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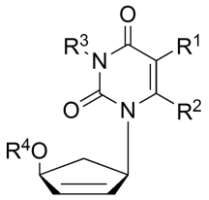

Trypanosoma brucei EC_{50} *8.0 - 38.3 µM*

Leishmania mexicana EC_{50} *11.8 - 97.0 µM*

Evaluation of the antiprotozoan properties of 5'-norcarbocyclic pyrimidine nucleosides

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Abstract

Carbocyclic nucleoside analogues have a distinguished history as anti-infectious agents, including key antiviral agents. Toxicity was initially a concern but this was reduced by the introduction of 5'-nor variants. Here, we report the result of our preliminary screening of a series of 5'-norcarbocyclic uridine analogues against protozoan parasites, specifically the major pathogens Leishmania mexicana and Trypanosoma brucei. The series displayed antiparasite activity in the low to mid-micromolar range and establishes a preliminary structure-activity relationship, with the 4',N(3)-di-(3,5-dimethylbenzoyl)-substituted analogues showing the most prominent activity. Utilizing an array of specially adapted cell lines, it was established that this series of analogues likely act through a common target. Moreover, the strong correlation between the trypanocidal and anti-leishmanial activities indicates that this mechanism is likely shared between the two species. EC50 values were unaffected by the disabling of pyrimidine biosynthesis in T. brucei, showing that these uridine analogues do not act directly on the enzymes of pyrimidine nucleotide metabolism. The lack of cross-resistance with 5-fluorouracil, also establishes that the carbocyclic analogues are not imported through the known uracil transporters, thus offering forth new insights for this class of nucleosides. The lack of cross-resistance with current trypanocides makes this compound class interesting for further exploration.

The protozoan parasites including *Trypanosoma brucei* and the various *Leishmania* species cause a spectrum of primarily tropical diseases in humans and animals. The subspecies *T. brucei gambiense* and *T. b. rhodesiense* cause African sleeping sickness, an infection that ultimately targets the central nervous system and is then invariably fatal.[1] Other trypanosome species infect domestic animals, resulting in a significant impact on agriculture.[2] *Leishmania* species cause various manifestations of leishmaniasis, divided in cutaneous, visceral and mucocutaneous forms.[3] The treatments for all of these diseases are outdated, often toxic and their use is threatened by drug resistance.[4]

Carbocyclic nucleoside analogues are compounds in which a methylene group replaces the oxygen atom in the furanose sugar moiety.[5] Because of this modification, carbocyclic nucleosides are more stable to enzymatic cleavage since the hemi-aminal of the glycosidic bond has been converted to a tertiary amine.[5] The first naturally occurring carbocyclic nucleosides were neplanocin A[6] and aristeromycin,[7] which were found to exhibit high antiviral activity due to their inhibition of S-adenosylhomocysteine hydrolase (SAHase), an enzyme involved in many biological methylation reactions.[8] Unfortunately, they also exhibited significant levels of toxicity due to the close resemblance of their triphosphate forms to ATP.[8]

One approach to overcoming the issue of carbocyclic toxicity was the removal of the CH_2 group of the 5'-hydroxymethyl, resulting in what are known as the 5'-norcarbocyclic nucleosides.[9] Due to the truncated nature of the 5'-hydroxyl group, they are not recognized and phosphorylated by kinases, and are thus not converted to their corresponding triphosphates; however, a number of them still proved to be inhibitors of SAHase.[10-14] This observation inspired the synthesis of numerous derivatives possessing a variety of different heterocyclic bases and other structural modifications/functional groups, which substantially extended their spectrum of biological activities.[15-38] Like other nucleoside analogues, carbocyclic nucleosides can function as substrates and/or inhibitors of a large number of biologically significant enzymes and, because they are immune to phosphorylation reactions, the 5'-norcarbocyclic nucleoside analogues can serve as versatile tools for studying new biological targets.[39]

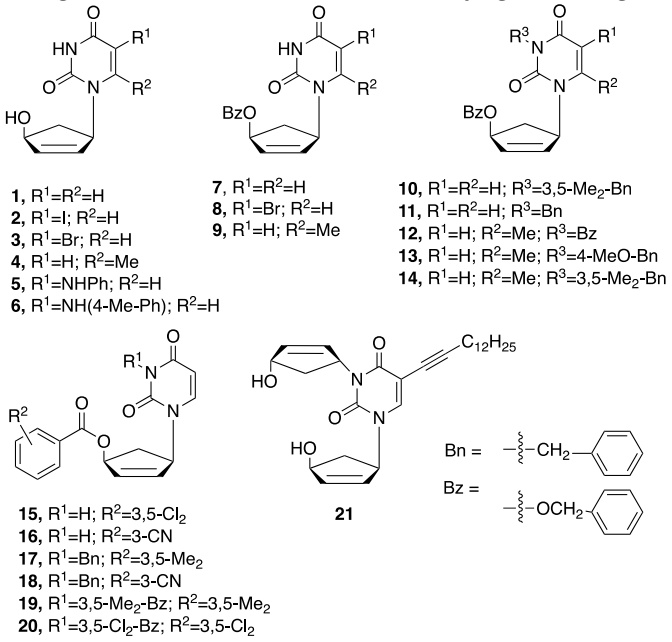


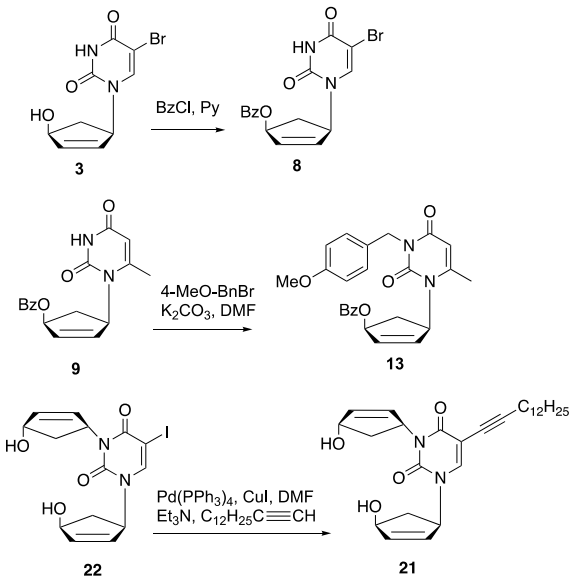
Figure 1. Proposed 5'-norcarbocylic uridine nucleosides.

In that regard, it was recently reported that 5'-norcarbocyclic nucleoside analogues can serve as HIV non-nucleoside reverse transcriptase inhibitors (NNRTIs) with K_i's of 5-19 μM for wild type and 1-55 μM for some mutant strains of HIV RT.[22, 40] Interestingly, some of the compounds from this group also proved to be potent inhibitors of *M. tuberculosis* H37Rv with MIC90 values between 10 to 40 μg/ml and MDR MS-115 with MIC90 values of 5 to 20 μg/ml. Importantly, both cases the 5'-norcarbocyclic nucleosides exhibited activity against resistant strains.[41]

Because of the different types of biological activity observed against these very different but important pathogens, we decided to further investigate their activity. In that regard, we decided to screen the compounds against parasites, particularly since other carbocyclic 5'-nor nucleosides had shown anti-parasitic activity.[13, 38, 42] A second impetus for our studies came from the observation that although pyrimidine biosynthesis is not essential in *Trypanosoma* and *Leishmania* parasites, many pyrimidine salvage enzymes have been validated as drug targets,[43-46] and the incorporation of some pyrimidine analogues into nucleic acids and/or metabolic intermediates has been demonstrated in both species.[47, 48] Moreover, 5'-Nor carbocyclic nucleosides have already demonstrated antitrypanosomal properties.[27, 28]

In an effort to further explore those observations, we opted to combine the two scaffolds and pursue a series of C-5 substituted 5'-norcarbocyclic uridine analogues (shown in Figure 1). New treatment options against leishmaniasis and trypanosomiasis are urgently needed, as the decades-old drugs have become ineffective from over-use.[4, 49] By testing the compounds on various wild-type and drug resistant *Trypanosoma* and *Leishmania* strains, we established that not only is there is no cross-resistance with existing trypanocides, but the compounds do not utilize uracil transporters, making the development of resistance to 5'-norcarbocyclic uridines much less likely.

The chemical routes to the various targets are straightforward and concise. Compounds **1**-**7**, **9**-**12** and **14**-**20** were synthesized as described earlier.[22, 40, 41, 50] Treatment of compound **3**[15] with benzoyl chloride in pyridine gave derivative **8** (80%, Scheme 1).[51] Benzylation of **9** by 4-methoxybenzyl bromide led to product **13** (80%).[52]



Scheme 1. Synthesis of new 5'-norcarbocyclic uridine analogues.

Compound **21** was synthesized from 1-(4′-hydroxy-2′-cyclopenten-1′-yl)-3-(4″-hydroxy-2″-cyclopent-en-1″-yl)-5-iodo- oxy-2″- uracil **22**[50] via Sonagashira coupling reaction (70%).[53] The structures of the synthesized compounds were elucidated by ^{1}H NMR and ^{13}C NMR[16-18] and the spectra were in accordance with the proposed structures.

Activity against *Trypanosoma brucei.* *T. b. brucei* bloodstream forms were grown in HMI-9 medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco) exactly as described,[54] and maintained in the log-phase of growth.

The 5'-norcarbocyclic pyrimidine nucleosides all displayed relatively similar activities against wild type *Trypanosoma brucei* (427WT)[55] in our standard viability test[56] based on the reduction of resazurin sodium salt (Sigma-Aldrich; blue, non-fluorescent) to resorufin (colorless, fluorescent) by live but not by dead cells.[57] Pentamidine and 5-fluorouracil (5-FU) were used as non-nucleoside controls (Sigma-Aldrich). EC_{50} values were generally in the mid-micromolar range (Table 1) for all compounds, potentially indicating a common mechanism of action for this class of compounds.

The core 5'-norcarbocyclic uridine analogue (**1**) displayed an EC_{50} value of 37.6 ± 0.2 μM, and halogenation at position 5 (I, Br) or methylation at position 6 did not significantly change this (**2**, **3**, **4**), whereas aminophenyl or 4-methyl-aminophenyl on position 5 slightly improved the activity (**5**, **6**). Addition of benzoic acid residue to the 4' position (**7** - **9**) likewise did not change the activity. We next explored the effect of substitutions on N3 of the 4'-benzoyl carbocyclic uridine analogues. N^3 addition of benzoyl (**12**), benzyl (**11**) or 4-methoxybenzyl (**13**) did not substantially change the antiparasitic activity, but the addition of the 3,5-dimethylbenzyl group reduced the EC_{50} to 15.2 μM and 15.1 μM, respectively, for **10** and **14**. A similar 3,5-dimethyl substitution on the 4'-benzoyl moiety was also highly favorable, yielding **17**, with an EC_{50} of just 8.6 μM.

The 3,5-dichloro substitution had a much lesser effect, however (compare **15** and **7**), as did a 3-cyano substitution (**16** and **18**). As 3,5-dimethyl additions on either the 4' and N^3-position aromatic substitutions both appeared favorable (**17** and **14**), it was then explored whether 3,5-dimethyl on both positions, yielding **19**, would further increase the activity, but this analogue only exhibited moderate activity in the series (EC_{50} = 23.8 μM), although this was still better than the equivalent chlorinated analogue (**20**, EC_{50} =31.8 μM). As a result, it was concluded that electron donating groups such as methyl are favored on the aromatic rings, and that electron-withdrawing groups such as chlorine are unfavorable.

Interestingly, compound **21**, substituted with a long aliphatic chain at the 5-position, proved to be the most active among the tested compounds with an EC_{50} of 8.0 μM. Although this is not a large improvement over some of the other compounds, it shows that further improvements can be made by expanding the SAR in this area, which will be the subject of a follow-on study. Perhaps the long lipophilic chain at C-5, as well as the two 5'-norcarbocyclic ring substitutions (on N^1 and N^3), all making the uracil core much more lipophilic, may serve to aid in cellular uptake by trans-membrane diffusion, and increased cellular penetration often correlates with the strength of cellular effects.[58]

The 5'-norcarbocyclic pyrimidine nucleosides were also screened against several other *T. brucei* cell lines in parallel. The clonal line B48 is highly resistant to the two main classes of trypanocides, the melaminophenyl arsenicals and the diamidines, due to the loss the TbAT1/P2 and HAPT1 drug transporters.[54] Only two of the 5'-norcarbocyclic nucleosides displayed a statistically significant difference in EC_{50} values compared to 427WT (Table 1) and even then the differences were minor, with only a 10-20% difference as compared to the 164-fold difference in EC_{50} for the control drug pentamidine. As a result, we can conclude from this result that (1) 5'-norcarbocyclic pyrimidines are highly unlikely to be cross-resistant with current trypanocidal treatments in human or veterinary use (i.e. pentamidine, diminazene, melarsoprol and cymelarsan), and (2) the mechanism of action of these analogues does not involve any of the currently known drug transporters of *T. brucei*, including TbAT1/P2, which is a nucleoside/nucleobase transporter that also recognizes a wide range of trypanocidal drugs.[59-61]

The next cell line explored was a pyrimidine-auxotrophic clone, PYR6-5$^{-/-}$, from which both alleles of the bifunctional gene *Pyr6-5* have been deleted, disrupting the last two steps of pyrimidine biosynthesis. As a result, the cells are solely dependent on pyrimidine salvage from the extracellular environment, which makes them hyper-sensitive to some pyrimidine analogues including 5-fluorouracil (5-FU).[62] Several 5'-norcarbocyclic analogues were indeed significantly more active against this cell line than against the unmodified

control 427WT, with up to 25% lower EC_{50} values. Again, these variations were relatively minor compared to 5-FU (30-fold sensitization; Table 1). Thus one can conclude that, unlike 5-FU[62] the mode of action of these nucleoside analogues does not competitively intersect with the trypanosome's pyrimidine salvage pathways, since the dearth of newly synthesized pyrimidine nucleotides would be expected to allow increased incorporation of the analogues, or increased binding to the enzymes of pyrimidine nucleotide metabolism.

Finally, we tested all of the analogues on a cell line that was adapted to very high levels of resistance to 5-FU, *T. brucei* FURes (59.2-fold; Table 1).[47] Surprisingly, almost all of the 5'-norcarbocyclics were in fact more active against this cell line. We consider this a strong indication that there is indeed a common mechanism of action for these compounds, as the adaptation to 5-FU has rendered the cells mildly more sensitive to almost all 5'-norcarbocyclic uridines. This hypothesis is further strengthened by the observation that there is a very good correlation between the resistance factors for each compound on PYR6-5$^{-/-}$ and FURes ($P<0.0001$; Fig. 2A, $r^2 = 0.66$), although 5-FU resistance in this line has been linked to changes in sugar nucleotide metabolism rather than *de novo* pyrimidine biosynthesis.[47]

Table 1. Effect of 5'-norcarbocyclic pyrimidine nucleosides on *Trypanosoma brucei*

	WT	B48		PYR6-5$^{-/-}$		Tbb-5FURes	
	EC_{50}	EC_{50}	RF	EC50	RF	EC_{50}	RF
1	37.6 ± 0.2	37.9 ± 1.5	1.0	34.6 ± 1.5	0.92	31.9 ± 1.9	0.85[1]
2	35.3 ± 0.7	36.2 ± 1.4	1.0	33.5 ± 1.8	0.95	30.8 ± 2.1	0.87
3	37.3 ± 0.6	36.7 ± 1.6	1.0	35.7 ± 2.5	0.96	32.4 ± 1.8	0.87[1]
4	35.1 ± 0.8	38.7 ± 2.0	1.1	32.2 ± 2.5	0.92	28.8 ± 2.6	0.82
5	28.1 ± 0.4	30.0 ± 1.0	1.1	21.4 ± 1.7	0.76[2]	13.8 ± 1.5	0.49[3]
6	31.7 ±0.4	31.0 ± 1.6	1.0	24.7 ± 1.4	0.78[2]	17.1 ± 1.2	0.54[3]
7	38.3 ± 0.7	38.1 ± 1.5	1.0	35.6 ± 1.6	0.93	32.3 ± 1.5	0.84[1]
8	29.8 ± 0.3	23.9 ± 1.5	0.8[2]	25.3 ± 1.2	0.85[2]	17.7 ± 1.7	0.59[3]
9	37.6 ± 0.9	38.1 ± 1.7	1.0	34.3 ± 1.5	0.91	32.0 ± 1.8	0.85[1]
10	15.2 ± 0.1	15.7 ± 0.2	1.0	15.9 ± 0.2	1.05	12.4 ± 1.1	0.82
11	29.1 ± 0.4	25.5 ± 1.3	0.9	27.7 ± 0.9	0.95	21.0 ± 1.9	0.72[2]
12	33.1 ± 0.2	34.0 ± 1.0	1.0	32.3 ± 1.3	0.98	27.7 ± 2.0	0.84[1]
13	30.5 ± 0.3	27.2 ± 2.1	0.9	28.3 ± 0.8	0.93[1]	20.3 ± 1.8	0.66[3]
14	15.1 ± 0.2	14.0 ± 0.8	0.9	15.6 ± 0.2	1.03	14.3 ± 0.5	0.95
15	33.2 ± 0.5	34.8 ± 0.6	1.0	31.6 ± 1.1	0.95	26.7 ± 1.8	0.80[1]
16	37.8 ± 0.7	36.4 ± 1.0	1.0	34.9 ± 1.4	0.92	31.3 ± 1.3	0.83[2]
17	8.6 ± 0.4	8.7 ± 0.2	1.0	11.1 ± 0.5	1.29[1]	8.7 ± 0.2	1.01
18	27.9 ± 1.3	25.0 ± 1.8	0.9	26.4 ± 1.4	0.94	21.4 ± 2.4	0.76[1]
19	23.8 ± 1.9	21.9 ± 0.4	0.9	24.4 ± 1.6	1.03	20.2 ± 2.2	0.85
20	31.8 ± 0.5	33.9 ± 1.1	1.1	29.5 ± 1.9	0.93	25.0 ± 2.5	0.79[1]
21	8.0 ± 0.1	9.0 ± 0.1	1.1[3]	9.2 ± 0.4	1.15	7.5 ± 0.1	0.94[2]
5-Fluorouracil	66.0 ± 5.4	74.6 ± 7.7	1.1	2.3 ± 0.3	0.034[3]	3905 ± 88	59.17[3]
Pentamidine	0.0057 ± 0.0011	0.938 ± 0.097	164[3]	0.0051 ± 0.0010	0.89	0.0061 ± 0.0009	1.06

All EC_{50} values were obtained using the Alamar blue assay and are given as averages in µM (±SEM), of 3-4 independent determinations. WT = wild-type sensitive control strain; B48 is a multi-drug resistant clone; PYR6-5$^{-/-}$ is a pyrimidine auxotrophic clone, hypersensitized to 5F-pyrimidines; Tbb-5FURes has been adapted to very high concentrations of 5F-uracil. Resistance Factor = EC_{50} (resistant clone)/EC_{50} (WT); $n \geq 4$. [1], $P<0.05$; [2], $P<0.01$; [3], $P<0.001$

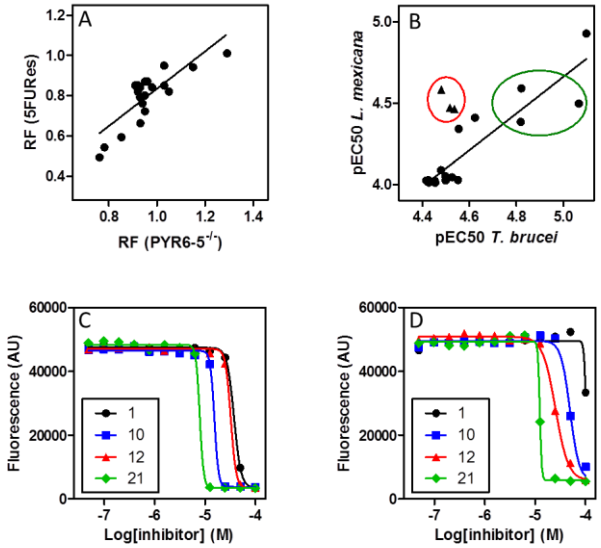


Figure 2. Analysis of the anti-protozoal activity of 5'-norcarbocylic uridine analogs. A. Correlation between the resistance factors (RF) of two *T. brucei* cell lines, 5FURes and PYR6-5-/-; the values were taken from Table 1. The correlation coefficient r^2 was 0.66 and the slope was significantly non-zero (F-test; P<0.0001). B. Correlation between the pEC_{50} values (i.e. -logEC_{50}) of the test compounds against *T. brucei* 427WT and *L. mexicana* wild-type (r^2 = 0.84; slope non-linearity F-test: P<0.0001). The points in the red circle, representing compounds **11-13** were excluded from the linear regression. The green circle represents compounds **10**, **14** and **17** as described in the body text. C. Representative cell viability experiment of several test compounds tested against *T. brucei* 427WT. The output is the fluorescence of the resazurin-metabolite resorufin, quantified in arbitrary units (AU). Highest concentration for each compound was 100 µM. D. Like frame C, but using wild-type *L. mexicana*. In this frame, the bottom value for the sigmoidal curve was fixed at the calculated lower value for compound **21**, in order to allow extrapolation of the incomplete curves for **1** and **10**.

Activity against *Leishmania mexicana.* The 5'-norcarbocyclic nucleosides were also tested against *Leishmania mexicana* promastigotes, in order to assess whether they might possess general activity against kinetoplastid parasites. Promastigotes (insect-stage cells) of *Leishmania mexicana* of strain MNYC/BZ/62/M37935 were cultured in HOMEM (GE Healthcare, Pasching, Austria) supplemented with 10% FBS at 25 ˚C exactly as described.[63] The viability assay for *Leishmania* was identical to that of *T. brucei*,[48] except for longer incubation times (72 h without resazurin versus 48 h, and 48 h with resazurin versus 24 h for *T. brucei*), owing to the slower rate of resazurin reduction by these cells.[57]

The antileishmanial activity followed the same general trends as the trypanocidal activity, highly consistent with the notion that the same mechanism of action underpins their activity against both parasite species. The correlation between either the EC_{50} or pEC_{50} values for *L. mexicana* and *T. brucei* was strong (r^2 = 0.84 in both cases); however, there were three notable outliers, compounds **11** – **13**, where the pEC_{50} against *Leishmania* was substantially higher than expected by the trend line (Figure 2B, red circle). These compounds are the 5'-norcarbocyclic uridine nucleosides with 4' and N^3 aromatic substitutions, but without substitutions on the 2 and 4 positions of either ring. Our analysis places them in a separate sub-category with improved activity against *L. mexicana*.

Interpretation of the data suggests that these unsubstituted analogues still act on the same target, but that the specific enzyme target in *Leishmania*, unlike *T. brucei*, does not favor the 3,5-dimethyl substitution (compounds **10**, **14**, **17**; green circle in Fig. 2B), probably because of a more favorable binding posture by the unsubstituted forms. The separate grouping of the unsubstituted 4'/N^3-diaromatic nucleosides is further visualized in Fig. 2C-D, showing the sigmoid viability curves in the same order of potency for both parasite species, for compounds **1**, **10**, and **21**, but not for **12**.

The compounds were also tested in parallel against a clonal line of *L. mexicana* that had been adapted to high levels of 5-FU (Lmex5FURes, 322-fold; Table 2). This cell line has a different adaptation to 5-FU than the Tbr5FURes cells, in that, instead of a metabolic adjustment, it has lost the capacity to take up uracil and 5-FU, due to the lack of the U1 uracil/5-FU transporter.[47, 48] All of the compounds displayed equal or near-equal activity against Lmex5FURes and the control wild-type strain (Table 2), showing that none of them relied on the previously characterized U1 transporter for uptake.[64]

Indeed, it is more likely that these nucleoside analogues are taken up through an NT1-type uridine/adenosine transporter, as these are far more permissive in allow uptake of uridine analogues (as opposed to the nucleobase uracil), allow at least some substitutions on position 5 of the pyrimidine ring without loss of affinity, and do not use N3 for interactions with the transporter binding pocket.[48] Regardless, it should be noted that the only compounds exhibiting a significant (although minor) increase in EC_{50} for a 5-FU adapted cell line were **11** and **13**, two of the three 'outliers' from Fig. 2B, again underlining that their behavior is slightly different from the rest of the series, but only for *L. mexicana*.

Table 2. Effect of 5'-norcarbocyclic pyrimidine nucleosides on *Leishmania mexicana*

	WT	Lmex-5FURes	
	EC_{50}	EC_{50}	RF
1	97.0 ± 1.0	97.9 ± 1.0	1.01

2	95.2 ± 0.6	95.6 ± 0.7	1.00
3	97.4 ± 1.2	97.3 ± 0.5	1.00
4	97.7 ± 1.7	102.0 ± 5.5	1.04
5	94.1 ± 1.1	95.3 ± 0.8	1.01
6	94.6 ± 0.8	96.7 ± 0.7	1.02
7	94.8 ± 0.6	95.7 ± 0.4	1.01
8	90.4 ± 0.7	91.9 ± 0.6	1.02
9	94.1 ± 0.5	94.9 ± 0.8	1.01
10	41.3 ± 6.4	38.6 ± 5.9	0.94
11	34.5 ± 1.4	46.3 ± 1.0	1.34[3]
12	26.1 ± 1.2	22.6 ± 1.2	0.86
13	33.8 ± 2.7	43.6 ± 1.3	1.29[1]
14	25.6 ± 0.9	28.5 ± 0.7	1.11
15	81.5 ± 8.2	89.4 ± 0.9	1.10
16	94.7 ± 0.9	96.3 ± 1.2	1.02
17	31.8 ± 2.8	25.1 ± 1.6	0.79
18	45.5 ± 3.4	52.6 ± 1.5	1.16
19	38.9 ± 2.6	23.6 ± 0.8	0.61[2]
20	89.1 ± 2.6	91.4 ± 0.7	1.03
21	11.8 ± 0.3	11.4 ± 0.3	0.97
5-Fluorouracil	7.7 ± 1.2	2470 ± 218	322[3]
Pentamidine	1.90 ± 0.16	1.63 ± 0.2	0.86

All EC_{50} values were obtained using the Alamar blue assay and are given as averages in µM (±SEM), of 3-4 independent determinations. WT = wild-type sensitive control strain of *L. mexicana*; Lmex-5FURes has been adapted to very high concentrations of 5F-uracil. Resistance Factor = EC_{50} (resistant clone)/EC_{50} (WT); $n \geq 4$. [1], $P<0.05$; [2], $P<0.01$; [3], $P<0.001$.

In conclusion, we have explored new chemical space for nucleoside analogues against the major kinetoplastid pathogens *Trypanosoma* and *Leishmania*. Several compounds displayed low micro-molar activity, with the two most potent ones, **17** and **21,** displaying a similar activity against both species. Earlier we reported that compound **17** can act as an HIV NNRTI and we now expand its antimicrobial range with activity against protozoan parasites.[22, 40]

Importantly, both **17** and **21** are effective against resistant forms of these pathogens (both resistant to other pyrimidines and to first-line clinical drugs), which makes them ideal leads for further structure optimization. Previous attempts have primarily focused on purine nucleotides,[38, 65-67] as all protozoan parasites lack the ability to synthesize purines *de novo*.[68] However, it has become clear that many of the enzymes of the pyrimidine interconversion and salvage pathways are excellent drug targets in these parasites[44-46, 69] and here we report a first-in-class example of antiparasitic activity against *T. brucei* and *L. mexicana* with an innovative series of 5'-norcarbocyclic uridine nucleosides.

Our findings suggest a common target for 5'-norcarbocyclic uridine nucleosides in the kinetoplastid parasites, which is not likely to be an enzyme directly involved in the principal pyrimidine pathways, as the level of activity was unaffected by the (absence of) *de novo* pyrimidine biosynthesis or very high levels of resistance to 5-FU in *T. brucei*. The anti-parasite activity was also independent of the U1 uracil transporter, as shown by the lack of cross-resistance in Lmex5FURes, and it is possible that (most of) these compounds, being quite lipophilic, diffuse across the parasite's plasma membrane. While the cellular target is still unknown at this point, we have been able to define a preliminary structure-activity relationship. Further cellular studies are currently in progress to potentially identify the target, which will facilitate the further optimization of these structurally unique nucleoside analogues. The results of those studies will be reported elsewhere as they become available.

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Supplementary Data

Supplementary data associated with this article can be found online at (insert link here).

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