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1 **Trypanotoxic activity of thiosemicarbazone iron**
2 **chelators**

3
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14

15 A B S T R A C T

16 Only a few drugs are available for treating sleeping sickness and nagana disease;
17 parasitic infections caused by protozoans of the genus *Trypanosoma* in sub-Saharan
18 Africa. There is an urgent need for the development of new medicines for
19 chemotherapy of these devastating diseases. In this study, three newly designed
20 thiosemicarbazone iron chelators, TSC24, Dp44mT and 3-AP, were tested for *in vitro*
21 activity against bloodstream forms of *T. brucei* and human leukaemia HL-60 cells. In
22 addition to their iron chelating properties, TSC24 and Dp44mT inhibit topoisomerase
23 II α while 3-AP inactivates ribonucleotide reductase. All three compounds exhibited
24 anti-trypanosomal activity, with minimum inhibitory concentration (MIC) values
25 ranging between 1 and 100 μ M and 50% growth inhibition (GI₅₀) values of around
26 250 nM. Although the compounds did not kill HL-60 cells (MIC values >100 μ M),
27 TSC24 and Dp44mT displayed considerable cytotoxicity based on their GI₅₀ values.
28 Iron supplementation partly reversed the trypanotoxic and cytotoxic activity of TSC24
29 and Dp44mT but not of 3-AP. This finding suggests possible synergy between the
30 iron chelating and topoisomerase II α inhibiting activity of the compounds. However,
31 further investigation using separate agents, the iron chelator deferoxamine and the
32 topoisomerase II inhibitor epirubicin, did not support any synergy for the interaction
33 of iron chelation and topoisomerase II inhibition. Furthermore, TSC24 was shown to
34 induce DNA degradation in bloodstream forms of *T. brucei* indicating that the
35 mechanism of trypanotoxic activity of the compound is topoisomerase II independent.
36 In conclusion, the data support further investigation of thiosemicarbazone iron
37 chelators with dual activity as lead compounds for anti-trypanosomal drug
38 development.

39

40 *Keywords:*

41 *Trypanosoma brucei*

42 Sleeping sickness

43 Topoisomerase

44 Thiosemicarbazone iron chelators

45 **1. Introduction**

46

47 African trypanosomes are the etiological agents of sleeping sickness in humans
48 and nagana disease in cattle (Steverding, 2008). The parasites are transmitted by the
49 bite of infected tsetse flies (*Glossina* spp.) and live and multiply in the blood and
50 tissue fluids of their mammalian host. Trypanosomiasis affects both humans and
51 animals mainly in rural sub-Saharan Africa where the disease imposes significant
52 burden on public health and economic development. Without treatment, both sleeping
53 sickness and nagana disease are fatal. Sadly, few drugs are available for
54 chemotherapy of African trypanosomiasis (Holmes et al. 2004; Steverding, 2010). In
55 addition, most drugs are outdated and difficult to administer. Moreover, drug
56 resistance in African trypanosomes is an increasing problem in the therapy of both
57 sleeping sickness and nagana disease (Matovu et al., 2001; Delespaux and de Koning,
58 2007). Thus, new strategies are needed if novel chemotherapies are to be developed.

59 One strategy to improve the activity of drugs is the conjugation of two bioactive
60 moieties. For instance, the conjugate of the iron chelator deferiprone and a
61 chloroquine fragment (7-chloro-4-aminoquinoline) has been shown to display higher
62 trypanotoxic activity than both parent compounds alone (Gehrke et al., 2013). Other
63 examples of compounds with dual activity are thiosemicarbazones. For instance, the
64 compounds Dp44mT and TSC24 (Fig. 1) possess both iron chelating and
65 topoisomerase II α inhibiting activity (Rao et al., 2009; Huang et al., 2010) while the
66 compound 3-AP (Fig. 1) exhibits iron chelating and ribonucleotide reductase
67 inhibiting activity (Finch et al., 1999; Aye et al., 2012). As topoisomerases and
68 ribonucleotide reductase are essential enzymes involved in the metabolism and
69 replication of DNA (Corbett and Berger, 2004; Nordlund and Reichard, 2006), and as
70 iron chelation has been shown to limit the proliferation of bloodstream form
71 trypanosomes (Breidbach et al., 2002; Merschjohann and Steverding, 2006),
72 inhibition of these enzymes in combination with iron depletion may be an interesting
73 option for the development of novel anti-trypanosomal chemotherapies. For this

74 reason, we studied the *in vitro* trypanotoxic activity of the thiosemicarbazones TSC24,
75 Dp44mT and 3-AP using bloodstream forms of *Trypanosoma brucei*. In addition, we
76 investigated whether the combination of iron chelation and topoisomerase inhibition
77 shows synergy.

78

79 **2. Materials and methods**

80

81 *2.1. Reagents*

82

83 Deferoxamine mesylate, di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone
84 (Dp44mT), 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP) and
85 ammonium ferric citrate were purchased from Sigma-Aldrich (Gillingham, U.K.). (E)-
86 N,N-dimethyl-2-(quinolin-2-ylmethylene)hydrazinecarbothioamide (TSC24) was
87 from Merck Chemicals Ltd. (Nottingham, U.K.). Epirubicin hydrochloride was
88 obtained from Cambridge Bioscience Ltd. (Cambridge, U.K.).

89

90 *2.2. Cell cultures*

91

92 Bloodstream forms of *T. brucei* clone 427-221a (Hirumi et al., 1980) and human
93 myeloid leukaemia HL-60 cells (Collins et al., 1977) were grown in Baltz medium
94 (Baltz et al., 1985) and RPMI medium (Moore et al., 1967), respectively. Both media
95 were supplemented with 16.7% (v/v) heat-inactivated foetal calf serum. All cultures
96 were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

97

98 *2.3. Toxicity assays*

99

100 Trypanosomes and HL-60 cells were seeded in 24-well plates in a final volume of
101 1 ml culture medium containing various concentrations of thiosemicarbazones
102 dissolved in 100% DMSO. Controls contained DMSO alone. In all experiments, the

103 final DMSO concentration was 1%. The seeding densities were 10^4 /ml trypanosomes
104 and 10^5 /ml HL-60 cells. For toxicity assays including iron supplementation, 10 μ l of
105 medium was replaced with 10 μ l of a 1.93 mg/ml ammonium ferric citrate solution to
106 give a final iron(III) concentration of 50 μ M. After 48 h of incubation, living cells
107 were counted with a Neubauer haemocytometer. The 50% growth inhibition (GI_{50})
108 values, i.e. the concentration of compounds necessary to reduce the growth rate of
109 cells by 50% to that of controls, was determined by linear interpolation according to
110 the method described in (Huber and Koella, 1993). The minimum inhibitory
111 concentration (MIC) values, i.e. the concentration of the compounds at which all cells
112 were killed, was determined microscopically.

113

114 2.4. Flow cytometric analysis

115

116 Flow cytometric analysis was performed as described previously (Phillips et al.,
117 2013). Bloodstream form trypanosomes ($1-5 \times 10^6$ /ml) were incubated with 500 nM
118 TSC24, 50 μ M ammonium ferric citrate, 5 μ M TSC plus 50 μ M ammonium ferric
119 citrate or 1% DMSO for 24 h. After harvesting by centrifugation at $850 \times g$ and
120 washing twice with PBS/1% glucose, cells were fixed in 100 μ l ice-cold methanol for
121 5 min and then diluted with 1 ml PBS. After centrifugation, the cell pellets were re-
122 suspended in PBS and stained with propidium iodide (final concentration 50 μ g/ml).
123 Cells were analysed on a BD Accuri C6 Flow Cytometer. Debris was excluded from
124 analysis through gating on forward scatter and side scatter properties. Singlets were
125 identified and doublets excluded through gating on FL-2 (585/40 nm) area versus
126 height. A minimum of 10,000 cells were collected for analysis. Data was analysed
127 using FlowJo version 10.

128

129 2.5. Isobolographic analysis

130

131 The interaction of the iron chelator deferoxamine and the topoisomerase II
132 inhibitor epirubicin was evaluated using the isobolographic method as described
133 previously (Steverding and Wang, 2009). First, the GI₅₀ value for each drug was
134 determined. Based on the GI₅₀ values, bloodstream form trypanosomes were
135 incubated with twofold serially diluted 1:1 ratios of drug combination. For controls,
136 trypanosomes were cultured with twofold serially diluted concentrations of each drug
137 alone. After 48 h incubation, live cells were counted and the GI₅₀ value for each drug
138 in the absence and in the presence of the other co-administered drug was determined.
139 The combination index (CI) for the drug combination was calculated using the
140 equation

141
142
$$CI = \frac{GI_{50(DFO,com)}}{GI_{50(DFO,sin)}} + \frac{GI_{50(EPI,com)}}{GI_{50(EPI,sin)}}$$

143
144

145
146 where GI_{50(DFO,com)} and GI_{50(EPI,com)} are the concentrations of deferoxamine and
147 epirubicin used in the combination to achieve 50% growth inhibition and GI_{50(DFO,sin)}
148 and GI_{50(EPI,sin)} are the concentrations of deferoxamine and epirubicin alone to achieve
149 the same effect. A CI value of <1, =1, and >1 indicates synergism, additive effect, and
150 antagonism, respectively.⁶

151

152 3. Results

153

154 The trypanotoxic activity of the thiosemicarbazones TSC24, Dp44mT and 3-AP
155 was determined with bloodstream forms of the *T. brucei* strain 427-221a while the
156 general cytotoxicity of the compounds was evaluated with human myeloid leukaemia
157 HL-60 cells. All three thiosemicarbazones showed a dose-dependent effect on the
158 inhibition of the growth of trypanosomes in cell culture with similar GI₅₀ values
159 ranging between 0.226 and 0.287 μM (Table 1). Statistical analysis revealed no
160 significant difference between the GI₅₀ values of the three compounds (ANOVA, p =

161 0.574). Both TSC24 and Dp44mT displayed a promising MIC value of 1 μ M while 3-
162 AP a less favourable value of 100 μ M (Table 1) demonstrating that all three
163 compounds are trypanocidal. **By comparison, clinically used anti-sleeping sickness**
164 **drugs display much higher anti-trypanosomal activities. For example, pentamidine,**
165 **melarsoprol and suramin exhibit GI₅₀ values of 0.001 μ M, 0.016 μ M and 0.032 μ M,**
166 **and MIC values of 0.006 μ M, 0.1 μ M and 1 μ M, respectively (Merschjohann et al.,**
167 **2001; Caffrey et al., 2007; Steverding et al., 2014). The thiosemicarbazones also**
168 **inhibited the proliferation of HL-60 cells but with GI₅₀ values varying between 0.005**
169 **and 0.673 μ M (Table 1). Dp44mT and TSC24 proved to be more effective in**
170 **inhibiting the growth of HL-60 cells than that of trypanosomes. However, all three**
171 **compounds had a MIC value of >100 μ M indicating that they were cytostatic rather**
172 **than cytotoxic. Anti-sleeping sickness drugs, on the other hand, are much less toxic to**
173 **HL-60 cells. For example, the GI₅₀ values of pentamidine, melarsoprol and suramin**
174 **are 33 μ M, 4 μ M and >100 μ M, respectively, while their MIC values are \geq 100 μ M**
175 **(Merschjohann et al., 2001; Caffrey et al., 2007; Steverding et al., 2014). As a result,**
176 **the GI₅₀ and MIC ratios of cytotoxic to trypanotoxic activities (selectivity indices) for**
177 **the thiosemicarbazones were much less favourable than those of anti-sleeping**
178 **sickness drugs. TSC24 and Dp44mT had a GI₅₀ ratio of <1 while their corresponding**
179 **MIC ratio was, at >100, more promising (Table 2). The GI₅₀ and MIC ratios for 3-AP**
180 **were 2.85 and >1 indicating poor selectivity of this drug. In contrast, the GI₅₀ and**
181 **MIC ratios of anti-sleeping sickness drugs are much higher (pentamidine: 9,800 and**
182 **13,000; melarsoprol: 267 and >1,000; suramin: >100 and >1,000) (Merschjohann et**
183 **al., 2001; Caffrey et al., 2007; Steverding et al., 2014).**

184 Supplementation of iron partially reversed the trypanotoxic activity of TSC24 and
185 Dp44mT causing a 13- and 100-fold increase of their GI₅₀ and MIC values,
186 respectively (Table 1). This finding supports the notion that both thiosemicarbazones
187 could chelate iron in cells, which may have contributed to the trypanotoxic activity of
188 the compounds. In contrast, addition of iron did not impair the anti-trypanosomal
189 activity of 3-AP (Table 1). Iron supplementation also reduced the cytotoxicity of the

190 compounds (Table 1). However, the GI₅₀ values for TSC24 and Dp44mT for HL-60
191 cells increased only 5- and 7-fold, respectively, which was lower than those observed
192 for the compounds for trypanosomes. As the addition of iron shifted the trypanotoxic
193 and the cytotoxic activity of the compounds in the same direction, no change in the
194 MIC and GI₅₀ ratios were observed apart from a 100-fold drop in the MIC ratios for
195 TSC24 and Dp44mT (Table 2).

196 As TSC24 and Dp44mT are inhibitors of topoisomerase II α and displayed almost
197 equal trypanotoxic activities indicating that their mechanism of anti-trypanosomal
198 action is identical, TSC24 was chosen to investigate the effect of this
199 thiosemicarbazone on the cell cycle progression in *T. brucei*. Bloodstream form
200 trypanosomes were incubated for 24 h in the absence or presence of iron with TSC24
201 at concentrations sufficient to inhibit the growth of the cells without killing them. The
202 iron supplementation control showed little change in the cell cycle distribution
203 compared to the DMSO control (Fig. 2A). TSC24 treatment increased the population
204 of cells with sub-G1 and post-G1 DNA content (Fig. 2B). This action of TSC24 is in
205 contrast to the effect of the compound on the cell cycle progression in mammalian
206 cells where the thiosemicarbazone has been reported to induce a G1-S arrest (Huang
207 et al., 2010). However, our finding is reminiscent of the action of idarubicin, a
208 classical topoisomerase II inhibitor, on *T. rangeli* where the drug has also been
209 demonstrated to lead to DNA degradation (Jobe et al., 2012). When bloodstream
210 forms of *T. brucei* were incubated with TSC24 in the presence of iron, an increase in
211 cells in the G1 phase was observed (Fig. 1C). This result resembles the action of
212 Dp44mT and TSC24 found for mammalian cells where the compounds induce a G1-S
213 cell cycle arrest (Rao et al., 2009; Huang et al., 2010).

214 To investigate whether the trypanotoxic action of TSC24 and Dp44mT was the
215 result from a synergistic effect of their iron chelating and topoisomerase II inhibiting
216 activity, a combination assay was carried out. Although the iron chelating properties
217 of TSC24 and Dp44mT is known to be due to their thiosemicarbazone scaffold, the
218 part of the molecules responsible for their topoisomerase inhibiting properties is not

219 known. Therefore, a combination assay was designed using two separate agents, the
220 iron chelator deferoxamine and the topoisomerase II inhibitor epirubicin. The
221 combination of deferoxamine with epirubicin showed an antagonistic effect with a CI
222 of 1.49 ± 0.25 (Fig. 3). Whereas the GI_{50} of deferoxamine dropped from $10.8 \pm 2.1 \mu\text{M}$
223 to $4.5 \pm 0.6 \mu\text{M}$, the GI_{50} of epirubicin remained unchanged ($108 \pm 17 \text{ nM}$ vs 113 ± 14
224 nM). This result suggests that iron chelation and topoisomerase inhibition probably do
225 not show trypanocidal synergy.

226

227 3. Discussion

228

229 As bloodstream forms of *T. brucei* contain only four iron-dependent enzymes
230 (aconitase, alternative oxidase, ribonucleotide reductase and superoxide dismutase)
231 and do not express any iron storage proteins, they are more prone to iron-depletion
232 than mammalian cells (Breidbach et al. 2002). Thus, iron chelation could be an
233 interesting approach for the development of new trypanocidal drugs. In this study, we
234 investigated the trypanotoxic activity of newly designed thiosemicarbazones that in
235 addition to their iron chelating properties display inhibitory activities against different
236 enzymes. Aiming simultaneously at two biological targets with one drug may achieve
237 greater therapeutic efficacy due to synergistic effects.

238 All three thiosemicarbazones studied showed similar trypanotoxic activities. The
239 addition of iron reduced the anti-trypanosomal action of TSC24 and Dp44mT but not
240 that of 3-AP. This may be explained by the different inhibitory mechanism of the
241 compounds. Whereas the anti-proliferate effect of Dp44mT and TSC24 have been
242 attributed to both iron chelation and inhibition of topoisomerase II α (Rao et al., 2009;
243 Huang et al., 2010), that of 3-AP is due to the destruction of the tyrosyl radical of the
244 $\beta 2$ subunit of ribonucleotide reductase through the active reductant [Fe(II)-(3-AP)]
245 (Aye et al., 2012). As the activity of 3-AP requires binding of iron, supplementation
246 of the metal would not be expected to significantly affect the toxic action of the
247 compound. **An alternative mode of action was reported for Dp44mT involving redox**

248 cycle of the iron-Dp44mT complex to generate reactive oxygen species (ROS) (Yuan
249 et al., 2004). Similar to 3-AP, iron supplementation should not affect this activity of
250 Dp44mT as the production of ROS requires the metal. However, as the addition of
251 iron reduces the anti-trypanosomal effect of Dp44mT, this additional mode of action
252 involving the production of ROS ~~doesseems~~ not seem to be responsible for the
253 trypanotoxic activity of the compound.

Comment [DS1]: 'Seems not' is perfectly correct but is more archaic and less used today. So my correct is only a suggestion.

254 The cytotoxic activity of Dp44mT and TSC24 has been associated with the
255 ability of the compounds to induce cell cycle arrest at the G1-S checkpoint (Rao et al.,
256 2009; Huang et al., 2010) which is consistent with previous reports of most iron
257 chelators (Brodie et al., 1993; Yu et al., 2007). Our results indicate that the
258 mechanism of action of the two thiosemicarbazones on the cell cycle in bloodstream
259 forms of *T. brucei* is different from that in cancer cells. In the absence of iron, the
260 compounds caused a reduction in the DNA content in many cells. This finding is
261 indicative for degradation of DNA suggesting a topoisomerase II independent
262 mechanism of trypanotoxic action for the compounds similar to that of idarubicin
263 described previously for *T. rangeli* (Jobe et al., 2012). In the presence of iron the
264 thiosemicarbazones caused an increase of bloodstream form trypanosomes in the G1
265 phase which suggests that some of the trypanosomes had undergone cell cycle arrest
266 at the G1-S boundary. It appears that in the absence of iron Dp44mT and TCS24
267 display different actions towards trypanosomes than to cancer cells. However, it
268 should be mentioned that bloodstream forms of *T. brucei* have a much lower iron
269 content than mammalian cells (Schell et al., 1991). Therefore, it is possible that the
270 thiosemicarbazones within cancer cells quickly bind iron and execute their activity
271 only as an iron complex while in bloodstream form trypanosomes they operate mainly
272 as iron-free compounds. This suggestion is supported by the fact that iron
273 supplementation has a much greater abrogating effect on the trypanotoxic activity of
274 Dp44mT and TSC24 (13-fold reduction) than on their cytotoxic activity (~6-fold
275 reduction). That iron supplementation has only a minor effect on the cytotoxic activity
276 of TSC24 has been previously demonstrated (Huang et al., 2010).

277 Although TCS24 has been demonstrated to have both iron chelating and
278 topoisomerase inhibiting activities with both actions believed to contribute to its
279 cytotoxicity against a range of cancer cell lines (Huang et al., 2010), it remains
280 unclear whether both activities contribute also to the trypanotoxic action of the
281 compound. The partial reversal of the anti-trypanosomal activity of TSC24 upon iron
282 addition may indicate that both actions play a role and act synergistically. However,
283 combination experiments carried out with the iron chelator, deferoxamine, and the
284 topoisomerase II inhibitor, epirubicin, showed no synergy between iron chelating and
285 topoisomerase inhibiting actions. As, in this test, two separate agents were used, it is
286 possible that the two compounds interfere with each other's activity reducing their
287 efficacy. Another explanation for a possible difference in the interaction of iron
288 chelating and topoisomerase II inhibiting activity of TSC24 and
289 deferoxamine/epirubicin combination may lie in the different topoisomerase
290 inhibition mechanism of TSC24 and epirubicin. Whereas TSC24 is a catalytic
291 inhibitor inactivating topoisomerase II via binding to the APTase domain and
292 blocking the ATP hydrolysis activity of the enzyme (Huang et al., 2010), epirubicin is
293 a topoisomerase poison that intercalates between DNA base pairs and stabilises the
294 DNA-enzyme complex (Coukell and Faulds, 1997).

295 In summary, the three thiosemicarbazones investigated in this study all show GI₅₀
296 values below 300 nM for bloodstream forms of *T. brucei*. These values are within the
297 range of GI₅₀ values reported previously for other topoisomerase inhibitors for
298 trypanosomes (Deterding et al., 2005). In addition, the MIC value of TSC24 and
299 Dp44mT was similar to that of suramin (1 µM), one of the current drugs used to treat
300 sleeping sickness (Merschjohann et al., 2001; Steverding et al., 2014). However, the
301 selectivity of the thiosemicarbazones was poor. While the MIC values showed that the
302 compounds did not kill human HL-60 cells, the GI₅₀ values indicated unsatisfactory
303 cytotoxicity of the agents. Nevertheless, the actual clinical selectivity of the
304 thiosemicarbazones may be much higher. As the thiosemicarbazones have been
305 selected for cytotoxic action against cancer cells, their anti-proliferative effect on HL-

306 | 60 cells may, therefore, be an overestimate for a healthy cell response. Whether
307 thiosemicarbazone iron chelators are interesting compounds for further anti-
308 trypanosomal drug development remains to be shown.
309

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311

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Table 1

MIC and GI₅₀ values of the thiosemicarbazones TSC24, Dp44mT and 3-AP for *T. brucei* bloodstream forms and human HL-60 cells.

Compound	<i>T. brucei</i>				HL-60			
	MIC (μM)		GI ₅₀ (μM)		MIC (μM)		GI ₅₀ (μM)	
	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe
TSC24	1	100	0.287±0.020	3.642±2.068	>100	>100	0.122±0.058	0.617±0.077
Dp44mT	1	100	0.226±0.082	3.069±0.436	>100	>100	0.005±0.002	0.036±0.025
3-AP	100	100	0.236±0.093	0.322±0.046	>100	>100	0.673±0.054	1.537±0.921

Data are mean values± SD of three experiments.

Table 2

MIC and GI₅₀ ratios of cytotoxic to trypanotoxic activities of the thiosemicarbazones TSC24, Dp44mT and 3-AP.

Compound	MIC _(HL-60) /MIC _(<i>T. brucei</i>)		GI ₅₀ (HL-60)/GI ₅₀ (<i>T. brucei</i>)	
	-Fe	+Fe	-Fe	+Fe
TSC24	>100	>1	0.43	0.17
Dp44mT	>100	>1	0.02	0.01
3-AP	>1	>1	2.85	4.77

MIC and GI₅₀ ratios were calculated from MIC and GI₅₀ values shown in Table 1.

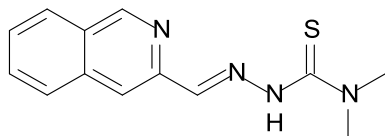
FIGURE LEGENDS

Fig. 1. Structures of the iron-chelating thiosemicarbazones TSC24, Dp44mT and 3-AP. The PubChem Compound Identifier (CID) for each compound is shown in parentheses.

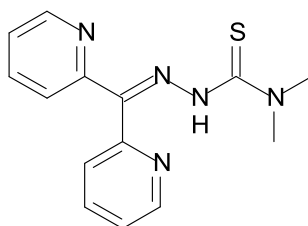
Fig. 2. Cell cycle distribution of *T. brucei* exposed to TSC24. Bloodstream form trypanosomes were treated with 50 μM iron(III) (A), 0.5 μM TSC (B) or 5 μM TSC plus 50 μM iron(III) (C). The dotted trace in each graph is the result of the DMSO control culture. After 24 h incubation, the trypanosomes were stained with propidium iodide and the DNA content analysed by flow cytometry.

Fig. 3. Isobolographic plot for the interaction between the iron chelator deferoxamine and the topoisomerase II inhibitor epirubicin. Bloodstream forms of *T. brucei* were incubated with twofold serial dilutions of the drug combination (1:1) or the drugs alone. After 48 h of incubation, live cells were counted and GI_{50} values determined. The dotted line that connects the GI_{50} points for the single drug treatments (filled squares) is the theoretical additive line. The GI_{50} of the combinations is indicated by the open circle. Each point represents the mean \pm SD of three independent experiments.

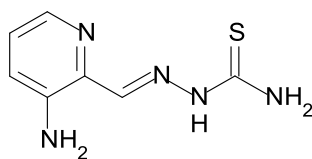
FIG. 1



TSC24 (CID: 46202546)



Dp44mT (CID: 10334137)



3-AP (CID: 9571836)

FIG. 2

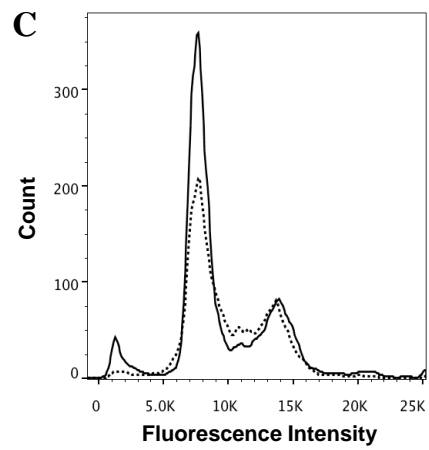
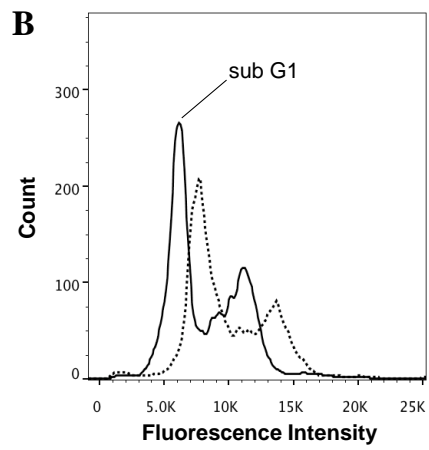
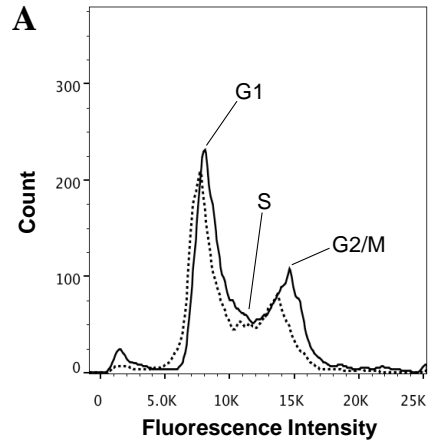


Fig. 3

