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M. El Ghozlani, L. Bouissane, M. Berkani, S. Mojahidi, A. Allam, et al.. Synthesis and biological evaluation against *Leishmania donovani* of novel hybrid molecules containing indazole-based 2-pyrone scaffolds. *MedChemComm*, 2019, 10 (1), pp.120-127. 10.1039/c8md00475g. hal-02059320

**HAL Id: hal-02059320**

**<https://hal.science/hal-02059320v1>**

Submitted on 16 Feb 2021

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# Synthesis and biological evaluation against *Leishmania donovani* of novel hybrid molecules containing indazole-based 2-pyrone scaffolds

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## Introduction

Hybridation of two different bioactive molecules with different mechanism of action is one of the methods that are being adopted to treat different diseases. From the last decades, the synthesis of hybrid molecules by the combination of different biologically relevant moieties has been under intense research along with their evaluation as pharmacological agents and potent drugs. A number of reports is focused on the biological potential of hybrid molecules with particular mention of those exhibiting anti-inflammatory, anti-cancer, anti-infectious like anti-fungal, anti-tuberculosis, anti-malarial, and antileishmanial activities.<sup>1-5</sup> For hybrid molecules containing indazole-based heterocycle scaffolds, only a few examples have been developed and reported in the literature as versatile chemotherapeutic agents and have found wide biological applications such as kinase inhibitors (pazopanib),<sup>6</sup> antibacterial,<sup>7</sup> receptor antagonist,<sup>8</sup> and anticancer agents<sup>9</sup>. Three papers are reported on the antileishmanial activity of 5-nitroindazole derivatives.<sup>10-12</sup> Biological evaluation of the studied indazoles has shown that some compounds exhibit adequate *in vitro* leishmanicidal activity against different parasite strains.

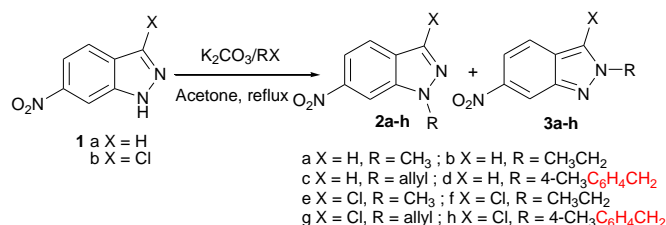
2-Pyrone is an important class heterocyclic scaffold. Due to its pharmacophore properties, synthesis of hybrid derivatives bearing 2-pyrone moiety has attracted much attention; most of the compounds display strong biological activities such as cytotoxic, antibiotic and antifungal.<sup>13-16</sup> It is also known that some pyrones exhibit antileishmanial activity.<sup>17,18</sup> In that context, we planned to explore the possibility of introducing a 2-pyrone into indazole ring. We wish to report here the reductive coupling of *N*-alkyl-6(5)-nitroindazoles and 4-hydroxy-6-methyl-2-pyrone in order to obtain indazole/pyrone hybrid compounds and their inhibition activity against the axenic and intramacrophage amastigotes of *Leishmania donovani*.

## Results and discussion

### Chemistry

#### Synthesis of compounds 2a-d and 3a-d

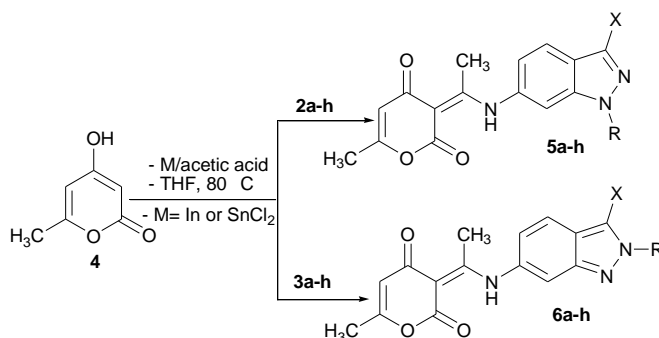
Preparation of the starting materials, *N*-alkyl-6-nitroindazoles **2a-d** and **3a-d** and *N*-alkyl-3-chloro-6-nitroindazoles **2e-g** and **3e-g** were accomplished by simple alkylation of 6-nitroindazoles **1a,b** using experimental conditions recently developed by our group<sup>19</sup> (Scheme 1).



**Scheme 1.** Synthesis of *N*-alkyl-6-nitroindazoles

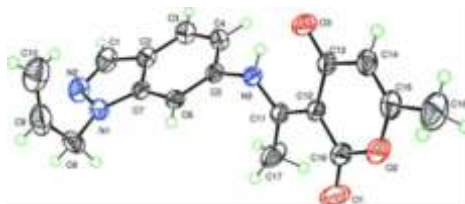
After chromatographic separation and isolation of each *N*-alkyl-6-nitroindazole isomer, we examined their reactivity and that of their chlorinated derivatives **2a-h** and **3a-h** with 4-hydroxy-6-methyl-2-pyrone **4** in the presence of different reducing agents (SnCl<sub>2</sub>/AcOH and In/AcOH in THF). By using an excess of anhydrous Indium and acetic acid respectively in comparison to reagents **2/3** and **4**, we obtained after reflux in THF the

corresponding (3*E*)-3-(1-(*N*-alkyl-1*H*-indazol-6-yl)amino)ethylidene)-6-methyl-3*H*-pyran-2,4-diones **5a–h** and **6a–h** in good yields 68–85% (Scheme 2). When we used SnCl<sub>2</sub> as the reductive system of nitroindazole derivatives, the yields of compounds **5a–h** and **6a–h** were slightly improved (72–91%).

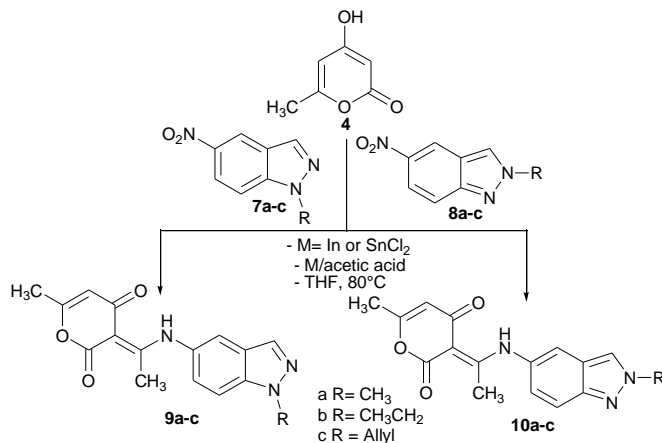


**Scheme 2.** Synthesis of compounds **5a–h** and **6a–h**.

All the structures of the obtained products **5a–h** and **6a–h** were determined by a detailed examination of their NMR data. Furthermore, the structure of product **5c** was unambiguously confirmed through X-ray crystallographic analysis (Figure 1).<sup>20</sup>

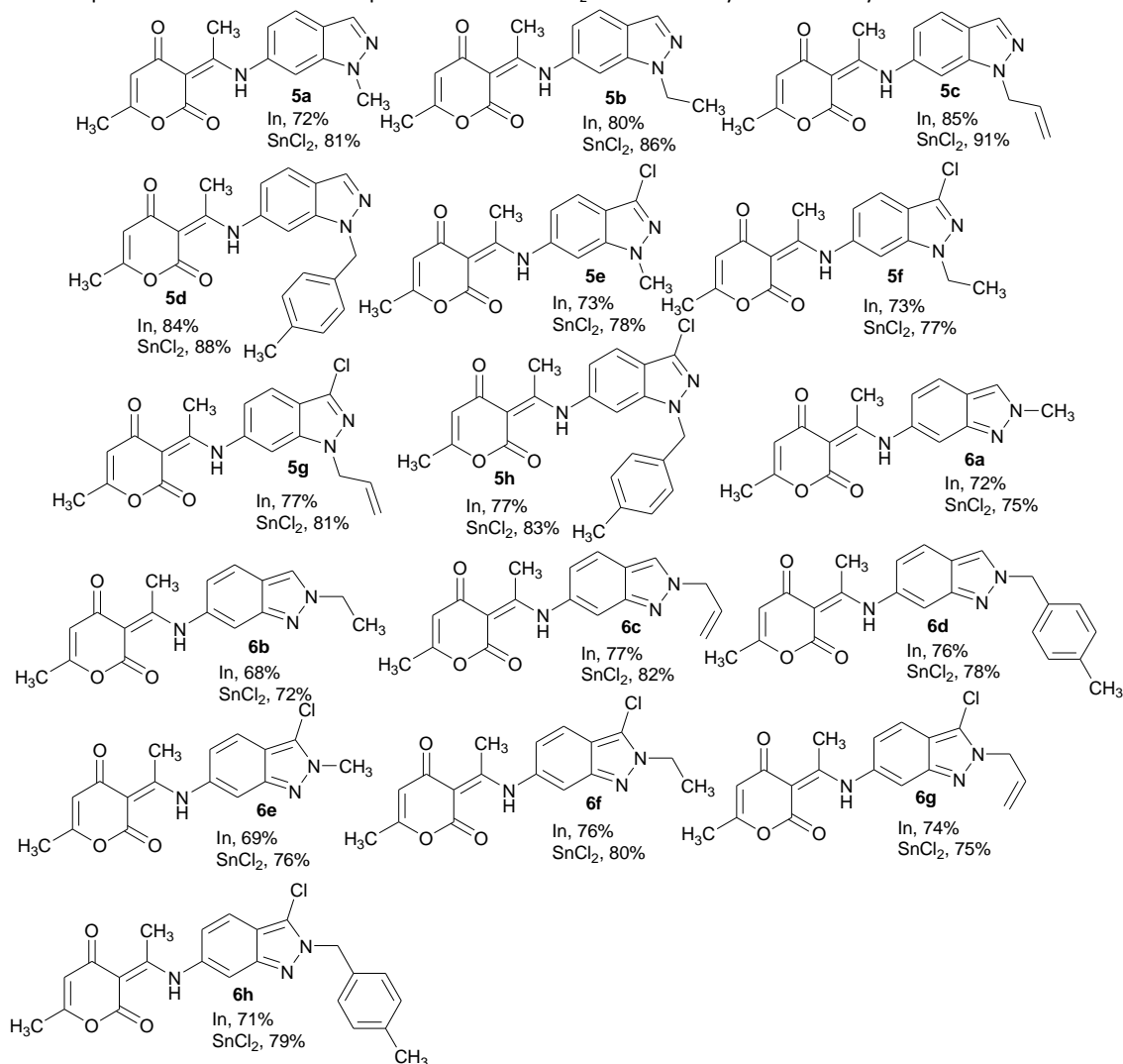


Encouraged by this result, we decided to enlarge the scope of this methodology to the synthesis of other hybrid molecules containing indazole and 2-pyrone moieties. Reaction of *N*-alkyl-5-nitroindazoles **7a–c** and **8a–c** with 4-hydroxy-6-methyl-2-pyrone **4** using the optimized reaction conditions (Scheme 4), afforded as expected compounds **9a–c** and **10a–c** with yields varying between 76%–92%. We obtained the (3*E*)-3-(1-((1-alkyl-indazol-5-yl)amino)ethylidene)-6-methyl-3*H*-pyran-2,4-diones **9a–c** in good yields (87–92%) while the *N*-2 alkylated indazoles **10a–c** were obtained in 76–84%. The highest yields were obtained by the reduction of nitroindazole derivatives with stannous chloride.



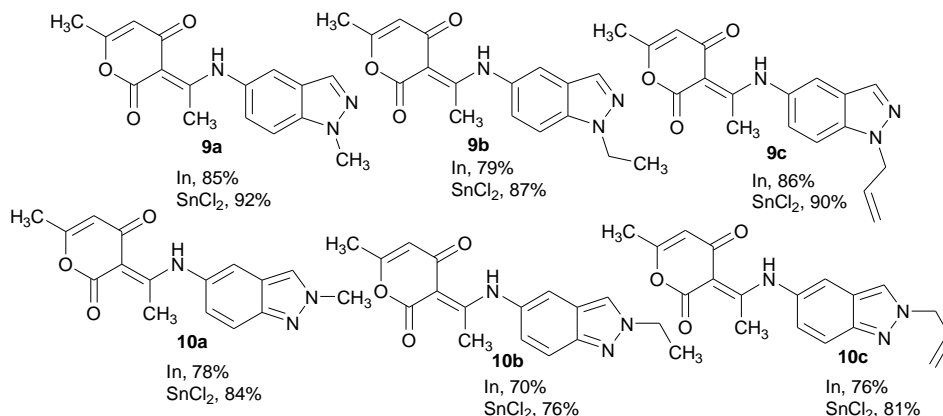
**Scheme 4.** Synthesis of compounds **9a–c** and **10a–c**.

Yields of compounds **5a–h** and **6a–h** in the presence of In or SnCl<sub>2</sub> as reductive system of *N*-alkyl-6-nitroindazole derivatives



\*Isolated yield after column chromatography

Yields of compounds **9a-c** and **10a-c** in the presence of In or SnCl<sub>2</sub> as reductive system of *N*-alkyl-5-nitroindazole derivatives



Isolated yield after column chromatography

### Biological evaluation against *L. donovani*

The synthesized compounds were tested first for their cytotoxicity. Among 19 compounds, only three of them exhibited cytotoxicity with CC<sub>50</sub> values around 6.25 μM and all the other were not cytotoxic at all at 100 μM. The compounds were then evaluated *in vitro* for antileishmanial activity against axenic and intramacrophage amastigotes of *L. donovani*. In this evaluation, the activity was expressed in IC<sub>50</sub> (concentration inhibiting the parasite growth by 50%).

**Table 1.** Antileishmanial activities of the synthesized compounds: Results are expressed as the concentration inhibiting parasite growth by 50% (IC<sub>50</sub>).

Compounds	IC <sub>50</sub> ± SD μM		CC <sub>50</sub> ± SD (μM)
	L. d. LV9 axenic amastigotes	L. d. LV9 intramacrophage amastigotes	Cytotoxicity on macrophages
<b>5a</b>	>100	>100	>100
<b>5b</b>	>100	>100	>100
<b>5c</b>	>100	>100	>100
<b>5d</b>	>100	>100	>100
<b>5e</b>	>100	>100	>100
<b>5f</b>	10.99 ± 1.79	6.50 ± 1.25	6.23±0.74
<b>5g</b>	>100	>100	>100
<b>5h</b>	62.56 ± 1.25	49.89 ± 4.69	>100
<b>6b</b>	>100	>100	>100
<b>6c</b>	>100	>100	>100
<b>6d</b>	49.64 ± 3.19	57.38 ± 6.75	>100
<b>6e</b>	11.89 ± 1.28	3.25 ± 1.99	6.26±0.39
<b>6f</b>	2.48 ± 1.02	2.25 ± 1.89	6.25±0.47
<b>6h</b>	>100	>100	>100
<b>9a</b>	>100	>100	>100
<b>9b</b>	>100	>100	>100
<b>9c</b>	>100	>100	>100
<b>10a</b>	>100	>100	>100
<b>10b</b>	>100	>100	>100
<b>AmB</b>	0.201±0.080	0.115±0,090	4.23±0.32

Amphotericin B (AmB) was used as the reference drug

Among the compounds **5a-h** possessing various substituents at the N-1 position and a hydrogen or chlorine atom at the C-3 position of the indazole ring, only **5f** and to a lesser extent **5h** presented interesting activities. As shown in table 1, **5h** showed moderate antileishmanial activity against both axenic and intramacrophage amastigotes (IC<sub>50</sub> of 62.56 ± 1.25 and

49.89 ± 4.69 μM) whereas derivative **5f** (substituted at C-3 with a chlorine atom and at *N*-1 with an ethyl group) showed the greatest activity (IC<sub>50</sub> of 10.99 ± 1.79 and 6.50 ± 1.25 μM).

Regarding the series of compounds **6b-h** (*N*-2 alkylated), we observed that three compounds **6d-f** displayed fair to very good activities on both intramacrophage and axenic amastigotes with IC<sub>50</sub> values about ten times higher than those of amphotericin B, the reference compound. The most active compound **6f** (substituted at C-3 with a chlorine atom and at *N*-2 with an ethyl group) displayed IC<sub>50</sub> of 2.48 ± 1.02 μM against *L. donovani* axenic amastigotes and, interestingly, an IC<sub>50</sub> of 2.25 ± 1.89 μM against the intramacrophage ones. Compound **6e** which is alkylated at *N*-2 with methyl group, presented an almost four times better activity against intramacrophage amastigote than axenic ones (IC<sub>50</sub> of 3.25 ± 1.99 vs 11.89 ± 1.28 μM).

In comparison between the two series **5a-h/6b-h** we can observe that *N*-2 alkylated derivatives are the most active and among them those bearing a chlorine atom at the C-carbon atom of the indazole ring. Nevertheless, cytotoxicity on macrophage of active compounds tested remains quite strong and in a log range of their antileishmanial activities (CC<sub>50</sub> = 6.25 μM).

Finally, looking at the influence of the position of pyrone attached to indazole on the structure activity relationship, we observed that compounds **9a-c** and **10a-c** (*N*-alkylated and indazole substituted at position 5 with pyrone) did not exhibit any antileishmanial activity (IC<sub>50</sub> >100 μM). When looking at the Selectivity Index (SI) values, defined as the ratio between CC<sub>50</sub>/IC<sub>50</sub> in intracellular amastigotes, the most promising compound is compound **6f** (SI=2.77), followed by compound **6e** (SI=1.92), then **5f** (SI=0.96), this last compound exhibiting no margin between activity and cytotoxicity.

## Conclusions

We have developed a simple and efficient synthesis of (3*E*)-3-(1-((*N*-alkyl-indazol-6-yl)amino)ethylidene)-6-methyl-3*H*-pyran-2,4-diones and (3*E*)-3-(1-((*N*-alkyl-indazol-5-yl)amino)ethylidene)-6-methyl-3*H*-pyran-2,4-diones by reductive coupling of *N*-alkyl-6(5)-nitroindazoles with 2-pyrone using indium or stannous chloride in THF in the presence of acetic acid. By using SnCl<sub>2</sub>/AcOH in THF at 80 °C with cheaper cost, we realized a methodology which provided high yields, short reaction time, easy work-up, purification of products by chromatographic method and clean reaction. Antileishmanial activity of 19 indazole-pyrone hybrid derivatives was studied, with compounds efficient against the intramacrophage and axenic form of the parasite. The remarkable antileishmanial activity obtained for **6f** (IC<sub>50</sub> values of 2.48 ± 1.02 μM and 2.25 ± 1.89 μM respectively on axenic and intramacrophage amastigotes) is moderated by its cytotoxicity. Considering the mechanism of action of both the series, the results obtained in this paper prompt us to develop further pharmacomodulations in order to diminish the cytotoxicity and enhance the antileishmanial activity. Therefore, compound **6f**, the most promising compound from this series, will be the basis for further pharmacomodulations.

## Experimental

### Chemistry

Melting points were determined using a Büchi-Tottoli apparatus. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded in CDCl<sub>3</sub>, DMSO-*d*<sub>6</sub> and solution (unless otherwise specified) with TMS as an internal reference using a Bruker AC 300 (<sup>1</sup>H) or 75MHz (<sup>13</sup>C) instruments. Chemical shifts are given in δ parts per million (ppm) downfield from TMS. Multiplicities of <sup>13</sup>C NMR resources were assigned by distortionless enhancement by polarization transfer (DEPT) experiments. Low-resolution mass spectra (MS) were recorded on a Perkin-Elmer Sciex API 3000 spectrometer. Column chromatography was carried out on SiO<sub>2</sub> (silica gel 60 Merck 0.063–0.200 mm). Thin-layer chromatography (TLC) was carried out on SiO<sub>2</sub> (silica gel 60, F 254 Merck 0.063–0.200 mm), and the spots were located with UV light. Commercial reagents were used without further purification unless stated.

#### General procedure for the synthesis of compounds **5a-h**, **6a-h**, **9a-c** and **10a-c**.

*N*-alkyl-6(5)-nitroindazoles (1.0 mmol) were added to a mixture of anhydrous SnCl<sub>2</sub> powder (460 mg, 4.0 mmol), and acetic acid (0.572 mL, 10 mmol) in tetrahydrofuran (10 mL), followed by the addition of 2-pyrone (1.0 mmol) in THF (15 mL). The reaction mixture was stirred at 80 °C for 4 to 6 h. After the reaction was completed, the mixture was diluted with ethyl acetate (30 mL), poured into 10% NaHCO<sub>3</sub> (30 mL), and then extracted with ethyl acetate (50 mL x 3). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered, and concentrated. The residue was purified by column chromatography on silica gel using ethyl acetate/hexane (3:7) to afford the desired products in good yields. <sup>1</sup>H NMR, <sup>13</sup>C NMR and DEPT copies of selected compounds are given in the supporting information.

**(3*E*)-3-(1-(1-Methyl-1*H*-indazol-6-ylamino)ethylidene)-6-methyl-3*H*-pyran-2,4-dione (5a):** Yield: 81%; mp 164–168 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 2.12 (s, 3H, CH<sub>3</sub>), 2.55 (s, 3H, CH<sub>3</sub>), 4.03 (s, 3H, NCH<sub>3</sub>), 5.85 (s, 1H, =CH), 7.08 (dd, 1H, J = 8.4 Hz, J = 1.5 Hz), 7.73 (d, 1H, J = 1.2 Hz), 7.84 (d, 1H, J = 8.4 Hz), 8.10 (s, 1H, H-3), 15.83 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 19.8 (CH<sub>3</sub>), 20.6 (CH<sub>3</sub>), 36.0 (NCH<sub>3</sub>), 97.1 (C), 107.2 (CH), 118.9 (CH), 122.4 (CH), 123.0 (C), 133.2 (CH-3), 134.4 (C), 139.9 (C), 162.8 (C),

163.9 (C), 175.7 (CO), 184.4 (CO); EI-MS ( $m/z$ ) = 298  $[M+1]^+$ , Anal. Calcd for  $C_{16}H_{15}N_3O_3$ ; C, 64.64; H, 5.09; N, 14.13. Found: C, 64.71; H, 5.02; N, 14.24.

**(3E)-3-(1-(2-Methyl-2H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (6a)**: Yield: 75%; 192–194 °C;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  2.21 (s, 3H,  $CH_3$ ), 2.66 (s, 3H,  $CH_3$ ), 4.43 (s, 3H,  $NCH_3$ ), 5.88 (s, 1H, =CH), 7.08 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.5$  Hz), 7.71 (d, 1H,  $J = 1.2$  Hz), 7.80 (d, 1H,  $J = 8.4$  Hz), 8.21 (s, 1H, H-3), 15.81 (s, 1H, NH);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  20.1 ( $CH_3$ ), 20.7 ( $CH_3$ ), 46.7 ( $NCH_3$ ), 97.9 (C), 107.0 (CH), 112.0 (CH), 120.4 (C), 121.8 (CH), 122.8 (CH), 123.7 (C), 125.2 (CH-3), 139.0 (C), 164.0 (C), 175.1 (CO), 184.9 (CO); EI-MS ( $m/z$ ) = 298  $[M+1]^+$ , Anal. Calcd for  $C_{16}H_{15}N_3O_3$ ; C, 64.64; H, 5.09; N, 14.13. Found: C, 64.75; H, 5.05; N, 14.18.

**(3E)-3-(1-(1-Ethyl-1H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (5b)**: Yield: 86%; mp 177–179 °C;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  1.54 (t, 3H,  $CH_3$ ,  $J = 7.2$  Hz), 2.21 (s, 3H,  $CH_3$ ), 2.68 (s, 3H,  $CH_3$ ), 4.46 (q, 2H,  $NCH_2$ ,  $J = 7.2$  Hz), 6.07 (s, 1H, =CH), 6.96 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.8$  Hz), 7.27 (d, 1H,  $J = 1.2$  Hz), 7.81 (d, 1H,  $J = 8.4$  Hz), 8.07 (s, 1H, H-3), 15.57 (s, 1H, NH);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  14.9 ( $CH_3$ ), 20.1 ( $CH_3$ ), 21.0 ( $CH_3$ ), 44.1 ( $NCH_2$ ), 98.2 (C), 106.1 (CH), 106.5 (CH), 118.7 (CH), 122.9 (CH), 123.4 (C), 132.7 (CH-3), 134.4 (C), 138.7 (C), 160.1 (C), 164.0 (C), 175.8 (CO), 184.0 (CO); EI-MS ( $m/z$ ) = 312  $[M+1]^+$ , Anal. Calcd for  $C_{17}H_{17}N_3O_3$ ; C, 65.58; H, 5.50; N, 13.50. Found: C, 65.67; H, 5.42; N, 13.64.

**(3E)-3-(1-(2-Ethyl-2H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (6b)**: Yield: 72%; mp 159–161 °C;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  1.75 (t, 3H,  $CH_3$ ,  $J = 7.2$  Hz), 2.19 (s, 3H,  $CH_3$ ), 2.66 (s, 3H,  $CH_3$ ), 4.72 (q, 2H,  $NCH_2$ ,  $J = 7.2$  Hz), 5.91 (s, 1H, =CH), 7.03 (dd, 1H,  $J = 9.0$  Hz,  $J = 1.2$  Hz), 7.65 (d, 1H,  $J = 1.2$  Hz), 7.83 (d, 1H,  $J = 9.0$  Hz), 8.23 (s, 1H, H-3), 15.88 (s, 1H, NH);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  15.6 ( $CH_3$ ), 20.0 ( $CH_3$ ), 20.7 ( $CH_3$ ), 49.0 ( $NCH_2$ ), 98.2 (C), 106.8 (CH), 111.4 (CH), 120.2 (C), 121.8 (CH), 122.6 (CH), 123.4 (C), 125.6 (CH-3), 138.7 (C), 164.2 (C), 175.6 (CO), 184.5 (CO); EI-MS ( $m/z$ ) = 312  $[M+1]^+$ , Anal. Calcd for  $C_{17}H_{17}N_3O_3$ ; C, 65.58; H, 5.50; N, 13.50. Found: C, 65.71; H, 5.38; N, 13.58.

**(3E)-3-(1-(1-Allyl-1H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (5c)**: Yield: 91%; mp 118–120 °C;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  2.21 (s, 3H,  $CH_3$ ), 2.68 (s, 3H,  $CH_3$ ), 5.04–5.06 (m, 2H,  $NCH_2$ ), 5.13–5.28 (m, 2H, = $CH_2$ ), 5.97–6.06 (m, 1H, =CH), 6.13 (s, 1H, =CH), 6.97 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.8$  Hz), 7.27 (d, 1H,  $J = 1.2$  Hz), 7.82 (d, 1H,  $J = 8.4$  Hz), 8.07 (s, 1H, H-3), 15.48 (s, 1H, NH);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  20.1 ( $CH_3$ ), 21.0 ( $CH_3$ ), 52.1 ( $NCH_2$ ), 98.2 (C), 106.5 (CH), 110.5 (CH), 118.5 (=CH<sub>2</sub>), 118.8 (CH), 122.7 (CH), 123.7 (C), 132.1 (CH), 133.3 (CH), 134.3 (C), 139.2 (C), 163.9 (C), 164.7 (C), 176.0 (CO), 184.0 (CO); EI-MS ( $m/z$ ) = 324  $[M+1]^+$ , Anal. Calcd for  $C_{18}H_{17}N_3O_3$ ; C, 66.86; H, 5.30; N, 13.00. Found: C, 66.74; H, 5.42; N, 13.08.

**(3E)-3-(1-(2-Allyl-2H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (6c)**: Yield: 82%; mp 96–98 °C;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  2.16 (s, 3H,  $CH_3$ ), 2.64 (s, 3H,  $CH_3$ ), 5.10–5.13 (m, 2H,  $NCH_2$ ), 5.35–5.42 (m, 2H, = $CH_2$ ), 5.83 (s, 1H, =CH), 6.09–6.18 (m, 1H, =CH), 6.90 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.5$  Hz), 7.54 (d, 1H,  $J = 1.0$  Hz), 7.74 (d, 1H,  $J = 8.4$  Hz), 8.10 (s, 1H, H-3), 15.71 (s, 1H, NH);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  19.9 ( $CH_3$ ), 20.6 ( $CH_3$ ), 56.3 ( $NCH_2$ ), 97.4 (C), 107.2 (CH), 113.5 (CH), 120.5 (CH), 120.7 (=CH<sub>2</sub>), 122.1 (CH), 122.7 (C), 123.9 (CH), 131.3 (CH), 147.3 (C), 163.4 (C), 163.8 (C), 175.6 (CO), 184.6 (CO); EI-MS ( $m/z$ ) = 324  $[M+1]^+$ , Anal. Calcd for  $C_{18}H_{17}N_3O_3$ ; C, 66.86; H, 5.30; N, 13.00. Found: C, 66.72; H, 5.38; N, 13.15.

**(3E)-3-[1-[1-(4-Methyl-benzyl)-1H-indazol-6-ylamino]-ethylidene]-6-methyl-3H-pyran-2,4-dione (5d)**: Yield: 88%; mp 98–100 °C;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  2.23 (s, 3H,  $CH_3$ ), 2.31 (s, 3H,  $CH_3$ ), 2.59 (s, 3H,  $CH_3$ ), 5.58 (s, 2H,  $NCH_2$ ), 5.79 (s, 1H, =CH), 6.94 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.8$  Hz), 7.11–7.15 (m, 5H), 7.82 (d, 1H,  $J = 8.4$  Hz), 8.11 (s, 1H, H-3), 15.37 (s, 1H, NH);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  20.1 ( $CH_3$ ), 21.1 (2 $CH_3$ ), 53.3 ( $NCH_2$ ), 98.2 (C), 106.0 (CH), 106.6 (CH), 118.7 (CH), 122.9 (CH), 123.8 (C), 127.3 (2CH), 129.5 (C), 129.7 (2CH), 132.7 (C), 133.2 (CH), 134.2 (C), 138.1 (C), 139.2 (C), 163.4 (C), 175.1 (CO), 184.1 (CO); EI-MS ( $m/z$ ) = 388  $[M+1]^+$ , Anal. Calcd for  $C_{23}H_{21}N_3O_3$ ; C, 71.30; H, 5.46; N, 10.85. Found: C, 71.18; H, 5.57; N, 10.74.

**(3E)-3-[1-[2-(4-Methyl-benzyl)-2H-indazol-6-ylamino]-ethylidene]-6-methyl-3H-pyran-2,4-dione (6d)**: Yield: 78%; mp 154–156 °C;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  2.16 (s, 3H,  $CH_3$ ), 2.34 (s, 3H,  $CH_3$ ), 2.64 (s, 3H,  $CH_3$ ), 5.59 (s, 2H,  $NCH_2$ ), 5.79 (s, 1H, =CH), 6.85 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.0$  Hz), 7.17 (d, 1H,  $J = 7.8$  Hz), 7.23 (d, 1H,  $J = 7.8$  Hz), 7.52 (d, 1H,  $J = 1.0$  Hz), 7.68 (d, 1H,  $J = 8.4$  Hz), 7.95 (s, 1H, H-3), 15.72 (s, 1H, NH);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  19.9 ( $CH_3$ ), 20.5 ( $CH_3$ ), 21.2 ( $CH_3$ ), 57.5 ( $NCH_2$ ), 97.5 (C), 107.2 (CH), 113.2 (CH), 120.6 (C), 120.7 (CH), 122.2 (CH), 124.3 (CH), 128.5 (2CH), 129.8 (2CH), 131.4 (C), 135.1 (C), 138.9 (C), 163.5 (C), 163.7 (C), 175.5 (CO), 184.6 (CO); EI-MS ( $m/z$ ) = 388  $[M+1]^+$ , Anal. Calcd for  $C_{23}H_{21}N_3O_3$ ; C, 71.30; H, 5.46; N, 10.85. Found: C, 71.21; H, 5.61; N, 10.78.

**(3E)-3-(1-(3-Chloro-1-methyl-1H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (5e)**: Yield: 78%; mp 185–187 °C;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  2.15 (s, 3H,  $CH_3$ ), 2.58 (s, 3H,  $CH_3$ ), 4.08 (s, 3H,  $NCH_3$ ), 5.91 (s, 1H, =CH), 7.05 (d, 1H,  $J = 8.4$  Hz), 7.20 (d, 1H,  $J = 1.0$  Hz), 7.81 (d, 1H,  $J = 8.4$  Hz), 15.65 (s, 1H, NH);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  20.0 ( $CH_3$ ), 20.7 ( $CH_3$ ), 47.6 ( $NCH_3$ ), 97.8 (C), 106.8 (CH), 107.1 (CH), 119.0 (CH), 121.0 (C), 121.4 (CH), 134.1 (C), 136.0 (C), 140.8 (C), 163.7 (C), 164.9 (C), 175.8 (CO), 184.6 (CO); EI-MS ( $m/z$ ) = 332 ( $^{35}Cl$ )  $[M+1]^+$ , 334 ( $^{37}Cl$ )  $[M+3]^+$  Anal. Calcd for  $C_{16}H_{14}ClN_3O_3$ ; C, 57.93; H, 4.25; N, 12.67. Found: C, 57.82; H, 4.36; N, 12.54.

**(3E)-3-(1-(3-Chloro-2-methyl-2H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (6e)**: Yield: 76%; mp 107–109 °C;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  2.16 (s, 3H,  $CH_3$ ), 2.63 (s, 3H,  $CH_3$ ), 4.17 (s, 3H,  $NCH_3$ ), 5.77 (s, 1H, =CH), 6.88 (dd, 1H,  $J = 8.7$  Hz,  $J = 1.5$  Hz), 7.42 (d, 1H,  $J = 1.5$  Hz), 7.62 (d, 1H,  $J = 8.7$  Hz), 15.88 (s, 1H, NH);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  19.9 ( $CH_3$ ), 20.5 ( $CH_3$ ), 37.8 ( $NCH_3$ ),



97.5 (C), 107.2 (C), 114.2 (CH), 111.8 (C), 118.1 (C), 120.1 (CH), 120.6 (CH), 135.0 (C), 147.1 (C), 163.4 (C), 175.5 (CO), 184.8 (CO); EI-MS ( $m/z$ ) = 332 ( $^{35}\text{Cl}$ ) [ $M+1$ ] $^+$ , 334 ( $^{37}\text{Cl}$ ) [ $M+3$ ] $^+$  Anal. Calcd for  $\text{C}_{16}\text{H}_{14}\text{ClN}_3\text{O}_3$ ; C, 57.93; H, 4.25; N, 12.67. Found: C, 57.78; H, 4.34; N, 12.52.

**(3E)-3-(1-(3-Chloro-1-ethyl-1H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (5f)**: Yield: 77%; mp 67–69 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.51 (t, 3H,  $\text{CH}_3$ ,  $J = 7.2$  Hz), 2.26 (s, 3H,  $\text{CH}_3$ ), 2.58 (s, 3H,  $\text{CH}_3$ ), 4.40 (q, 2H,  $\text{NCH}_2$ ,  $J = 7.2$  Hz), 5.81 (s, 1H, =CH), 6.90 (dd, 1H,  $J = 8.7$  Hz,  $J = 1.5$  Hz), 7.45 (d, 1H,  $J = 1.5$  Hz), 7.66 (d, 1H,  $J = 8.7$  Hz), 15.82 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.8 ( $\text{CH}_3$ ), 20.2 ( $\text{CH}_3$ ), 21.0 ( $\text{CH}_3$ ), 44.5 ( $\text{NCH}_2$ ), 97.8 (C), 107.4 (C), 114.1 (CH), 111.9 (C), 118.5 (C), 120.3 (CH), 120.8 (CH), 135.1 (C), 146.9 (C), 163.8 (C), 175.6 (CO), 184.2 (CO); EI-MS ( $m/z$ ) = 346 ( $^{35}\text{Cl}$ ) [ $M+1$ ] $^+$ , 348 ( $^{37}\text{Cl}$ ) [ $M+3$ ] $^+$  Anal. Calcd for  $\text{C}_{16}\text{H}_{14}\text{ClN}_3\text{O}_3$ ; C, 59.05; H, 4.66; N, 12.15. Found: C, 59.18; H, 4.54; N, 12.22.

**(3E)-3-(1-(3-Chloro-2-ethyl-2H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (6f)**: Yield: 80%; mp 62–64 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.45 (t, 3H,  $\text{CH}_3$ ,  $J = 7.2$  Hz), 2.20 (s, 3H,  $\text{CH}_3$ ), 2.64 (s, 3H,  $\text{CH}_3$ ), 4.72 (q, 2H,  $\text{NCH}_2$ ,  $J = 7.2$  Hz), 5.81 (s, 1H, =CH), 6.91 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.5$  Hz), 7.45 (d, 1H,  $J = 1.5$  Hz), 7.68 (d, 1H,  $J = 8.4$  Hz), 15.78 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  15.7 ( $\text{CH}_3$ ), 20.0 ( $\text{CH}_3$ ), 20.6 ( $\text{CH}_3$ ), 48.8 ( $\text{NCH}_2$ ), 97.8 (C), 107.1 (C), 113.9 (CH), 112.0 (C), 118.2 (C), 120.4 (CH), 120.8 (CH), 134.9 (C), 146.8 (C), 163.5 (C), 175.7 (CO), 184.5 (CO); EI-MS ( $m/z$ ) = 346 ( $^{35}\text{Cl}$ ) [ $M+1$ ] $^+$ , 348 ( $^{37}\text{Cl}$ ) [ $M+3$ ] $^+$  Anal. Calcd for  $\text{C}_{16}\text{H}_{14}\text{ClN}_3\text{O}_3$ ; C, 59.05; H, 4.66; N, 12.15. Found: C, 59.24; H, 4.50; N, 12.28.

**(3E)-3-(1-(1-Allyl-3-chloro-1H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (5g)**: Yield: 81%; mp 146–148 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.23 (s, 3H,  $\text{CH}_3$ ), 2.66 (s, 3H,  $\text{CH}_3$ ), 4.95–4.98 (m, 2H,  $\text{NCH}_2$ ), 5.18–5.30 (m, 2H, = $\text{CH}_2$ ), 5.82–6.02 (m, 1H, =CH), 6.14 (s, 1H, =CH), 7.02 (d, 1H,  $J = 8.4$  Hz), 7.24 (d, 1H,  $J = 1.0$  Hz), 7.77 (d, 1H,  $J = 8.4$  Hz), 15.54 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  20.1 ( $\text{CH}_3$ ), 21.1 ( $\text{CH}_3$ ), 52.5 ( $\text{NCH}_2$ ), 97.1 (C), 106.3 (CH), 106.9 (CH), 118.9 (=CH $_2$ ), 119.3 (CH), 121.0 (C), 121.6 (CH), 131.7 (CH), 133.5 (C), 135.3 (C), 140.5 (C), 163.8 (C), 165.0 (C), 176.2 (CO), 184.0 (CO); EI-MS ( $m/z$ ) = 358 ( $^{35}\text{Cl}$ ) [ $M+1$ ] $^+$ , 360 ( $^{37}\text{Cl}$ ) [ $M+3$ ] $^+$  Anal. Calcd for  $\text{C}_{18}\text{H}_{16}\text{ClN}_3\text{O}_3$ ; C, 60.43; H, 4.51; N, 11.74. Found: C, 60.32; H, 4.64; N, 11.63.

**(3E)-3-(1-(2-Allyl-3-chloro-2H-indazol-6-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (6g)**: Yield: 75%; mp 70–72 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.18 (s, 3H,  $\text{CH}_3$ ), 2.67 (s, 3H,  $\text{CH}_3$ ), 5.08–5.12 (m, 2H,  $\text{NCH}_2$ ), 5.32–5.40 (m, 2H, = $\text{CH}_2$ ), 5.86 (s, 1H, =CH), 6.10–6.17 (m, 1H, =CH), 6.92 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.5$  Hz), 6.91 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.5$  Hz), 7.45 (d, 1H,  $J = 1.5$  Hz), 7.61 (d, 1H,  $J = 8.4$  Hz), 15.77 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  20.0 ( $\text{CH}_3$ ), 20.6 ( $\text{CH}_3$ ), 56.4 ( $\text{NCH}_2$ ), 97.6 (C), 106.8 (C), 113.6 (CH), 112.0 (C), 118.2 (C), 120.4 (CH), 120.7 (=CH $_2$ ), 121.0 (CH), 131.5 (CH), 135.0 (C), 146.2 (C), 164.0 (C), 175.4 (CO), 184.8 (CO); EI-MS ( $m/z$ ) = 358 ( $^{35}\text{Cl}$ ) [ $M+1$ ] $^+$ , 360 ( $^{37}\text{Cl}$ ) [ $M+3$ ] $^+$  Anal. Calcd for  $\text{C}_{18}\text{H}_{16}\text{ClN}_3\text{O}_3$ ; C, 60.43; H, 4.51; N, 11.74. Found: C, 60.28; H, 4.60; N, 11.61.

**(3E)-3-(1-(1-Methyl-1H-indazol-5-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione 9a**: Yield: 92%; mp 173–175 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.23 (s, 3H,  $\text{CH}_3$ ), 2.65 (s, 3H,  $\text{CH}_3$ ), 4.13 (s, 3H,  $\text{NCH}_3$ ), 6.24 (s, 1H, =CH), 7.20 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.8$  Hz), 7.53 (d, 1H,  $J = 8.4$  Hz), 7.57 (d, 1H,  $J = 1.2$  Hz), 8.05 (s, 1H, H-3), 15.22 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  20.1 ( $\text{CH}_3$ ), 21.0 ( $\text{CH}_3$ ), 35.9 ( $\text{NCH}_3$ ), 98.2 (C), 106.2 (CH), 110.5 (CH), 118.1 (CH), 123.8 (C), 124.1 (CH), 128.8 (C), 133.0 (CH-3), 138.9 (C), 164.1 (C), 164.9 (C), 176.3 (CO), 183.6 (CO); EI-MS ( $m/z$ ) = 298 [ $M+1$ ] $^+$ , Anal. Calcd for  $\text{C}_{16}\text{H}_{15}\text{N}_3\text{O}_3$ ; C, 64.64; H, 5.09; N, 14.13. Found: C, 64.75; H, 5.18; N, 14.20.

**(3E)-3-(1-(2-Methyl-2H-indazol-5-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (10a)**: Yield: 84%; mp 142–144 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.16 (s, 3H,  $\text{CH}_3$ ), 2.61 (s, 3H,  $\text{CH}_3$ ), 4.29 (s, 3H,  $\text{NCH}_3$ ), 5.81 (s, 1H, =CH), 7.09 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.8$  Hz), 7.48 (d, 1H,  $J = 1.8$  Hz), 7.77 (d, 1H,  $J = 8.4$  Hz), 8.02 (s, 1H, H-3), 15.59 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  19.9 ( $\text{CH}_3$ ), 20.5 ( $\text{CH}_3$ ), 40.6 ( $\text{NCH}_3$ ), 97.3 (C), 107.3 (CH), 116.8 (CH), 118.6 (CH), 121.4 (C), 124.9 (CH), 125.5 (CH-3), 130.5 (C), 146.8 (C), 163.4 (C), 163.8 (C), 175.7 (CO), 184.6 (CO); EI-MS ( $m/z$ ) = 298 [ $M+1$ ] $^+$ , Anal. Calcd for  $\text{C}_{16}\text{H}_{15}\text{N}_3\text{O}_3$ ; C, 64.64; H, 5.09; N, 14.13. Found: C, 64.72; H, 5.21; N, 14.24.

**(3E)-3-(1-(1-Ethyl-1H-indazol-5-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (9b)**: Yield: 87%; mp 156–158 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.54 (t, 3H,  $\text{CH}_3$ ,  $J = 7.2$  Hz), 2.22 (s, 3H,  $\text{CH}_3$ ), 2.65 (s, 3H,  $\text{CH}_3$ ), 4.48 (q, 2H,  $\text{NCH}_2$ ,  $J = 7.2$  Hz), 6.21 (s, 1H, =CH), 7.19 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.8$  Hz), 7.51 (d, 1H,  $J = 8.4$  Hz), 7.58 (d, 1H,  $J = 1.5$  Hz), 8.06 (s, 1H, H-3), 15.25 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.9 ( $\text{CH}_3$ ), 20.1 ( $\text{CH}_3$ ), 20.9 ( $\text{CH}_3$ ), 44.2 ( $\text{NCH}_2$ ), 96.8 (C), 106.3 (CH), 110.4 (CH), 118.2 (CH), 123.9 (C), 124.1 (CH), 128.8 (C), 133.0 (CH-3), 138.0 (C), 164.1 (C), 164.8 (C), 176.2 (CO), 183.7 (CO); EI-MS ( $m/z$ ) = 312 [ $M+1$ ] $^+$ , Anal. Calcd for  $\text{C}_{17}\text{H}_{17}\text{N}_3\text{O}_3$ ; C, 65.58; H, 5.50; N, 13.50. Found: C, 65.75; H, 5.41; N, 13.64.

**(3E)-3-(1-(2-Ethyl-2H-indazol-5-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (10b)**: Yield: 76%; mp 113–115 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.69 (t, 3H,  $\text{CH}_3$ ,  $J = 7.2$  Hz), 2.17 (s, 3H,  $\text{CH}_3$ ), 2.62 (s, 3H,  $\text{CH}_3$ ), 4.59 (q, 2H,  $\text{NCH}_2$ ,  $J = 7.2$  Hz), 5.83 (s, 1H, =CH), 7.13 (dd, 1H,  $J = 8.4$  Hz,  $J = 1.8$  Hz), 7.51 (d, 1H,  $J = 1.0$  Hz), 7.82 (d, 1H,  $J = 8.4$  Hz), 8.10 (s, 1H, H-3), 15.61 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  15.7 ( $\text{CH}_3$ ), 19.9 ( $\text{CH}_3$ ), 20.5 ( $\text{CH}_3$ ), 48.9 ( $\text{NCH}_2$ ), 97.3 (C), 107.2 (CH), 117.0 (CH), 118.3 (CH), 121.0 (C), 124.0 (CH), 125.5 (CH-3), 130.7 (C), 145.7 (C), 163.5 (C), 163.8 (C), 175.7 (CO), 184.6 (CO); EI-MS ( $m/z$ ) = 312 [ $M+1$ ] $^+$ , Anal. Calcd for  $\text{C}_{17}\text{H}_{17}\text{N}_3\text{O}_3$ ; C, 65.58; H, 5.50; N, 13.50. Found: C, 65.72; H, 5.38; N, 13.60.

**(3E)-3-(1-(1-Allyl-1H-indazol-5-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (9c)**: Yield: 90%; mp 90–92 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.22 (s, 3H,  $\text{CH}_3$ ), 2.65 (s, 3H,  $\text{CH}_3$ ), 5.05–5.08 (m, 2H,  $\text{NCH}_2$ ), 5.15–5.26 (m, 2H, = $\text{CH}_2$ ), 5.97–6.08 (m, 1H, =CH),



6.19 (s, 1H, =CH), 7.17 (dd, 1H, J = 8.4 Hz, J = 1.8 Hz), 7.50 (d, 1H, J = 9.0 Hz), 7.58 (d, 1H, J = 1.8 Hz), 8.07 (s, 1H, H-3), 15.27 (s, 1H, NH); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 20.1 (CH<sub>3</sub>), 20.8 (CH<sub>3</sub>), 52.1 (NCH<sub>2</sub>), 96.9 (C), 106.5 (CH), 110.7 (CH), 118.1 (CH), 118.4 (=CH<sub>2</sub>), 124.1 (CH), 124.3 (C), 128.9 (C), 132.2 (CH), 133.5 (CH), 138.5 (C), 164.1 (C), 164.7 (C), 176.2 (CO), 183.8 (CO); EI-MS (m/z) = 324 [M+1]<sup>+</sup>, Anal. Calcd for C<sub>18</sub>H<sub>17</sub>N<sub>3</sub>O<sub>3</sub>; C, 66.86; H, 5.30; N, 13.00. Found: C, 66.70; H, 5.37; N, 13.16.

**(3E)-3-(1-(2-Allyl-2H-indazol-5-ylamino)ethylidene)-6-methyl-3H-pyran-2,4-dione (10c):** Yield: 81%; mp 78–80 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.18 (s, 3H, CH<sub>3</sub>), 2.63 (s, 3H, CH<sub>3</sub>), 5.16–5.19 (m, 2H, NCH<sub>2</sub>), 5.40–5.46 (m, 2H, =CH<sub>2</sub>), 5.92 (s, 1H, =CH), 6.05–6.20 (m, 1H, =CH), 7.15 (dd, 1H, J = 8.4 Hz, J = 1.5 Hz), 7.53 (d, 1H, J = 1.0 Hz), 7.68 (d, 1H, J = 8.4 Hz), 8.12 (s, 1H, H-3), 15.56 (s, 1H, NH); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 19.9 (CH<sub>3</sub>), 20.6 (CH<sub>3</sub>), 56.2 (NCH<sub>2</sub>), 97.6 (C), 106.9 (CH), 117.2 (CH), 118.3 (CH), 121.4 (=CH<sub>2</sub>), 124.8 (CH), 121.7 (C), 128.8 (CH), 130.7 (CH), 132.4 (C), 163.8 (C), 163.9 (C), 175.8 (CO), 184.4 (CO); EI-MS (m/z) = 324 [M+1]<sup>+</sup>, Anal. Calcd for C<sub>18</sub>H<sub>17</sub>N<sub>3</sub>O<sub>3</sub>; C, 66.86; H, 5.30; N, 13.00. Found: C, 66.77; H, 5.43; N, 13.21.

## Biology

**Parasite and cell cultures.** Promastigotes of the *Leishmania donovani* (MHOM/ET/67/HU3/LV9) were cultured in the dark at 26°C with 5% CO<sub>2</sub> in M199 complete medium containing M199 medium supplemented with 100 μM adenosine, 0.5 mg/L hemin, 40 mM Hepes pH 7.4 and 10 % heat inactivated foetal bovine serum (HIFBS). Cultures of axenic amastigotes of *L. donovani* were obtained from late log promastigotes diluted at 1 x 10<sup>6</sup>/mL in M199 complete medium acidified at pH 5.5 and cultured at 37°C with 5% CO<sub>2</sub>.

**Macrophage.** The macrophages RAW 264.7 were cultured at 37°C with 5% CO<sub>2</sub> in DMEM complete medium containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/mL penicillin-streptomycin, and 10% HIFBS.

**Evaluation of compounds cytotoxicity.** Cytotoxicity was evaluated on RAW 264.7 macrophages. Cells were plated in 96-well microplates at a density of 2 x 10<sup>4</sup> cells per well. After an incubation of 24 h at 37°C with 5% CO<sub>2</sub>, the medium was removed in each well, and 100 μL of DMEM complete medium containing two fold serial dilutions of the compounds was added to each well. After 48 h of incubation at 37°C with 5% CO<sub>2</sub>, 10 μL of resazurin (450 μM) was added to each well, and further incubated in the dark for 4 h at 37°C with 5% CO<sub>2</sub>. Cell viability was then monitored as described above. The cytotoxicity of the compounds was expressed as CC50 (Cytotoxic Concentration 50%: concentration inhibiting the macrophages growth by 50%).

**In vitro antileishmanial evaluation of compounds on axenic and intramacrophage amastigotes.** The evaluations of activity on axenic and intramacrophage amastigotes of *L. donovani* were adapted from the protocols previously described [19]. Briefly, for the evaluation on axenic amastigotes, two fold serial dilutions of the compounds were performed in 100 μL of complete medium (see above) in 96-well microplates. Axenic amastigotes were then added to each well at a density of 10<sup>6</sup>/mL in a 200 μL final volume. After 72 h of incubation at 37°C with 5% CO<sub>2</sub>, 20 μL of resazurin (450 μM) was added to each well and further incubated in the dark for 24 h at 37°C with 5% CO<sub>2</sub>. In living cells, resazurin is reduced in resorufin and this conversion is monitored by measuring OD570nm (resorufin) and OD600nm (resazurin; Lab systems Multiskan MS). The activity of the compounds was expressed as IC<sub>50</sub> in μM. Amphotericin B (AmB) was used as the reference drug.

Concerning the evaluation on intramacrophage amastigotes, RAW 264.7 macrophages were plated in 96-well microplates at a density of 2 x 10<sup>4</sup> cells per well and incubated for 24 h at 37°C with 5% CO<sub>2</sub>. Axenic amastigotes were differentiated as described above, centrifuged at 2,000 g for 10 min, resuspended in DMEM complete medium, and added to each well to reach a 16 :1 parasite to macrophage ratio. After 24 h of infection at 37°C with 5% CO<sub>2</sub>, extracellular parasites were removed, and DMEM complete medium (100 μL) containing two fold serial dilutions of the compounds from a maximal concentration of 100 μM was added to each well. After 48 h of treatment, the medium was removed and replaced by Direct PCR Lysis Reagent (100 μL; Euromedex) before 3 freeze-thaw cycles at room temperature, addition of 50 μg/mL proteinase K, and a final incubation at 55°C overnight to allow cell lysis. 10 μL of each cell extract was then added to 40 μL of Direct PCR Lysis reagent containing Sybr Green I (0.05%; Invitrogen). DNA fluorescence was monitored using Mastercycler® realplex (Eppendorf). The activity of the compounds was expressed as IC<sub>50</sub> in μM. Amphotericin B (AmB) was used as the reference drug.

## Acknowledgements

We thank the University of Sultan Moulay Slimane, Beni-Mellal, for financial support and the National Centre for Scientific and Technical Research (CNRST), Rabat, for providing the NMR and X-ray crystallography of our compounds.

## Conflict of Interest

The authors declare no competing interests

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