235, 1667, 1526, 1280,
302 1132.
303
304 *(E)-N′-(6-fluoro-2-methylthiochroman-4-ylidene)isonicotinohydrazide* **(17).** Yield 85%, mp: 210-212°C. ^{1}H
305 NMR (300 MHz, DMSO) δ 11.16 (s, 1H), 8.77 (br s, 2H), 7.89 (d, *J* = 10.1 Hz, 1H), 7.80 (br s, 2H), 7.29
306 (dd, *J* = 17.4, 11.5 Hz, 1H), 7.21 (t, *J* = 9.1 Hz, 1H), 3.51 – 3.22 (m, *J* = 28.2 Hz, 2H), 2.69 (dd, *J* = 17.0, 11.0
307 Hz, 1H), 1.35 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (75 MHz, DMSO) δ 162.9, 159.9 (d, *J* = 241.2 Hz), 152.5,
308 150.2, 140.9, 132.4 (d, *J* = 7.3 Hz), 131.7, 129.9 (d, *J* = 7.6 Hz), 122.1, 117.7 (d, *J* = 22.8 Hz), 112.5 (d, *J* =
309 23.3 Hz), 35.5, 34.9, 20.2. HRMS (ESI) calculated for $C_{16}H_{15}FN_3OS$ $[M + H]^+$ 316.0914 found 316.0923.
310 IR ν: 3183, 1646, 1376, 1269.
311
312 *(E)-4-amino-N′-(6-fluoro-2-methylthiochroman-4-ylidene)benzohydrazide* **(18).** Yield 60%, mp: 187-189°C.
313 ^{1}H NMR (300 MHz, DMSO) δ 10.42 (s, 1H), 7.85 (dd, *J* = 11.0, 3.2 Hz, 1H), 7.65 (s, 2H), 7.27 (dt, *J* =
314 10.1, 5.0 Hz, 1H), 7.16 (tt, *J* = 8.6, 4.3 Hz, 1H), 6.60 (d, *J* = 8.6 Hz, 2H), 5.79 (s, *J* = 19.0 Hz, 1H), 3.58 –
315 3.14 (m, 2H), 2.67 (dd, *J* = 17.1, 10.4 Hz, 1H), 1.35 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, DMSO)* δ
316 164.9, 160.1 (d, *J* = 241.3 Hz), 152.5, 148.7, 133.2 (d, *J* = 7.5 Hz), 131.0 (d, *J* = 2.4 Hz), 130.2, 129.9 (d, *J* =
317 7.5 Hz), 119.7 (s), 117.1 (d, *J* = 22.4 Hz), 112.7, 112.3 (d, *J* = 23.8 Hz), 35.1, 35.0, 20.3. HRMS (ESI)
318 calculated for $C_{17}H_{17}FN_3OS$ $[M + H]^+$ 330.1071 found 330.1093. IR ν: 3381, 3065, 2955, 1623, 1604,
319 1566, 1372.
320
321 *(E)-2-(2-phenylthiochroman-4-ylidene)hydrazinecarboxamide* **(19).** Yield 47%, mp: 194-195°C. ^{1}H NMR
322 (300 MHz, DMSO) δ 9.46 (s, 1H, NH), 8.26 (d, *J* = 7.7 Hz, 1H, H5), 7.49 (d, *J* = 7.3 Hz, 2H), 7.35 (dt, *J* =
323 19.1, 7.0 Hz, 3H), 7.28 – 7.09 (m, 3H), 6.56 (s, 2H), 4.51 (dd, *J* = 11.9, 3.0 Hz, 1H, H2), 3.56 (cae en
324 solvente1H, H3), 3.03 (dd, *J* = 17.4, 12.0 Hz, 1H, H3). ^{13}C NMR (75 MHz, DMSO) δ 157.6, 142.7, 139.9,
325 135.3, 131.9, 129.2, 129.1, 128.4, 128.1, 128.0, 127.0, 126.1, 43.9, 34.8. HRMS (ESI) calculated for
326 $C_{16}H_{16}N_3OS$ $[M + H]^+$ 298.1009 found 298.1024. IR ν: 3465, 3136, 1696, 1664, 1412, 746.
327
328 *(E)-2-(2-phenylthiochroman-4-ylidene)hydrazinecarbothioamide* **(20).** Yield 38%, mp: 198-200°C. ^{1}H NMR
329 (300 MHz, DMSO) δ 10.42 (s, 1H,), 8.39 (d, *J* = 7.8 Hz, 1H), 8.29 (s, 1H), 8.04 (s, 1H), 7.74 – 7.21 (m,
330 7H), 7.17 (t, 1H) 4.52 (d, *J* = 9.8 Hz, 1H), 3.54 – 3.51 (m, 1H), 3.16 (dd, *J* = 17.4, 12.4 Hz, 1H). ^{13}C NMR
331 (75 MHz, DMSO) δ 179.4, 146.0, 139.7, 136.4, 131.1, 130.0, 129.1, 128.5, 128.1, 128.0, 127.7, 126.0, 43.8,
332 35.1. HRMS (ESI) calculated for $C_{16}H_{16}N_3S_2$ $[M + H]^+$ 314.0780 found 314.0779. IR ν: 3423, 3240, 2925,
333 1593, 1504, 1461.
334
335 *(E)-N′-(2-phenylthiochroman-4-ylidene)benzohydrazide* **(21).** Yield 45%, mp: 232-233°C. ^{1}H NMR (300
336 MHz, DMSO) δ 10.90 (s, 1H, NH), 7.81 (d, *J* = 7.2 Hz, 2H), 7.64 – 7.15 (br m, 12H), 4.57 (dd, *J* = 12.3, 2.9
337 Hz, 1H), 3.57 (m, 1H), 3.21 (dd, *J* = 17.2, 12.4 Hz, 1H). ^{13}C NMR (75 MHz, DMSO) δ 164.9 (C=O), 152.7
338 (C=N), 139.9, 136.6, 134.2, 132.1, 131.3, 130.3, 129.2, 128.8, 128.6, 128.4, 128.1, 128.0, 127.3, 126.0, 44.1
339 (C3), 35.2 (C2). HRMS (ESI) calculated for $C_{22}H_{19}N_2OS$ $[M + H]^+$ 359.1213 found 359.1232. IR ν: 3059,
340 1633, 1567, 763.

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(E)-N'-(2-phenylthiochroman-4-ylidene)isonicotinohydrazide **(22).** Yield 45%, mp: 240-242°C. ^{1}H NMR (300 MHz, DMSO) δ 11.12 (s, 1H), 8.71 (d, *J* = 4.0 Hz, 2H), 8.23 (d, *J* = 7.7 Hz, 1H), 7.73 (d, *J* = 3.8 Hz, 2H), 7.50-7.10 (m, 8H), 4.58 (dd, *J* = 12.3, 2.7 Hz, 1H), 3.58 (d, *J* = 16.1 Hz, 1H), 3.20 (dd, *J* = 16.8, 12.8 Hz, 1H). ^{13}C NMR (75 MHz, DMSO) δ 163.5, 153.9, 150.5, 141.5, 139.8, 136.9, 131.1, 130.6, 129.2, 128.6, 128.2, 128.1, 127.4, 126.0, 122.4, 44.0, 35.4. HRMS (ESI) calculated for $C_{21}H_{18}N_3OS$ $[M + H]^+$ 360.1165 found 360.1184. IR ν: 3167, 1635, 1534, 756.

4.2. Biological Activity

4.2.1. Cytotoxic Activity

Cytotoxicity of the compounds was evaluated over human monocytes (U-937 ATCC CRL-1593.2) in exponential growing phase and, adjusted at 1 × 105 cells/mL in RPMI-1640 enriched with 10% fetal bovine serum (FBS). One hundred microliters of cell suspension were dispensed in each well of a 96-wells microplate and then, 100 µL of each compound or standard drug (amphotericin B) at four serial dilution concentrations (200, 50, 12.5 and 3.125 µg/mL) were added dissolved in pbs with 0.5% DMSO. Cell exposed to compounds or standard drugs were incubated 72 h at 37 °C and 5% of CO_2. Cytotoxic activity of each compound was determined according to the effect on the cell viability by the MTT microenzymatic method in which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is reduced to a purple product named formazan by mitochondrial enzyme succinate dehydrogenase. Thus, 10 µL/well of MTT solution (5 mg/mL) were added to each well of exposed and unexposed cells, and plates were incubated at 37 °C, 5% CO_2 during 3 h. The reaction was stopped by adding 100 µL/well of isopropanol with 50% and 10% of SDS (sodium dodecyl sulfate). The concentration of formazan was determined spectrophotometrically at 570 nm (Varioskan Flash Multimode Reader, Thermo Scientific, Waltham, MA, USA) and intensity of color (absorbance) was registered as O.D. Cells exposed to control drug (amphotericin B) were used as control for toxicity (positive control) while cell incubated in absence of any compound or drug were used as control for viability (negative control). Non-specific absorbance was corrected by subtracting absorbance (O.D) of the blank. Determinations were done by triplicate in at least two independent experiments [31].

4.2.2. Antileishmanial Activity

Antileishmanial activity of compounds was determined according to the ability of the compound to reduce the infection by *L. panamensis* parasites. For this, the antileishmanial activity was tested on intracellular amastigotes of *L. panamensis* transfected with the green fluorescent protein gene (MHOM/CO/87/UA140-EGFP strain) [24]. Briefly, U-937 human cells at a density of 3 × 105 cells/mL in RPMI 1640 and 0.1 µg/mL of PMA (phorbol-12-myristate-13-acetate) were dispensed on 24-wells microplate and then infected with stationary phase growing *L. panamensis* promastigotes in 15:1 parasites per cell ratio. Plates were incubated at 34 °C and 5% CO_2 for 3 h and then cells were washed twice with phosphate buffer solution (PBS) to eliminate not internalized parasites. Fresh RPMI-1640 was added into each well (1 mL) and plates were incubated again. After 24 h of infection, the RPMI-1640 medium was replaced by fresh culture medium containing each compound at four serial dilutions (50, 12.5, 3.125 and 0.78 µg/mL) and plates were then incubated at 37 °C and 5% CO_2 during 72 h, then, cells were removed from the bottom plate with 100 µL of EDTA/Trypsin (250 mg) solution. The cells were centrifuged at 1100 rpm during 10 min at 4 °C, the supernatant was discarded and cells were washed with 1 mL of cold PBS and centrifuged at 1100 rpm for 10 min at 4 °C. Cells were washed two times employing PBS, as previously, and after the last wash, the supernatant was discarded and cells were suspended in 500 µL of PBS.

Cells were analyzed by flow cytometry employing a flow cytometer (cytomics FC 500MPL, Beckman Coulter. Pasadena, CA, USA) reading at 488 nm (exciting) and 525 nm (emitting) over an

argon laser and counting 10,000 events. Infected cells were determined according the events for green fluorescence (parasites). All determinations for each compound and standard drug were carried out by triplicate, in two experiments. Infected cells exposed to control drug (amphotericin B) were used as control for antileishmanial activity (positive control) while infected cells incubated in absence of any compound or drug were used as control for infection (negative control). Nonspecific fluorescence was corrected by subtracting fluorescence of unstained cells. Determinations were done by triplicate in at least two independent experiments [24,32].

4.2.2. Statistical Analysis

Cytotoxicity was determined according to viability and mortality percentages obtained for each experimental condition (synthetized compounds, amphotericin B and culture medium). Results were expressed as the mean lethal concentrations (LC_{50}), concentration necessary to kill 50% of cells, calculated by the parametric method of linear regression that permits doses-response analysis (Probit analysis) [32].

Initially, viability percentages were calculated by Equation (1), where the O.D of control well, corresponds to 100% of viability.

$$\% \text{ viability} = (\text{O.D exposed cells}/\text{O.D unexposed cells}) \times 100 \quad (1)$$

Then, the percentage of cell growth inhibition was calculated using Equation (2):

$$\% \text{ inhibition} = 100 - (\% \text{ Viability}) \quad (2)$$

The toxicity was defined according to LC_{50} values, using the follow scale: Toxic; $LC_{50} < 100$ µM; moderately toxic; $LC_{50} > 100$ µM and <200 µM and potentially nontoxic; $LC_{50} > 200$ µM.

Antileishmanial activity was determined according reduction of percentage of fluorescent parasites determined according to the median fluorescence intensity (MFI), obtained for each experimental condition by cytometry. The parasite values for each concentration of compound were calculated by Equation (3), where the % of parasites in the control well, corresponds to 100% of parasites.

$$\% \text{ parasites} = (\text{MFI exposed parasites}/\text{MFI unexposed parasites}) \times 100 \quad (3)$$

Then, inhibition percentage was calculated with Equation (4):

$$\% \text{ inhibition of parasites} = 100 - (\% \text{ parasites}) \quad (4)$$

Results of antileishmanial activities were expressed as the median effective concentrations (EC_{50}) measured by Probit method. The activity of each compound was established according to EC_{50} values as: high activity: $EC_{50} < 25$ µM; moderate activity: $EC_{50} > 25$ µM and <100 µM and low activity: $EC_{50} > 100$ µM.

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Conflicts of Interest: The authors declare no conflict of interest.

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513 **Sample Availability:** Samples of the acyl hydrazone compounds are available from the authors.