Independence from Kinetoplast DNA Maintenance and Expression Is Associated with Multidrug Resistance in *Trypanosoma brucei In Vitro*

Matthew K. Gould,* Achim Schnaufer
Centre for Immunity, Infection & Evolution, Institute of Immunology & Infection Research, University of Edinburgh, Edinburgh, United Kingdom

It is well known that several antitrypanosomatid drugs accumulate in the parasite’s mitochondrion, where they often bind to the organelar DNA, the kinetoplast. To what extent this property relates to the mode of action of these compounds has remained largely unquantified. Here we show that single point mutations that remove the dependence of laboratory strains of the sleeping sickness parasite *Trypanosoma brucei* on a functional kinetoplast result in significant resistance to the diamidine and phenanthridine drug classes.

Trypanosomes are protist parasites that are the causative agents of a range of pathogenic infections in humans and in animals. A defining characteristic of these organisms is the presence of the kinetoplast, a unique structure comprising the cell’s mitochondrial genome (kDNA). In the disease-causing long slender bloodstream forms (BF) of *Trypanosoma brucei*, subspecies of which cause human African trypanosomiasis (HAT) (or sleeping sickness, caused by *T. brucei rhodesiense* and *T. brucei gambiense*) and animal African trypanosomiasis (AAT) (caused by *T. brucei brucei*), the presence of a functional kinetoplast is essential (1–3).

However, the closely related animal pathogens *Trypanosoma equiperdum* and *Trypanosoma evansi* are dyskinetoplastic (DK) (i.e., lacking all or critical parts of their kDNA), with no apparent detrimental effect (4, 5). Single point mutations had been identified in the nucleolarly encoded subunit γ of the mitochondrial F₁F₀-ATPase of the naturally DK trypanosomes *T. evansi* and *T. equiperdum* (6) and in a laboratory strain of *T. brucei brucei* (7) that was chemically induced to lose its kinetoplast through long-term exposure to the DNA intercalator acriflavine (8). We recently reported that some of these mutations are sufficient to fully compensate for the complete loss of kDNA in BF *T. brucei* (9). The mechanism involves F₀-independent generation of the essential mitochondrial membrane potential (ΔΨm), obviating the need for expression of the kDNA-encoded F₉ subunit (9).

A number of current and potential trypanocidal chemotherapies, and their related compounds, have been shown to accumulate in the mitochondrion and/or interact with kDNA (10–13). Other compounds have been proposed to act, at least in part, by disrupting the replication of kDNA through the inhibition of key enzymes, such as the topoisomerases (14–18). Until now, attempts to quantify the degree to which compounds target the kinetoplast have relied upon comparing the sensitivities of divergent strains and species (16, 19–21). Consequently, the ability to draw definitive conclusions was limited by unknown effects of interstrain/species variations acquired through differing evolutionary selection pressures or culturing histories.

We generated BF *T. brucei brucei* strain Lister 427 trypanosomes ectopically expressing F₁F₀-ATPase subunit γ with an L262P or A281del mutation with both endogenous alleles knocked out. We generated a reference cell line that constitutively expressed ectopic wild-type (WT) subunit γ in a double-endogenous knockout background. DK versions of each mutant γ-expressing cell line were obtained by exposure to 20 nM acriflavine for 7 days (9).

The generation of otherwise isogenic cell lines, either dependent or not on functional kDNA, allowed us to conclusively establish to what extent the mode of action of antitrypanosomatid compounds is related to the parasite’s dependence on kDNA maintenance or expression. The 50% effective concentrations (EC₅₀s) were determined for a panel of trypanocidal compounds using a slightly modified version of the alamarBlue method (22) as described previously (9).

For the topoisomerase inhibitors etoposide, camptothecin, norfloxacin, and enoxacin, no major change in sensitivity was observed in the kinetoplast-independent γL262P-expressing trypanosomes, either with or without the kinetoplast, compared to that of the γWT-expressing controls (see Table S1 in the supplemental material). These data suggest that for these compounds, the dominant mode of action is not related to kDNA maintenance or expression, which contrasts with a study in *Trypanosoma cruzi* that reported a kDNA-specific effect for norfloxacin and enoxacin (17). Similarly, for the antimalarial atovaquone, a compound that targets the mitochondrial cytochrome bc₁ complex in *Plasmodium falciparum* (23), no differences in sensitivity were observed (see Table S1). This is not surprising, since this complex is not expressed in BF *T. brucei* (24). Interestingly, proguanil, a compound that acts synergistically with atovaquone, presumably by inhibiting an alternative mode of generating the essential mitochondrial membrane potential (ΔΨm) that is independent of the mitochondrial electron transport chain (23), also displayed no differences in potency. Hence, the alternative mechanism for generating the...
dependence on a functional kinetoplast, at least (25). The average EC50s for to the phenanthridine class of drugs, used for AAT chemotherapy have EC50s close to those of pound-induced loss of kinetoplast expression and consequently /H9253 A281del mutants do not have enough time to adapt to test com-
assay
Likewise, (Table 1). The highest resistance shown by
nylindole (DAPI), also a diamidine, resistance factors of 32.0 and
trypanosomes with and without kDNA, respectively, were ob-
plast, respectively) (Table 1).
kinetoplast-specific activity (see Table S2 in the supplemental ma-
to proguanil.
The lack of resistance to DB75 by the /H9253 L262P mutants
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
of the long-term effects on parasite growth by con-
replication initiation of free minicircles (16).
Expressing trypanosomes with both endogenous alleles knocked out, with or without (DK) their kinetoplast, compared to WT ATP synthase (9); how-
replication is conducted over only 3 days, the kinetoplast-retaining
replicating cumulative growth curves over 7 days in the presence of acriflavine, the
WT-expressing trypanosomes (see Fig. S1A and B in the supplemental
material). In the DK version or the
kinetoplast, compared to WT ATP synthase
replicase (9); however, after a period of ~7 days in the presence of acriflavine, the
replicase is no longer functional under cumulative selection, suggesting that the Poty of this DNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression. Exposure to diami-
slightly enhanced by the presence of kDNA, even though the cells
are no longer dependent on its expression.

The DB compounds and isometamidium were kind gifts from David Boykin and Michael Barrett/Harry de Koning, respectively. All other compounds were purchased from Sigma.

Asterisks indicate statistically significant resistance, as calculated using a one-tailed Student’s t-test: *P < 0.05; **P < 0.01; ***P < 0.001.

TABLE 1 Cross-resistance characterization of L262P or A281 del mutated ATP synthase (γ-expressing) trypanosomes with both endogenous alleles knocked out, with or without (DK) their kinetoplast, compared to WT ATP synthase (γ-expressing) control

<table>
<thead>
<tr>
<th>Compound</th>
<th>γL262P</th>
<th>γL262P (DK)</th>
<th>γA281del</th>
<th>γA281del (DK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine</td>
<td>2.1 ± 0.1</td>
<td>2.9 ± 0.2</td>
<td>17.6 ± 0.5</td>
<td>6.1**</td>
</tr>
<tr>
<td>Dimanes</td>
<td>5.0 ± 0.3</td>
<td>5.0 ± 0.1</td>
<td>15.6 ± 1.2</td>
<td>3.1**</td>
</tr>
<tr>
<td>Pentamidine</td>
<td>35 ± 6</td>
<td>23 ± 2</td>
<td>817 ± 196</td>
<td>35.5*</td>
</tr>
<tr>
<td>Dimanes</td>
<td>0.8 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>2.7*</td>
</tr>
<tr>
<td>DAPI</td>
<td>5.0 ± 0.7</td>
<td>3.4 ± 0.4</td>
<td>27.6 ± 5.7</td>
<td>8.1*</td>
</tr>
<tr>
<td>Phenanthridine</td>
<td>0.32 ± 0.01</td>
<td>0.40 ± 0.02</td>
<td>32.9 ± 7.3</td>
<td>82.3**</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>239 ± 8.0</td>
<td>217.3***</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>2.6 ± 0.5</td>
<td>0.8 ± 0.1</td>
<td>112 ± 14</td>
<td>140.0*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Resistance factor vs γ WT</th>
<th>Resistance factor vs γ L262P</th>
<th>Resistance factor vs γ A281del</th>
<th>Resistance factor vs γ A281del (DK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine</td>
<td>16.1 ± 0.4</td>
<td>5.6**</td>
<td>0.9</td>
</tr>
<tr>
<td>Dimanes</td>
<td>18.2 ± 0.7</td>
<td>3.6**</td>
<td>1.2</td>
</tr>
<tr>
<td>Pentamidine</td>
<td>958 ± 167</td>
<td>41.7**</td>
<td>1.2</td>
</tr>
<tr>
<td>Dimanes</td>
<td>2.5 ± 0.4</td>
<td>2.5*</td>
<td>0.9</td>
</tr>
<tr>
<td>DAPI</td>
<td>28.8 ± 5.1</td>
<td>8.5*</td>
<td>1.0</td>
</tr>
<tr>
<td>Phenanthridine</td>
<td>97.8 ± 8.9</td>
<td>31.5**</td>
<td>1.0</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>5.8 ± 1.0</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>1389.8***</td>
<td>1.9**</td>
<td>1.9</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>289.1***</td>
<td>1.3**</td>
<td>0.9</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>208 ± 13</td>
<td>260.0**</td>
<td>1.9</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>114 ± 28</td>
<td>1041.3***</td>
<td>92.5**</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>140.0*</td>
<td>875.7***</td>
<td>92.5**</td>
</tr>
</tbody>
</table>
ingly, the only difference between DB75 and DB829 is the substitution in DB829 of one carbon in each of the two benzene rings for nitrogen (26). This single change partially links the mode of action of DB829 to the parasite’s dependence on kDNA.

In order to test whether the cell lines with mutated ATP synthase γ remained viable in vivo and still retained drug resistance, MF1 mice were inoculated intraperitoneally with each of the cell lines that had been tested in vitro (1 × 10^6 trypanosomes per infection) and treated with normally curative doses of EtBr (Fig. 1; see also Fig. S2 in the supplemental material). For γWT-expressing cells, parasitemia in the mice peaked around 48 h postinoculation, and trypanosomes were entirely absent from the blood after 4 days (by microscopic inspection of blood smears [29]). In contrast, the mutant γ-expressing cell lines were apparently unaffected by EtBr treatment, with parasitemia increasing exponentially until the experiment was ended, clearly demonstrating that trypanosomes with single point mutations in ATP synthase γ that circumvent dependence on kDNA are viable and drug resistant in animal models. In vivo, no noticeable adaptation period was apparent upon EtBr-induced loss of the kinetoplast for the γA281del mutant, with parasitemia progressing at rates similar to those for the other mutants and with no discernible difference to that of its DK cell line (Fig. 1; see also Fig. S2). This suggests that, in vivo, either the A281del mutation is fully sufficient to compensate for kDNA loss or any secondary adaptation occurs much faster than in vitro. A third mutation conferring independence from the kinetoplast, γA273P (in this case, ectopically expressed in a single endogenous knockout background) (9), showed resistance to EtBr similar to that of the γWT-expressing cell line.

Finally, we investigated the possibility of a change in drug accumulation being responsible for the observed resistance phenotypes. Whole-cell uptake of the naturally fluorescent phenanthridines isomers (with or without kinetoplast) was monitored using a previously described oil-stop method to halt uptake at specific time points (30). Over 20 min, no significant differences were observed in the rates of uptake of either compound by γL262P-expressing trypanosomes (with or without kinetoplast) compared to that of γWT-expressing trypanosomes (see Fig. S3 in the supplemental material). Thus, mutations to ATP synthase γ do not affect the overall uptake of phenanthridines into the cell, although differences in the rate of accumulation by the mitochondrion cannot be ruled out.

In summary, we have exploited single point mutations in the F_{1}F_{0}-ATPase to demonstrate that the uncoupling of viability of laboratory strains of T. brucei from a functional kinetoplast results in >80-fold resistance to isometamidium, >200-fold resistance to EtBr, and >30-fold resistance to some diamidines. While an obvious explanation for these findings could be that these drugs interfere with the maintenance and/or expression of kinetoplast-encoding genes, we presently cannot rule out that they have additional, relevant mitochondrial targets and that the altered mechanisms for generating a mitochondrial membrane potential (Δψm) in the mutant cells affect drug accumulation in the mitochondrion. Although the overall drug accumulation of at least phenanthridines was unaffected, a potentially lower Δψm in cells expressing the L262P or A281del mutation could change the intracellular drug distribution. This requires further study.

It seems likely that the levels of resistance we observed can be relevant in the field. For example, it has been reported that Chinese T. evansi isolates (most, if not all, of which have the A281del mutation) have innate resistance to isometamidium (31), and our results strongly suggest that independence from the kinetoplast plays an important role in this resistance. A report concluding that dyskinetoplasticity is not associated with isometamidium resistance was misled by comparing the sensitivities of T. evansi and T. equiperdum strains before and after complete deletion of kDNA (21). As we now know, all strains of these species are already preadapted to kDNA loss (9); whether certain parts of kDNA remain or not is largely irrelevant for phenanthridine resistance.

ACKNOWLEDGMENTS

We thank Michael Barrett, Harry de Koning, and David Boykin for chemical compounds and critical reading of the manuscript, Akhil Vaidya for proparganil, Heidrun Interthal for camptothecin and etoposide, and the de Koning group for assistance with the uptake assays.

This work was funded by Medical Research Council grant G0600129 and Wellcome Trust grant WT094899MA (to A.S.).

REFERENCES

t.2010.08.001.
8. Stuart KD. 1971. Evidence for the retention of kinetoplast DNA in an


