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MECHANISMS OF RESISTANCE TO ANTIPARASITIC DRUGS IN \textit{TRYPANOSOMA CRUZI}. CORRELATIONS BETWEEN GENOTYPE AND RESISTANCE (*)

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ABSTRACT

The nitroheterocyclic compounds benznidazole and nifurtimox are the only drugs licensed for treatment of \textit{Trypanosoma cruzi} infections. Both are pro-drugs and do not have significant trypanocidal properties until they have undergone intra-parasitic activation. The enzyme responsible is a nitroreductase (TcNTR), which initiates a reductive cascade that leads to the generation of the toxic metabolites that kill the parasite. Processes that act to down-regulate this enzyme lead to cross-resistance against both front line drugs. These include the loss of one of the chromosomes containing the TcNTR gene, or point mutations which inactivate the enzyme. TcNTR heterozygotes are infectious, do not display an obvious deleterious phenotype, and are up to 5-fold more resistant to benznidazole and nifurtimox. Complete loss of TcNTR activity however, renders \textit{T.cruzi} largely non-infectious suggesting that there may be a limit to the level of resistance by this mechanism. In natural populations of \textit{T. cruzi}, we found no evidence that the extensive variations in benznidazole-sensitivity were linked to mutations in TcNTR. This, together with evidence that resistance to benznidazole and nifurtimox is not always linked, indicates that there may be other mechanisms independent of TcNTR that can operate. New advances in technology provide opportunities to explore this further.

Keywords: \textit{Trypanosoma cruzi}. Resistance. Antiparasitic drugs.

RESUMEN

Correlación entre genotipo y resistencia a los antiparasitarios en la Enfermedad de Chagas

El benznidazol y el nifurtimox, compuestos nitroheterocíclicos, son los únicos medicamentos aprobados para el tratamiento de las infecciones por \textit{Trypanosoma cruzi}. Ambos son profármacos y no tienen importantes propiedades tripanocidas hasta su activación intraparasitaria. La enzima responsable es una nitro-reductasa (TcNTR), que inicia una cascada reductora que conduce a la generación de los metabolitos tóxicos que matan al parásito. Los procesos que actúan para regular esta enzima conducen a la resistencia cruzada contra ambos fármacos. Estos incluyen la pérdida de uno de los cromosomas que contienen el gen TcNTR o mutaciones puntuales que inactivan la enzima. Los parásitos TcNTR heterocigotos son infectiosos, no muestran un fenotipo nocivo obvio y son hasta 5 veces más resistente a benznidazol y el nifurtimox. Sin embargo, la pérdida completa de la actividad TcNTR hace que T. cruzi no sea infectioso, lo que sugiere que puede haber un límite para el nivel de resistencia por este mecanismo. En las poblaciones naturales de T. cruzi no se encontraron pruebas de que las amplias variaciones en la sensibilidad al benznidazol estén vinculadas a las mutaciones en TcNTR, lo que, junto con la evidencia de que la resistencia a benznidazol y nifurtimox no siempre es conjunta, indica que existen otros mecanismos independientes de TcNTR. Los nuevos avances en tecnología ofrecen la oportunidad de explorar más a fondo esta cuestión.

Palabras clave: \textit{Trypanosoma cruzi}. Resistencia. Fármacos antiparasitarios.
INTRODUCTION

Chagas disease is one of the world’s major “Neglected Diseases”. It is caused by infection with the protozoan parasite *T. cruzi*, which is spread primarily by blood-sucking triatomine bugs. Other means of transmission include the congenital route, contaminated food and drink, organ transplantation and blood transfusion. Over 10 million people in Latin America are infected, with 10-20,000 deaths annually. As a result of migration, the disease is also undergoing globalisation, with for example, more than 300,000 infected individuals in the USA. In 2010, the WHO announced that Chagas disease had become a serious public health challenge in Europe, where 4,000 cases have been confirmed in the last 10 years and the estimated number of infections exceeds 80,000. This represents a major hidden disease burden. The WHO report recommended that “the capacity of national health systems to correctly diagnose, manage and treat the disease should be ensured”.

Chagas disease has three phases; acute, indeterminate and chronic. The ‘acute’ stage is typically asymptomatic or relatively mild, and normally undiagnosed. In children the infection can be more severe, presenting as a generalised febrile illness, with death from myocarditis or meningoencephalitis in up to 5% of diagnosed cases. With the development of a cellular immune response parasitemia is suppressed, although sterile immunity is not achieved. This ‘indeterminate’ stage is asymptomatic and can last throughout life. However, these individuals remain a source of infection. About 30% of those infected with *T. cruzi* proceed to the ‘chronic’ stage, sometimes decades later. This symptomatic phase is characterised by clinical manifestations which include cardiomyopathy, damage to the digestive tract (mainly megacolon and megaoesophagus) and/or lesions in the peripheral nervous system. Corrective surgery costs $750 million/annum in Brazil alone. Chagas disease is a leading cause of premature heart disease in many parts of Latin America, often resulting in sudden cardiac failure. Reactivation of latent Chagas disease in HIV/AIDS patients is also observed, often with unusual clinical manifestations, including CNS involvement.

THE MECHANISMS OF ACTION OF DRUGS USED TO TREAT *T. CRUZI* INFECTIONS

There is unlikely to be a vaccine against Chagas disease in the foreseeable future. The main prevention strategy has been to break the transmission cycle by focusing on the insect vectors. These approaches have had significant success, although problems have been encountered with sustainability and re-infestation. For treatment of infected individuals, only two drugs are available, nifurtimox and benznidazole, nitroheterocyclic compounds which contain a nitro-group linked, respectively, to a furan and an imidazole (figure 1). Both have been in use for more than 40 years. Although effective against the acute stage, their efficacy against chronic disease is still under investigation. Treatment courses are long, often stretching over several months, with frequent side effects, which can result in failure to complete the therapeutic schedule. *T. cruzi* strains refractory to treatment are encountered throughout South America and laboratory-generated resistance is readily achievable. Given the large cohort of infected individuals, new drugs remain a major research goal. Two parallel research strategies are being prioritised. First, the development of new drugs to cure or alleviate chronic stage symptoms is being widely pursued, with increasing input from public-private partnerships. Second, there have been concerted attempts to fully catalogue and
**Figure 1**
**Structures of the nitroheterocyclic drugs used to treat *T. cruzi* infections**

The highlighted regions in nifurtimox and benznidazole correspond to the 5-nitrofuran and the 2-nitroimidazole groups respectively.

Nifurtimox and benznidazole are pro-drugs that require to be activated within the parasite to have trypanocidal effects, a process that is mediated by nitroreductases (NTRs). For many years, the specific enzyme(s) involved were unknown, and there was no information on the nature of the toxic metabolites that were involved in parasite killing. In the case of nifurtimox, initial experiments had hinted that there could be a role for reactive oxygen species, which can be produced in parasite lysates following one-electron reduction of nifurtimox by type II NTR activity. Under aerobic conditions, this leads to the production of superoxide radicals and the regeneration of nifurtimox, a process that has been termed “futile cycling”. Although several parasite flavin-dependent reductases have been linked with this mechanism, there has been no direct evidence for a functionally significant role in drug activity. In addition, parasites that have been genetically manipulated to enhance their oxidative defence capacity do not display increased resistance to nifurtimox.

Other nitrofuran pro-drugs have antimicrobial activity, for example nitrofurantoin, which can be used to treat urinary tract infections. Drug-resistance in bacteria is conferred by mutations to flavin-dependent oxidoreductases belonging to the type I NTR family. These enzymes catalyse the O₂-insensitive NAD(P)H-dependent two-electron reduction of the drug nitro group. This results in the generation of a hydroxylamine product, which can react further to produce nitrenium ions, leading to DNA breakage and damage to other macromolecules. In *T. cruzi*, two enzymes have been identified that display type I NTR-like activity. However, evidence suggests that one of these, prostaglandin F2α synthase, does not have a significant role in drug activation, as it is only capable of promoting nifurtimox reduction under anaerobic conditions. Recently, it has been demonstrated that the second, which has been designated *TcNTR*, is primarily responsible for the activation of nifurtimox, benznidazole and other nitroheterocyclic drugs in *T. cruzi*. The reduction of nifurtimox by this enzyme generates an unsaturated open-chain nitrile, which is responsible for the trypanocidal effect. In the case of benznidazole, drug metabolism leads to the formation of glyoxal, a metabolite with diverse cytotoxic properties.
THE T. CRUZI NITROREDUCTASE (TcNTR)

TcNTR is a NADH-dependent type-I nitroreductase which utilises FMN as a co-factor\(^9,10,30\). Although this class of enzyme is typically bacterial, paralogues are found in *Trypanosoma brucei*, *Leishmania* species and some other protozoan parasites. TcNTR can metabolise a range of nitroheterocycle drugs including nitrofurans (such as nifurtimox) and nitroimidazoles (such as benznidazole)\(^9,30\). Deletion of one copy of the gene confers resistance to these drugs. Similar findings have been made in the African trypanosome *T. brucei*\(^9\), where a genome-wide RNAi screen for genes associated with nifurtimox and benznidazole resistance by loss-of-function mechanisms identified TbNTR as the major candidate\(^31\).

Cross-resistance to nitroheterocyclic drugs is easily achievable in the laboratory, using either nifurtimox or benznidazole as the selective agent. When we examined the genetic profile of cross-resistant parasites we found that loss of one copy of the chromosome containing the *TcNTR* gene was a common feature\(^9,10\). Chromosome plasticity is widely observed in trypanosomatids\(^33\) and in vitro, resistance against heterocyclic drugs by this mechanism appears to occur without any obvious phenotypic consequences. Interestingly, sequence analysis of the remaining *TcNTR* allele in one group of benznidazole-resistant parasites, revealed the presence of three distinct mutant genes in different resistant clones (figure 2)\(^10\). These mutations were restricted to a region of the enzyme associated with flavin-binding\(^24\) (figure 2) and had arisen independently within a single population. When each of the mutant proteins were expressed as a recombinant protein, they were unable to activate either benznidazole or nifurtimox, a defect that correlated with loss of FMN-binding capacity. The drug-resistant phenotype could be reversed by transfection with wild-type *TcNTR*.

The biological role of *TcNTR*s has yet to be unequivocally defined. However, it has been inferred from a functional screen in *T. brucei* that the corresponding enzyme may be involved in ubiquinone biosynthesis and that it mediates the transfer of electrons from NADH to ubiquinone (UQ\(_9\)) to generate ubiquinol\(^31\). Consistent with this, the trypanosome enzyme preferentially uses NADH as an electron donor and quinones as an electron acceptor, suggesting that in vivo, it functions as a NADH:ubiquinone oxidoreductase\(^30\). When one copy of the gene encoding the *T. cruzi* enzyme was disrupted by targeted gene deletion, the virulence phenotype of the drug-resistant parasites in vitro was found to be the same as *TcNTR* homozygotes\(^9,10\). This potential for nifurtimox/benznidazole cross-resistance by loss of one copy of *TcNTR*, coupled with the absence of haploid insufficiency, may be an explanation for some of the treatment failures observed with these drugs. Complete loss of *TcNTR* activity does however have a significant detrimental effect\(^9,10\). Null mutants display a considerably impaired ability to infect mammalian cells. Even when they do, there is a major reduction in the number of amastigotes produced. This implies that in vivo, there could be a limit to the extent of resistance to nitroheterocyclic drugs achievable by mechanisms involving *TcNTR* (approximately 5-fold), since parasites are essentially non-infectious in the absence of a residual level of enzyme activity.

THE COMPLEX NATURE OF DRUG-RESISTANCE IN T. CRUZI

Mechanisms can act in concert to promote drug-resistance in *T. cruzi*. As described above, in a single drug-selection experiment with benznidazole, we were able to identify two different mechanisms that led to reduced intracellular *TcNTR* activity and concurrent drug-resistance; chromosome loss and point mutation\(^10\). Three distinct mutations were detected which had arisen inde-
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Figure 2

Mutations identified in *TcNTR* following selection of benznidazole-resistant *T. cruzi*

![Diagram showing mutations in TcNTR](image)

Clones were isolated from a population of benznidazole-resistant parasites and the *TcNTR* genes sequenced. Differences in amino acid sequence compared to the parental sensitive strain were restricted to a single region of the protein (highlighted in turquoise). Mutations in the corresponding region of *E. coli* nfS associated with nitrofurantoin-resistance are indicated with asterisks. The cartoon model of *TcNTR* identifies the FMN-binding regions by analogy with *E. coli* nfS. A box identifies the relevant residues in the *TcNTR* sequence.

Although, resistance mediated by *TcNTR* is a readily acquired trait, other experimental evidence also indicates that additional mechanisms can impinge on drug efficacy. This was apparent from a study that we undertook to investigate possible associations between the sequence of *TcNTR* and susceptibility to benznidazole. We analyzed the genes from 28 *T. cruzi* Colombian strains derived from a variety of biological and geographical backgrounds. 17 synonymous polymorphisms were detected in this sample, although these were restricted to just 7 of the strains. None of the polymorphisms were located in regions of *TcNTR* implicated in enzyme activity. The major haplotype grouping, which encompassed the other 21 strains, displayed a wide range of benznidazole-sensitivities (IC$_{50}$ 4-35µM). This naturally occurring variation in sensitivity was therefore independent of *TcNTR* sequence. Other studies have also shown that resistance to nifurtimox can occur independently of resistance to nitroheterocyclic drugs must exist. Identifying these, using the full complement of post-genome technologies, must be regarded as a priority for Chagas disease researchers.

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