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Research paper

Antichagasic and trichomonacidal activity of 1-substituted 2-benzyl-5-nitroindazolin-3-ones and 3-alkoxy-2-benzyl-5-nitro-2*H*-indazoles☆

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ABSTRACT

Two series of new 5-nitroindazole derivatives, 1-substituted 2-benzylindazolin-3-ones (**6**–**29**, series **A**) and 3-alkoxy-2-benzyl-2*H*-indazoles (**30**–**37**, series **B**), containing differently functionalized chains at position 1 and 3, respectively, have been synthesized starting from 2-benzyl-5-nitroindazolin-3-one **5**, and evaluated against the protozoan parasites *Trypanosoma cruzi* and *Trichomonas vaginalis*, etiological agents of Chagas disease and trichomonosis, respectively. Many indazolinones of series **A** were efficient against different morphological forms of *T. cruzi* CL Brener strain (compounds **6**, **7**, **9**, **10** and **19**–**21**: IC_{50} = 1.58–4.19 μM for epimastigotes; compounds **6**, **19**–**21** and **24**: IC_{50} = 0.22–0.54 μM for amastigotes) being as potent as the reference drug benznidazole. SAR analysis suggests that electron-donating groups at position 1 of indazolinone ring are associated with an improved antichagasic activity. Moreover, compounds of series **A** displayed low unspecific toxicities against an *in vitro* model of mammalian cells (fibroblasts), which were reflected in high values of the selectivity indexes (SI). Compound **20** was also very efficient against amastigotes from Tulahuen and Y strains of *T. cruzi* (IC_{50} = 0.81 and 0.60 μM, respectively), showing low toxicity towards cardiac cells (LC_{50} > 100 μM). In what concerns compounds of series **B**, some of them displayed moderate activity against trophozoites of a metronidazole-sensitive isolate of *T. vaginalis* (**35** and **36**: IC_{50} = 9.82 and 7.25 μM, respectively), with low unspecific toxicity towards Vero cells. Compound **36** was also active against a metronidazole-resistant isolate (IC_{50} = 9.11 μM) and can thus be considered a good prototype for the development of drugs directed to *T. vaginalis* resistant to 5-nitroimidazoles.

1. Introduction

Our research group has been pursuing during the last decade the identification of alternative therapeutic options for parasitic protozoan infections through the design, synthesis and biological activity analysis of novel nitroheterocycles. In these previous studies we reported the activity against *Trypanosoma cruzi* [1–8], *Trypanosoma brucei* [9], *Leishmania* spp. [2,10] and *Trichomonas vaginalis* [1,11–13] of 5-nitroindazole derivatives, mainly 1-substituted indazol-3-ols [11,12], 2-substituted indazolin-3-ones [11], 1-substituted 3-alkoxyindazoles [1–5,7,9], 1,2-disubstituted

☆ Dedicated to our dear colleague Dr. María Teresa García López, former director of Instituto de Química Médica (IQM, CSIC, Madrid) for her outstanding contribution to Medicinal Chemistry, on the occasion of her retirement.

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indazolin-3-ones [5,6,8,13] and 2-substituted 3-alkoxyindazoles [5,6,13].

In the present study our efforts have been centered on new indazole derivatives focusing on two parasitic infections, Chagas disease and trichomonosis. The reference drugs for both diseases, i.e., nifurtimox and benznidazole for the former, and metronidazole for the latter, are nitroheterocyclic compounds [14], and furthermore our 5-nitroindazole derivatives have previously shown, as mentioned above, activity against both protozoan parasites.

Chagas disease is a silent chronic infection caused by the trypanosomatid (Kinetoplastid) protozoan parasite *T. cruzi*. This parasitosis, originally endemic to poor rural areas of Latin America, from Mexico to Argentina, is an anthropozoonosis, mainly transmitted in this area by hematophagous triatomine insects [15,16]. The disease is currently endemic to 21 countries, where causes more than 7000 deaths per year and maintains over 25 million people at risk for the infection [17]. Now, owing to intense international migrations and secondary routes of transmission (i.e., transfusion of infected blood, contaminated organ transplantation or congenitally), it can also be found in non-endemic areas such as Western Europe, USA, or Australia [18], currently affecting about 7 million people worldwide [17].

The initial acute phase of Chagas disease, which appears after 1 week of *T. cruzi* infection, has low (<10%) mortality usually related to heart failure and/or meningoencephalitis, but it is often asymptomatic [15,19]. After 1–2 months, immune response partially controls the infection but cannot eradicate it completely. Although the infection persists for life, about 60–70% of untreated infected people never develop clinical manifestations, entering in an indeterminate phase of the disease lacking organ affectation. However, the remaining 30–40%, 10–30 years after initial infection, evolve the symptomatic chronic phase of the disease, characterized by a life-threatening cardiomyopathy and/or severe digestive problems (e.g. megacolon and megaesophagus). In fact, Chagas infection significantly contributes to the global burden of cardiovascular disease [15,16]. The differences noticed in clinical presentations occur as a consequence of six discrete typing units (DTU) of *T. cruzi* (TcI–TcVI), which along the endemic area show differences in their geographical distribution, ecotopes and mammalian host [20].

The drugs currently used to treat Chagas disease, nifurtimox and benznidazole, are decades old and have many limitations, including severe side-effects, low efficacy specially at the later chronic stage of the disease [21], and other important inconveniences such as the different response to the treatment registered among divergent *T. cruzi* strains [20]. Although benznidazole is the first-line treatment in most countries [17], the drawbacks of the current therapy make the search for new drugs urgently needed; it is especially necessary to find effective and safe compounds for the treatment of prevalent late chronic stage of the disease, where the effectiveness of benznidazole, despite some controversial reports [19,21], seems to be very low, especially to prevent heart damage progression [22].

In recent years many compounds exhibiting anti-*T. cruzi* activity, as well as different potential molecular targets of parasite, have been reported [21,23–26].

Antifungal azoles acting as sterols biosynthesis inhibitors such as posaconazole or ravuconazole seemed to be very promising candidates but recent clinical studies have been rather disappointing, showing that azoles are less effective than benznidazole, at least when used as monotherapy [15,27]. In fact, it has recently been pointed out that nitroheterocyclic derivatives still represent the only real alternative in the antichagasic fight [28].

Regarding trichomonosis, *T. vaginalis* (fam. Trichomonadidae) is the causative agent of this sexually transmitted infection (STI) accounting for over 50% of all curable STIs worldwide [29]. According to recent data from the World Health Organization, the number of new cases in adults in 2008 was estimated to be 276.4 million [30].

This protozoan is transmitted only through sexual contact. It is characterized by a wide range of clinical manifestations that can cause severe inflammation of genitourinary passages accompanied by a characteristic vaginal discharge, erythema, pruritus, dysuria, infertility or the formation of small lesions in the cervix. In men, the infection can result in non-gonococcal urethritis (NGU) and impaired sperm viability and motility [31,32]. Human trichomonosis has been associated with various complications such as problems during pregnancy, “low birth weight” (LBW), preterm births, etc. [33]. This infection also increases the risk of development of cervical [34] and prostate [35] neoplasia, and it is also related to a greater predisposition to co-infection with other STIs of bacterial or viral origin [36–38]. However, epidemiological studies have shown that at least half of the infected women and 80% of men do not show symptoms, becoming asymptomatic carriers and potential transmitters of this infection [39].

Trichomonosis is preferably treated with metronidazole, introduced to the market by 1960. Currently, metronidazole and tinidazole, both from the same family of 5-nitroimidazoles [40], are the only two drugs approved by the Food and Drug Administration (FDA) to treat this STI [41]. However, there are no effective alternatives for patients who develop side effects or hypersensitivity, or when its use is contraindicated. Moreover, it is estimated that approximately 5–10% of diagnosed cases of trichomonosis are caused by nitroimidazoles-resistant isolates [42,43].

Therefore, the search for alternative drugs for the treatment of both parasitic pathologies is urgently needed.

In the present study we have synthesized and phenotypically evaluated *in vitro* against *T. cruzi* and *T. vaginalis* two main series of new indazole derivatives: 1-substituted 2-benzyl-5-nitroindazolin-3-ones **6**–**29** (Scheme 1, series **A**) and 3-alkoxy-2-benzyl-2*H*-indazoles **30**–**37** (Scheme 1, series **B**), as well as some other products, i.e., bisindazoles **38** and **39**, and quinazolinones **40**–**43**, arising from the synthetic procedures.

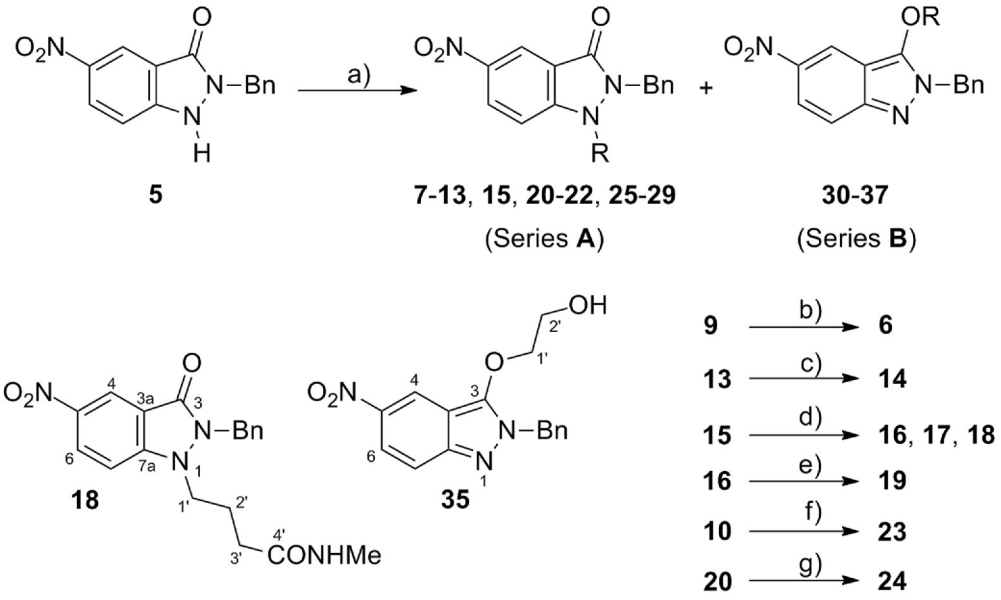
The preparation and study of both series of compounds were planned considering the previously reported activity against *T. cruzi* [5,6,8] and *T. vaginalis* [13] of related compounds containing different moieties at position 2 (Me, Ph, Bn, phenethyl and 2-naphthylmethyl) and alkyl or aryl substituents (Me, Pr, iPr, Bu, Pe, Bn and Ph) at position 1 of indazolin-3-one ring (related to series **A**) or at 3-*O* of 2*H*-indazol-3-ol system (related to series **B**). The most active compounds against *T. cruzi* epimastigotes (**1**–**3**; IC_{50} = 0.93–2.39 μM) [5] and *T. vaginalis* trophozoites (**4**; IC_{50} = 18.51 μM) [13] from previous studies are gathered in Fig. 1.

The current new compounds **6**–**37** have been designed to explore the antiprotozoal activity of indazoles containing at the mentioned 1 or 3-*O* positions unsaturated moieties, ω-substituted alkyl chains supporting different functional groups or acyl and sulfonyl residues. Taking in mind that in previous reports [5,6] 2-benzyl group led to the best antichagasic activities, this substituent was maintained in the current series **A** and **B**. In relation to physicochemical properties, some of these chemical modifications have also been planned in order to improve the low water solubility of the previously studied indazole derivatives [5].

2. Results and discussion

2.1. Chemistry

Many compounds gathered in Scheme 1 were obtained by alkylation of the previously reported [44] 2-benzyl-5-nitroindazolin-3-one **5** with the required functionalized halides. These alkylation reactions afforded, as previously described [5],



Reagents and conditions: a_1) for **7**, **9**/**30**, **10**/**31**, **15**/**34**, **20**/**35**, **21**/**36** and **22**/**37**: RBr, K_2CO_3, DMF, 100 ºC, 30 min-ca. 1 day, 31-91% (series **A**), 10-32% (series **B**); a_2) for **8**: RBr, K_2CO_3, acetone, reflux, 12 h, 54%; a_3) for **11**/**32** and **12**: RBr, $NaHCO_3$, acetone, 35 ºC, 5 days, 93% (**11**), 73% (**12**), 3% (**32**); a_4) for **13**/**33**: RBr, K_2CO_3, $BnBu_3N^+$ Br^-, toluene/water, 100 ºC, 2 days, 42% (**13**), 11% (**33**); a_5) for **25**, **26**, **28** and **29**: RCl, pyridine, rt, 1 h, 95-98%; a_6) for **27**: Ac_2O, 100 ºC, 3 h, 89%. b) piperidine, ethanol, reflux, 48 h, 93%. c) conditions a_4, 26%. d) for **16**: LiOH, THF/H_2O, rt, 12 h, 91%; for **17**: NH_3 (sat.)/MeOH, rt, 10 days, 98%; for **18**: $MeNH_2$/EtOH (8 M), rt, 24 h, 94%. e) $SOCl_2$, $CHCl_3$, reflux, 1 h, and then Me_2NH x HCl, K_2CO_3, water, rt, 12 h, 90%. f) NaOH, EtOH/H_2O, rt, 3 days, 71%. g) Ac_2O, 100 ºC, 2 h, 88%.

Compound	R	Compound	R
6	$CH{=}CH_2$	**18**	$[CH_2]_3CONHMe$
7	$CH_2CH{=}CH_2$	**19**	$[CH_2]_3CONMe_2$
8	$CH_2C{\equiv}CH$	**20**, **35**	$[CH_2]_2OH$
9, **30**	$[CH_2]_2Br$	**21**, **36**	$[CH_2]_3OH$
10, **31**	$[CH_2]_3Br$	**22**, **37**	$[CH_2]_2OMe$
11, **32**	CH_2COOMe	**23**	$[CH_2]_3OEt$
12	CH_2CN	**24**	$[CH_2]_2OAc$
13, **33**	$[CH_2]_2COOMe$	**25**	COOEt
14	$[CH_2]_2COOH$	**26**	COOBn
15, **34**	$[CH_2]_3COOEt$	**27**	Ac
16	$[CH_2]_3COOH$	**28**	Bz
17	$[CH_2]_3CONH_2$	**29**	Ts

Scheme 1. Synthesis of 1-substituted 2-benzyl-5-nitroindazolin-3-ones **6**–**29** and 3-alkoxy-2-benzyl-5-nitro-2*H*-indazoles **30**–**37**.

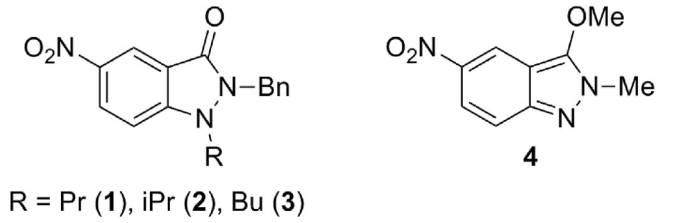


Fig. 1. Structure of previously studied antichagasic (**1**–**3**) and trichomonacidal (**4**) 5-nitroindazole derivatives.

mixtures of the corresponding 1,2-disubstituted indazolin-3-ones (**7**–**13**, **15** and **20**–**22**) and 3-alkoxy-2-alkyl-2*H*-indazoles (**30**–**37**); the latter were usually minor reaction products which in some cases were not isolated. Most alkylation reactions were carried out in potassium carbonate/DMF at 100 °C. Under these conditions, however, alkylation with methyl bromoacetate or bromoacetamide afforded quinazolinones **40** and **41**, respectively (Scheme 2). This rearrangement, due to the acidity of CH_2 protons of methyl acetate or acetamide moieties, has been previously observed for other 1,2-disubstituted indazolinones [45,46] and for indazole-derived betaines [47]. Since the alkylation reactions were carried out with an excess of the corresponding bromoacetic acid derivative (methyl ester or amide), further alkylation of compounds **40** and **41** at position 1 to yield derivatives **42** and **43** took also place. Nevertheless, alkylation of compound **5** with methyl bromoacetate or bromoacetonitrile in sodium hydrogen carbonate/acetone at 35 °C afforded the desired compounds **11**/**32** and **12**, respectively.

Propargylation reaction leading to compound **8** gave best results working in potassium carbonate/refluxing acetone; during alkylation of compound **5** to derivatives **13**/**33** with methyl 3-bromopropionate in DMF at 100 °C, dehydrogenation of the latter to methyl acrylate was observed; best results were obtained in potassium carbonate/toluene/water under phase transfer catalysis; nevertheless, large amounts of the corresponding acid **14**, arising from hydrolysis of ester moiety of compound **13**, were also produced.

From the reaction of **5** with 1,2-dibromoethane, *O*,*O*'- (**38**) and *O*,*N*$_1$'-bisindazole (**39**) derivatives (Fig. 2) could also be isolated.

On the other hand, treatment of compound **5** with the required

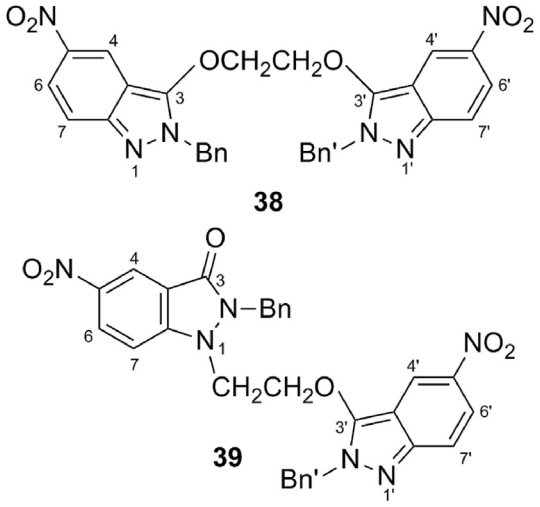
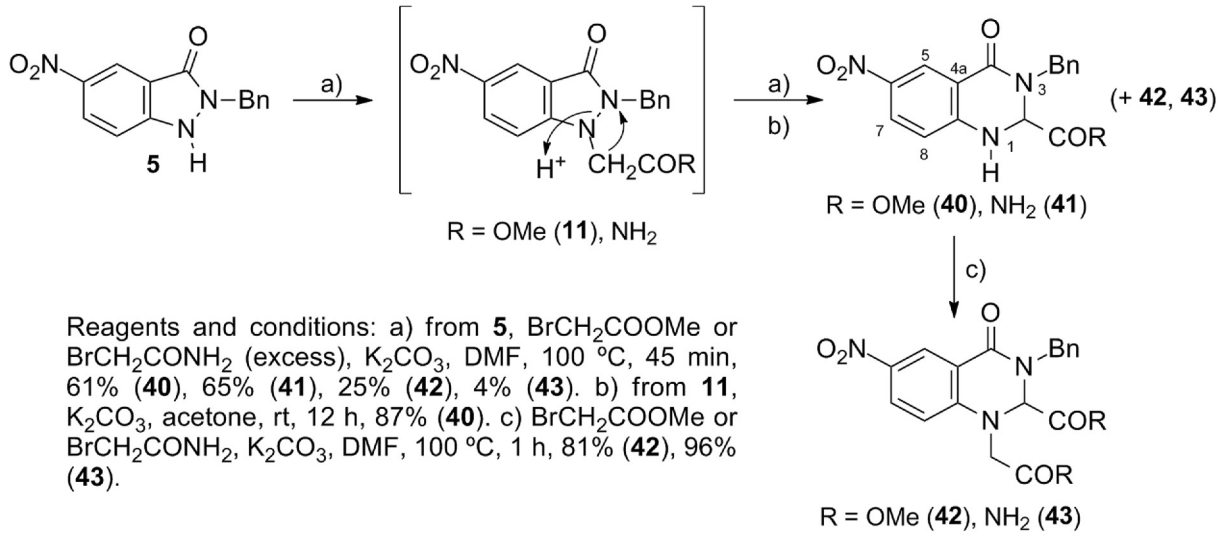


Fig. 2. Structure of bisindazole derivatives **38** and **39**.

chloroformates or with the corresponding acyl or sulfonyl chlorides in pyridine afforded 1-alkoxycarbonyl- (**25**, **26**), 1-benzoyl- (**28**) and 1-tosyl- (**29**) indazolinones. 1-Acetyl derivative **27** was prepared from compound **5** and acetic anhydride. In agreement with previous reports, only the corresponding 1,2-disubstituted derivatives could be isolated from these processes [45].

Compounds **6**, **14**, **16**–**19**, **23** and **24** (Scheme 1) were obtained following different procedures from 1,2-disubstituted indazolinones arising from the above mentioned alkylation reactions. Thus, 1-vinyl derivative **6** was prepared from 2-bromoethyl derivative **9** through a dehydrohalogenation reaction. Butyric acid derivative **16** was obtained by hydrolysis (LiOH) of ester **15**. Butyramide **17** and *N*-methylbutyramide **18** were obtained by treatment of ethyl ester **15** with ammonia or methylamine, respectively; *N*,*N*-dimethylbutyramide **19** was prepared from the chloride ($SOCl_2$) of acid **16** and dimethylamine. 3-Ethoxypropyl derivative **23** was isolated from an attempted preparation of 3-hydroxypropyl derivative **21** from the corresponding bromide **10** and sodium hydroxide in ethanol/water. Finally, 2-acetoxyethyl derivative **24** was prepared by acetylation with acetic anhydride of the corresponding 2-hydroxyethyl compound **20**.



Scheme 2. Synthesis of 6-nitroquinazolin-4-ones **40**–**43**.

The corresponding mixtures of isomeric 1-substituted 2-benzylindazolin-3-ones (series **A**) and 3-alkoxy-2-benzyl-2*H*-indazoles (series **B**) obtained in some case (pairs **9**/**30**, **10**/**31**, **11**/**32**, **13**/**33**, **15**/**34**, **20**/**35**, **21**/**36** and **22**/**37**), as well as some byproducts (e.g., **38** and **39**), were easily separated by column chromatography; the same procedure was used to purify crude compounds (**8**, **12**, **17** and **23**) containing minor impurities. In most cases, products included in this work are crystalline solids which were easily purified by recrystallization from an appropriate solvent.

The structure of all compounds has been established on the basis of analytical and spectral data. The patterns of ^{1}H and ^{13}C NMR signals of indazole ring found for compounds of series **A** and for the isomeric derivatives of series **B** are rather different, in agreement with previously reported data [5]. Furthermore, signals of 1-CH_2 and Bn CH_2 protons of compounds **A** always appear, respectively, at higher field than those of OCH_2 and Bn CH_2 protons of the corresponding isomers **B**. On the other hand, NMR signals of quinazolin-4-one **40** are also rather different from those of its indazole-derived isomers **11** (series **A**) and **32** (series **B**); a remarkable feature of compounds **40**–**43** is that their Bn CH_2 protons, as well as 1-CH_2 protons of **42** and **43**, are anisochronic and have been distinguished in the description of ^{1}H NMR spectra as H_A and H_B.

2.2. Biology

2.2.1. In vitro evaluation of anti-T. cruzi activity

In vitro assays against *T. cruzi* CL Brener strain (DTU TcVI) were performed according to a sequential protocol of drug screening in which activity against epimastigotes (extracellular insect vector stage of parasite), unspecific cytotoxicity against L929 murine fibroblasts (amastigotes host cells) and selectivity indexes (SI) are simultaneously evaluated. Compounds with selectivity indexes (SI) on epimastigotes similar or higher than that of benznidazole, were selected for further testing of activity on amastigotes (intracellular mammalian host forms) [48].

In vitro activity of compounds **6**–**43** against epimastigotes and amastigotes of *T. cruzi* [49,50], unspecific cytotoxicity against murine fibroblasts and their corresponding selectivity indexes (SI) are collected in Table 1. These compounds as well as the standard drug benznidazole were assayed at concentrations of 0.125–256 μM and from the obtained data IC_{50} values shown in Table 1 were calculated. It can be seen that most 3-alkoxy-2-benzylindazoles (**30**–**37**) exhibited poor activity towards epimastigotes, while many 1,2-disubstituted indazolinones were much more efficient that the reference drug benznidazole (IC_{50} = 22.73 μM). Special mention deserve indazolinones containing at position 1 unsaturated groups such as vinyl (**6**) or allyl (**7**), or ω-substituted alkyl groups such as 2-bromoethyl (**9**), 3-bromopropyl (**10**), 3-(dimethylcarbamoyl)propyl (**19**), 2-hydroxyethyl (**20**) and 3-hydroxypropyl (**21**), with IC_{50} = 1.58–4.19 μM. On the other hand, indazolinones with a carboxylic acid side-chain (**14**, **16**), those acylated at position 1 (**27**, **28**) and quinazolinones (**40**–**43**), do not exhibit significant activity. The low antichagasic efficiency of the latter against epimastigotes of *T. cruzi* Dm28c clone (DTU TcI) has previously been observed [51].

Most compounds showing high activity against epimastigotes (**6**, **7**, **9**, **19**, **20** and **21**) display low unspecific cytotoxicity against murine fibroblasts, giving SI (46 to > 162) much higher than that of the reference drug benznidazole (SI > 11.26).

In relation to activities and selectivity indexes against amastigotes, 2-benzyl-1-(ω-hydroxyalkyl)indazolinones **20** and **21** as well as acetoxy derivative **24** (IC_{50} = 0.22–0.25 μM; SI > 1024) were also more effective than benznidazole (IC_{50} = 0.68 μM; SI > 376.47) (Table 1). Taking in mind the metabolic instability of ester moiety, 2-acetoxyethyl derivative **24** may act as a prodrug of 2-hydroxyethyl compound **20**. It is important to consider that amastigotes are the intracellular forms present in the mammalian host cells, thus the high efficiency of these indazolinones is especially relevant from the point of view of human disease.

Since 1-(2-hydroxyethyl) derivative **20** achieved the best trypanocidal profile on both epimastigotes (IC_{50} = 1.58 μM) and intracellular amastigotes (IC_{50} = 0.22 μM) of CL-B5, further *in vitro* studies were carried out to explore its potential activity on other *T. cruzi* strains. The Tulahuen strain was selected to confirm the activity of **20** on parasites belonging to DTU TcVI (drug-sensitive *T. cruzi*), as well as the Y strain (DTU TcII) to evaluate it over moderately drug-resistant parasites [52]. The obtained results (Table 2) show that this compound was rather more efficient than benznidazole against epimastigotes of Y strain; however, its activity against the clinically significant forms, i.e., amastigotes of both strains of *T. cruzi*, was only slightly higher than that of the reference drug. Nevertheless, according to recently proposed criteria [53], compounds displaying IC_{50} values lower than 10 μM on intracellular amastigotes of DTUs TcII and TcVI, may be considered at an initial stage of the drug pipeline as promising hits for Chagas disease chemotherapy. Moreover, the low unspecific toxicity (LC_{50} > 100 μM, Table 2) of compound **20** against cardiac cells was also determined. The absence of cardiotoxicity characteristics supports the specific trypanocidal activity of compound **20**, according to the importance this target cell has in the pathology of Chagas disease [8].

In previous works on related 1,2-disubstituted indazolinones we found that these compounds do not support much variation at position 2, benzyl group being the best substituent [5,6]; however, in relation to position 1, our current findings demonstrate that simple alkyl groups (Me, Pr, iPr, Bu and Pe) present in the previously reported antichagasic indazolinones are not strictly necessary, and that a large number of differently substituted groups can be accept at this position without considerable loss of activity. This fact may allow in the future improving the physicochemical and pharmacokinetic properties of 5-nitroindazolinones, especially their low solubility in water.

2.2.2. In vitro evaluation of anti-T. vaginalis activity

Initial studies of activity against *T. vaginalis* were carried out on trophozoites of the metronidazole-sensitive JH31A#4 isolate. The studied compounds were assayed at concentrations of 9.37–300 μM, and the activity results (IC_{50} values) are collected in Table 3. Activity of all compounds was lower than that of the reference drug metronidazole (IC_{50} = 1.43 μM), but some of them showed a moderate to good trichomonacidal activity. Indazoles showing IC_{50} < 50 μM included 1,2-disubstituted indazolinones **19** and **20** (series **A**) and 3-alkoxy-2-alquilindazoles **31** and **33**–**36** (series **B**). Compounds **35** and **36**, 3-(2-hydroxyethoxy)- and 3-(3-hydroxypropoxy)indazole derivatives, had a relevant activity against the parasite with IC_{50} values of 9.82 and 7.25 μM, respectively. These results are in agreement with the previously reported [13] potential of 3-alkoxy-2-alkyl-2*H*-indazole scaffold in the field of trichomonacidal drugs. Other compounds, including quinazolinones **40**–**43**, had low activity or even showed no effect on the parasite growth and, in this case, their IC_{50} values could not be calculated (Table 3).

Further cytotoxicity studies against Vero cells as well as the determination of the corresponding selectivity indexes (SI) were only conducted for most indazoles displaying IC_{50} < 50 μM. The data, also included in Table 3, show that compounds **19**, **20**, **31**, **33**, **35** and **36** display low unspecific cytotoxicity, with percentages of inhibition of Vero cells growth <20% at 300 μM and SI values between >6.1 and > 41.4. Once again, 3-alkoxy-1-alkyl-2*H*-indazoles **35** and **36** (series **B**), which showed very low percentages of cytotoxicity (%C) at the highest concentration tested (300 μM) (%

Table 1
In vitro activities against *T.cruzi* CL-B5 epimastigotes and amastigotes (IC_{50}), unspecific cytotoxicities against murine fibroblasts (LC_{50}) and selectivity indexes (SI) found for compounds **6**–**43**.

Compound	IC_{50} epimastigotes (μM)[a]	LC_{50} fibroblasts (μM) [a]	SI[b] epimastigotes	IC_{50} amastigotes (μM) [a]	SI[c] amastigotes
6	2.75 ± 0.09	160.46 ± 14.89	58.35	0.47 ± 0.10	341.40
7	4.19 ± 0.95	192.36 ± 21.85	45.91	1.58 ± 0.60	121.75
8	10.79 ± 4.80	195.27 ± 9.70	18.10	3.02 ± 0.55	64.66
9	2.13 ± 0.72	>256	>120.19	1.14 ± 0.14	>224.56
10	2.90 ± 0.37	25.19 ± 3.16	8.69	–	–
11	26.17 ± 11.31	128.89 ± 10.32	4.92	–	–
12	11.66 ± 2.13	>256	>21.95	3.50 ± 0.83	>73.14
13	9.19 ± 1.20	>256	>27.86	2.93 ± 1.68	>87.37
14	>256	>256	ND	–	–
15	11.14 ± 0.59	78.07 ± 8.02	7.01	–	–
16	>256	>256	ND	–	–
17	30.98 ± 3.94	>256	>8.26	–	–
18	13.29 ± 0.92	>256	>19.26	1.50 ± 0.23	>170.67
19	3.86 ± 0.37	>256	>66.32	0.54 ± 0.04	>474.07
20	1.58 ± 0.06	>256	>162.02	0.22 ± 0.06	>1163.64
21	1.68 ± 0.36	>256	>152.38	0.25 ± 0.12	>1024
22	6.56 ± 0.87	>256	>39.02	2.29 ± 0.79	>111.79
23	6.57 ± 0.27	170.41 ± 9.94	25.94	3.31 ± 0.52	51.48
24	5.43 ± 1.89	>256	>47.14	0.25 ± 0.14	>1024
25	49.01 ± 14.58	122.03 ± 15.98	2.49	–	–
26	43.59 ± 5.41	86.06 ± 13.66	1.97	–	–
27	>256	>256	ND	–	–
28	>256	145.52 ± 6.10	<0.57	–	–
29	28.76 ± 2.67	>256	>8.90	–	–
30	>256	>256	ND	–	–
31	39.04 ± 13.42	59.04 ± 4.61	1.51	–	–
32	144.71 ± 9.30	>256	>1.77	–	–
33	>256	>256	ND	–	–
34	25.05 ± 1.75	>256	>10.22	25.36 ± 4.50	>10.09
35	>256	>256	ND	–	–
36	64.91 ± 8.34	>256	>3.94	–	–
37	>256	>256	ND	–	–
38	>256	>256	ND	–	–
39	>256	>256	ND	–	–
40	216.34 ± 14.99	>256	>1.18	–	–
41	>256	>256	ND	–	–
42	>256	205.14 ± 17.32	<0.8	–	–
43	>256	>256	ND	–	–
Benznidazole	22.73 ± 3.33	>256	>11.26	0.68 ± 0.08	>376.47

–: Not evaluated against amastigotes due to a SI on epimastigotes lower than that of benznidazole or a SI which could not be calculated (ND) owing to the very low activity on epimastigotes.
ND: Not determined owing to the low activity values (IC_{50} epimastigotes > 256 μM).
[a] Results of IC_{50} and LC_{50} are expressed as the mean ± SD.
[b] Selectivity indexes for epimastigotes (SI = LC_{50} L929/IC_{50} epimastigotes).
[c] Selectivity indexes for amastigotes (SI = LC_{50} L929/IC_{50} amastigotes).

Table 2
In vitro activities (IC_{50}) of compound **20** on intracellular amastigotes of Tulahuen (DTU TcVI), epimastigotes and intracellular amastigotes of Y strain (DTU TcII) and toxicities (LC_{50}) on primary cultures of cardiac cells.

Compound	IC_{50} Tulahuen amastigotes (μM)[a]	IC_{50} Y strain epimastigotes (μM)[a]	IC_{50} Y strain amastigotes (μM)[a]	LC_{50} cardiac cells (μM)
20	0.81 ± 0.29	3.18 ± 0.37	0.60 ± 0.03	>100
Benznidazole	1.66 ± 0.10	28.11 ± 1.77	1.77 ± 1.11	>100

[a] Results of IC_{50} are expressed as the mean ± SD.

C_{35} = 14.7 ± 4.3; $\%C_{36}$ = 14.5 ± 3.2), were the most interesting compounds.

Indazoles with the highest SI values against *T. vaginalis* JH31A#4, **31**, **35** and **36**, were then assayed against the metronidazole-resistant isolate IR78 following similar protocols and the obtained results are gathered in Table 4. Values found for compound **36** (IC_{50} = 9.11 μM; SI > 32.9) are very similar to those obtained for the metronidazole-sensitive isolate (IC_{50} = 7.25 μM; SI > 41.4), evidencing the lack of cross-resistance between this compound and the reference drug. Thus, compound **36** can be considered a good prototype for the future development of drugs against *T. vaginalis* isolates resistant to 5-nitroimidazoles.

2.3. Physicochemical and pharmacokinetic parameters, electrostatic potential and structure–activity relationships

In order to get further insights onto the new indazole derivatives of both series **A** and **B**, some physicochemical and pharmacokinetic parameters (QikProp software), as well as the electrostatic potentials maps (Spartan software) of selected molecules, were calculated (Supplementary material, Table S1 and Fig. S1).

Neither indazolin-3-one derivatives (series **A**) nor 3-alkoxy-5-nitroindazoles (series **B**) violate Lipinski's and Jorgensen's rules. The calculated predictions suggest a good human oral bioavailability [71–100%, except for acids **14** (57%) and **16** (64%), and for

Table 3
In vitro activities against *T. vaginalis* JH31A#4 trophozoites (IC_{50}), unspecific cytotoxicities against Vero cells (CC_{50}) and selectivity indexes (SI) found for compounds **6**–**31**, **33**–**37** and **40**–**43**.

Compound	IC_{50} (μM)[a]	CC_{50} (μM)	SI[b]	Compound	IC_{50} (μM)[a]	CC_{50} (μM)	SI[b]
6	258.43 [200.15–363.67]	–	–	**24**	265.63 [196.68–402.26]	–	–
7	358.86 [197.50–498.70]	–	–	**25**	418.86 [324.24–615.79]	–	–
8	260.97 [171.51–500.54]	–	–	**26**	588.88 [386.67–733.84]	–	–
9	347.74 [242.80–587.54]	–	–	**27**	212.91 [174.24–271.41]	–	–
10	80.21 [61.22–112.81]	–	–	**28**	197.42 [130.21–326.63]	–	–
11	534.57 [406.21–843.18]	–	–	**29**	151.47 [101.14–289.79]	–	–
12	ND	–	–	**30**	64.75 [45.00–96.83]	–	–
13	375.54 [304.84–507.72]	–	–	**31**	18.57 [12.98–24.49]	538.85[c]	29.0
14	ND	–	–	**33**	43.02 [25.11–71.78]	>300	>7.0
15	192.42 [120.05–429.96]	–	–	**34**	20.63 [17.08–24.54]	–	–
16	ND	–	–	**35**	9.82 [8.58–11.11]	>300	>30.5
17	104.13 [91.47–119.55]	–	–	**36**	7.25 [6.49–8.04]	>300	>41.4
18	182.01 [151.62–227.51]	–	–	**37**	107.45 [97.90–117.89]	–	–
19	48.94 [38.82–61.41]	>300	>6.1	**40**	ND	–	–
20	17.94 [14.30–21.68]	>300	>16.7	**41**	ND	–	–
21	128.44 [110.59–152.07]	–	–	**42**	58.20 [40.78–84.25]	–	–
22	284.62 [162.44–402.26]	–	–	**43**	ND	–	–
23	362.18 [237.01–515.79]	–	–	Metronidazole	1.43 [1.13–1.79]	>600	>100

–: Not evaluated against Vero cells owing to the low IC_{50} values.
ND: IC_{50} values could not be determined owing to the very low activity.
Values of growth inhibition used to calculate IC_{50} and CC_{50} displayed a standard deviation of less than 10%.
[a] In brackets, 95% confidence intervals.
[b] Selectivity indexes (SI = CC_{50} Vero cells/IC_{50} trophozoites).
[c] 95% confidence interval: 399.77–942.38.

Table 4
In vitro activities against *T. vaginalis* IR78 trophozoites (IC_{50}), unspecific cytotoxicities against Vero cells (CC_{50}) and selectivity indexes (SI) found for compounds **31**, **35** and **36**.

Compound	IC_{50} (μM)[a]	CC_{50} (μM)	SI[b]
31	39.12 [34.24–44.55]	538.85[c]	13.8
35	49.82 [24.21–71.01]	>300	>6.0
36	9.11 [7.16–11.48]	>300	>32.9
Metronidazole	5.78 [4.38–9.43]	>600	>100

Values of growth inhibition used to calculate IC_{50} and CC_{50} displayed a standard deviation of less than 10%.
[a] In brackets, 95% confidence intervals.
[b] Selectivity indexes (SI = CC_{50} Vero cells/IC_{50} trophozoites).
[c] 95% confidence interval: 399.77–942.38.

amide **17** (55%)] and they do not detect side effects associated with CNS (supplementary material, Table S1).

We have previously published that many 1,2-disubstituted indazolinones (series **A** analogues) are efficient against *T. cruzi*, while most isomeric 2-substituted 3-alkoxy-2*H*-indazoles (series **B** analogues) show no antichagasic activity [5]. As commented in section 2.4, antiparasitic nitroheterocycles are in fact prodrugs activated by the parasites metabolism after bioreduction. Taking in mind that the reduction potentials corresponding to the formation of nitro anion radicals in aprotic solvents are similar for the previously described analogues of series **A** (−1.04 to −1.10 V) and **B** (−1.13 to −1.22 V) [54], it is likely that the former are better substrates for trypanosome nitroreductases than the latter. In fact, the structures and properties of both kinds of compounds, derived from two distinct indazole tautomers, are rather different; e.g., derivatives of series **B** (log P = 3.09–5.09) are always significantly more lipophilic than the corresponding isomers of series **A** (log P = 1.38–3.39) according to the calculated log P values (e.g., 1.52 for **20** vs 3.09 for **35**) (supplementary material, Table S1). Compounds of both series show also clearly distinct electrostatic potential maps, e.g., **11**, **20** and **27** (series **A**) vs **35** (series **B**) (supplementary material, Fig. S1).

In relation to trypanocidal properties, one of the aims of the current work was to obtain indazolinone derivatives with improved water solubility; in this sense, values of calculated log S show that some of the more efficient antichagasic 1,2-disubstituted derivatives (e.g., **6**, **7**, **19**, **20**, **22** and **23**; log S = −2.53 to −3.26) are more water soluble than the previously reported [5] 2-benzylindazolinones carrying propyl- (**1**), isopropyl- (**2**) or butyl (**3**) substituents at position 1 (log S = −3.51 to −4.00) (Supplementary material, Table S1).

Among compounds of series **A**, we have not found a clear relationship between lipophilicity and trypanocidal properties; in fact anti-epimastigote activity of 3-bromopropyl derivative **10**, one of the more lipophilic compounds (IC_{50} = 2.90 μM; log P = 3.39), is only slightly lower than that of 2-hydroxyethyl derivative **20**, one of the less lipophilic derivatives (IC_{50} = 1.58 μM; log P = 1.52).

On the contrary, electron-donating or -withdrawing properties of substituents at position 1 of series **A** derivatives seem to play a crucial role with regard to activity. In fact, indazolinones containing at this position strong electron-withdrawing substituents, with the higher values of Taft's polar substituent constants (σ^*), e.g., COOEt (**25**; σ^* = 2.26), Ac (**27**; σ^* = 1.81) or Bz (**28**; σ^* = 2.20) moieties, show little or no activity (Supplementary material, Table S1). Compounds containing groups with intermediate σ^* values, e.g., CH_2COOMe (**11**; σ^* = 1.06) or CH_2CN (**12**; σ^* = 1.30) display moderate activity and, finally, the most active compounds support substituents with low σ^* values, e.g., CH=CH_2 (**6**; σ^* = 0.56), CH_2CH=CH_2 (**7**; σ^* = 0.00), $[CH_2]_2Br$ (**9**; σ^* = 0.41), $[CH_2]_2OH$ (**20**; σ^* = 0.09) or $[CH_2]_3OH$ (**21**; σ^* = −0.04). The low antichagasic activities observed for acids **14** and **16** and for amide **17**, despite the appropriate values of σ^*, are probably due to pharmacokinetic issues; compound **17** has the lowest calculated value of log P, and **14** and **16** must be ionized in the culture medium (for epimastigotes, LIT medium; pH = 7.4), this hindering the penetration across the parasite membranes.

The molecular electrostatic potential maps of selected compounds from series **A** (**11**, **20** and **27**) show an overall similarity for all derivatives. In a more specific analysis, subtle changes in the electronic density of the carbonyl oxygen can be observed depending on substituents at position 1 (Supplementary material, Fig. S1). Thus, the carbonyl oxygen of the inactive 1-acetyl

derivative **27** presents lower maximum values of electronegativity potential (−161.4 kJ/mol) than the oxygen of the moderately and highly active compounds **11** (−201.2 kJ/mol) and **20** (−191.0 kJ/mol), respectively. These differences suggest that 3-CO group of these compounds participates in some important interaction in the active site of nitroreductases, which is affected by the electronegativity of the carbonyl oxygen.

Regarding trichomonacidal activity, the best compounds, 3-(ω-hydroxyalkoxy)indazoles **35** and **36** (series **B**), are more active against JH31A#4 isolate than the previously described and more hydrophilic analogue **4** [13] (supplementary material, Table S1). Unfortunately, only a few products have shown activity among derivatives of series **B**, and these limited data do not allow us to establish a structure–activity relationship.

2.4. Considerations on the mode of action of antiparasitic nitroheterocycles

Classical antichagasic drugs have been accepted to act, after initial metabolic reduction by trypanosome nitroreductases, through the final production of reactive oxygen species (ROS) (nifurtimox) or electrophilic metabolites (benznidazole), able to damage essential biomolecules of parasites such as thiols, lipids, proteins, DNA, etc. [55,56]. However, a report showed that the induction of oxidative stress in *T. cruzi* by nifurtimox was not able to explain its activity [57]. Effectively, it has recently been proposed that the trypanocidal effects of nifurtimox can be attributed to the reactivity of an open chain α,β-unsaturated nitrile arising from reductive and hydrolytic metabolism, probably acting as a Michael acceptor [58]. On the other hand, it has been suggested that the antichagasic effects of benznidazole probably rely on the combined effects of several of its metabolites, especially those of the final product, the highly reactive dialdehyde glyoxal, able to produce adducts, e.g., with guanosine [59]; furthermore, the detection of covalent conjugates of reduced metabolites of benznidazole with low molecular weight thiols (e.g., cysteine, glutathione, trypanothione, etc.) and other parasite biomolecules has recently been published; in this study, low molecular weight glyoxal-derived adducts could not be however detected [60].

Regarding trichomonacidal agents, it has been assumed that metronidazole and other nitroimidazole drugs act against *T. vaginalis* after reduction of NO_2 group at hydrogenosomal level; this process has been suggested to be carried out by reduced ferredoxin, which is in turn produced by pyruvate:ferredoxin oxidoreductase or by NAD:ferredoxin oxidoreductase [61]. Furthermore, the involvement of a flavin-dependent thioredoxin reductase displaying nitroreductase activity towards nitromidazoles has recently been proposed [62]. It has been reported that the reduction of metronidazole yields nitro radicals and, especially, cytotoxic intermediates leading to the formation of adducts with parasite proteins and to DNA disruption. A recent study has shown that metronidazole and tinidazole metabolites deplete intracellular thiol levels, and that metabolite-protein adducts are not formed indiscriminately; in fact, only ten non-hydrogenosomal proteins, most of them associated with thioredoxin-mediated redox regulation, seem to be mainly affected [62].

The mode of action of the present 5-nitroindazole derivatives has not been studied in detail, but for several antiprotozoal 1,2-disubstituted indazolin-3-ones and 1-substituted indazol-3-ols the involvement of 5-nitro group seems evident according to previous reports [5,12,13]. A study of electrochemical and enzymatic reduction of related 1,2-disubstituted indazolinones (analogues of series **A**) and 2-substituted 3-alkoxyindazoles (analogues of series **B**) [54] suggested that these compounds could induce oxidative stress in the parasites, i.e., a mode of action similar to that initially accepted for nifurtimox. On the other hand, it has also been proposed that the inhibition of trypanothione reductase [63,64] or the interference with glycosomal or mitochondrial enzymes involved in the catabolism of *T. cruzi* [7] could contribute to the activity of other 5-nitroindazole derivatives with different substitution patterns. According to these discrepant proposals and taking in consideration the mentioned recent studies carried out with the antichagasic and trichomonacidal reference drugs, it is evident that the mode of action of our indazole derivatives needs to be investigated more thoroughly.

3. Conclusions and future outlooks

The previously proposed antichagasic potential of 1,2-disubstituted 5-nitroindazolin-3-one scaffold [5,6,8] is confirmed in the current study. Compounds **20**, **21** and **24** (series **A**) are very efficient against amastigotes of *T. cruzi* CL Brener strain ($IC_{50} < 1$ μM, $SI > 1000$) and, additionally, the former displays activities of the same order against amastigotes of Tulahuen and Y strains. Although a 2-benzyl group seems convenient for optimized activity [5], many substituents, e.g. unsaturated chains or ω-substituted alkyl moieties, are accepted at position 1 without severe loss of activity. Inactive compounds are the result of strong electron-withdrawing substituents at position 1, e.g., acyl groups, or the presence of a carboxylic acid moiety in the side chain, i.e., compounds **14** and **16**, which leads to unsuitable pharmacokinetic issues.

In what refers to trichomonacidal activity, some of the new 3-alkoxy-1-alkyl-2*H*-indazoles (series **B**) have shown moderate efficiency. Compound **36** displays similar activity for a metronidazole-sensitive (JH31A#4) and for a metronidazole-resistant (IR78) isolate of *T. vaginalis* ($IC_{50} = 7.25$ and 9.11 μM, respectively). Thus, 3-alkoxy-1-alkyl-2*H*-indazole can be considered a good scaffold for further chemical modifications directed to treat infections caused by 5-nitroimidazoles-resistant parasites. Unfortunately, in this case, the structure–activity relationship is not clear, and much work remains to be done to understand the effect of the different substituents on trichomonacidal activity.

In order to better understand the structure–activity relationships and the mode of action of nitroindazoles, further studies are underway aimed to study the biological effect of some analogues carrying the nitro group at different positions, or supporting other substituents including ω-aminoalkyl chains directed to improve their activity and physicochemical and pharmacokinetic properties, or even containing fluorescent moieties potentially able to label storage sites and/or the organelles constituting the primary cellular target of the studied compounds. On the other hand, taking into account the recently published outstanding articles on the mode of action of nifurtimox [58], benznidazole [59,60] and metronidazole [62], studies will be directed to the identification of products arising from the bioreduction of 5-nitroindazoles mediated by reductases and also to the detection of potential adducts of nitroindazole metabolites with parasites biomolecules.

4. Experimental section

4.1. Chemistry

4.1.1. General methods

Melting points (mp) were determined in a Stuart Scientific melting point apparatus SMP3. ^1H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded at room temperature (~20 °C) on a Bruker Avance 300 spectrometer. The chemical shifts are reported in ppm from TMS (δ scale) but were measured against the solvent signal. The assignments have been performed by means of different standard homonuclear and heteronuclear correlation experiments

(NOE, gHSQC and gHMBC). Numbering used in the description of NMR spectra is indicated in Schemes 1 and 2 and in Fig. 2. Electron impact (EI) and electrospray (ES^+) mass spectra were obtained on a Hewlett Packard 5973 MSD (70 eV) and on a Hewlett Packard 1100 MSD spectrometer, respectively. DC-Alufolien silica gel 60 PF_{254} (Merck, layer thickness 0.2 mm) was used for TLC, and silica gel 60 (Merck, particle size 0.040–0.063 mm) for flash column chromatography. Solvents and reagents were obtained from different commercial sources and used without further purification. Microanalyses were performed on a Heraeus CHN-O-RAPID analyzer and were within ±0.3% of the theoretical values.

4.1.2. Alkylation of 2-benzyl-5-nitroindazolin-3-one ***5***

*4.1.2.1. Preparation of allyl (**7**), 3-(ethoxycarbonyl)propyl (**15**/**34**), 2-hydroxyethyl (**20**/**35**), 3-hydroxypropyl (**21**/**36**) and 2-methoxyethyl (**22**/**37**) derivatives.* A stirred mixture of the starting 2-benzylindazolinone **5** [44] (1.00 g, 3.71 mmol), the required bromide (4.00 mmol) and potassium carbonate (0.55 g, 4.00 mmol) in DMF (20 mL) was heated at 100 °C until completion of reaction [TLC; 30 min (for **7**), 12 h (for **22**/**37**) and 12 h followed by 1–3 additions of further amounts of bromide (0.3 mmol) and, eventually, if needed, base (0.3 mmol) each 6 h (for **15**/**34**, **20**/**35** and **21**/**36**)]. The mixture was then evaporated to dryness and, after addition of water (200 mL), extracted with chloroform (3 × 50 mL). For compound **7**, the organic phase was dried ($MgSO_4$) and concentrated to dryness; the residue was triturated with 2-PrOH (10 mL) and the insoluble material recovered by filtration and air-dried. For the remaining compounds, the concentrated chloroform phase was applied to the top of a chromatography column which was eluted with chloroform/acetone mixtures (50:1 to 25:1) (for **15**/**34** and **22**/**37**) or with the same mixtures (10:1 to 5:1) followed by chloroform/methanol (50:1 to 25:1) (for **20**/**35** and **21**/**36**). In all cases, 3-alkoxy-2-benzyl-2*H*-indazoles (**34**–**37**) eluted first, followed by the corresponding 1-substituted 2-benzylindazolinones (**15**, **20**–**22**).

Spectral and analytical data of compounds **15**, **21**, **22**, **34**, **36** and **37** are included as supplementary material.

*4.1.2.1.1. 1-Allyl-2-benzyl-5-nitro-1,2-dihydro-3H-indazol-3-one (**7**).* Yield: 1.04 g (91%). Mp 137–139 °C (2-PrOH). The preparation of this compound from the corresponding 1-allylindazol-3-ol and benzyl bromide has been claimed in a patent [65]; however, mp is not given and the reported ^{1}H NMR spectrum is not in agreement with that recorded by us in the same solvent. ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.51 (d, J = 2.1 Hz, 1H, 4-H), 8.38 (dd, J = 9.0, 2.1 Hz, 1H, 6-H), 7.70 (d, J = 9.0 Hz, 1H, 7-H), 7.26 (m, 5H, Bn aromatic H), 5.48 (m, 1H, 2′-H), 5.21 (dd, J = 17.1, 1.1 Hz, 1H, 3′-H_{trans}), 5.15 (s, 2H, Bn CH_2), 5.10 (dd, J = 10.5, 1.1 Hz, 1H, 3′-H_{cis}), 4.67 (d, J = 6.0 Hz, 2H, 1′-H); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 161.0 (C-3), 149.3 (C-7a), 141.7 (C-5), 136.1 (Bn C-1), 130.3 (C-2′), 128.6 (Bn C-3, -5), 127.8 (Bn C-4), 127.4 (Bn C-2, -6), 127.2 (C-6), 120.3 (C-4, -3′), 116.4 (C-3a), 112.6 (C-7), 49.4 (C-1′), 44.8 (Bn CH_2); MS (EI): *m/z* (%) 309 (100) (M^+), 268 (19), 232 (3), 218 (2), 164 (3), 131 (4), 103 (7). Anal. $C_{17}H_{15}N_3O_3$ (C, H, N).

*4.1.2.1.2. 2-Benzyl-1-[3-(ethoxycarbonyl)propyl]-5-nitro-1,2-dihydro-3H-indazol-3-one (**15**).* Yield: 1.00 g (70%). Oil which solidifies on standing; mp 51–53 °C.

*4.1.2.1.3. 2-Benzyl-1-(2-hydroxyethyl)-5-nitro-1,2-dihydro-3H-indazol-3-one (**20**).* Yield: 0.91 g (78%). Mp 137–139 °C (2-PrOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.49 (d, J = 2.1 Hz, 1H, 4-H), 8.31 (dd, J = 9.3, 2.1 Hz, 1H, 6-H), 7.60 (d, J = 9.3 Hz, 1H, 7-H), 7.29 (m, 3H) and 7.20 (m, 2H) (Bn aromatic H), 5.20 (s, 2H, Bn CH_2), 4.73 (t, J = 5.1 Hz, 1H, OH), 4.12 (t, J = 4.9 Hz, 2H, 1′-H), 3.45 (m, 2H, 2′-H); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 160.9 (C-3), 149.2 (C-7a), 140.7 (C-5), 136.1 (Bn C-1), 128.7 (Bn C-3, -5), 127.8 (Bn C-4), 127.2 (Bn C-2, -6), 126.4 (C-6), 120.3 (C-4), 114.6 (C-3a), 112.4 (C-7), 58.4 (C-2′), 49.2 (C-1′), 44.8 (Bn CH_2); MS (EI): *m/z* (%) 313 (100) (M^+), 282 (11), 236 (6), 192 (11), 177 (9), 162 (5), 146 (8), 131 (11), 103 (7). Anal. $C_{16}H_{15}N_3O_4$ (C, H, N).

*4.1.2.1.4. 2-Benzyl-1-(3-hydroxypropyl)-5-nitro-1,2-dihydro-3H-indazol-3-one (**21**).* Yield: 0.58 g (48%). Mp 141–143 °C (2-PrOH).

*4.1.2.1.5. 2-Benzyl-1-(2-methoxyethyl)-5-nitro-1,2-dihydro-3H-indazol-3-one (**22**).* Yield: 0.78 g (64%). Mp 116–118 °C (2-PrOH).

*4.1.2.1.6. 2-Benzyl-3-[3-(ethoxycarbonyl)propoxy]-5-nitro-2H-indazole (**34**).* Yield: 0.40 g (28%). Mp 87–89 °C (2-PrOH).

*4.1.2.1.7. 2-Benzyl-3-(2-hydroxyethoxy)-5-nitro-2H-indazole (**35**).* Yield: 0.21 g (18%). Mp 167–169 °C (2-PrOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.90 (d, J = 2.1 Hz, 1H, 4-H), 7.91 (dd, J = 9.6, 2.1 Hz, 1H, 6-H), 7.53 (d, J = 9.6 Hz, 1H, 7-H), 7.31 (m, 5H, Bn aromatic H), 5.50 (s, 2H, Bn CH_2), 5.16 (t, J = 5.5 Hz, 1H, OH), 4.72 (t, J = 4.5 Hz, 2H, 1′-H), 3.81 (m, 2H, 2′-H); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 150.0 (C-3), 146.9 (C-7a), 140.2 (C-5), 135.9 (Bn C-1), 128.6 (Bn C-3, -5), 128.0 (Bn C-2, -6), 127.9 (Bn C-4), 120.9 (C-4), 119.9 (C-6), 118.0 (C-7), 105.3 (C-3a), 76.4 (C-1′), 59.8 (C-2′), 51.5 (Bn CH_2); MS (EI): *m/z* (%) 313 (100) (M^+), 269 (58), 252 (14), 222 (7), 191 (41), 164 (7), 149 (4), 103 (14). Anal. $C_{16}H_{15}N_3O_4$ (C, H, N).

*4.1.2.1.8. 2-Benzyl-3-(3-hydroxypropoxy)-5-nitro-2H-indazole (**36**).* Yield: 0.12 g (10%). Mp 122–124 °C (2-PrOH).

*4.1.2.1.9. 2-Benzyl-3-(2-methoxyethoxy)-5-nitro-2H-indazole (**37**).* Yield: 0.39 g (32%). Mp 109–111 °C (2-PrOH).

4.1.2.2. Preparation of propargyl derivative ***8****.* A stirred mixture of 2-benzylindazolinone **5** [44] (1.00 g, 3.71 mmol), propargyl bromide (80% w/w in toluene) (4.00 mmol) and potassium carbonate (0.55 g, 4.00 mmol) in acetone (50 mL) was refluxed for 12 h. The mixture was then evaporated to dryness and, after addition of water (200 mL), extracted with chloroform (3 × 50 mL). The concentrated chloroform phase was applied to the top of a chromatography column which was eluted with chloroform/acetone mixtures (50:1 to 30:1) to afford compound **8**.

*4.1.2.2.1. 2-Benzyl-5-nitro-1-propargyl-1,2-dihydro-3H-indazol-3-one (**8**).* Yield: 0.62 g (54%). Mp 163–165 °C (EtOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.52 (d, J = 2.1 Hz, 1H, 4-H), 8.47 (dd, J = 9.0, 2.1 Hz, 1H, 6-H), 7.80 (d, J = 9.0 Hz, 1H, 7-H), 7.27 (m, 5H, Bn aromatic H), 5.12 (s, 2H, Bn CH_2), 4.96 (d, J = 2.1 Hz, 2H, 1′-H), 3.16 (t, J = 2.1 Hz, 1H, 3′-H); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 161.5 (C-3), 151.6 (C-7a), 143.1 (C-5), 136.1 (Bn C-1), 128.6 (Bn C-3, -5), 127.7 (Bn C-4), 127.6 (C-6, Bn C-2, -6), 119.9 (C-4), 118.8 (C-3a), 114.0 (C-7), 76.6 (C-2′), 75.9 (C-3′), 44.7 (Bn CH_2), 38.5 (C-1′); MS (EI): *m/z* (%) 307 (100) (M^+), 268 (13), 249 (5), 230 (3), 203 (4), 128 (5), 115 (4), 103 (11). Anal. $C_{17}H_{13}N_3O_3$ (C, H, N).

*4.1.2.3. Preparation of 2-bromoethyl (**9**/**30**) and 3-bromopropyl (**10**/**31**) derivatives and related compounds (**38**, **39**).* A stirred mixture of the starting 2-benzylindazolinone **5** [44] (2.00 g, 7.43 mmol), the required α,ω-dibromoalkane (40.00 mmol) and potassium carbonate (1.10 g, 8.00 mmol) in DMF (50 mL) was heated at 100 °C for 3 h. The mixture was then evaporated to dryness and, after addition of water (200 mL), extracted with chloroform (3 × 50 mL). The organic phase was dried ($MgSO_4$), concentrated and applied to the top of a chromatography column which was eluted with chloroform/acetone mixtures (50:1 to 25:1).

Starting from 1,2-dibromoethane, the following compounds were obtained in this elution order: 2,3-disubstituted indazole **30**, 1-vinylindazolinone **6** [70 mg (3%); mp and spectral and analytical data are given below, section 4.1.4.1.1], 1,2-disubstituted indazolinone **9**, and the corresponding *O*,*O*′- (**38**) and *O*,N_1′- (**39**) ethylene derivatives.

Analogously, starting from 1,3-dibromopropane, the corresponding 2,3-disubstituted indazole **31** and 1,2-disubstituted indazolinone **10** could be obtained.

Spectral and analytical data of compounds **10**, **30**, **38** and **39** are

included as supplementary material.

*4.1.2.3.1. 2-Benzyl-1-(2-bromoethyl)-5-nitro-1,2-dihydro-3H-indazol-3-one (**9**).* Yield: 1.20 g (43%). Mp 172–174 °C (2-PrOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.52 (d, J = 2.4 Hz, 1H, 4-H), 8.37 (dd, J = 9.0, 2.4 Hz, 1H, 6-H), 7.75 (d, J = 9.0 Hz, 1H, 7-H), 7.26 (m, 5H, Bn aromatic H), 5.19 (s, 2H, Bn CH_2), 4.53 (t, J = 6.3 Hz, 2H, 1′-H), 3.51 (t, J = 6.3 Hz, 2H, 2′-H); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 161.4 (C-3), 149.3 (C-7a), 141.5 (C-5), 135.9 (Bn C-1), 128.7 (Bn C-3, -5), 127.9 (Bn C-4), 127.3 (Bn C-2, -6), 127.0 (C-6), 120.4 (C-4), 115.4 (C-3a), 112.6 (C-7), 47.7 (C-1′), 45.2 (Bn CH_2), 29.2 (C-2′); MS (EI): *m/z* (%) 377 (99) ($[M+2]^+$), 375 (100) (M^+), 361 (5), 359 (5), 347 (7), 345 (7), 256 (9), 254 (9), 192 (8), 177 (9), 164 (9), 145 (8), 131 (17), 103 (36). Anal. $C_{16}H_{14}BrN_3O_3$ (C, H, N).

*4.1.2.3.2. 2-Benzyl-1-(3-bromopropyl)-5-nitro-1,2-dihydro-3H-indazol-3-one (**10**).* Yield: 0.90 g (31%). Mp 111–113 °C (2-PrOH).

*4.1.2.3.3. 2-Benzyl-3-(2-bromoethoxy)-5-nitro-2H-indazole (**30**).* Yield: 0.84 g (30%). Mp 140–142 °C (2-PrOH).

*4.1.2.3.4. 2-Benzyl-3-(3-bromopropoxy)-5-nitro-2H-indazole (**31**).* Yield: 0.84 g (29%). Mp 124–126 °C (2-PrOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.88 (d, J = 2.4 Hz, 1H, 4-H), 7.90 (dd, J = 9.6, 2.4 Hz, 1H, 6-H), 7.54 (d, J = 9.6 Hz, 1H, 7-H), 7.31 (m, 5H, Bn aromatic H), 5.47 (s, 2H, Bn CH_2), 4.82 (t, J = 5.8 Hz, 2H, 1′-H), 3.66 (t, J = 6.6 Hz, 2H, 3′-H), 2.35 (m, 2H, 2′-H); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 149.4 (C-3), 146.8 (C-7a), 140.3 (C-5), 135.8 (Bn C-1), 128.6 (Bn C-3, -5), 127.9 (Bn C-4), 127.7 (Bn C-2, -6), 120.8 (C-4), 119.9 (C-6), 118.1 (C-7), 104.9 (C-3a), 71.7 (C-1′), 51.8 (Bn CH_2), 32.0 (C-2′), 30.5 (C-3′); MS (EI): *m/z* (%) 391 (99) ($[M+2]^+$), 389 (100) (M^+), 280 (7), 269 (98), 252 (43), 222 (12), 191 (38), 164 (14), 123 (48), 121 (50), 103 (27). Anal. $C_{17}H_{16}BrN_3O_3$ (C, H, N).

*4.1.2.3.5. 1,2-Bis(2-benzyl-5-nitro-2H-indazol-3-yloxy)ethane (**38**).* Yield: 51 mg (3%). Mp 205–207 °C (1-PrOH).

*4.1.2.3.6. 2-Benzyl-1-[2-(2-benzyl-5-nitro-2H-indazol-3-yloxy) ethyl]-5-nitro-1,2-dihydro-3H-indazol-3-one (**39**).* Yield: 0.24 g (14%). Mp 207–209 °C ($MeNO_2$).

*4.1.2.4. Preparation of (methoxycarbonyl)methyl derivatives **11**/**32** and cianomethyl derivative **12**.* A stirred mixture of 2-benzylindazolinone **5** [44] (1.00 g, 3.71 mmol), methyl bromoacetate or bromoacetonitrile (4.5 mmol) and sodium hydrogen carbonate (0.42 g, 5.00 mmol) in acetone (30 mL) was heated at 35 °C for 5 days. The mixture was then evaporated to dryness and, after addition of water (50 mL), the precipitated solid was collected by filtration. For **11**/**32**, the obtained solid was washed with acetone (2 × 5 mL) affording pure compound **11** (1.03 g). The filtrate was concentrated to dryness, dissolved in chloroform and applied to the top of a chromatography column which was eluted with chloroform and a chloroform/acetone mixture (50:1) to afford, following this elution order, compound **32** and then an additional amount of compound **11**.

For compound **12**, the solid obtained after filtration was directly chromatographed with chloroform/acetone mixtures (50:1 to 30:1) to afford pure 1-cyanomethyl derivative.

Alkylation of compound **5** with methyl bromoacetate or bromoacetamide in potassium carbonate/DMF at 100 °C afforded the rearranged quinazolinones **40**–**43** (see below, section 4.1.5).

Spectral and analytical data of compound **12** are included as supplementary material.

*4.1.2.4.1. 2-Benzyl-1-(methoxycarbonyl)methyl-5-nitro-1,2-dihydro-3H-indazol-3-one (**11**).* Yield: 1.18 g (93%). Crystals (2-PrOH) of this compound soften at 150–170 °C, then the compound resolidifies showing a further mp at 195–197 °C, corresponding (TLC) to that of the rearranged quinazolinone **40**. ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.54 (d, J = 2.1 Hz, 1H, 4-H), 8.40 (dd, J = 9.3, 2.1 Hz, 1H, 6-H), 7.67 (d, J = 9.3 Hz, 1H, 7-H), 7.27 (m, 5H, Bn aromatic H), 5.15 (s, 2H, Bn CH_2), 5.09 (s, 2H, 1′-H), 3.41 (s, 3H, CH_3); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 167.3 (C-2′), 160.8 (C-3), 150.1 (C-7a), 141.8 (C-5), 135.9 (Bn C-1), 128.5 (Bn C-3, -5), 127.7 (Bn C-4), 127.4 (Bn C-2, -6), 127.3 (C-6), 120.1 (C-4), 116.3 (C-3a), 112.0 (C-7), 52.0 (CH_3), 47.8 (C-1′), 44.9 (Bn CH_2); MS (ES^+): *m/z* (%) 705 (23) ($[2M + Na]^+$), 683 (25) ($[2M + H]^+$), 364 (34) ($[M+Na]^+$), 342 (100) ($[M+H]^+$). Anal. $C_{17}H_{15}N_3O_5$ (C, H, N).

*4.1.2.4.2. 2-Benzyl-1-cyanomethyl-5-nitro-1,2-dihydro-3H-indazol-3-one (**12**).* Yield: 0.84 g (73%). Mp 146–148 °C (2-PrOH).

*4.1.2.4.3. 2-Benzyl-3-(methoxycarbonyl)methoxy-5-nitro-2H-indazole (**32**).* Yield: 40 mg (3%). Mp 94–96 °C (2-PrOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.83 (d, J = 2.2 Hz, 1H, 4-H), 7.91 (dd, J = 9.6, 2.2 Hz, 1H, 6-H), 7.57 (d, J = 9.6 Hz, 1H, 7-H), 7.33 (m, 5H, Bn aromatic H), 5.54 (s, 2H, Bn CH_2), 5.49 (s, 2H, 1′-H), 3.74 (s, 3H, CH_3); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 168.4 (C-2′), 148.7 (C-3), 146.8 (C-7a), 140.6 (C-5), 135.6 (Bn C-1), 128.6 (Bn C-3, -5), 127.9 (Bn C-2, -4, -6), 120.1 (C-4), 119.9 (C-6), 118.2 (C-7), 105.1 (C-3a), 69.5 (C-1′), 52.2 (CH_3), 51.8 (Bn CH_2); MS (EI): *m/z* (%) 341 (100) (M^+), 325 (1), 282 (2), 268 (16), 252 (2), 236 (2), 222 (2), 191 (2), 164 (2), 103 (3). Anal. $C_{17}H_{15}N_3O_5$ (C, H, N).

*4.1.2.5. Preparation of 2-(methoxycarbonyl)ethyl derivatives **13**/**33** and 2-carboxyethyl derivative **14**.* A stirred solution of the starting 2-benzylindazolinone **5** [44] (1.00 g, 3.71 mmol), potassium carbonate (1.38 g, 10.00 mmol) and benzyltributylammonium bromide (0.10 g) in a mixture of water (20 mL) and toluene (20 mL) was heated at 100 °C for 30 min, and then, methyl 3-bromopropionate (0.84 g, 5.03 mmol) was added. Further amounts of methyl 3-bromopropionate (ca. 10 × 0.25 g) and, eventually, if needed, potassium carbonate to have a basic pH, were added during 2 days. The reaction mixture was allowed to reach room temperature and the toluene phase was then separated, dried ($MgSO_4$) and evaporated to dryness. The obtained residue was chromatographed on a column using chloroform/acetone mixtures (50:1 to 10:1) to afford, following this elution order, compound **33** and then compound **13**.

The remaining water phase was acidified with conc. HCl (pH 1) and the precipitated solid collected by filtration, dried and applied to the top of a column which was eluted first with chloroform/methanol (50:1 to 10:1) mixtures and then with a chloroform/methanol (10:1) mixture containing 0.5% of acetic acid to afford, in this elution order, recovered starting indazolinone **5** [70 mg (7%)] and then acid **14**.

Spectral and analytical data of compounds **13** and **33** are included as supplementary material.

*4.1.2.5.1. 2-Benzyl-1-[2-(methoxycarbonyl)ethyl]-5-nitro-1,2-dihydro-3H-indazol-3-one (**13**).* Yield: 0.56 g (42%). Mp 127–129 °C (2-PrOH).

*4.1.2.5.2. 2-Benzyl-1-(2-carboxyethyl)-5-nitro-1,2-dihydro-3H-indazol-3-one (**14**).* Yield: 0.33 g (26%). Mp 224–226 °C (EtOH/H_2O). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 12.39 (br s, 1H, COOH), 8.50 (d, J = 2.1 Hz, 1H, 4-H), 8.37 (dd, J = 9.3, 2.1 Hz, 1H, 6-H), 7.69 (d, J = 9.3 Hz, 1H, 7-H), 7.26 (m, 5H, Bn aromatic H), 5.17 (s, 2H, Bn CH_2), 4.27 (t, J = 6.9 Hz, 2H, 1′-H), 2.26 (t, J = 6.9 Hz, 2H, 2′-H); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 171.9 (C-3′), 161.3 (C-3), 149.0 (C-7a), 141.6 (C-5), 136.0 (Bn C-1), 128.7 (Bn C-3, -5), 127.8 (Bn C-4), 127.3 (Bn C-2, -6), 127.1 (C-6), 120.3 (C-4), 116.2 (C-3a), 112.7 (C-7), 45.0 (Bn CH_2), 43.1 (C-1′), 30.5 (C-2′); MS (ES^+): *m/z* (%) 705 (51) ($[2M+Na]^+$), 683 (35) ($[2M+H]^+$), 364 (65) ($[M+Na]^+$), 342 (100) ($[M+H]^+$). Anal. $C_{17}H_{15}N_3O_5$ (C, H, N).

*4.1.2.5.3. 2-Benzyl-3-[2-(methoxycarbonyl)ethoxy]-5-nitro-2H-indazole (**33**).* Yield: 0.15 g (11%). Mp 130–132 °C (2-PrOH).

*4.1.3. Alkoxycarbonylation, acylation and sulfonylation of 2-benzyl-5-nitroindazolin-3-one **5**: preparation of alkoxycarbonyl (**25**, **26**), acyl (**27**, **28**) and tosyl (**29**) derivatives*

To a stirred solution of the starting 2-benzylindazolin-3-one **5**

[44] (1.00 g; 3.71 mmol) in pyridine (15 mL), the required alkyl chloroformate (4.00 mmol) (for **25**, **26**), acetic anhydride (2.0 mL, excess) (for **27**) or the corresponding acid chloride (3.90 mmol) (for **28**, **29**) were slowly added. The reaction mixture was heated at 100 °C during 3 h (for **27**), or stirred at room temperature during 1 h (for **25**, **26**, **28**, **29**), and then poured into water (100 mL). The precipitated solid was collected by filtration, washed with 2% aq. HCl (50 mL) and with plenty water, and air-dried.

Spectral and analytical data of compounds **26**, **28** and **29** are included as supplementary material.

*4.1.3.1. 2-Benzyl-1-ethoxycarbonyl-5-nitro-1,2-dihydro-3H-indazol-3-one (**25**).* Yield: 1.20 g (95%). Mp 135–137 °C (EtOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.56 (d, $J = 2.2$ Hz, 1H, 4-H), 8.52 (dd, $J = 9.0$, 2.2 Hz, 1H, 6-H), 8.00 (d, $J = 9.0$ Hz, 1H, 7-H), 7.26 (m, 3H) and 7.11 (m, 2H) (Bn aromatic H), 5.33 (s, 2H, Bn CH_2), 4.39 (q, $J = 7.0$ Hz, 2H, Et CH_2), 1.30 (t, $J = 7.0$ Hz, 3H, CH_3); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 162.6 (C-3), 149.5 (COO), 145.6 (C-7a), 144.2 (C-5), 135.3 (Bn C-1), 128.9 (C-6), 128.7 (Bn C-3, -5), 128.0 (Bn C-4), 127.4 (Bn C-2, -6), 119.6 (C-4), 118.2 (C-3a), 116.3 (C-7), 64.7 (Et CH_2), 50.0 (Bn CH_2), 13.8 (CH_3); MS (ES^+): *m/z* (%) 705 (39) ($[2M+Na]^+$), 683 (75) ($[2M+H]^+$), 364 (15) ($[M+Na]^+$), 342 (100) ($M+H]^+$). Anal. $C_{17}H_{15}N_3O_5$ (C, H, N).

*4.1.3.2. 2-Benzyl-1-benzyloxycarbonyl-5-nitro-1,2-dihydro-3H-indazol-3-one (**26**).* Yield: 1.45 g (97%). Mp 143–145 °C (2-PrOH).

*4.1.3.3. 1-Acetyl-2-benzyl-5-nitro-1,2-dihydro-3H-indazol-3-one (**27**).* Yield: 1.03 g (89%). Mp 165–167 °C (2-PrOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.56 (d, $J = 2.1$ Hz, 1H, 4-H), 8.50 (dd, $J = 9.3$, 2.1 Hz, 1H, 6-H), 8.30 (d, $J = 9.3$ Hz, 1H, 7-H), 7.25 (m, 3H) and 7.11 (m, 2H) (Bn aromatic H), 5.33 (s, 2H, CH_2), 2.57 (s, 3H, CH_3); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 168.4 (Ac CO), 164.0 (C-3), 145.9 (C-7a), 144.1 (C-5), 135.1 (Bn C-1), 128.9 (C-6), 128.7 (Bn C-3, -5), 128.0 (Bn C-4), 127.6 (Bn C-2, -6), 119.7 (C-4), 118.5 (C-3a), 116.2 (C-7), 51.1 (CH_2), 25.0 (CH_3); MS (ES^+): *m/z* (%) 645 (44) ($[2M+Na]^+$), 623 (3) ($[2M+H]^+$), 334 (62) ($[M+Na]^+$), 312 (100) ($[M+H]^+$). Anal. $C_{16}H_{13}N_3O_4$ (C, H, N).

*4.1.3.4. 1-Benzoyl-2-benzyl-5-nitro-1,2-dihydro-3H-indazol-3-one (**28**).* Yield: 1.36 g (98%). Mp 164–166 °C (2-PrOH).

*4.1.3.5. 2-Benzyl-5-nitro-1-tosyl-1,2-dihydro-3H-indazol-3-one (**29**).* Yield: 1.54 g (98%). Mp 162–164 °C (1-PrOH).

4.1.4. Transformation of alkylation products

*4.1.4.1. Preparation of vinyl derivative **6**.* A solution of 1-(2-bromoethyl)indazolinone **9** (0.56 g, 1.49 mmol) and piperidine (0.35 g, 4.11 mmol) in ethanol (20 mL) was refluxed for 48 h. The solution was evaporated to dryness and, after addition of 2% aq. HCl (50 mL), extracted with chloroform (3 × 50 mL). The organic phase was dried ($MgSO_4$) and evaporated to dryness to afford the title compound; similar results were obtained using methylamine (33% w/w solution in ethanol).

Compound **6** was also obtained as a byproduct arising from alkylation of 2-benzylindazolinone **5** with 1,2-dibromoethane (see above, section 4.1.2.3).

*4.1.4.1.1. 2-Benzyl-5-nitro-1-vinyl-1,2-dihydro-3H-indazol-3-one (**6**).* Yield: 0.41 g (93%). Mp 116–118 °C (2-PrOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.53 (d, $J = 3.0$ Hz, 1H, 4-H), 8.40 (dd, $J = 9.3$, 3.0 Hz, 1H, 6-H), 7.71 (d, $J = 9.3$ Hz, 1H, 7-H), 7.26 (m, 3H) and 7.15 (m, 2H) (Bn aromatic H), 7.08 (dd, $J = 14.7$, 9.0 Hz, 1H, 1′-H), 5.25 (dd, $J = 14.7$, 1.4 Hz, 1H, 2′-H_{trans}), 5.20 (s, 2H, Bn CH_2), 5.10 (dd, $J = 9.0$, 1.4 Hz, 1H, 2′-H_{cis}); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 162.0 (C-3), 146.7 (C-7a), 142.1 (C-5), 135.5 (Bn C-1), 129.9 (C-1′), 128.7 (Bn C-3, -5), 128.1 (C-6), 127.9 (Bn C-4), 127.4 (Bn C-2, -6), 120.4 (C-4), 115.5 (C-3a), 112.1 (C-7), 104.7 (C-2′), 47.0 (Bn CH_2); MS (EI): *m/z* (%) 295 (100) (M^+), 280 (2), 265 (2), 218 (2), 158 (3), 145 (3), 116 (7), 104 (9). Anal. $C_{16}H_{13}N_3O_3$ (C, H, N).

*4.1.4.2. Preparation of 4-(indazol-1-yl)butiric acid **16**.* A mixture of ethyl ester **15** (0.50 g, 1.30 mmol) and lithium hydroxide (0.16 g, 6.68 mmol) in THF/H_2O (1:1, 20 mL) was stirred at room temperature for 12 h. THF was then evaporated and the aqueous solution acidified with conc. aq. HCl (pH 1). The precipitated solid was collected by filtration, washed with 1% aq. HCl (3 × 3 mL) and air-dried.

*4.1.4.2.1. 2-Benzyl-1-(3-carboxypropyl)-5-nitro-1,2-dihydro-3H-indazol-3-one (**16**).* Yield: 0.42 g (91%). Mp 202–204 °C (EtOH/H_2O). Spectral and analytical data of this compound are included as supplementary material.

*4.1.4.3. Preparation of amide **17** and N-methylamide **18**.* For compound **17**, a mixture of ethyl ester **15** (0.50 g, 1.30 mmol) and a saturated solution of ammonia in methanol (15 mL) was allowed to stand at room temperature for 10 days. After evaporation of the solvent and ammonia, in order to remove some minor byproducts, the residue was directly applied to the top of a column which was eluted with a chloroform/methanol (25:1) mixture to afford the required product.

For compound **18**, a mixture of ethyl ester **15** (0.50 g, 1.30 mmol) and 8 M methylamine in ethanol (20 mL) was allowed to stand at room temperature for 24 h. The reaction mixture was evaporated to dryness to afford the desired amide.

*4.1.4.3.1. 2-Benzyl-1-(3-carbamoylpropyl)-5-nitro-1,2-dihydro-3H-indazol-3-one (**17**).* Yield: 0.45 g (98%). Oil which solidifies after trituration with 2-PrOH. Mp 106–108 °C. Spectral and analytical data of this compound are included as supplementary material.

*4.1.4.3.2. 2-Benzyl-1-[3-(methylcarbamoyl)propyl]-5-nitro-1,2-dihydro-3H-indazol-3-one (**18**).* Yield: 0.45 g (94%). Mp 159–161 °C (2-PrOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.52 (d, $J = 2.1$ Hz, 1H, 4-H), 8.36 (dd, $J = 9.3$, 2.1 Hz, 1H, 6-H), 7.67 (br q, $J = 4.5$ Hz, 1H, NH), 7.61 (d, $J = 9.3$ Hz, 1H, 7-H), 7.25 (m, 5H, Bn aromatic H), 5.20 (s, 2H, Bn CH_2), 4.03 (t, $J = 7.4$ Hz, 2H, 1′-H), 2.52 (d, $J = 4.5$ Hz, 3H, CH_3), 1.97 (t, $J = 7.2$ Hz, 2H, 3′-H), 1.53 (m, 2H, 2′-H); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 171.3 (C-4′), 160.8 (C-3), 148.4 (C-7a), 141.2 (C-5), 136.1 (Bn C-1), 128.7 (Bn C-3, -5), 127.8 (Bn C-4), 127.2 (Bn C-2, -6), 127.1 (C-6), 120.5 (C-4), 115.5 (C-3a), 111.9 (C-7), 46.2 (C-1′), 44.6 (Bn CH_2), 31.3 (C-3′), 25.4 (CH_3), 22.0 (C-2′); MS (ES^+): *m/z* (%) 759 (17) ($[2M+Na]^{+)}$, 737 (53) ($[2M+H]^{+)}$, 391 (22) ($[M+Na]^{+)}$, 369 (100) ($[M+H]^+$). Anal. $C_{19}H_{20}N_4O_4$ (C, H, N).

*4.1.4.4. Preparation of N,N-dimethylamide **19**.* A suspension of acid **16** (0.53 g, 1.49 mmol) in a mixture of thionyl chloride (1.00 mL) and chloroform (30 mL) was refluxed for 1 h. After evaporation of the solvent and excess of thionyl chloride, a solution of dimethylamine hydrochloride (1.63 g, 19.99 mmol) and potassium carbonate (2.80 g, 20.26 mmol) in water (20 mL) was added to the oily residue. The mixture was vigorously stirred for 12 h and then the precipitated product **19** was collected by filtration, washed with water (3 × 5 mL) and air dried.

*4.1.4.4.1. 2-Benzyl-1-[3-(dimethylcarbamoyl)propyl]-5-nitro-1,2-dihydro-3H-indazol-3-one (**19**).* Yield: 0.51 g (90%). Mp 144–146 °C (2-PrOH). Spectral and analytical data of this compound are included as supplementary material.

*4.1.4.5. Preparation of 3-ethoxypropyl derivative **23**.* To a solution of 1-(3-bromopropyl) derivative **10** (0.59 g, 1.51 mmol) in ethanol (100 mL), sodium hydroxide (0.40 g) in water (20 mL) was added, and the mixture was stirred at room temperature for 3 days.

Ethanol was then evaporated and the basic solution extracted with chloroform (3 × 50 mL). The organic phase was dried ($MgSO_4$), concentrated and applied to the top of a chromatography column; elution with chloroform/acetone mixtures (50:1 to 25:1) afforded first 1-allyl derivative **7** [28 mg (6%)] and then ethyl ether **23**.

*4.1.4.5.1. 2-Benzyl-1-(3-ethoxypropyl)-5-nitro-1,2-dihydro-3H-indazol-3-one (**23**).* Yield: 0.38 g (71%). Mp 93–95 °C (2-PrOH). Spectral and analytical data of this compound are included as supplementary material.

*4.1.4.6. Preparation of 2-acetoxyethyl derivative **24**.* A suspension of 1-(2-hydroxyethyl) derivative **20** (0.47 g, 1.50 mmol) in acetic anhydride (5 mL) was heated at 100 °C for 2 h. The reaction was evaporated to dryness and the residue triturated with 2-PrOH (2 mL); the insoluble title compound was collected by filtration, washed with 2-PrOH (2 × 2 mL) and air-dried.

*4.1.4.6.1. 1-(2-Acetoxyethyl)-2-benzyl-5-nitro-1,2-dihydro-3H-indazol-3-one (**24**).* Yield: 0.47 g (88%). Mp 168–170 °C (2-PrOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.51 (d, J = 2.4 Hz, 1H, 4-H), 8.38 (dd, J = 9.0, 2.4 Hz, 1H, 6-H), 7.66 (d, J = 9.0 Hz, 1H, 7-H), 7.25 (m, 5H, Bn aromatic H), 5.19 (s, 2H, Bn CH_2), 4.36 (t, J = 4.8 Hz, 2H, 1′-H), 4.07 (t, J = 4.8 Hz, 2H, 2′-H), 1.57 (s, 3H, CH_3); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 169.5 (Ac CO), 161.2 (C-3), 149.9 (C-7a), 141.3 (C-5), 136.0 (Bn C-1), 128.7 (Bn C-3, -5), 127.9 (Bn C-4), 127.3 (Bn C-2, -6), 126.8 (C-6), 120.3 (C-4), 115.5 (C-3a), 112.5 (C-7), 60.2 (C-2′), 45.9 (C-1′), 44.8 (Bn CH_2), 20.1 (CH_3); MS (EI): *m/z* (%) 355 (100) (M^+), 325 (2), 295 (5), 282 (6), 204 (6), 177 (9), 131 (10), 103 (7). Anal. $C_{18}H_{17}N_3O_5$ (C, H, N).

*4.1.5. Preparation of quinazolinones **40**–**43***

a) *Compounds **40**–**43** from starting indazolinone **5**.* A stirred mixture of the starting 2-benzylindazolinone **5** [44] (1.00 g, 3.71 mmol), the required bromoacetic acid derivative (methyl ester or amide) (5.00 mmol) and potassium carbonate (0.83 g, 6.00 mmol) in DMF (20 mL) was heated at 100 °C for 45 min. The reaction was evaporated to dryness and, after addition of water (150 mL), the precipitated solid was collected by filtration, washed with water and air-dried. For compounds **40**/**42**, the obtained solid was triturated with chloroform (15 mL); the insoluble material was recovered by filtration, washed with chloroform (2 × 4 mL) and air-dried to afford 0.63 g of pure compound **40**. The filtrate was concentrated and applied to the top of a column which was eluted with a chloroform/methanol mixture (50:1) to afford, following this elution order, compound **42** [0.38 g, 25%] and an additional amount of compound **40** [overall yield: 0.77 g (61%)].

For compounds **41**/**43**, the solid obtained after addition of water was directly chromatographed using chloroform/methanol mixtures (30:1 to 10:1) to afford, following this elution order, compound **41** [0.79 g, 65%] and compound **43** [0.06 g (4%)].

b) *Rearrangement of indazolinone **11** to quinazolinone **40**.* A mixture of indazolinone **11** (0.51 g, 1.49 mmol) and potassium carbonate (0.22 g, 1.59 mmol) in acetone (40 mL) was stirred at room temperature for 12 h. After evaporation of acetone and addition of water (20 mL), the precipitated solid was collected by filtration, dried and chromatographed using chloroform/acetone mixtures (30:1 to 20:1) to afford compound **40** [0.44 g, 87%].

c) *Compounds **42** and **43** from **40** and **41**, respectively.* A mixture of the corresponding quinazolinone **40** or **41** (1.50 mmol), the required bromoacetic acid derivative (methyl ester or amide) (1.70 mmol) and potassium carbonate (0.27 g, 1.95 mmol) in DMF (20 mL) was heated at 100 °C for 1 h. The reaction was evaporated to dryness and, after addition of water (100 mL), the precipitated solid was collected by filtration, washed with water and air-dried to afford the corresponding 1-substituted quinazolinones **42** [0.50 g (81%)] and **43** [0.55 g (96%)].

Spectral and analytical data of compounds **42** and **43** are included as supplementary material.

*4.1.5.1. 3-Benzyl-2-methoxycarbonyl-6-nitro-1,2,3,4-tetrahydroquinazolin-4-one (**40**).* Mp 194–196 °C (1-PrOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.84 (br s, 1H, 1-H), 8.46 (d, J = 2.7 Hz, 1H, 5-H), 8.14 (dd, J = 9.0, 2.7 Hz, 1H, 7-H), 7.31 (m, 5H, Bn aromatic H), 6.91 (d, J = 9.0 Hz, 1H, 8-H), 5.49 (s, 1H, 2-H), 5.12 (d, J = −15.3 Hz, 1H, Bn CH_A), 4.33 (d, J = −15.3 Hz, 1H, Bn CH_B), 3.56 (s, 3H, CH_3); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 169.4 (COOMe), 160.6 (C-4), 151.2 (C-8a), 138.3 (C-6), 136.5 (Bn C-1), 128.9 (C-7), 128.5 (Bn C-3, -5), 127.8 (Bn C-2, -6), 127.5 (Bn C-4), 124.3 (C-5), 114.8 (C-8), 113.4 (C-4a), 67.0 (C-2), 52.8 (CH_3), 47.9 (CH_2); MS (ES^+): *m/z* (%) 705 (41) ($[2M+Na]^+$), 683 (9) ($[2M+H]^+$), 364 (43) ($[M+Na]^+$), 342 (100) ($M+H]^+$), 102 (87). Anal. $C_{17}H_{15}N_3O_5$ (C, H, N).

*4.1.5.2. 3-Benzyl-2-carbamoyl-6-nitro-1,2,3,4-tetrahydroquinazolin-4-one (**41**).* Mp 219–221 °C (EtOH). ^{1}H NMR [300 MHz, $(CD_3)_2SO$]: δ 8.46 (br s, 1H, 1-H), 8.43 (d, J = 2.7 Hz, 1H, 5-H), 8.10 (dd, J = 9.0, 2.7 Hz, 1H, 7-H), 7.66 (br s, 1H, $CONH_A$), 7.46 (br s, 1H, $CONH_B$), 7.33 (m, 5H, Bn aromatic H), 6.82 (d, J = 9.0 Hz, 1H, 8-H), 5.31 (d, J = −15.6 Hz, 1H, Bn CH_A), 5.17 (s, 1H, 2-H), 3.87 (d, J = −15.6 Hz, 1H, Bn CH_B); ^{13}C NMR [75 MHz, $(CD_3)_2SO$]: δ 169.7 ($CONH_2$), 161.0 (C-4), 151.7 (C-8a), 137.5 (C-6), 136.6 (Bn C-1), 128.7 (C-7), 128.6 (Bn C-3, -5), 127.6 (Bn C-2, -6), 127.4 (Bn C-4), 124.0 (C-5), 114.2 (C-8), 113.8 (C-4a), 67.6 (C-2), 47.2 (CH_2); MS (ES^+): *m/z* (%) 675 (13) ($[2M+Na]^+$), 653 (24) ($[2M+H]^+$), 349 (5) ($[M+Na]^+$), 327 (82) ($[M+H]^+$), 102 (100). Anal. $C_{16}H_{14}N_4O_4$ (C, H, N).

*4.1.5.3. 3-Benzyl-2-methoxycarbonyl-1-(methoxycarbonyl)methyl-6-nitro-1,2,3,4-tetrahydroquinazolin-4-one (**42**).* Mp 158–160 °C (EtOH).

*4.1.5.4. 3-Benzyl-2-carbamoyl-1-carbamoylmethyl-6-nitro-1,2,3,4-tetrahydroquinazolin-4-one (**43**).* Mp 244–246 °C ($MeNO_2$).

4.2. Biology

4.2.1. Study of antichagasic activity

4.2.1.1. T. cruzi strains culture. CL-B5 and Tulahuen C4 strains, both stably transfected with *Escherichia coli* β-galactosidase gene (lacZ) [66] and the Y strain of *T. cruzi*, originally isolated from an acute human case [67] were used throughout the experiments.

Axenic cultures of *T. cruzi* CL-B5 *lacZ* epimastigotes were grown at 28 °C in liver infusion tryptose (LIT) medium, supplemented with 10% heat-inactivated FBS and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL) and continuously maintained in logarithmic growth by weekly passages. Tissue culture-derived trypomastigotes (TCT) of this strain were obtained by infecting L929 cells with epimastigotes at the stationary phase (14-day-old cultures) and incubated 24 h at 33 °C in a humidified 5% CO_2 atmosphere. Non-penetrated parasites were removed by washing infected cultures with phosphate-buffered saline (PBS). After the addition of fresh medium, infected cultures were incubated in similar conditions for 7 days and then TCT obtained in the supernatant.

Similarly, TCT of *T. cruzi* Tulahuen C4 *lacZ* were harvested in the supernatant of L929 cultures previously infected with invasive forms of the parasite and maintained in RPMIS at 37 °C in a humidified 5% CO_2 atmosphere [68].

Regarding the Y strain, epimastigotes were maintained in the conditions described above. Bloodstream trypomastigotes (BT) were obtained by heart puncture from infected Swiss mice at the parasitemia peak day and after their purification, resuspended in RPMI medium supplemented with 5% heat-inactivated FBS. All procedures involving mice were carried out in accordance with the

guidelines established by the Fiocruz Committee for the Use of Animals (CEUA LW16/14).

4.2.1.2. Mammalian cell cultures. Murine L929 fibroblasts were grown in plastic culture flasks (75 cm^2) and sustained either in MEM or RPMIS without phenol-red, supplemented as reported [8]. Cell cultures were maintained in a humidified 5% CO_2 atmosphere at 37 °C and sub-passaged once a week using a solution 0.03% EDTA and 0.05% trypsin in PBS for cell-detachment.

Primary cultures of embryonic cardiac cells were obtained from Swiss mice as previously described [69]. After their purification, cultures were sustained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2.5 mM $CaCl_2$, 1 mM L-glutamine, 5% heat-inactivated foetal bovine serum (FBS) (30 min, 56 °C) and 2% chicken embryo extract. Finally, cardiac cells cultures were maintained at 37 °C in an atmosphere of 5% CO_2.

4.2.1.3. Epimastigote susceptibility assays. The activity over *T. cruzi* CL-B5 *lacZ* was evaluated in presence of the substrate CPRG [66,68], according to the colorimetric method optimized by our research group [70], with some modifications [71]. The assay was performed in 96-well microtitre plates by seeding 2.5 × 10^5 log-phase epimastigotes/mL (200 μL/well), which were incubated within the compounds for 72 h at 28 °C. Stock solutions of the studied compounds and the reference drug benznidazole [kindly provided by LAFEPE (Laboratório Farmacêutico de Pernambuco), Brazil] were prepared in $(CH_3)_2SO$ (DMSO) and added to the cultures to give 0.125–256 μM final concentrations. Concentration of DMSO in the cultures was always <0.2% v/v. Each concentration was tested in triplicate and growth, medium and drug controls included in all the plates. Afterwards, 50 μL of CPRG in 0.9% Triton X-100 was added per well (final concentration 200 μM, pH 7.4) and the plates incubated 3 h at 37 °C. Finally, absorbance was read at 595 nm in an ELx808 ELISA reader (Biotek Instruments Inc.) and percentages of epimastigote growth inhibition (%EGI) estimated as previously reported [71].

The activity on Y strain epimastigotes was evaluated by applying the resazurin assay previously standardized by our group [72]. Log-phase cultures were seeded at a density of 3 × 10^6 epimastigotes/mL in culture tubes and maintained at 28 °C overnight to allow homogeneous growth. Afterwards, 200 μL per well of cultures were distributed in 96-well microplates and incubated within the compounds for 48 h at 28 °C. Each concentration was tested in triplicate and growth, medium and drug controls included in all the plates. Then, 20 μL/well of resazurin in PBS (3 mM, pH 7) was added and the plates maintained at 28 °C for another 5 h. Finally, fluorescence intensity was measured (AlamarBlue® Assay, U.S. Patent No. 5,501,959; λ_{exc} 535 nm, λ_{em} 590 nm) in an Infinite 200 multifunctional microplate reader (Tecan) and %EGI estimated.

For both assays, the experiments were run in the same conditions three times separately (n = 3). IC_{50} values, i.e., the concentrations causing 50% of epimastigote growth inhibition (EGI), were estimated from the dose–response curves obtained by plotting drug concentrations vs %EGI. Results are expressed as the mean value of IC_{50} ± SD (standard deviation) (SPSS, v20, IBM).

4.2.1.4. Amastigote susceptibility assays. The activity on intracellular amastigotes was tested by infecting either L929 fibroblasts with TCT (CL-B5 and Tulahuen strains) or cardiac cells with BT (Y strain).

The assay on CL-B5 *lacZ* amastigotes was performed in 48-well tissue culture plates by using CPRG [72]. According to this, 120 μL of MEM containing 10,000 L929 cells were seeded per well and the plates incubated 2 h at 37 °C in a humidified 5% CO_2 atmosphere. After the attachment, cells were infected with TCT at a 1:6 ratio (cell:parasite) and incubated overnight at 33 °C with 5% CO_2. Following the infection, the medium was discarded and cultures washed with PBS to remove non-penetrated TCT. Infected cultures were incubated within compounds diluted in fresh MEM for 7 days in similar conditions of temperature and humidity. Each concentration was assayed in triplicate and controls of compounds, medium, cell growth and infection included in all the plates. By this time, 50 μL of CPRG in 3% Triton X-100 (final concentration 400 μM, pH 7.4) was added per well and the plates incubated 3 h at 37 °C. Absorbance was read at 595 nm in an Infinite 200 multifunctional microplate reader (Tecan) and percentages of amastigote growth inhibition (%AGI) calculated as reported [71].

The assay on Tulahuen amastigotes was conducted in 96-well tissue culture plates by distributing 100 μL/well of RPMIS containing 4000 L929 cells. The plates were maintained for 24 h at 37 °C with 5% CO_2 and afterwards, cells were incubated within TCT (ratio 1:10) for another 2 h. Next, non-penetrated parasites were discarded replacing culture medium by fresh one. In order to establish the infection, the plates were maintained during 48 h at 37 °C with 5% CO_2. Then, medium was replaced by solutions of each compound in fresh RPMIS and the plates incubated for 96 h at the same conditions of temperature and humidity. Each concentration was evaluated by triplicate and controls of infection and cell growth included in all the plates. Afterwards, 50 μL of 500 μM CPRG in 0.5% Nonidet P40 was added to each and the plates returned to the incubator for 18 h. Finally, absorbance was read at 570 nm in a SpectraMax 190 microplate reader (Molecular Devices) and the results expressed as %AGI [68].

Regarding the assay over Y strain amastigotes, 100,000 cardiac cells/well were seeded in 24-well tissue culture plates provided with round coverslips previously coated with gelatin and then, maintained overnight in DMEM at 37 °C with 5% CO_2. After 24 h of cell–parasite interaction (ratio 1:10), infected cultures were washed to remove non-internalized BT and incubated 48 h within compounds diluted in fresh DMEM. Next, cultures were fixed with Bouin's fixative and stained with Giemsa. The mean number of infected cells and the mean number of parasites per infected cell were scored in 400 host cells by duplicate and thus the endocytic index (EI) calculated by multiplying these two parameters. Only parasites with characteristic nuclei and kinetoplast were counted, since irregular ones were considered as parasites undergoing death. Activity results were estimated by calculating the percentage of inhibition of the EI (% EI) [73].

For each strain, the assays were run at the same conditions three times separately (n = 3). IC_{50} values were estimated from the dose–response curves obtained by plotting drug concentrations vs %AGI or %EI. Results are expressed as the mean value of IC_{50} ± SD (standard deviation) (SPSS, v20, IBM).

4.2.1.5. Unspecific cytotoxicity assays. The cytotoxic profile on L929 cells was studied in 96-well flat bottom microplates containing 100 μL of MEM with 15 × 10^3 cells/well. After cell attachment (3 h at 37 °C, 5% CO_2), fibroblasts were incubated for 48 h at 37 °C and 5% CO_2 within compounds diluted in MEM. Each concentration was tested in triplicate and controls of the compounds, medium and cultures included in all the plates. Afterwards, 20 μL of resazurin in PBS solution (2 mM, pH 7) was added to each well and the plates incubated similarly for 3 h. Fluorescence intensity was measured as aforementioned (λ_{exc} 535 nm, λ_{em} 590 nm) and percentages of unspecific cytotoxicity (%C) calculated [71].

The toxic effect on cardiac cell cultures was evaluated by seeding per well 6 × 10^4 cardiac cells/100 μL DMEM in 96-well microplates previously coated with gelatin and then, incubated 24 h at 37 °C in a humidified 5% CO_2 atmosphere. Afterwards, medium was replaced by solutions of each compound in fresh DMEM and plates

incubated 48 h in similar conditions of temperature and humidity. Each concentration was tested in triplicate and controls included in all the plates. Finally, cell morphology and contractibility were examined by light microscopy and viability evaluated in presence of the redox indicator PrestoBlue® according to manufacturer's instructions. After 5 h of incubation at 37 °C and 5% CO_2, absorbance was measured at 570 nm and 600 nm in a SpectraMax 190 microplate reader (Molecular Devices) and %C estimated as described previously [68].

For both assays, all the experiments were developed similarly for three (n = 3). LC_{50} values, i.e., the concentrations causing 50% of cellular lethality, were estimated from de dose–response curves obtained by plotting drug concentrations vs %C. Results are expressed as the mean value of LC_{50} ± SD (standard deviation) (SPSS, v20, IBM).

4.2.2. Study of trichomonacidal activity

4.2.2.1. T. vaginalis culture. The *in vitro* assays were conducted with two different isolates from the American Type Culture Collection (ATCC, Maryland, USA): JH31A#4 (metronidazole-sensitive) and IR78 (metronidazole-resistant). Trophozoites were cultivated in TYM (Tripticase-Yeast-Maltose) medium supplemented with 10% fetal bovine serum (FBS) and 5% of antibiotic solution (100 IU penicillin and 100 μg/mL streptomycin), in a humified chamber at 37 °C and 5% CO_2.

4.2.2.2. Mammalian cell cultures. The cellular line employed for the unspecific cytotoxic assays were Vero CCL-81 (ATCC, Maryland, USA). The cells were cultured in RPMI medium (Sigma–Aldrich), supplemented with 10% FBS and antibiotics solution (100 IU penicillin and 100 μg/mL streptomycin) in a humidified 95% air/5% CO_2 atmosphere at 37 °C.

4.2.2.3. Trophozoites susceptibility assays. Initially, the studied compounds were evaluated against the sensitive isolate JH31A#4. Stock solutions of the studied compounds were prepared in DMSO and added to glass tubes containing log-phase growth cultures after 5 h of seeding 10^5 trophozoites/mL, to achieve 9.37–300 μM final concentrations of the products. Final concentration of DMSO was always <0.2% v/v. The biological activity was determined after 24 h of incubation at 37 °C by fluorimetric determination using resazurin (Sigma–Aldrich) as redox dye as previously described [74]. Metronidazole (Sigma–Aldrich) at 25 μM concentration was used as the reference drug.

Trichomonacidal activity against the resistant isolate IR78 was evaluated only for compounds showing a relevant activity against JH31A#4 isolate and absence of unspecific cytotoxic effect against Vero cells [75].

The experiments were performed at least two times in triplicate. IC_{50} values and the 95% confidence limits were calculated by probit analysis (SPSS v20, IBM).

4.2.2.4. Unspecific cytotoxicity assays. Vero cells were seeded (50,000 cells/well) in 96-well flat-bottom microplates (Nunc) with 100 μL of medium. After cell attachment for 6 h at 37 °C, 100 μL of medium containing the studied products were added. The unspecific toxicity was determined after 24 h of incubation with the compounds. Resazurin (1 mM stock solution) was used as redox dye. After 3 h of incubation, fluorescence was measure in a fluorimeter (Infinite 200, Tecan) (λ_{exc} 535 nm, λ_{em} 590 nm) following a method previously published by our group [75].

Each concentration was assayed per triplicate and in two independent assays. The concentration causing 50% of Vero cells growth inhibition (CC_{50}) and the 95% confidence limits was determined by probit analysis (SPPS v20, IBM).

4.3. Physicochemical and pharmacokinetic parameters

4.3.1. In silico calculation of parameters

A set of 34 physicochemical descriptors was computed using QikProp version 3.5 integrated in Maestro (Schrödinger, LLC, New York, USA). Relevant QikProp descriptors are presented as supplementary material (Table S1). The 3D conformations used in the calculation of QikProp descriptors were generated using the program Spartan '08 (Wave function, Inc., Irvine CA) as follows: the structure of each molecule was built from the fragment library available in the program. Then, *ab initio* energy minimizations of each structure at the Hartree-Fock 6-31G* level were performed. A conformational search was next implemented using Molecular Mechanics (Monte Carlo method) followed by a minimization of the energy of each conformer calculated at the Hartree-Fock 6-31G* level. The global minimum energy conformer of each compound was used as input for ADME studies with QikProp.

4.3.2. Taft's σ^* constants

Most values of σ^* constants for substituents (supplementary material, Table S1) have been obtained from the literature [76]; in some cases, σ^* values have been calculated from pK_as found in the literature for the corresponding carboxylic acids [77] using the equation $pK_a = 4.66–1.62\sigma^*$ [76].

4.3.3. Conformational analysis and electrostatic potential maps calculation

Global minimum energy analysis of the compounds was performed using *ab initio* Hartree-Fock (HF) calculations at the 6-31G* level, within the Spartan '08 (Wave function, Inc., Irvine, CA). A conformational search was next implemented using Molecular Mechanics (Monte Carlo method). Local energy minima were identified by rotation of a subject torsion angle through 360° in 60° increments (6-fold search), followed by HF 6-31G* energy minimization of each rotamer generated. The electrostatic potential of the global minimum energy conformer was calculated using the Hatree-Fock method at the 6-31G* level of theory and was mapped on the 0.002 isodensity surface of each molecule. The surface was color-coded according to the potential, with electron-rich regions colored red and electron-poor regions colored blue (supplementary material, Fig. S1).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.03.036.

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