Amaryllidaceae alkaloids with anti-<i>Trypanosoma cruzi</i> activity

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Abstract

**Background:** Chagas disease, caused by the protozoan <i>Trypanosoma cruzi</i>, is a neglected disease that affects ~7 million people worldwide. Development of new drugs to treat the infection remains a priority since those currently available have frequent side effects and limited efficacy at the chronic stage. Natural products provide a pool of diversity structures to lead the chemical synthesis of novel molecules for this purpose. Herein we analyzed the anti-<i>T. cruzi</i> activity of nine alkaloids derived from plants of the family Amaryllidaceae.

**Methods:** The activity of each alkaloid was assessed by means of an anti-<i>T. cruzi</i> phenotypic assay. We further evaluated the compounds that inhibited parasite growth on two distinct cytotoxicity assays to discard those that were toxic to host cells and assure parasite selectivity.

**Results:** We identified a single compound (hippeastrine) that was selectively active against the parasite yielding selectivity indexes of 12.7 and 35.2 against Vero and HepG2 cells, respectively. Moreover, it showed specific activity against the amastigote stage (IC<sub>50</sub> = 3.31 μM).

**Conclusions:** Results reported here suggest that natural products are an interesting source of new compounds for the development of drugs against Chagas disease.

**Keywords:** Chagas disease, <i>Trypanosoma cruzi</i>, Alkaloids, Amaryllidaceae, Hippeastrine, Phenotypic assays, Cytotoxicity

Background

Chagas disease (or American trypanosomiasis) is a neglected infectious disease caused by the protozoan parasite <i>Trypanosoma cruzi</i> (order Kinetoplastida; family Trypanosomatidae). It is estimated that ~7 million people are affected by the disease, mainly in Latin America where <i>T. cruzi</i> infection is endemic [1].

The disease progresses in two phases. There is first a short acute phase that is usually asymptomatic and thus goes unnoticed. This is followed by a chronic phase characterized by absent or slow progression of clinical manifestations [2]. Nonetheless, it is estimated that ~40% of those chronically infected will ultimately develop disruptive damage to the heart and/or digestive tract (esophagus and colon) tissues, which can lead to the formation of mega-syndromes and death if untreated [2, 3].

Since the 1970s only two drugs have been available to treat <i>T. cruzi</i> infections: benznidazole (BNZ) and nifurtimox (NFX) [1]. Both have good efficacy and tolerability when administered to infected new-borns [4]. But their efficacy diminishes at the chronic stage, which is usually diagnosed at adulthood with serological tests that detect specific anti-<i>T. cruzi</i> type G immunoglobulins [1]. Moreover, both drugs have long regimens of administration that entail the advent of frequent adverse events which often drive to treatment discontinuation [5–7]. There
is thus an urgent unmet need of safer and more efficacious drugs for the treatment of chronic Chagas disease, for which natural products may represent a promising approach to discover new lead compounds [8–10].

In this regard, members of the family Amaryllidaceae have attracted considerable attention in the last few years due to their unique alkaloid composition with multiple biological activities [11]. Amaryllidaceae plants have been studied for their potential application as a source of anticancer, anti-inflammatory, antimicrobial, anti-parasitic and anticholinesterase activities [12]. In fact, they have been used for centuries as part of traditional treatments for fever, swelling, cancer or malaria [12]. Remarkably, in 2001 the Food and Drug Administration (FDA) approved the use of galanthamine (trade name Razadyne), an alkaloid identified from the Amaryllidaceae plant Galanthus woronowii, to treat Alzheimer’s disease [13].

Alkaloid constituents found in these plants are classified in eight groups based on structure and biogenesis from the common precursor O-methylorobelladine: galanthamine; lycorine; crinine; haemanthamine; homolycorine; narciscamine; tazettine; and montanine [14]. The unique structure of this set of alkaloids provides a viable platform for phytochemical-based drug discovery [8]. With the aim to identify prospective drug development starting points that could eventually become new therapeutic solutions for Chagas disease we have adapted an in vitro anti-T. cruzi phenotypic assay based on the parasite Tulahuen strain engineered to express a bacterial β-galactosidase gene [15] and green monkey epithelial cells (Vero) as hosts. We evaluated the anti-T. cruzi activity of nine crystalized alkaloid compounds extracted from members of the Amaryllidaceae family: lycorine, hippeastrine, crinine, haemanthamine, narciscamine, tazettine, montanine, sanguinine and 1-O-acetylcaranine (Fig. 1) [16]. In all the assays performed we always included the standard anti-parasitic drug BNZ for comparison.

In order to unveil the specific T. cruzi growth inhibitory capacity of those compounds that were found active in the anti-parasitic assay, we further used two secondary biological assays to determine the compounds’ level of cytotoxicity. These were respectively based on the same host Vero cells and in the human hepatocellular carcinoma cell line HepG2. Finally, we determined the anti-amastigote specific activity of the only compound that was revealed to hold selective anti-parasitic activity. Results obtained were particularly promising for the compound hippeastrine from Narcissus cv. Salome [17] and they are discussed herein.

**Methods**

**Collection of purified alkaloid compounds from Amaryllidaceae plants**

Lycorine, hippeastrine, crinine, haemanthamine, narciscamine, tazettine, montanine, sanguinine and 1-O-acetylcaranine alkaloids were isolated from extracts of different Narcissus species [16, 18]. The information of all the compounds studied can be found in the extensive chapter by Bastida et al. [16]. In brief, the procedure followed to identify the alkaloids within the corresponding plant extract was as follows: plant material (60 mg) was macerated with MeOH; the mix was filtered and the solvent evaporated to dryness. After that, extracts were acidified with 500 µl of H2SO4 (2%, v/v). The neutral material was removed with diethyl ether and basified with 200 µl NH4OH (25%, v/v). Then, 750 µl of diethyl ether was added to separate the organic phase, this was repeated twice, and the solvent evaporated to dryness. All compounds were crystals, obtained after three successive crystallization rounds to ensure maximum purity. They were subjected to a combination of chromatographic techniques and alkaloids were identified by GC-MS and NMR [16, 17] (Additional file 1: Figures S1, S2). In order to obtain milligrams of product, the corresponding scale-up was performed as previously described [19, 20].

**Host cells cultures**

Vero (green monkey kidney epithelial cells), LLC-MK2 (Reshus monkey kidney epithelial cells) and HepG2 (human liver epithelial cells) cultures were maintained with DMEM supplemented with 1% penicillin-streptomycin (100 units/ml of penicillin and 100 µg/ml of streptomycin; P-S) and 10% heat inactivated fetal bovine serum (FBS) at 37 °C, 5% CO2 and > 95% humidity as described by Buckner et al. [15]. HepG2 were also supplemented with 1 x non-essential amino acids (ref. 01-340-1B; Biological Industries, Beit-Haemek, Israel).

**Culture of T. cruzi parasites**

Trypanosoma cruzi parasites from the Tulahuen strain (discrete typing unit, DTU VI) expressing β-galactosidase were kindly provided by Dr Fred Buckner (University of Washington, Seattle, USA) and maintained using LLC-MK2 cells as hosts in DMEM supplemented with 2% FBS and 1% P-S as previously described [15]. Free-swimming trypomastigotes were purified by centrifugation of the cell culture supernatant for 7 min at 2500 × rpm using a low break speed, then allowing them to swim out of the pellet [21]. Purified trypomastigotes were used to keep the parasite
cycle in LLC-MK2 cells and for the performance of the anti-parasitic assays. In the last case, an extra round of centrifugation was performed to remove phenol red from the maintenance DMEM and replace with a phenol red-free DMEM medium, which was supplemented with 1% P-S-glutamine, 2% FBS, 1 mM sodium-pyruvate and 25 mM HEPES [21].

**Assay to detect T. cruzi growth inhibition in 96-well plates**

Our assay is based on Vero cells as hosts and infective trypomastigotes from the Tulahuen strain that express the bacterial β-galactosidase enzyme as reporter activity [15]. First, alkaloids were added in the first column of a 96-well tissue culture treated plates at an initial concentration of 100 µM and diluted in assay medium into the next columns of the plate to conform dose-response plate-maps following either a 1:2 or 1:3 fold pattern. Then, Vero cells were detached from their growing flasks, counted and diluted at a concentration of 1 × 10⁶ cells per ml. Trypan blue staining was used to check their viability, which had to be >95% to proceed. In conjunction, purified trypomastigotes were counted and diluted at a concentration of 1 × 10⁶ cells per ml. We directly mixed Vero cells and trypomastigotes in a falcon tube in a sufficient volume so as to add 100 µl of the mix per well (50,000 Vero cells and 50,000 trypomastigote cells per well; with the multiplicity of infection, MOI = 1). The percentage of DMSO in all wells was always kept below 0.5%.

The reference drug BNZ was used as a control of drug growth inhibition in each run, whereas each plate contained its own negative (maximum parasite growth; Vero cells plus parasites without drugs) and positive (minimum parasite growth; trypomastigote forms alone marking an enzymatic zero time or baseline

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**Fig. 1** Chemical structures of the alkaloids evaluated in this study
galactosidase activity) controls. Note that trypomastigotes are unable to multiply in the absence of susceptible host cells. Plates were incubated for 4 days at 37 °C [21], with the readout performed by adding 50 μl per well of a PBS solution containing 0.25% NP40 and 500 μM chloroferol red-β-β-d-galactoside (CPRG) substrate, as previously described [21]. Upon addition of the substrate, plates were further incubated at 37 °C for another 4 h and the absorbance read out at 590 nm using an Epoch Gene5 spectrophotometer (Biotek, Winooski, USA). All experiments were performed at least in triplicate.

Anti-amastigote specific activity of progressed compound (hippeastrine)
Using Vero cells as hosts and the recombinant T. cruzi strain expressing β-galactosidase, we further adapted the anti-parasitic assay described above to determine whether the anti-parasitic activity was specific against the intracellular amastigote forms. In brief, we plated 50,000 Vero cells per well in a 96-well plate and allowed them to attach for 1.5 h. Then, we infected the monolayers with 50,000 purified trypomastigotes per well (MOI = 1) that were allowed for 1 h to adsorb and enter the cells before being washed with PBS three times. Finally, assay medium was added and used to dilute hippeastrine and BNZ in a dose-response pattern. In each plate we included the same controls as for the anti-T. cruzi assay. Test plates were incubated for 96 h and the assay readout was performed as described above.

Toxicity assays with Vero and HepG2 cells
For the cell toxicity assays, compounds were added to tissue culture treated 96-well plates following a dose-response dilution pattern, 1:2 or 1:3, with a starting concentration of 400 μM or 800 μM per well in the first column of the plate. Cell viability was checked upon cell counting with Trypan blue and we only proceeded if viability was > 95%. Vero cell suspension was diluted at 10% Alamar Blue reagent (Thermo Fisher Scientific, Eugene, USA) and incubated the plates for 6 h at 37 °C before reading the fluorescence intensity in a Tecan Infinite M Nano® reader (Tecan, Männedorf, Switzerland) (excitation: 530 nm, emission: 590 nm). The percentage of DMSO in all wells was always kept below 0.5%. All experiments were performed at least in triplicate.

Data analysis
Absorbance and fluorescence values derived from the anti-T. cruzi and cell toxicity assays were normalized to the controls [22]. IC$_{50}$ and TC$_{50}$ values were determined using GraphPad Prism 7 software (version 7.00, 2016) using a non-linear regression analysis model defined by the equation:

$$Y = 100 \div \left( \left(1 + \frac{X^{\text{HillSlope}}}{\text{IC}_{50}^{\text{HillSlope}}} \right) \div \left(1 + \frac{X^{\text{HillSlope}}}{\text{TC}_{50}^{\text{HillSlope}}} \right) \right)$$

These IC$_{50}$ and TC$_{50}$ values are the compound concentrations capable of inhibiting growth of parasites and cells by 50%, respectively. Z$'$-values were calculated as described previously [23]. Values provided are the mean and standard deviation (SD) of at least three independent experiments.

Results and discussion
Quality assessment of the anti-T. cruzi and cell toxicity assays
As part of the process of setting up the biological assays we calculated their Z$'$ parameter to assess reproducibility and statistical robustness [23]. In general, assays with a Z$'$ between 0.5 and 1 are considered appropriate for the screening of compounds [23]. Remarkably, our anti-parasitic assay had a very good performance and its Z$'$-value remained consistently > 0.5 with an average value of 0.89 (0.097) (Fig. 2a). Regarding the two cytotoxicity assays used in this study, we retrieved a Z$'$ of 0.76 (0.067) for the assay based on Vero cells, and a Z$'$ of 0.73 (0.054) for the assay based on HepG2 cells (Fig. 2c–e).

Additionally, in every run of the T. cruzi growth inhibition assay and Vero cell toxicity assay performed, we included the reference drug BNZ as a control, whereas the reference drug digitoxin (DTX) [24] was included in all the HepG2 cells toxicity assays. Overall, averaged IC$_{50}$ and TC$_{50}$ values for BNZ were 1.56 (0.39) μM and 173.4 (43.57) μM, respectively (Fig. 2b–d, Additional file 1: Figure S3), which correlates with previous reports [21, 25]. The digitoxin TC$_{50}$ mean value in the HepG2 cell assay was 0.29 (0.14) μM (Fig. 2f, Additional file 1: Figure S3).

Anti-T. cruzi activity of the alkaloids extracted from Amaryllidaceae
As in other widely used anti-T. cruzi assays [21, 22, 26] we relied on the genetically robust T. cruzi Tulahuen strain expressing beta-galactosidase activity as a surrogate of parasite growth [15]. However, because the amastigote replicative stage of T. cruzi is obligatory intracellular,
quality controls of the T. cruzi growth inhibition assay (a, b), toxicity assay with Vero cells (c, d) and toxicity assay with HepG2 cells (e, f). Z’-values for each of the rounds launched are represented on the left (a, c, e); dashed line marks the 0.5 threshold. IC_{50} and TC_{50} values of the reference drugs BNZ and DTX are represented on the right (b, d, f); continuous lines indicate the average values, whereas the dashed lines indicate ± 3 SD limits.
promising results against ZIKA virus infection have recently been reported with hippeastrine hydrobromide [38]. This was shown to remove ZIKA virus from infected human neural progenitors, recover a ZIKV-induced microcephaly phenotype in human forebrain organoids and even suppress virus propagation in infected adult mice [38]. Antiviral [37, 38], antibacterial and antifungal [39] activities have been reported for hippeastrine, despite this, little information is available about its antiparasitic activity. Cedron et al. [40] tested 21 hippeastrine derivatives that included functional group transformations, structural simplification and dimer formation against *Plasmodium falciparum* (strain F-32 Tanzania). The anti-malarial activity increased by 10-fold when dimers were evaluated compared to the single alkaloid activity, suggesting an improved binding to the related target or the hydrolysis of the dimer onto two molecules [40]. To our knowledge, this is the first time that anti-*T. cruzi* activity is reported for hippeastrine. Results reported by Cedron et al. [40] would suggest to further pursue research with hippeastrine derivatives against *T. cruzi*.

**Identification of alkaloid compounds with specific anti-*T. cruzi* activity**

With the aim of further selecting those alkaloids with specific activity against the parasite and discard those that were toxic to host cells, we used two secondary cell toxicity assays with monkey (Vero) and human (HepG2) cells. Since the compounds activity might vary depending on the characteristics of the cell line used, performing the cytotoxicity assay in two cell lines will provide a more robust readout. Moreover, HepG2 cells are a widespread cellular model used to anticipate potential liver toxicity of drug metabolism [41, 42]. We determined a selectivity index (SI; or TC$_{50}$ to IC$_{50}$ ratio) > 10 to consider whether an alkaloid was suitable for further progression, as described elsewhere [22].

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**Fig. 3** Anti-*T. cruzi* phenotypic assay dose-response curves. Graphs represent mean results and SD of at least three biological replicates.
Thereafter, all the alkaloids reported as active against *T. cruzi* were analysed through both cell toxicity assays. All of them were more toxic to Vero cells than to HepG2 cells (Fig. 4). The cytotoxicity values registered against Vero and HepG2 cells indicated that narciclasine activity was not specific against *T. cruzi*, as it respectively showed TC$_{50}$ = 0.66 (0.082) µM against Vero cells, and TC$_{50}$ = 2.73 (0.67) µM against HepG2 cells, resulting in a SI < 10 in both cases (Table 1). In addition, montanine with a SI = 2.53 in relation to Vero cells was not specific to *T. cruzi* either (Table 1), even though it showed low toxicity to HepG2 cells [TC$_{50}$ = 46.1 (11.99) µM] (Table 1). Something similar occurred with lycorine, which was weakly active against HepG2 cells [TC$_{50}$ = 21.87 (4.16) µM] (Table 1). Lycorine presented a SI versus this cell line over ten times its registered anti-*T. cruzi* activity, but turned to be toxic to Vero cells [TC$_{50}$ = 5.21 (0.80) µM] with a SI < 10 and thus was discarded from further progression (Table 1). We generally observed an increase sensitivity of Vero cells to the alkaloids when compared to HepG2 cells. This may have been in part due to the fact that compounds were incubated for a longer time (four versus two days) on Vero cells than on HepG2 cells. Haemanthamine has been reported to present a TC$_{50}$ = 13 µg/ml, i.e. 43.14 µM for HepG2 cells [43], which correlates with the TC$_{50}$ value obtained in our study with the same cell line [TC$_{50}$ = 42.48 (6.96) µM] (Table 1). However, although this compound showed a SI > 10 with respect to HepG2 cells, when evaluated on

**Table 1** Alkaloid average IC$_{50}$, TC$_{50}$ and SI values for Vero and HepG2 cells

| Alkaloid     | IC$_{50}$ (µM) | TC$_{50}$ (µM)$^a$ | SI$^a$ | TC$_{50}$ (µM)$^b$ | SI$^b$ |
|--------------|----------------|---------------------|--------|---------------------|--------|
| BNZ          | 1.56           | 173.4               | 111.15 | 168.76              | 108.18 |
| Lycorine     | 0.70           | 5.21                | 7.44   | 21.87               | 31.24  |
| Hippeastrine$^c$ | 3.63        | 45.99               | 12.67  | 128.10              | 35.29  |
| Crinine      | 57.93          | –                   | –      | –                   | –      |
| Haemanthamine| 1.59           | 11.52               | 7.25   | 42.48               | 26.72  |
| Narciclasine | 0.49           | 0.66                | 1.33   | 2.73                | 5.52   |
| Tazettine    | 83.03          | –                   | –      | –                   | –      |
| Montanine    | 1.99           | 5.04                | 2.53   | 46.10               | 23.17  |
| Sanguinine   | 213.40         | –                   | –      | –                   | –      |
| 1-O-acetylcaranine | 35.49     | –                   | –      | –                   | –      |

$^a$ Vero cell toxicity assay
$^b$ HepG2 cell toxicity assay
$^c$ The only alkaloid evaluated in this study that showed specific anti-*T. cruzi* activity

*Note*: The standard drug BNZ is included in the first line for comparison

**Fig. 4** Dose-response curves obtained from the Vero and HepG2 cell toxicity assays. Vero cells toxicity assays are represented by circles and straight lines while HepG2 cell toxicity assays are represented by triangles and dashed lines. Graphs represent mean results and SD of at least three biological replicates.
Vero cells its TC\textsubscript{50} to IC\textsubscript{50} ratio was below that threshold and thus its anti-parasitic activity could not be considered specific (Table 1).

In contrast to all the aforementioned results, hippeastrine did show low toxicity against Vero cells [TC\textsubscript{50} = 45.99 (6.32) µM] and HepG2 cells [TC\textsubscript{50} = 128.1 (12.26) µM], and complied with the SI window > 10 against both cell lines (Table 1). It was the only compound that had a SI > 10 versus Vero cells (SI = 12.67; Table 1). Additionally, hippeastrine presented the highest SI against HepG2 cells (SI = 35.29), with a TC\textsubscript{50} value similar to that previously reported by Weniger et al. [43] (TC\textsubscript{50} = 40 µg/ml, i.e. 126.85 µM). Therefore, in a subsequent anti-amastigote biological assay we assessed whether hippeastrine anti-\textit{T. cruzi} activity was indeed specific against this replicative form of the parasite. We found that the observed anti-amastigote activity (IC\textsubscript{50}) was 3.31 (0.39) µM, which was again within 5× that of the reference drug BNZ in the same assay [IC\textsubscript{50} = 1.2 (0.22) µM] (Fig. 5). Moreover, the corresponding SI for hippeastrine with respect to its anti-amastigote activity will yet be > 10 (SI = 13.89 against Vero cells, and 38.69 against HepG2 cells).

Cytotoxicity results together with in vivo assays reported by Zhou et al. [38] may predict a low toxicity of hippeastrine in a future evaluation of its anti-\textit{T. cruzi} activity in animal models. However, before performing in vivo studies, additional in vitro studies should be pursued to better validate this alkaloid. For example, assessing its effect on a \textit{T. cruzi} CYP51 target [44], since this drug target has been invalidated in the clinic [45, 46], and identifying whether it can kill dormant parasite forms [47]. Moreover, it would be of interest to determine key in vitro pharmacokinetic (PK) parameters such as its solubility, permeability and clearance.

**Conclusions**

We identified one compound with specific anti-\textit{T. cruzi} activity, upon the evaluation of nine alkaloids purified from extracts of different \textit{Narcissus} species (family Amaryllidaceae) [16], proving that natural products are an interesting source to potentially identify new chemical structures for Chagas disease drug discovery. Our findings suggest that hippeastrine [17] is a relevant compound to be further studied. The analysis of its capacity to kill parasite dormant forms, and identification of its main target deserve further investigation in the future.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04171-6.

**Additional file 1:** Figure S1. EIMS spectra of the nine compounds used in the study. Figure S2. 1H NMR spectra of hippeastrine. Figure S3. BNZ and DTX dose-response curves. Both reference drugs were included in every assay as a control of drug inhibition. Anti-\textit{T. cruzi} assays are represented by circles while Vero and HepG2 cell toxicity assays by squares and triangles, respectively.

**Abbreviations**

BNZ: benznidazole; NFX: nifurtimox; FDA: Food and Drug Administration; GC-MS: gas chromatography-mass spectrometry; NMR: nuclear magnetic resonance; DMEM: Dulbecco’s Modified Eagle’s Medium; P-S: penicillin-streptomycin; FBS: fetal bovine serum; DMSO: dimethyl sulfoxide; PBS: phosphate-buffered saline; CPRG: chlorophenol red-β-d-galactoside; SD: standard deviation; SI: selectivity index.

**Acknowledgements**

Not applicable.

**Authors’ contributions**

NMP, NCS, JB, MJP, JG and JAP conceptualized the study. NMP performed all biological assays. NCS helped with the assays. LTC and JB purified the alkaloids and provided the collection. MJP and JG provided funds. NMP and JAP wrote the article. All authors read and approved the final manuscript.
Funding
We thank the support by the Departament d'Universitats i Recerca de la Generalitat de Catalunya, Spain (GA2UR; 2017SGR000924), and funding by the Instituto de Salud Carlos III RETICS Network for Cooperative Research in Tropical Diseases (ISCIII RD12/0018/0010) and FEDER. JAP was funded by a Juan de la Cierva - Incorporación contract from the Spanish Science Ministry. LTC and JB (UR research group 2017SGR604) thank CYTED (416RT0511) for financial support. MJP research is supported by the Ministry of Health, Government of Catalonia (PERIS 2016-2010 SLT008/18/00132). We acknowledge support from the Spanish Ministry of Science and Innovation through the "Centro de Excelencia Severo Ochoa 2019-2023" Program (CEX2018-000286-S), and support from the Generalitat de Catalunya through the CERCA Program.

Availability of data and materials
Data supporting the conclusions of this article are included within the article and its additional file. Data and materials can be made available upon reasonable request to the authors.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 18 January 2020    Accepted: 4 June 2020

Published online: 10 June 2020

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