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# 6-Methyl-7-deazapurine nucleoside analogues as broad-spectrum antikinetoplastid agents

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ABSTRACT

Kinetoplastid parasites are the causative agents of Chagas disease (CD), leishmaniasis and human African trypanosomiasis (HAT). Despite a sustained decrease in the number of HAT cases, more efforts are needed to discover safe and effective therapies against CD and leishmaniasis. Kinetoplastid parasites lack the capability to biosynthesize purines *de novo* and thus critically depend on uptake and processing of purines from host cells. As such, modified purine nucleoside analogues may act as broad-spectrum antikinetoplastid agents. This study assessed the *in vitro* activity profile of 7-modified 6-methyl tubercidin derivatives against *Trypanosoma cruzi*, *Leishmania infantum*, *Trypanosoma brucei brucei* and *T. b. rhodesiense*, and led to the identification of analogues that display activity against all these species, such as 7-ethyl (**13**) and 7-chloro (**7**) analogues. These selected analogues also proved sufficiently stable in liver microsomes to warrant *in vivo* follow-up evaluation.

## 1. Introduction

Kinetoplastid protozoan infections are responsible for a number of neglected tropical diseases (NTDs) that cause significant mortality and morbidity. These NTDs include Chagas disease (CD), leishmaniasis and human African trypanosomiasis (HAT), of which the former two also impose a growing threat to non-endemic areas owing inter alia to global migration (Bilbe, 2015; Filardy et al., 2018). CD is caused by *Trypanosoma cruzi* (*T. cruzi*) and transmitted primarily by triatomine bugs (Perez-Molina and Molina, 2018), leishmaniasis is caused by more than 20 *Leishmania* species which are transmitted by female sandflies (Burza et al., 2018), and HAT or sleeping sickness is caused by *T. brucei* species upon transmission by tsetse flies (Buscher et al., 2017). These infections mainly affect poor communities in Latin America, Asia and Africa where an estimated 30,000 people succumb every year (Rao et al., 2019). Extensive efforts from the World Health Organization (WHO) and Drugs for Neglected Diseases *initiative* (DND*i*) have catalyzed the introduction of the nifurtimox-eflornithine combination (Kuemmerle et al., 2020; Priotto et al., 2009) and the approval of fexinidazole (Deeks, 2019; Mesu et al., 2018), an oral drug for advanced-stage HAT. A possible drawback is that nifurtimox and fexinidazole share a common bioactivation mechanism, enhancing the risk of cross-resistance (Wilkinson et al., 2008).

The encouraging progress in combating HAT contrasts with that of CD and leishmaniasis (Croft et al., 2017). Approved drugs for both diseases have important limitations and often fail to result in sterile cure. Neither of the available CD dugs (benznidazole and nifurtimox) is effective against the chronic state (Bermudez et al., 2016) and potentially serious adverse effects restrict their use (Croft et al., 2006). Drugs for leishmaniasis have generally been repurposed from other indications, but display variable efficacy against the different species (Uliana et al., 2018). In summary, new, safe, effective and short-course therapies for these diseases are still urgently awaited and the number of drugs in the clinical development pipelines remains rather scarce.

Although parasites are highly diverse in structure and replication strategies, many eukaryotic parasites share similar metabolic pathways and housekeeping genes, as evident from their protein sequence conservation (Marilyn Parsons, 2016). The aforementioned target kinetoplastids share a conserved core proteome encoded by about 6200 genes (El-Sayed et al., 2005). This is reflected by fairly similar protein sequences and remarkable sequence and structural congruence in the active sites of enzymes (Naula et al., 2005). Besides, the three target protozoa are critically dependent on the acquisition of purine nucleobases and nucleosides from host cells to grow and multiply. Towards

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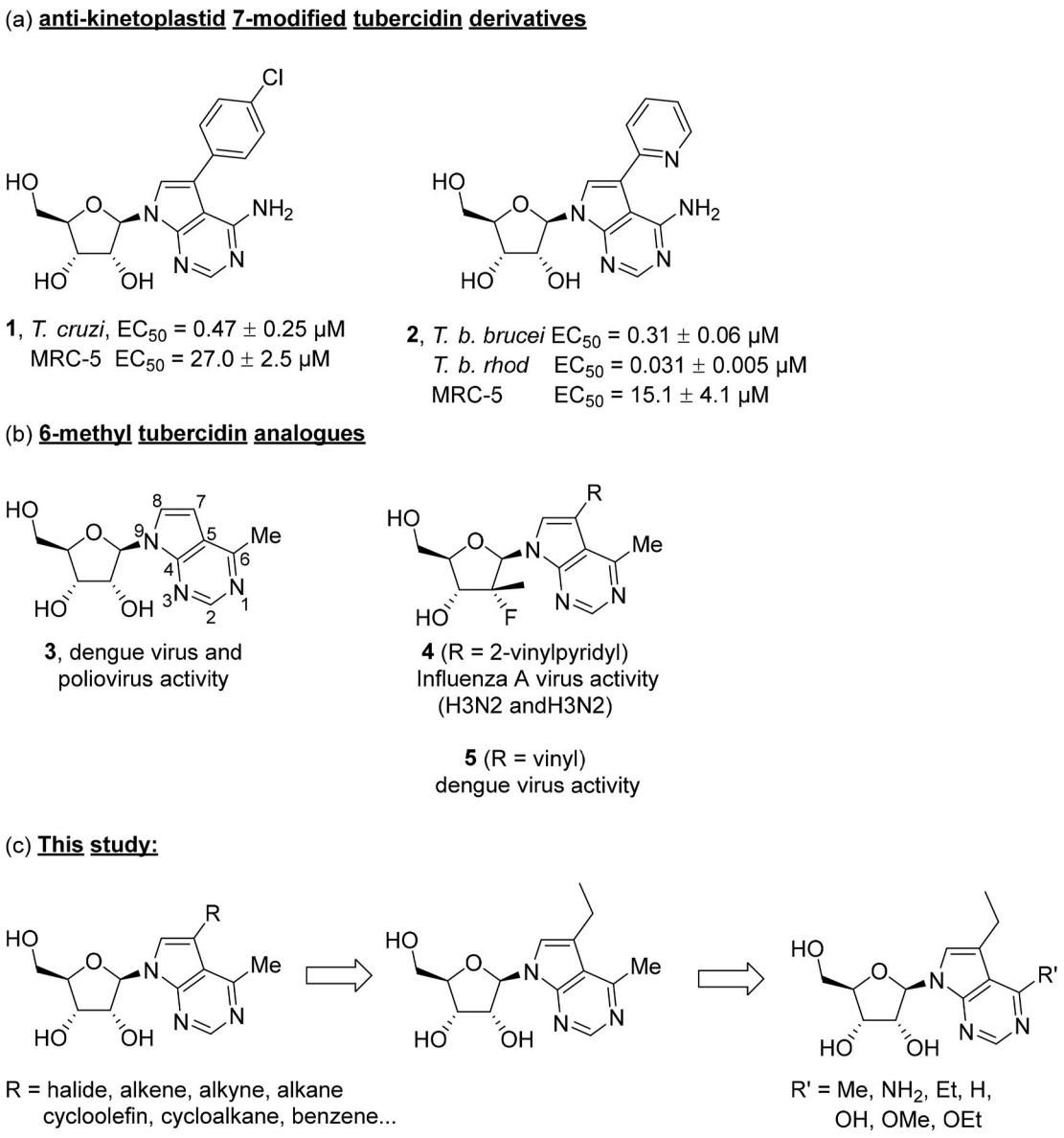



**Fig. 1.** (a) Antikinetoplastid 7-modified tubercidin derivatives; (b) Examples of antiviral 6-methyl tubercidin analogues; the indicated numbering (purine numbering) was used in the text; (c) 6-Methyl tubercidin analogues investigated in this study as antikinetoplastid agents.

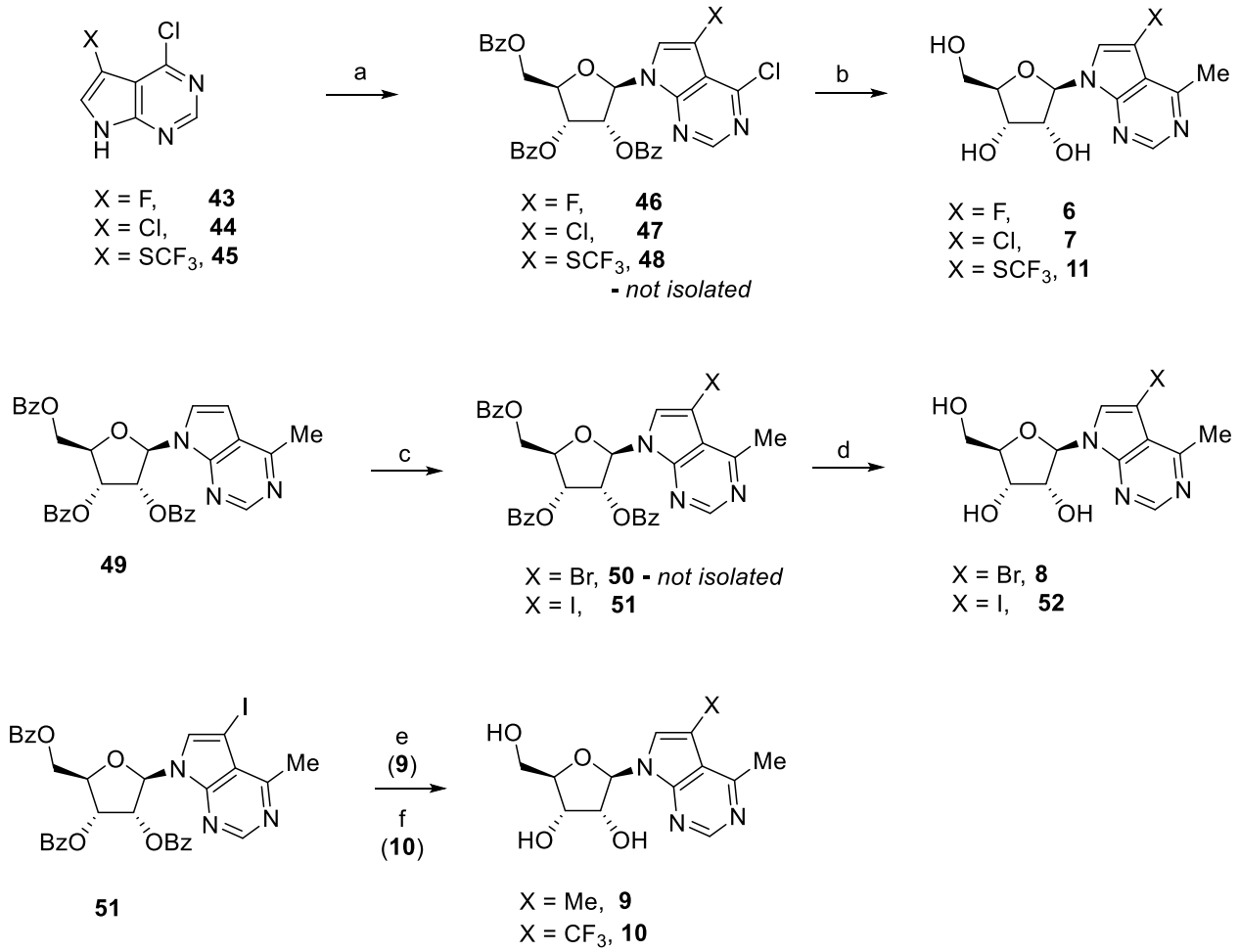
that goal they have developed dedicated intracellular salvage pathways and transporter proteins. These features make these parasites vulnerable to inhibitors of purine salvage pathways or subversive substrates (Berg et al., 2010). Indeed, modified nucleosides provide a rich source of hits against kinetoplastids as we previously identified tubercidin analogues with potent anti-*T. brucei* and anti-*T. cruzi* activity (Hulpia et al., 2018, 2019a) (Fig. 1 (a)). Hitherto, we were less successful in identifying nucleoside-like antileishmania hits.

In this study, we explored nucleoside analogues derived from 7-deaza-6-methyl-9-β-D-ribofuranosylpurine (6-methyl tubercidin analogues). 6-Methyl-7-deazapurine ribonucleoside itself is a potent inhibitor of dengue virus (DENV) and poliovirus (PV) replication, but toxicity hampered its application (Wu et al., 2010). An extended series of 6-substituted 7-(het)aryl-7-deazapurine ribonucleosides afforded 7-substituted 6-Methyl-7-deazapurine ribonucleoside analogues with activity against *Mycobacterium bovis*, HCV, as well as anti-cancer activity (Naus et al., 2014; Perlikova et al., 2013). Several 2′-modified analogues showed potent *in vitro* activity against Influenza A virus (H3N2 and H1N1) (Lin et al., 2016) and DENV (serotypes 1–4) (Lin et al., 2018) without apparent cytotoxicity (Fig. 1 (b)). A recent screening of a panel of tubercidin analogues with a privileged 7-(4-chlorophenyl) substituent indicated that upon substitution of a 6-methyl for a 6-amino group the anti-T. cruzi activity was largely retained, while the selectivity (vs. MRC-5 fibroblasts) was improved (Lin et al., 2021). To expand the SAR, the present study aimed to synthesize mostly novel 7-substituted 6-methyl-7-deazapurine ribonucleosides with emphasis on non-aromatic substituents and assess their activity against the stated protozoan parasites.

## 2. Methods

### 2.1. Chemistry

All reagents and solvents were in the analytical grade from standard commercial sources. All were used directly without further purification unless indicated. Reactions were performed under the monitor of analytical TLC or HRMS. The TLC was precoated F254 aluminum plates obtained from Machery-Nagel® and were visualized by UV followed by staining with basic aq. $KMnO_4$. HRMS were equipped with a Waters LCT Premier XE™ Time of Flight (ToF) mass spectrometer, a standard




**Scheme 1.** Reagents and conditions: (a) 1-*O*-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose, BSA, TMSOTf, MeCN, 80 °C for **46** and **47**; 65 °C for **48**, 63% (**46**), 69% (**47**); (b) (i) $Me_3Al$, $Pd(PPh_3)_4$, THF, 100 °C; (ii) 7 N $NH_3$/MeOH, 66% (**6**), 62% (**7**) for two steps; 27% (**11**) for three steps (c) NIS or NBS, DMF, 85% (**50**), 82% (**51**); (d) 7 N $NH_3$/MeOH, 90% (**8**); 88% (**52**); (e) (i) $Me_3Al$, $Pd(PPh_3)_4$, THF, 100 °C; (ii) 7 N $NH_3$/MeOH, 22% (**9**) for two steps, (f) (i) KF, CuI, $TMSCF_3$, DMF/NMP (1:1 ratio), 100 °C; (ii) 7 N $NH_3$/MeOH, 65% (**10**) for two steps.

electrospray (ESI) and modular Lockspray™ interface. For analytical LC-MS, the conditions were that: a C18 column (2.7 mm, 100 × 4.6 mm) from Waters Cortecs; a gradient system consisting of 0.2% formic acid in $H_2O$ (v/v)/MeCN; a gradient from 95:5 to 0:100 in 10 min with a flow rate of 1.44 mL/min. The gradient system was MeCN/water (1:1) + 0.1% formic acid mixture and a flow rate was 100 μL/min. All purification was completed via silica gel column chromatography or preparative RP-HPLC. Column chromatography was performed manually using Machery-Nagel® 60 M silica gel (40–63 μm) or on an automated Flash unit (Reveleris X2) from Grace/Büchi® using pre-packed silica columns. Preparative RP-HPLC was performed on a Waters AutoPurification system, equipping with a Phenomenex Luna Omega Polar column (250 × 21 mm, 5 μm). The gradient system was aq. 10 mM $NH_4CO_3$/MeCN and a flow rate was 20 mL/min. NMR spectra were recorded on a 300 MHz spectrometer from Varian Mercury® or a 400 MHz spectrometer from Bruker Avance Neo®. Chemical shifts (δ) were presented in ppm and spectra were referenced to the solvent peak of deuterated NMR solvents. Coupling constants were presented in Hz. Purity of the compounds for biological assays was tested via analytic LCMS. All obtained purity was up to 95%, as characterized by UV. The synthesis details and copies of NMR spectra of target compounds were found in the supporting information.

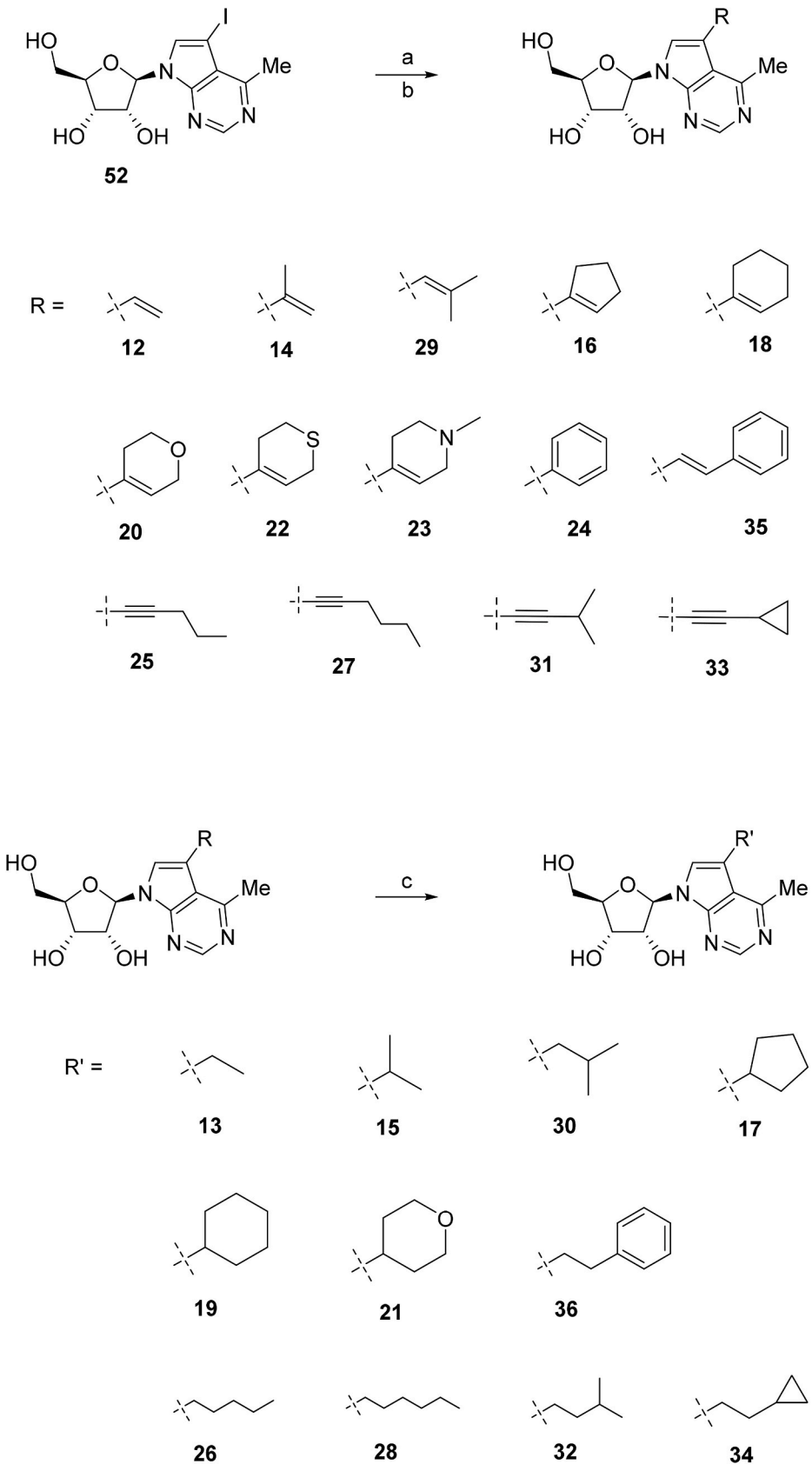
### 2.2. Biology

#### 2.2.1. Test compounds

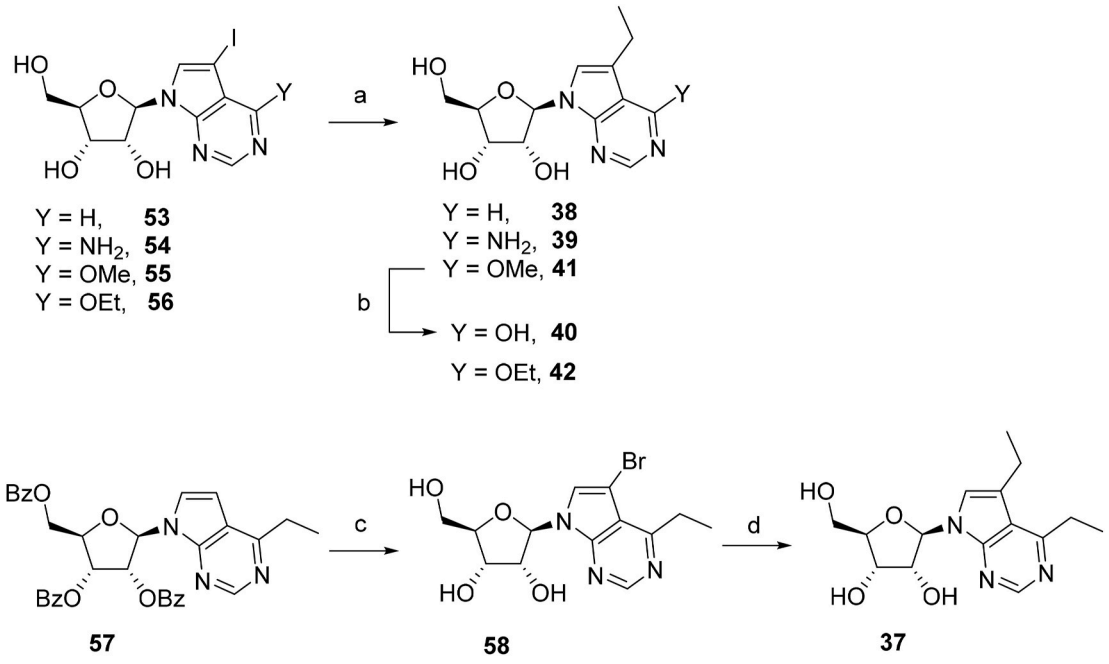
Test compounds were prepared as 20 mM solutions in 100% DMSO and stored at 4 °C until use. Further serial dilutions in demineralized water were performed to assure a final in-test concentration of <1%. Ten concentrations of 4-fold compound dilutions were performed in the *in vitro* assay with the highest concentration of 64 μM.

#### 2.2.2. Trypanosoma cruzi

Compounds were assayed against *T. cruzi* Tulahuen CL2 β-galactosidase strain that is nifurtimox-sensitive. This strain was maintained in MRC-$5_{SV2}$ human lung fibroblast cells in MEM medium. This medium was supplemented with 200 mM L-glutamine, 16.5 mM $NaHCO_3$, and 5% inactivated fetal calf serum. The condition of cultures and assays were conducted at 37 °C under an atmosphere of 5% $CO_2$. Assays were performed in sterile 96-well microtiter plates with $4 \times 10^3$ MRC-5 cells/well and $4 \times 10^4$ parasites/well. Parasite growth was determined after 7 days incubation. Untreated-infected controls represented 100% growth. Parasite burdens were analyzed via adding the substrate CPRG (chlorophenolred ß-D-galactopyranoside). After 4 h incubation at 37 °C, the color change was measured spectrophotometrically at 540 nm. The results were expressed as percentage of reduction in parasite growth compared to control wells and $IC_{50}$ values were calculated. Benznidazole was used as a positive control.




**Scheme 2.** Reagents and conditions: (a) R–B$(OH)_2$, $Pd(OAc)_2$, TPPTS, $Na_2CO_3$, MeCN/water (1:2 ratio), 100 °C, 28%–94% or Potassium trifluoroborate, $Pd(OAc)_2$, TPPTS, $Cs_2CO_3$, MeCN/water (1:2 ratio), 100 °C for **12** (67%) and **14** (41%); (b) Alkynes, $Pd(PPh_3)_2Cl_2$, CuI, TEA, DMF, r. t.; (c) Pd/C or $Pd(OH)_2$/C, $H_2$, MeOH, r. t. 70%–95%.




**Scheme 3.** Reagents and conditions: (a) (i) tributylvinylstanne, $Pd(PPh_3)_4$, DMF, 100 °C; (ii) $Pd(OH)_2/C$, $H_2$, MeOH, r. t., 29% (**38**), 21% (**39**), 33% (**41**), 31% (**42**) for two steps; (b) NaI, TMSCl, MeCN, 58%; (c) (i) NBS, DMF; (ii) 7 N $NH_3$/MeOH, 63% for two steps; (d) (i) Potassium vinyl trifluoroborate, TPPTS, $Cs_2CO_3$, MeCN/water (1:2 ratio), 100 °C; (ii) $Pd(OH)_2/C$, $H_2$, MeOH, r. t., 17% for two steps.

*2.2.3. MRC-5$_{SV2}$ cytotoxicity*

MRC-$5_{SV2}$ cells were cultured in MEM medium with Earl's salts. This medium was supplemented with L-glutamine, $NaHCO_3$ and 5% inactivated fetal calf serum. The condition of cultures and assays were conducted at 37 °C under an atmosphere of 5% $CO_2$. Compound dilutions (10 μL) were added to 190 μL of MRC-$5_{SV2}$ ($3 \times 10^4$ cells/mL) in each well of sterile 96-well microtiter plates. Cell growth was compared to untreated-infected controls as 100% growth and medium-controls as 0% growth. After 3 days incubation, 50 μL resazurin was added per well. After 4 h incubation at 37 °C, cell viability was determined fluorimetrically at $\lambda_{ex}$ 550 nm, $\lambda_{em}$ 590 nm. The results were expressed as percentage of reduction in cell growth or viability compared to control wells and $CC_{50}$ values were calculated.

*2.2.4. Leishmania infantum and PMM cytotoxicity*

Compounds were assayed against intracellular amastigotes of *L. infantum* MHOM/MA (BE)/67 (WT). Spleen-derived amastigotes from an infected donor hamster were used to infect primary peritoneal mouse macrophages (PMM) from Swiss mice after a 2-day peritoneal stimulation with a 2% potato starch suspension. The condition of cultures and assays were conducted at 37 °C under an atmosphere of 5% $CO_2$. Assays were performed in sterile 96-well microtiter plates with $4 \times 10^3$ MRC-5 cells/well and $4.5 \times 10^5$ parasites/well. The culture was maintained in RPMI-1640 medium supplemented with 200 mM L-glutamine, 16.5 mM $NaHCO_3$ and 5% inactivated fetal calf serum. The infection of macrophages was performed for 48 h. Compound dilutions (10 μL) were added after 2 h of infection. Parasite growth was compared to untreated-infected controls as 100% growth and medium-controls as 0% growth. After 5 days incubation, parasite burdens (mean number of amastigotes/macrophage) were microscopically measured after staining with a 10% Giemsa solution. The results were expressed as percentage of reduction in growth compared to control wells and $IC_{50}$ values were calculated. Miltefosine was used as a positive control.

PMM toxicity was recorded when the toxicity to the macrophages was observed in the lowest concentration via microscopic evaluation of cell detachment, lysis, and granulation, and this was used as a qualitative phenotypic assessment. The results were expressed as percentage of reduction in cell growth or viability compared to untreated controls and $CC_{50}$ values were calculated.

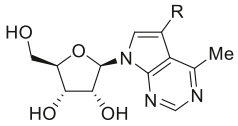
*2.2.5. Trypanosoma brucei spp*

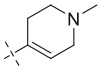
Compounds were assayed against *T. b. brucei (*Squib 427 strain) and *T. b. rhodesiense* (STIB-900 strain). Both species were maintained axenically in HMI-9 medium. This medium was supplemented with 10% fetal bovine serum. The cultures and assays were conducted at 37 °C under an atmosphere of 5% $CO_2$. Assays were performed in sterile 96-well microtiter plates. T. b. brucei was seeded at $1.5 \times 10^4$ parasites/well and *T. b. rhodesiense* at $4 \times 10^3$ parasites/well. Parasite growth was determined after 24 h (*T. b. brucei*) or 6 h (*T. b. rhodesiense*). Untreated-infected controls were compared as 100% growth. Parasite burdens were analyzed upon adding the substrate resazurin. After 24 h incubation at 37 °C, fluorescence was measured using a FLUOstar Optima (BMG Labtech, Durham, NC) and the results fitted to a sigmoid curve with variable slope using Prism 5.0 (GraphPad, San Diego, Ca). The results were expressed as percentage of reduction in parasite growth compared to control wells and $IC_{50}$ values were calculated. Suramin was used as a positive control.

*2.2.6. Microsomal stability assays*

Mouse, rat and pooled human liver microsomes were obtained commercially (Corning®) and stored at −80 °C until use. NADPH generating system solutions A and B and UGT reaction mix solutions A and B (Corning) were kept at −20 °C. The test compound and the reference diclofenac were prepared in 10 mM DMSO. The microsomal stability assay was carried out based on the BD Biosciences Guidelines for Use (TF000017 Rev1.0) with minor adaptations. The *in vitro*

**Table 1**
*In vitro* antikinetoplastid activity of 6-methyl-7-modified nucleoside analogues against *T. cruzi, L. inf., T. b. bruc.* and *T. b. rhod.*[a].




| Cpd. | R | *T. cruzi*[#] | *L. inf.*[#] | *T. b. bruc.* | *T. b. rhod.* | MRC-5 $CC_{50}$ (μM) | PMM $CC_{50}$ (μM) |
|---|---|---|---|---|---|---|---|
| | | $EC_{50}$ (μM) | $EC_{50}$ (μM) | $EC_{50}$ (μM) | $EC_{50}$ (μM) | | |
| **6** | -F | <0.25 | <0.25 | *0.41* | <0.25 | *0.30* | *32* |
| **7** | -Cl | **3.61 ± 1.24** | **3.00 ± 1.23** | **4.78 ± 2.19** | **0.84 ± 0.63** | **54.8 ± 2.52** | **> 64; 32** |
| **8** | -Br | *5.28* | *24.1* | *> 64* | *3.84* | *39.6* | *32* |
| **9** | -Me | **1.54 ± 0.52** | **15.3 ± 7.88** | **0.82 ± 0.32** | **0.45 ± 0.08** | **> 64** | **> 64** |
| **10** | -$CF_3$ | *5.06* | *3.17* | *0.58* | *1.89* | *1.68* | *8.00* |
| **11** | -$SCF_3$ | *30.9* | >64 | >64 | >64 | *36.8* | >64 |
| **12** |  | 6.95 ± 1.29 | >64 | 2.73 ± 0.28 | 2.20 ± 0.11 | >64 | >64 |
| **13** |  | **2.54 ± 0.47** | **1.79 ± 0.78** | **0.75 ± 0.18** | **0.43 ± 0.06** | **> 64** | **> 64** |
| **14** |  | *5.04* | *0.79* | *0.50* | <0.25 | *1.49* | >64 |
| **15** |  | *29.1* | *22.6* | *0.67* | *0.51* | *0.30* | >64 |
| **16** |  | >64 | >64 | >64 | >64 | >64 | >64 |
| **17** |  | *48.9* | >64 | >64 | *53.6* | >64 | >64 |
| **18** |  | >64 | >64 | >64 | >64 | >64 | >64 |
| **19** |  | >64 | >64 | >64 | >64 | >64 | >64 |
| **20** |  | 2.87 ± 0.28 | 2.45 ± 0.027 | 12.3 ± 2.45 | 9.75 ± 0.02 | >64 | >64 |
| **21** |  | 3.98 ± 0.94 | 5.31 ± 3.73 | 15.1 ± 1.02 | 12.0 ± 1.75 | >64 | >64 |
| **22** |  | *20.8* | *6.96* | >64 | >64 | >64 | >64 |
| **23** |  | >64 | >64 | >64 | >64 | >64 | >64 |
| **24** |  | *40.3* | >64 | >64 | >64 | >64 | >64 |

[a] $EC_{50}$ values were expressed in μM concentrations as mean ± SEM which was based on 2 to 4 independent experiments; Benznidazole was employed as a reference for *T. cruzi* (2.28 ± 0.046 μM) and miltefosine was employed as a reference for *L. infantum* (8.30 ± 1.64 μM) and suramin was employed as a reference for *T. b. bruc.* and *T. b. rhod.* (0.13 ± 0.09 and 0.07 ± 0.01 μM);[#]: intracellular amastigote stages; Values *in italics* indicated the data of the first (single) screening because of low or no potency or non-specific effect; Cytotoxicity was determined in assays against MRC-$5_{SV2}$ cells and primary peritoneal mouse macrophages (PMM).

metabolic stability assays were performed as described before(Hulpia et al., 2018, 2019a). Samples were collected after 0, 15, 30 and 60 min. Two replicates were included.

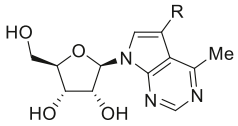
## 3. Results and discussion

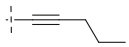
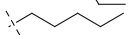
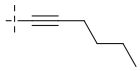
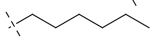
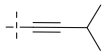
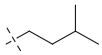
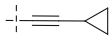
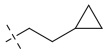
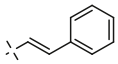
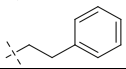
### 3.1. Chemistry

The synthesis of the 7-modified 6-methyl-7-deazapurineribofuranosyl nucleosides is presented in Schemes 1 and 2. The previously reported 3-modified pyrrolo [2,3-*b*]pyrimidines (3-F (**43**) (Hu et al., 2010), 3-Cl (**44**) and 3-$SCF_3$ (**45**) (Hulpia et al., 2020b)) were applied as starting materials for Vorbrüggen glycosylation with perbenzoylated 1-*O*-acetylribofuranose. A Pd-catalyzed methylation (Hocek and Dvorakova, 2003; Naus et al., 2014) with $AlMe_3$ provided the corresponding 6-methyl nucleoside analogues **6**, **7** and **11** after deprotection with methanolic $NH_3$ solution (Scheme 1). The 7-bromo (**8**) and 7-iodo (**52**) derivatives were obtained via halogenation of **49** (Naus et al., 2014) with *N*-bromosuccinimide (NBS) and *N*-bromosuccinimide (NIS), respectively, followed by deprotection. Attempted methylation of the 7-iodo product **51** using the above procedure (Pd/$AlMe_3$) resulted in a mixture of the desired product (**9**) and the dehalogenated product, which were separated by preparative HPLC. The 7-trifluoromethyl analogue **10** was obtained upon reaction of the 7-iodo intermediate **51** with CuI/$TMSCF_3$ (Lin et al., 2019), followed by final deprotection.

The introduction of various alkenyl and alkynyl substituents at C-7 was achieved via Suzuki or Sonogashira cross-coupling reactions of 7-deaza-7-iodo-6-methyl-9-β-D-ribofuranosylpurine (Scheme 2).(Lin et al.,

**Table 2**
*In vitro* antikinetoplastid activity of 6-methyl-7-modified nucleoside analogues against *T. cruzi, L. inf., T. b. bruc.* and *T. b. rhod.* [a].




| Cpd. | R | *T. cruzi*[#] | *L. inf.*[#] | *T. b. bruc.* | *T. b. rhod.* | MRC-5 $CC_{50}$ (μM) | PMM $CC_{50}$ (μM) |
|---|---|---|---|---|---|---|---|
| | | $EC_{50}$ (μM) | $EC_{50}$ (μM) | $EC_{50}$ (μM) | $EC_{50}$ (μM) | | |
| 13 |  | 2.54 ± 0.47 | 1.79 ± 0.78 | 0.75 ± 0.18 | 0.43 ± 0.06 | >64 | >64 |
| **25** |  | 20.4 ± 0.39 | >64 | 0.89 ± 0.21 | 0.37 ± 0.08 | >64 | >64 |
| **26** |  | >64 | >64 | >64 | >64 | >64 | >64 |
| **27** |  | *2.08* | *2.38* | *0.52* | *1.19* | *7.58* | *8.00* |
| **28** |  | *42.1* | >64 | >64 | >64 | >64 | >64 |
| **29** |  | >64 | >64 | >64 | >64 | >64 | >64 |
| **30** |  | >64 | >64 | >64 | >64 | >64 | >64 |
| **31** |  | >64 | >64 | *23.5* | *16.0* | >64 | >64 |
| **32** |  | >64 | >64 | >64 | *4.00* | >64 | >64 |
| **33** |  | 4.59 ± 0.89 | >64 | 1.36 ± 0.41 | 0.34 ± 0.11 | 27.5 ± 19.0 | >64 |
| **34** |  | >64 | >64 | >64 | >64 | >64 | >64 |
| **35** |  | 1.94 ± 0.09 | 3.27 ± 2.01 | 6.19 ± 0.57 | 4.54 ± 0.93 | 26.6 ± 1.42 | 32.0 ± 0.00 |
| **36** |  | *40.3* | >64 | >64 | *38.7* | >64 | >64 |

[a] $EC_{50}$ values were expressed in μM concentrations as mean ± SEM which was based on 2 to 4 independent experiments.
[#] Intracellular amastigote stages; Values *in italics* indicated the data of the first (single) screening because of low or no potency or non-specific effect; Cytotoxicity was determined in assays against MRC-$5_{SV2}$ cells and primary peritoneal mouse macrophages (PMM).

2019) Suzuki coupling in aqueous $Pd(OAc)_2$/TPPTS conditions involved the use of appropriate boronic acids or potassium trifluoroborates as alkenyl donors (Naus et al., 2014; Snasel et al., 2014). Catalytic hydrogenation then furnished the corresponding 7-alkyl analogues.

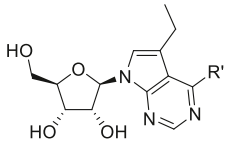
To combine the preferred 7-ethyl substituent with various groups at position 6 (Naus et al., 2014), the known 7-iodo precursors were subjected to Stille coupling (Bourderioux et al., 2011) with tributylvinylstanne (Scheme 3). Subsequent $Pd(OH)_2$-catalyzed hydrogenation gave access to the desired analogues **38**, **39**, **41** and **42**. Demethylation of **41** was achieved under NaI/TMSCl conditions (Naus et al., 2014) to furnish the inosine analogue **40**. The brominated intermediate **58**, obtained in two steps from **57** (Naus et al., 2010), served as a precursor for the 6, 7-diethyl analogue **37**.

### 3.2. Biological evaluation

The *in vitro* activity of all target compounds was determined against *T. cruzi* (Tulahuen CL2 β-galactosidase strain), *L. infantum* (*L. inf,* MHOM/MA (BE)/67), *T. brucei brucei* (*T. b. bruc,* Squib 427) and *T. b. rhodesiense* (*T. b. rhod,* STIB-900) (Tables 1–3). Benznidazole (BZ), miltefosine (MIL) and suramin were included as control compounds. Cytotoxicity was assessed against MRC-5 (host cells for *T. cruzi*) and primary mouse macrophages (PMM; host cells for *L. infantum).*

To probe the structure-activity relationship (SAR), different substituents were introduced on position 7 of nucleoside **3**. The 7-fluoro analogue **6** displayed activity against both *T. brucei* subspecies, but suffered from cytotoxicity (MRC-5 fibroblasts), while the 7-chloro analogue **7** showed broad-spectrum activity with superior activity against *L. infantum* than MIL and without apparent cytotoxicity. The 7-bromo analogue **8** possessed good activity against *T. cruzi* but with suboptimal selectivity. 7-Trifluoromethyl substitution (**10**) conferred good anti-parasitic activity but also host cell toxicity, while a trifluoromethylthio substituent (**11**) was detrimental for kinetoplastid activity. The 6,7-dimethyl analogue **9** and the vinyl analogue **12** combined potent activity against *T. cruzi*, *T. b. brucei* and *T. b. rhodesiense* with moderate or no activity against *L. infantum*. Interestingly, compared to the 7-methyl analogue **9** the 7-ethyl homologue **13** displayed 8-fold improved activity against *L. infantum*, whilst showing comparable potency and selectivity against *T. cruzi*, *T. b. brucei* and *T. b. rhodesiense*. Further branching of the vinyl and ethyl (**14** and **15**) caused MRC-5 toxicity. Analogues involving five and six-membered cycloalkene (**16** and **18**) and cycloalkane (**17** and **19**) substituents on C7 were devoid of antiparasitic activity. Interestingly, introduction of an oxygen in the 6-membered rings of **18** and **19** was associated with broad-spectrum activity, with the 3,6-dihydro-2*H*-pyranyl analogue **20** favorably competing with the reference drugs for *T. cruzi* and *L. infantum*.

**Table 3**
*In vitro* antikinetoplastid activity of 6-modified-7-ethyl nucleoside analogues against *T. cruzi, L. inf., T. b. bruc.* and *T. b. rhod.* [a].




| Cpd. | R′ | *T. cruzi*[#] | *L. inf.*[#] | *T. b. bruc.* | *T. b. rhod.* | MRC-5 $CC_{50}$ (μM) | PMM $CC_{50}$ (μM) |
|---|---|---|---|---|---|---|---|
| | | $EC_{50}$ (μM) | $EC_{50}$ (μM) | $EC_{50}$ (μM) | $EC_{50}$ (μM) | | |
| 13 | -Me | 2.54 ± 0.47 | 1.79 ± 0.78 | 0.75 ± 0.18 | 0.43 ± 0.06 | >64 | >64 |
| **37** | -Et | >64 | >64 | *2.03* | *2.02* | *20.2* | >64 |
| **38** | -H | *5.38* | >64 | *1.54* | *1.73* | >64 | >64 |
| **39** | -$NH_2$ | 0.99 ± 0.25 | 21.6 ± 2.92 | 0.65 ± 0.02 | 0.50 ± 0.03 | 50.4; >64 | >64 |
| **40** | -OH | >64 | >64 | *48.5* | *40.3* | >64 | >64 |
| **41** | -OMe | >64 | >64 | 0.47 ± 0.01 | 1.31 ± 0.44 | >64 | >64 |
| **42** | -OEt | 7.71 ± 0.91 | 43.1; >64 | 1.25 ± 0.06 | 2.06 ± 0.51 | 52.5; >64 | >64 |

[a] $EC_{50}$ values were expressed in μM concentrations as mean ± SEM which was based on 2 to 4 independent experiments.
[#] Intracellular amastigote stages; Values *in italics* indicated the data of the first (single) screening because of low or no potency or non-specific effect; Cytotoxicity was determined in assays against MRC-$5_{SV2}$ cells and primary peritoneal mouse macrophages (PMM).

Replacement of the oxygen with a sulfur or methylamino led to an activity drop or loss. The 7-phenyl analogue **24** proved inactive.

Based on the encouraging activities obtained with alkyl (7-methyl or 7-ethyl) or alkenyl (7-(3,6-dihydro-2*H*-pyranyl)) substituents, we examined the effect of longer alkyl, alkenyl or alkynyl substituents (Table 2). However, none of these analogues displayed sufficiently potent (or selective) antiparasitic activity.

Next, we decided to leave the 7-ethyl substituent of **13** unaltered and verify the effect of alternative groups at position 6. Homologation of the 6-methyl to a 6-ethyl (**37**) resulted in a significant *T. cruzi* and *L. infantum* activity drop, while removal of the methyl group (**38**) particularly impacted on activity against *L. infantum*. Compared to its 6-Me congener 7-ethyl-7-deazaadenosine (**39**) showed enhanced activity against *T. cruzi*, remained equipotent against *T. b. brucei* and *T. b. rhodesiense*, but was inferior against *L. infantum*. The corresponding inosine analogue **40** proved weakly active or inactive against all parasites, while the 6-methoxy analogue **41** only retained potency against *T. b. brucei* and *T. b. rhodesiense*. The 6-ethoxy analogue **42** suffered from an inferior or no activity. The antikinetoplastid effect summarized from the above SAR investigations was outlined in Fig. 2.

To assess their metabolic stability, **7** and **13** were incubated with mouse, hamster and human microsomal (S9) fractions (Table 5). Both compounds proved to largely remain intact up to 1 h of incubation with CYP-NADPH (Phase-I) and UGT enzymes (Phase-II) independent of the species, indicating they can be considered suitable candidates for *in vivo* follow-up evaluation.

## 4. Conclusion

Previous evaluation of 7-deazapurine nucleoside analogues has uncovered several series of anti-*T. brucei* spp. and anti-*T. cruzi* agents resulting from modifications on the C7 position (Hulpia et al., 2018, 2019a), 3′-deoxygeneration (Hulpia et al., 2018, 2019b, 2020b), 3′-fluorination (Bouton et al., 2021), C6–O-alkylation (Hulpia et al., 2020a) and 1,7-dideazapurine nucleoside analogues (Lin et al., 2019), whereas this was less successful in generating hits against *Leishmania*.

Building further on our recent findings that 6-methyl-7-deazapurine nucleoside analogues may display improved selectivity (Lin et al., 2021), the present study focused on expanding the SAR of 6-methyl-7-deazapurine ribonucleosides with non-aromatic substituents. Variation of the substituents at position 7 of 7-deaza-6-methyl-9-β-D-ribofuranosylpurine revealed that chloro (**7**), methyl (**9**) and ethyl (**13**) groups afforded low micromolar activity against *T. cruzi*, *L. infantum* and *T. brucei* spp. without apparent cytotoxicity. The pan-kinetoplastid hits **7** and **13** possessed acceptable metabolic stability to warrant further follow-up evaluation in laboratory animal models. Of note, a related 6-methylpurine 2′-deoxyribonucleoside was found to selectively lyse mouse macrophages infected with amastigotes of *L. donovani* and *L. Mexicana*, possibly due to Leishmania-mediated generation of the toxic 6-methylpurine metabolite (Carson and Chang, 1981; Koszalka and Krenitsky, 1979).

While the development of a broadly active pan-kinetoplastid drug might not be achievable, the identification of hits active against multiple kinetoplastids might facilitate further exploration/development, since the resources for drug development in these disease areas are scarce. A similar phenotypic multi-parasite screening strategy enabled the discovery of the selective kinetoplastid proteasome inhibitor GNF6702 with unprecedented *in vivo* efficacy in three models of infection (Khare et al., 2016). While the mechanism of action of the pan-kinetoplastid agents discovered in this study remains largely unknown, identification of a common chemical scaffold with potential across multiple kinetoplastid infections provides new hope for finding novel leads and ultimately result in improved treatment options for NTDs that affect the world's poorest people.

## Notes

The authors declare no competing financial interest.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

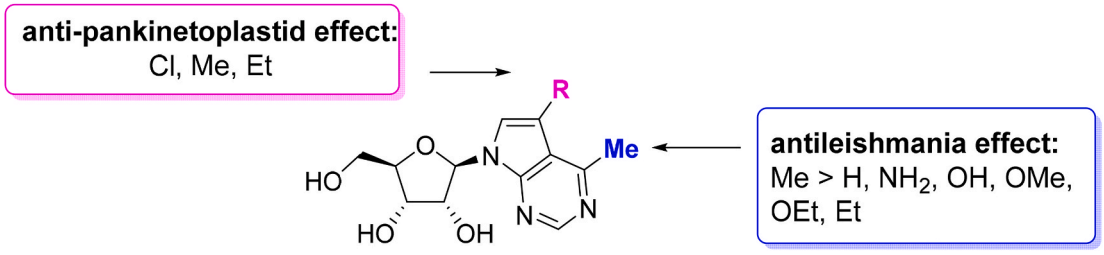



**Fig. 2.** Summary of antikinetoplastid effect.

**Table 5**
*In vitro* metabolic stability of selected nucleoside analogues in mouse, hamster and pooled human microsomal fractions.[a].

| Microsomes | Phase I/II | Time | % remaining parent compound | | | |
|---|---|---|---|---|---|---|
| | | | 7 | | 13 | |
| | | | Mean | STDEV | Mean | STDEV |
| **Mouse** | **CYP – NADPH** | 0 | 100 | – | 100 | – |
| | | 15 | 97 | 6.2 | 96 | 8.0 |
| | | 30 | 96 | 4.4 | 95 | 6.6 |
| | | 60 | 87 | 4.4 | 94 | 8.8 |
| | **UGT Enz.** | 0 | 100 | – | 100 | – |
| | | 15 | 99 | 2.3 | 99 | 2.5 |
| | | 30 | 104 | 3.8 | 105 | 2.2 |
| | | 60 | 98 | 6.6 | 106 | 2.0 |
| **Hamster** | **CYP – NADPH** | 0 | 100 | – | 100 | – |
| | | 15 | 95 | 2.0 | 93 | 1.9 |
| | | 30 | 93 | 2.2 | 102 | 13.9 |
| | | 60 | 93 | 1.9 | 91 | 5.5 |
| | **UGT Enz.** | 0 | 100 | – | 100 | – |
| | | 15 | 96 | 1.9 | 101 | 8.6 |
| | | 30 | 100 | 7.6 | 103 | 1.6 |
| | | 60 | 102 | 0.4 | 108 | 3.2 |
| **Human** | **CYP – NADPH** | 0 | 100 | – | 100 | – |
| | | 15 | 96 | 6.3 | 89 | 1.7 |
| | | 30 | 97 | 3.7 | 92 | 10.9 |
| | | 60 | 96 | 2.0 | 96 | 3.6 |
| | **UGT Enz.** | 0 | 100 | – | 100 | – |
| | | 15 | 102 | 2.1 | 105 | 6.4 |
| | | 30 | 97 | 3.4 | 102 | 2.7 |
| | | 60 | 96 | 1.7 | 96 | 3.0 |

[a] Values (mean and STDEV) are the remaining percentage (%) of parent compound at 0, 15, 30 and 60 min of incubation calculated from 2 independent repeats. CYP–NADPH refers to Phase-I while UGT enzyme refers to Phase-II metabolism. Diclofenac (susceptible to Phase-I and Phase-II metabolism) was included as the reference drug (*data not shown*). Mouse and hamster microsomal fractions were applied since these laboratory rodent species are respectively used for *T. cruzi* infection and *L. infantum* infection.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.08.001.

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