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Suramin: Effectiveness of analogues reveals structural features that are important for the potent trypanocidal activity of the drug

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ABSTRACT

Suramin was the first effective drug for the treatment of human African sleeping sickness. Structural analogues of the trypanocide have previously been shown to be potent inhibitors of several enzymes. Therefore, four suramin analogues lacking the methyl group on the intermediate rings and with different regiochemistry of the naphthalenetrisulphonic acid groups and the phenyl rings were tested to establish whether they exhibited improved antiproliferative activity against bloodstream forms of *Trypanosomes brucei* compared to the parent compound.

The four analogues exhibited low trypanocidal activity and weak inhibition of the antitrypanosomal activity of suramin in competition experiments. This indicates that the strong trypanocidal activity of suramin is most likely due to the presence of methyl groups on its intermediate rings and to the specific regiochemistry of naphthalenetrisulphonic acid groups. These two structural features are also likely to be important for the inhibition mechanism of suramin because DNA distribution and nucleus/kinetoplast configuration analyses suggest that the analogues inhibit mitosis while suramin inhibits cytokinesis.

1. Introduction

Suramin is regarded as one of the first chemotherapeutic agents to have been systematically developed in a drug discovery program. The German company Bayer synthesized the molecule in 1917 as a drug against trypanosomes (Steverding, 2010; Steverding and Troeberg, 2024). It was introduced in 1922 for the treatment of human African trypanosomiasis (Steverding, 2010; Steverding and Troeberg, 2024) and is still being used for the therapy of the early stage of sleeping sickness caused by *Trypanosoma brucei rhodesiense* (Steverding, 2017) (*T. brucei* SRA+ (Steverding and Tyler, 2021)). Suramin is highly active against bloodstream forms of *T. brucei* but not very cytotoxic against mammalian cells (Merschjohann et al., 2001). This selectivity can be explained by suramin's structure (Fig. 1). As a large molecule with six negative charges, the drug does not passively enter cells and is not internalized via nutrient transporters or other channels. However, bloodstream forms of *T. brucei* take up suramin via receptor-mediated endocytosis (Zoltner et al., 2020; Makarov et al., 2023), which may explain why the drug displays strong trypanocidal activity but low cytotoxicity. The mechanism of action of the drug is, however, still unclear. Due to its six negative charges at physiological pH, suramin is able to bind and inactivate various proteins. In fact, a large number of enzymes have been identified in *T. brucei* as targets of suramin (Wiedemar et al., 2020). Hence, the strong trypanocidal activity of suramin may be the result of its polypharmacology (Wiedemar et al., 2020). Since the late 1980s, numerous structural analogues of suramin and related compounds have been synthesized and tested as inhibitors of different enzymes and as antagonists of receptors (Jentsch et al., 1987; Damer et al., 1998; McCain et al., 2004; Munkonda et al., 2007; Chanalaris et al., 2017; Green et al., 2023). Compared to suramin, the analogues were found to be generally more potent in inhibiting HIV reverse transcriptase, protein-tyrosine phosphatases, and NTPDases (Jentsch et al., 1987; McCain et al., 2004; Munkonda et al., 2007), and in blocking P2X and LRP1 receptors (Damer et al., 1998; Chanalaris et al., 2017; Green et al., 2023). This prompted us to ask whether suramin analogues also exhibit greater anti-proliferative activity against bloodstream forms of *T. brucei*

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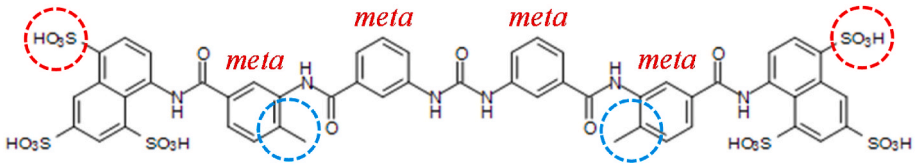
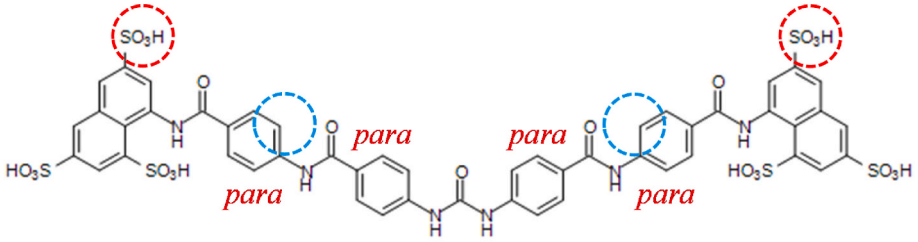
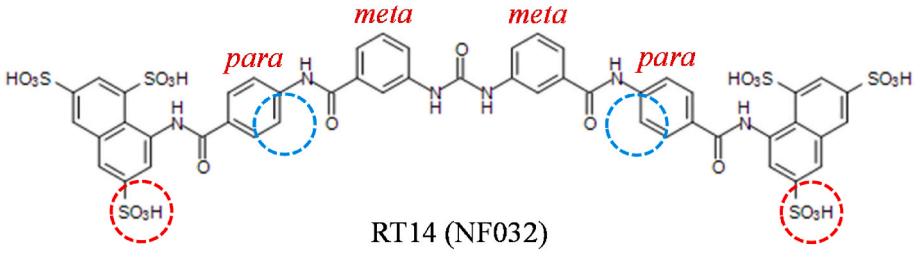


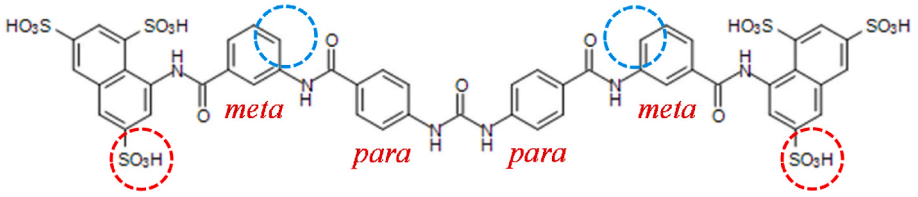
Suramin



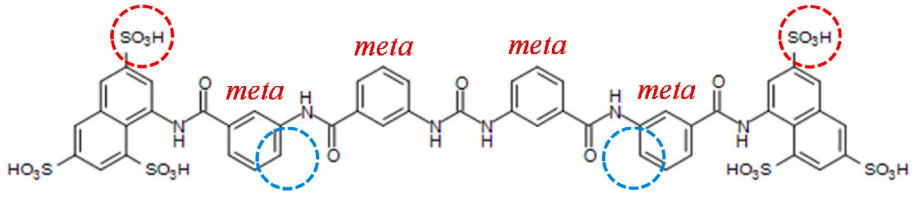
RT5 (NF031)



RT14 (NF032)



RT31



RT58

Fig. 1. Structures of suramin and its analogues RT5 (NF031), RT14 (NF032), RT31, and RT58. The four analogues were synthesized using an "outside-in approach" (Kassack et al., 2004; Croci et al., 2014) and the details of their synthesis is described by Green et al. (2023). The structural differences between suramin and the analogues are indicated as follows: the different arrangement of the sulphonic acid groups at the positions 5 and 6, respectively, on the naphthalene rings is highlighted with red dashed circles; the presence and the lack of the methyl group, respectively, is highlighted with blue dashed circles; the regiochemistry of the phenyl rings is indicated with *meta* and *para* above or below the rings.

than the anti-sleeping sickness drug.

In this study, four suramin analogues (RT5 (also known as NF031 (Jentsch et al., 1987), RT14 (also known as NF032 (Jentsch et al., 1987)), RT31, and RT58 (Fig. 1)) previously synthesized as inhibitors to prevent cartilage degeneration in osteoarthritis (Green et al., 2023) were investigated for their trypanocidal activity. The effect of the analogues to impede the uptake of suramin and on DNA distribution and nucleus/kinetoplast configuration was also studied.

2. Methods and materials

2.1. Trypanosomes

Bloodstream forms of *T. brucei* 427-221a (Hirumi et al., 1980) were grown in Baltz medium (Baltz et al., 1985) supplemented with 16.5% heat-inactivated bovine serum. The cells were maintained in a humified CO_2 incubator at 37 °C.

2.2. Toxicity assay

The trypanocidal activity of suramin analogues was determined as previously described (Merschjohann et al., 2001; Antoszczak et al., 2019). In brief, bloodstream-form trypanosomes were seeded at an initial cell density of 1×10^4 cells/ml in 96-well plates in a final volume of 200 μl medium containing various concentrations of RT5 (NF031), RT14 (NF032), RT31, RT58, and suramin (tenfold dilutions from 100 μM to 1 nM), respectively. Wells containing medium and 0.9% DMSO served as controls. After incubation for 24 h, 20 μl of a 0.5 mM resazurin solution prepared in sterile PBS was added and the cells were incubated for a further 48 h. Thereafter, the absorbance of wells was read on a BioTek ELx808 microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. The 50% growth inhibition (GI_{50}) value, i.e., the concentration of a compound necessary to reduce the growth rate of cells by 50% compared to the control, was determined by linear interpolation (Huber and Koella, 1993). The minimum inhibitory concentration (MIC) value, i.e., the concentration of a compound at which all cells were killed, was determined microscopically.

2.3. Competition assay

Bloodstream-form trypanosomes were incubated at an initial cell density of 1×10^4 cells/ml in 96-well plates with 10 μM RT5 (NF031), RT14 (NF032), RT31, and RT58 in the absence or presence of 0.2 μM suramin in 200 μl medium containing 0.9% DMSO. Controls were incubated in medium containing only DMSO without or with suramin. After incubation for 24 h, 20 μl of a 0.5 mM resazurin solution prepared in sterile PBS was added and the cells were incubated for a further 48 h. Thereafter, the absorbance of wells was read on a BioTek ELx808 microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm.

2.4. Cell cycle analysis

The cell cycle distribution was determined by flow cytometry as previously outlined (Steverding and Rushworth, 2017). In short, bloodstream-form trypanosomes were incubated with each of the suramin analogues at $5 \times GI_{50}$ (RT5 (NF031) = 105.5 μM; RT31 = 152.5 μM; RT58 = 149.5 μM) in medium containing 1.37% DMSO at a cell density of 5×10^5/ml for 21 h. At the end of the incubation period, cells started to die. Controls were incubated with 0.2 μM suramin ($5 \times GI_{50}$) or 1.37% DMSO alone under the same incubation conditions. The cells were harvested by centrifugation at 850×*g* for 10 min and washed once with PBS/1% glucose (PBSG). After another centrifugation at 850×*g* for 5 min, the cell pellets were resuspended in 300 μl PBSG and fixed with 700 μl ice-cold ethanol for 30 min. Then, 500 μl PBSG was added and the samples were centrifuged at 16,800×*g* for 5 min. Subsequently, 1.4 ml was removed, 900 μl PBSG was added, the cell pellets were resuspended, and centrifuged again at 16,800×*g* for 5 min. This washing step was repeated once more by removing 900 μl and adding 900 μl fresh PBSG. After centrifugation at 16,800×*g* for 5 min, 900 μl was removed and the cell pellets were resuspended by adding 350 μl PBSG. Then, 2 μl 10 mg/ml RNase A and 50 μl 0.5 mg/ml propidium iodide were added and the cells were incubated overnight at 4 °C. Finally, the cells were analyzed on a BD FACSymphony A1 flow cytometer.

2.5. Nucleus/kinetoplast configuration analysis

The distribution of the nucleus/kinetoplast configuration was determined by DAPI staining and fluorescence microscopy. Bloodstream-form trypanosomes were treated with DMSO, suramin, and suramin analogues as described in Section 2.4. After harvesting and washing, cells were, however, fixed in 2% formaldehyde and 0.05% glutaraldehyde in PBS, applied to poly-L-lysine-coated microscope slides, and stained with 0.0001% DAPI in PBS. Slides were covered in Vectashield mounting medium and examined with Leica DMi microscope using a 100X oil immersion objective.

3. Results and discussion

The four suramin analogues differ only slightly in their structure from the parent compound. The differences are (i) the lack of the methyl groups on the intermediate rings, (ii) the replacement of the two 1,3,5-naphthalenetrisulphonic acid groups with 1,3,6- naphthalenetrisulphonic acid groups, and (iii) the regiochemistry of the phenyl rings (RT5 (NF031): *para/para*; RT14 (NF032): *meta/para*; RT31: *para/meta*; RT58: *meta/meta* like suramin). Based on the close structural similarity, the predicted physicochemical and pharmacokinetic properties of the analogues and suramin differ only slightly (Table 1). All compounds are highly water-soluble substances as their predicted Log *P* values are smaller than −5. The predicted topological polar surface area (TPSA) of all five molecules is identical while the predicted molecular volume differs only marginally between the analogues and suramin (Table 1). Based on predicted Log P_{app} values (Caco2 permeability) and predicted Log K_P values (skin permeability), all five compounds also show very similar low predicted permeability properties (Table 1). The four analogues and suramin are thus very similar molecules in terms of their predicted physicochemical and pharmacokinetic properties.

The trypanocidal activity of the RT compounds and suramin was determined with the resazurin vital dye assay as previously described (Merschjohann et al., 2001; Antoszczak et al., 2019). While the suramin analogues RT5 (NF031), RT31, and RT58 suppressed the proliferation of bloodstream forms of *T. brucei* with minimum inhibitory concentration (MIC) values of 100 μM and 50% growth inhibition (GI_{50}) value of 20–30 μM, RT14 (NF032) affected the growth of the parasite only marginally (Table 2). However, RT5 (NF031), RT31 and RT58 were 1000 times (MIC values) and more than 500 times (GI_{50} values), respectively, less potent in inhibiting the growth of bloodstream-form trypanosomes than suramin (Table 2). Thus, compared to suramin, the

Table 1
Predicted physiochemical and pharmacokinetic properties of RT5, RT14, RT 31, RT58, and suramin.

Compound	Log *P*[a]	TPSA (Å^2)[a]	Volume (Å^3)[a]	Log P_{app}[b]	Log K_P[b]
RT5 (NF031)	−5.84	483.74	935.10	−3.365	−2.735
RT14 (NF032)	−5.85	483.74	935.10	−3.232	−2.735
RT31	−5.85	483.74	935.10	−3.216	−2.735
RT58	−5.86	483.74	935.10	−3.198	−2.735
Suramin	−5.72	483.74	982.22	−3.255	−2.735

[a] Log *P*, TPSA, and Volume were determined with Molinspiration interactive logP calculator (Molinspiration Cheminformatics, 2022).

[b] Log P_{app} and Log K_P were determined with the pkCSM web servers (Pires et al., 2015).

Table 2
MIC and GI_{50} values of RT5, RT14, RT31, RT58, and suramin.

Compound	MIC (μM)[a]	GI_{50} (μM)[b]	Fold change in MIC compared to suramin	Fold change in GI_{50} compared to suramin
RT5 (NF031)	100	21.7 ± 4.0	1000	529
RT14 (NF032)	>100	>100 (32 ± 6%)[c]	>1000	>2439
RT31	100	30.5 ± 3.3	1000	744
RT58	100	29.9 ± 1.7	1000	729
Suramin	0.1	0.04 ± 0.01	–	–

[a] As the compounds were tested at 10-fold dilutions starting from 100 μM, the MIC could only take values of 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, and 1 nM. As the same MIC value was determined for each compound in three independent experiments, only that MIC value is shown.

[b] Mean values and standard deviations of three independent experiments are shown.

[c] Value in brackets refers to the percentage of inhibition at 100 μM.

four analogues displayed substantially lower trypanocidal activity. Given the close structural similarity between the analogues and suramin, such a dramatic difference in trypanocidal activity was unexpected. The reason for this difference in activity may be due to either (i) low uptake, (ii) lower inhibitory activity, or (iii) a combination of both low uptake and lower inhibition potency of the suramin analogues.

To see whether the low trypanocidal activity of the suramin analogues is due to a low uptake rate, competition assays were performed. As negatively charged substances, the analogues cannot passively enter cells. Like suramin, they must be taken up by bloodstream-form trypanosomes via receptor-mediated endocytosis. For the cellular uptake of suramin, the invariant surface glycoprotein 75 (ISG75) has been suggested as the receptor for the entry of the drug in bloodstream forms of *T. brucei* (Alsford et al., 2012; Zoltner et al., 2020; Makarov et al., 2023). However, ISG75 knockout trypanosomes were not resistant to suramin indicating that other routes for the uptake of suramin exist (Makarov et al., 2023). Either way, given the close structural similarity between suramin and RT compounds, it is reasonable to assume that the cellular entry of the analogues occurs via the same routes as that for the trypanocide. If the four analogues are indeed taken up via the same route as suramin, then an excess of RT compounds over the trypanocide should prevent the toxic action of the drug. To this end, bloodstream forms of *T. brucei* were co-incubated with 0.2 μM suramin (5 × GI_{50}), a concentration that will kill the parasite (2 × MIC), and 10 μM analogues, a concentration that would inhibit the growth of the cells by less than 50%. As the excess of the analogues over suramin is 50-fold, only 2% of the drug would enter the trypanosomes. This equates to an accessible concentration of 0.004 μM of suramin which would not affect the growth of bloodstream-form trypanosomes. Thus, if the RT compounds compete with suramin for cellular entry, the excess of analogues should almost completely protect the trypanosomes from any growth-inhibitory effect induced by suramin. However, the observed protection by the analogues was distinctly lower (Fig. 2). While incubation of bloodstream-form trypanosomes with 0.2 μM suramin led to the killing of the parasite, the addition of 10 μM analogues prevented the death of the parasites and provided protection rates of 56.1%, 46.1%, and 22.4% in the case of RT5 (NF031), RT58, and RT31, respectively. The analogue RT14 (NF032) did not provide any protection and all parasites were dead at the end of the experiment. Although the analogues did not provide full protection against the trypanocidal effect of suramin, the result of the competition assay showed clearly that the analogues RT5 (NF031), RT31, and RT58 impede the trypanocidal action of suramin. This finding supports the suggestion that the analogues are also taken up by bloodstream forms of *T. brucei* via the same entry route(s) as suramin but probably bind to the

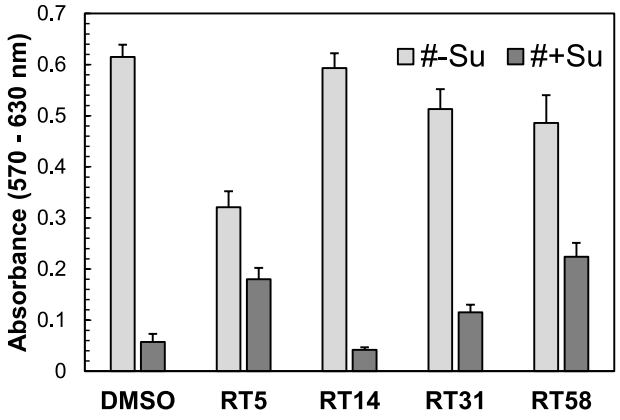


Fig. 2. Competition assay between suramin and suramin analogues. Bloodstream forms of *T. brucei* were incubated with 10 μM suramin analogues RT5 (NF031), RT14 (NF032), RT31, and RT58 in the absence (light grey columns) and in the presence (dark grey columns) of 0.2 μM suramin, respectively. Controls were incubated in medium containing only DMSO without or with suramin. After 72 h of culture, cell viability and proliferation were determined with the colorimetric dye resazurin. Mean values plus the standard deviation of three independent experiments are shown.

respective receptor(s) with lower affinity. These conclusions are further supported by the observation that the trypanocidal activity of the analogues correlates with their efficiency in blocking the trypanocidal action of suramin: the lower their GI_{50} value the higher their protection.

Although the trypanocidal mechanism of action of suramin remains unresolved, the drug has been shown to inhibit cytokinesis in bloodstream forms of *T. brucei* (Thomas et al., 2018). To determine whether the RT compounds also inhibit cytokinesis, cell cycle distribution and nucleus/kinetoplast configuration of bloodstream-form trypanosomes exposed to the analogues were analyzed. To this end, cells were incubated with each of the compounds at 5 × GI_{50} (except RT14 (NF032) as for this compound no GI_{50} could be established). After staining with propidium iodide or DAPI, the DNA content and nucleus/kinetoplast configuration of the cells were analyzed by flow cytometry and fluorescence microscopy, respectively (Figs. 3 and 4). Control cells that were only treated with DMSO showed the characteristic DNA distribution profile with about half of the cells in the G1 phase (Fig. 3, top-left) and a nucleus/kinetoplast configuration with around 90% of the cell displaying a 1n/1k pattern (Fig. 4). On the other hand, the DNA distribution and nucleus/kinetoplast configuration of trypanosomes treated with suramin was distinct from that of the control cells. The percentage of cells in the G1 phase dramatically decreased to 5.2%, while 60.6% of the cells accumulated in the G2/M phase (Fig. 3, top-right). In addition, 31.8% of cells were observed with a >2n DNA content (Fig. 3, top-right). As for the nucleus/kinetoplast configuration, only a fourth of the cells displayed a 1n/1k pattern while 60% of the cells had aberrant nucleus/kinetoplast patterns indicative of inhibition of cytokinesis (Fig. 4). This DNA distribution and nucleus/kinetoplast configuration profile conform to that previously reported for suramin-treated trypanosomes (Thomas et al., 2018). In contrast, trypanosomes treated with the RT compounds all showed similar changes in the DNA distribution and nucleus/kinetoplast configuration profile. Parasites treated with RT5 and RT58 had almost equal G1 and G2/M populations (RT5 (NF031): 41.3% and 41.5%; RT58: 45.7% and 47.9%), while for cells exposed to RT31, the G1 population was somewhat smaller than the G2/M population (32.9% vs 47.5%) (Fig. 3, bottom row). Also unlike suramin, the analogues only slightly affected the nucleus/kinetoplast configuration in trypanosomes. Around 70% of cells displayed the 1n/1k pattern with

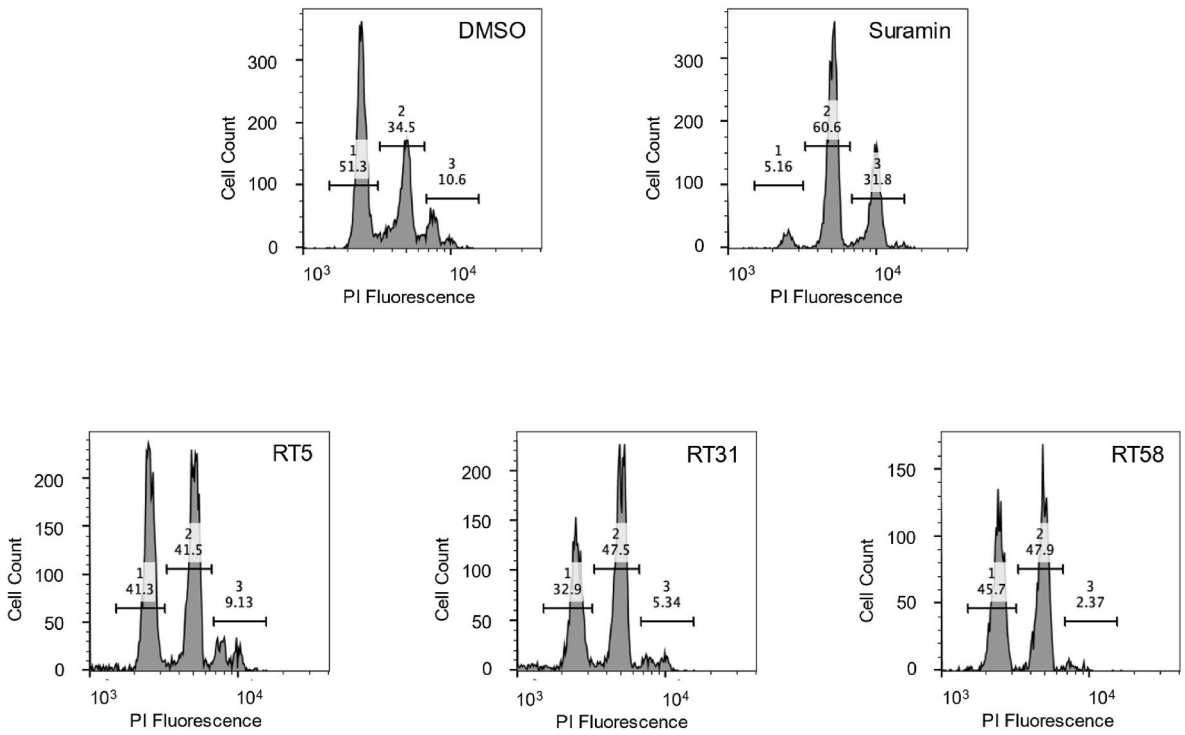


Fig. 3. Cell cycle distribution of bloodstream-form trypanosomes exposed to suramin analogues. Cell cycle distribution determined by flow cytometry. Bloodstream forms of *T. brucei* were incubated with each of the suramin analogues at 5 × GI_{50} (RT5 (NF031) = 105.5 μM; RT31 = 152.5 μM; RT58 = 149.5 μM). Controls were incubated with 0.2 μM suramin (5 × GI_{50}) or DMSO alone. After 21 h incubation, the cells were stained with propidium iodide and the DNA content analyzed by flow cytometry. Peak 1, G1 population; peak 2, G2/M population; peak 3, cells with >2n DNA.

many cells having enlarged nuclei (most likely from trypanosomes of the G2/M population) which is also indicative of inhibition of mitosis (Fig. 4). Only between 10 and 13% of trypanosomes had an aberrant nucleus/kinetoplast configuration (Fig. 4) This DNA-staining pattern resembles that previously reported for trypanosomes treated with the trypanocidal drug melarsoprol, which was suggested as an indication for inhibition of mitosis (Thomas et al., 2018). Because of the similarity in the DNA-staining pattern of trypanosomes treated with melarsoprol and the RT compounds, it is reasonable to assume that the suramin analogues also inhibit mitosis while the parent compound suramin inhibits cytokinesis (Thomas et al., 2018).

As RT5 (NF031), RT31, and RT58 seem to share the same mode of inhibition which appears to be different from that of suramin, it can be concluded that the different regiochemistry of the phenyl rings is not crucial for the altered inhibitory activity of the suramin analogues. This conclusion is supported by the fact that RT58 and suramin have the same regiochemistry of the phenyl rings, viz. *meta/meta* (Fig. 1). From this follows that only the regiochemistry of the naphthalenetrisulphonic acid groups and the missing methyl groups on the intermediate rings are the main cause for the different mode of inhibition of the suramin analogues. To determine whether the methyl groups on the intermediate rings and/or the regiochemistry of the naphthalenetrisulphonic acid groups are responsible for the superior inhibitory activity of suramin, analogues of the drug without the methyl groups on the intermediate rings (but with 1,3,5-naphthalenetrisulphonic acid groups) and with 1,3,6-naphthalenetrisulphonic acid groups (but with the methyl groups on the intermediate rings), respectively, would be needed to be tested. In contrast to the mode of inhibition, the regiochemistry of the phenyl rings seems to play some role in the uptake of the suramin analogues. While RT5 (NF031) and RT58 with all *para* and *meta* regiochemistry, respectively, showed moderate inhibition of the uptake of suramin, RT31 and RT14 (NF032) with mixed regiochemistry showed low or no inhibition of the internalisation of the drug. However, the methyl groups on the intermediate rings and/or the regiochemistry of the naphthalenetrisulphonic acid groups are also important factors in the uptake of suramin. This is clear from the competition experiment where RT58, which has, like suramin, all *meta* regiochemistry of the phenyl rings but is lacking the methyl groups on the intermediate rings and has the 1,3,5-naphthalenetrisulphonic acid groups, at a 50-fold excess protects the trypanosomes only partially from the toxic effect of suramin.

4. Conclusion

This study has shown that the inferior trypanocidal activity of the suramin analogues RT5 (NF031), RT31, and RT58 seems to be due to a combination of reduced uptake and different modes of inhibition. It was also found that the methyl groups on the intermediate rings and the regiochemistry of the naphthalenetrisulphonic acid groups are important structural features for the superior trypanocidal activity of suramin. However, to demonstrate this with certainty, more suramin analogues with different regiochemistry of the naphthalenetrisulphonic acid groups and with or without methyl groups on the intermediate rings need to be evaluated.

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CRediT authorship contribution statement

Dietmar Steverding: Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Ryan A.J. Tinson:** Writing – review & editing, Validation, Methodology, Investigation. **Monica Piras:** Writing – review & editing, Validation, Methodology, Investigation. **Stephen P. Wren:** Writing – review & editing, Validation, Methodology. **Stuart A. Rushworth:** Writing – review & editing,

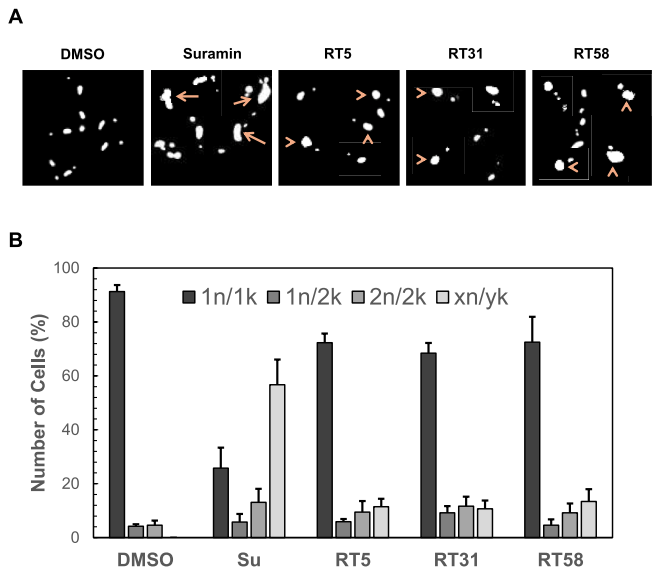

Fig. 4. Nucleus/kinetoplast configuration of bloodstream-form trypanosomes exposed to suramin analogues. Nucleus/kinetoplast configuration determined by DAPI staining and microscopy. Bloodstream forms of *T. brucei* were treated with DMSO, suramin, and suramin analogues as described in Fig. 3. After 21 h incubation, the cell were stained with DAPI and nucleus/kinetoplast configuration was determined by fluorescence microscopy. (A) Microscope images as examples of the DAPI staining. Compared to the control (DMSO), numerous trypanosomes exposed to suramin have aberrant nucleus/kinetoplast configuration (indicated by orange arrows) while quite a few parasites treated with the suramin analogues have an enlarged nucleus with one kinetoplast (indicated by orange arrow heads). (B) Percentage of nucleus/kinetoplast configurations. The designation xn/yk refers to aberrant nucleus/kinetoplast configurations with x = 2 or more nuclei and y = any number of kinetoplast; note that the configuration 2n/2k is scored separately. Data are the mean values plus standard deviation of two independent experiments each analyzed in two technical replicates.

Visualization, Validation, Methodology, Investigation, Data curation. **Mark Searcey:** Writing – review & editing, Supervision, Resources, Data curation. **Linda Troeberg:** Writing – original draft, Supervision, Resources, Data curation.

Declaration of competing interest

All authors declare that there were no commercial or financial interests or any other conflicts of interest.

Data availability

No data was used for the research described in the article.

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