Genotoxicity profile of fexinidazole—a drug candidate in clinical development for human African trypanomiasis (sleeping sickness)

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Received on August 31, 2011; revised on February 22, 2012; accepted on March 2, 2012

The parasitic disease human African trypanomiasis (HAT), also known as sleeping sickness, is a highly neglected fatal condition endemic in sub-Saharan Africa, which is poorly treated with medicines that are toxic, no longer effective or very difficult to administer. New, safe, effective and easy-to-use treatments are urgently needed. Many nitroimidazoles possess antibacterial and antiprotozoal activity and examples such as tinidazole are used to treat trichomoniasis and guardiasis, but concerns about toxicity including genotoxicity limit their usefulness. Fexinidazole, a 2-substituted 5-nitroimidazole rediscovered by the Drugs for Neglected Diseases initiative (DNDi) after extensive compound mining of public and pharmaceutical company databases, has the potential to become a short-course, safe and effective oral treatment, curing both acute and chronic HAT. This paper describes the genotoxicity profile of fexinidazole and its two active metabolites, the sulfoxide and sulfone derivatives. All the three compounds are mutagenic in the *Salmonella*/Ames test; however, mutagenicity is either attenuated or lost in Ames *Salmonella* strains that lack one or more nitroreductase(s). It is known that these enzymes can nitroreduce compounds with low redox potentials, whereas their mammalian cell counterparts cannot, under normal conditions. Fexinidazole and its metabolites have low redox potentials and all mammalian cell assays to detect genetic toxicity, conducted for this study either *in vitro* (micronucleus test in human lymphocytes) or *in vivo* (*ex vivo* unscheduled DNA synthesis in rats; bone marrow micronucleus test in mice), were negative. Thus, fexinidazole does not pose a genotoxic hazard to patients and represents a promising drug candidate for HAT. Fexinidazole is expected to enter Phase II clinical trials in 2012.

Introduction

An estimated 60 million people in sub-Saharan African countries are at risk for human African trypanomiasis (HAT), especially poor and neglected populations living in remote areas (1,2). In west and central Africa where it is endemic, *Trypanosoma brucei gambiense* causes a chronic form of sleeping sickness, whereas in eastern and southern Africa, *T. b. rhodesiense* causes an acute form of the disease. HAT occurs in two stages: Stage 1 (early haemolymphatic) is characterised by non-specific clinical symptoms, such as malaise, headache, fever and peripheral oedema, whereas Stage 2 (late meningoencephalic) is characterised by neurological symptoms, including behavioural changes, severe sleeping disturbances and convulsions, which, if left untreated, lead to coma and death in 100% of cases. There are few current treatments for HAT and these are old, limited due to toxicity, diminishing in efficacy and can be complex to use. Treatment is stage-specific, and the only therapies available for Stage 2 are the more difficult to administer or more toxic drugs (3,4). There is a pressing need for new, safe and effective treatments for HAT, preferably oral drugs that can treat both stages.

There are many nitroheterocyclic compounds, including nitroimidazoles, which possess good oral therapeutic activity against protozoal parasites; however, concerns over toxicity and in particular genotoxicity has made drug development problematical. A typical anti-trypanosomal nitroimidazole is megazol (5) but its development was abandoned because of genotoxicity in bacteria and in mammalian systems with the resulting concerns about carcinogenicity (6). Another example is metronidazole (7), which is widely used as an antibiotic and antiprotozoal treatment. While the potential risks posed by the genotoxicity and carcinogenicity of metronidazole are still areas of active controversy (8), the therapeutic usefulness of this compound, including the lack of evidence of clinical toxicity, despite widespread use, has kept the drug in the arsenal of key antimcicrobial drugs, included on the World Health Organisation (WHO) list of Essential Medicines (9). Tinidazole, which is closely related to metronidazole, is used for the treatment of a variety of parasitic and bacterial infections (10). Nifurtimox and benznidazole used for the treatment of Chagas disease, also caused by a trypanosome (11), are further members of the class. These compounds possess both bacterial and mammalian cell genotoxicity (12,13) and thus generate concern in terms of adverse long-term health effects (14). However, when no safer alternative therapeutic options are available, the medical benefit of treating patients with these drugs is considered to outweigh their risks.

Compounds containing nitro groups are known to be activated by various enzymes, including nitroreductases, to form reactive radicals. This bioreduction requires low redox electron transfer systems. In general, the single-electron reduction potential of nitroimidazoles lies outside the normal range of mammalian redox systems (15). However, if a one-electron reduction of these compounds does occur, this can lead to the formation of a hydroxylamine intermediate, an unstable nitro radical anion that can go on to form a nitrite anion and an imidazole radical or be further reduced by accepting a second electron (16). In the presence of oxygen, this can lead to the generation of a superoxide radical and then

on to the formation of highly genotoxic nitroso derivatives (17). Most of these reactive chemical species have the potential to interact with DNA.

The bacterial mutagenicity of many members of this group of compounds (often in both base-substitution strains such as TA100 and in frameshift-detecting strains such as TA98) has been known for many years (18). Nitroreduction is essential for the expression of this mutagenicity, as shown for example in tests of the anti-schistosomal drug niridazole, such that activity is reduced 10-fold in nitroreductase-deficient TA100 and TA98 *Salmonella* strains compared to their nitroreductase-proficient counterparts. In addition, the desnitro analogue is not mutagenic (19). These results were confirmed by Speck *et al.* (20) who extended the findings to show niridazole is also mutagenic for TA102, which has an A–T mutational site in the target of his G gene compared to the G–T sites in the target genes for mutation in TA100 and TA98. Again, these authors showed that mutagenicity is attenuated in a TA102 strain with a nitroreductase deficiency.

In view of the notable oral anti-infective or anti-parasitic activities observed in this class of compounds, there have been many attempts to separate the anti-parasitic activity, of relevant nitroheterocyclic compounds, from genotoxicity, with varying degrees of success (21–24). The difficulty remains that the anti-parasitic mode of action of these compounds is to produce genotoxic compounds as a result of nitroreduction within the parasite, with subsequent lethal effects on the parasite. In a study on the resistance of trypanosomes to nifurtimox and benznidazole, it was shown that both drugs are activated by an nicotinamide adenine dinucleotide reduced-dependent, mitochondrially located, bacterial-like type 1 nitroreductase. Resistance appears to be due to downregulation of this enzyme. Loss of a single copy of this gene is sufficient to cause cross-resistance to a wide variety of nitroheterocyclic compounds (25,26).

Thus, bacteria and parasites including trypanosomes possess low redox electron transfer systems capable of carrying out the nitroreduction and generating toxic compounds. The promise and the challenge were to find a compound that can be efficiently nitroreduced by the target parasites but not by equivalent mammalian enzymes.

Several new nitroimidazole drug candidates have emerged recently, of which PA-824 (27) and OPC-67683 (28) are currently in clinical development for the treatment of tuberculosis. Published data have indicated that neither of these compounds appears to possess genotoxicity, including in the Ames test, showing that it is possible and feasible to synthesise nitroimidazoles devoid of mutagenicity.

This paper describes a genotoxicity evaluation of fexinidazole (1H-imidazole,1-methyl-2-[[4-methylthio) phenoxy] methyl] 5-nitroimidazole), a 2-substituted 5-nitroimidazole, rediscovered by the Drugs for Neglected Diseases initiative (DNDi) after extensive compound mining of drug companies and published databases. It was first synthesised by Hoechst and described in 1978 (29). It was shown to possess potent *in vitro* and *in vivo* activity against trichomonads, *Entamoeba histolytica*, *Trypanosoma cruzi* and *Trypanosoma brucei* infections in mice (30). The principal metabolites, sulfoxide and sulfone derivatives, were shown to have equivalent anti-parasitic activity to the parent molecule (31). However, the development of this drug candidate was not pursued by Hoechst beyond preclinical studies. A detailed reassessment of the pharmacological and toxicological profile of fexinidazole, according to the current state of the art, has validated the compound as a promising drug candidate for HAT (32), and Phase I clinical studies are currently ongoing.

Materials and methods

All studies described in this paper were carried under good laboratory practice (GLP) regulations, except where indicated.

Test compounds

Fexinidazole, 1H-imidazole,1-methyl-2-[[4-methylthio)phenoxy] methyl] 5-nitroimidazole, was manufactured for DNDi by Centipharm Ltd, Grasse, France, under current good manufacturing practice (cGMP) conditions and had a purity of >99.0%. Fexinidazole sulfone, 1-methyl-2-(4-methylsulfonyl phenoxymethyl)-5-nitroimidazole was manufactured by Centipharm Ltd and had a nominal purity of 99.9%. The structures of these compounds are shown in Figure 1.

Positive control compounds used were as follows:

Ames tests: 2-nitrofluorene (2NF), sodium azide, 4-nitroquinoline 1-oxide (4NQO), metronidazole (MTZ), 9-aminoacridine (9-AC), mitomycin C (MMC), benzo(a)pyrene (B(a)P), 2-aminoanthracene(2-AA) and nitrofurantoin (NFu).

In vitro micronucleus test: 4NQO, cyclophosphamide and vinblastine.

In vivo bone marrow micronucleus test in the mouse: cyclophosphamide.

Ex vivo unscheduled DNA synthesis (UDS) study in the rat: 2-acetamidofluorene and dimethylnitrosamine.

All positive control compounds were of high grade and obtained from Sigma–Aldrich Chemical Company, Poole, Dorset, UK.

Drug preparation

Fexinidazole and the sulfone metabolite were prepared for *in vitro* studies as stock solutions in dimethyl sulphoxide (DMSO), further diluted with water or 0.5% methylcellulose in water to appropriate concentration required for the assays. For *in vivo* studies, fexinidazole was prepared as an optimised suspension comprising 5% w/v Tween 80/0.5% w/v Methocel in water.

Test systems

Salmonella typhimurium strains TA98, TA100, TA1535 and TA1537 were originally obtained from the UK NCTC. Strain TA102 was derived from a culture obtained from GlaxoSmithKline Limited. Strain TA98NR was derived from a culture obtained from the University of York Cancer Research Unit and TA100NR was derived from a culture obtained from Novartis Pharma AG, Switzerland. Strains TA1535NR, YG7167 YG7168 and YG7127 were obtained from the National Institute of Health Sciences (NIHS), Japan. Strains

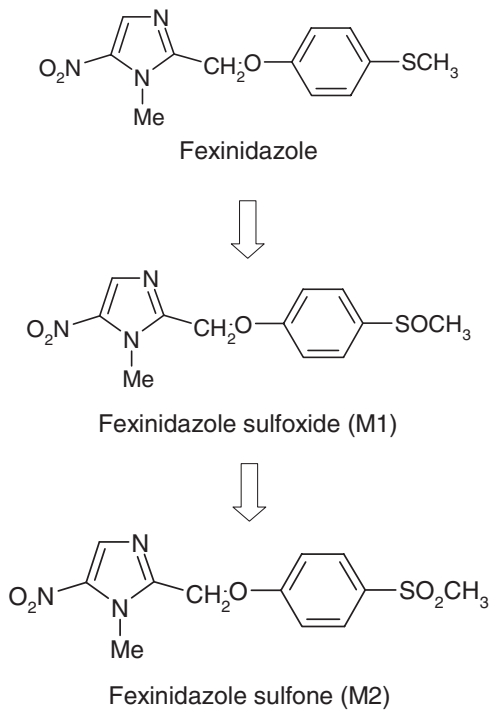

Fig. 1. Fexinidazole and its primary metabolites.

TA98NR, TA100NR and TA1535NR are variants of strain TA98, TA100 and TA1535, respectively, that are deficient in 'classical' nitroreductase activity. Strain YG7127 is a further variant of strain TA1535 that has the classical nitroreductase gene deleted. Similarly, strainsYG7167 and YG7168 are variants of the strains TA1537 and TA102, respectively, with the classical nitroreductase gene deleted. These latter strains were specifically constructed for this study by targeted disruption of the *cnr* gene (classical nitroreductase) using techniques published previously (33).

Young adult male Crl:CD-1 (ICR) mice were used for the bone marrow micronucleus test and young male Sprague–Dawley rats for the *ex vivo* liver UDS test.

Bacterial mutation assays (Ames test)

When the *Salmonella typhimurium* TA strains were first constructed by Bruce Ames and colleagues, *uvrB* mutants were selected, removing the main DNA excision repair pathway, by scoring cultures for chlorate resistance. The gene conferring chlorate resistance is in close proximity to the *uvrB* gene; thus, those cells acquiring chlorate resistance as a result of a deletion were likely to also have lost the *uvrB* gene as well. As each strain was selected independently, the commonly used *Salmonella* strains used in the Ames test have differently sized deletions. Thus, 47 genes are missing in TA100, 87 in TA1537 and 119 in TA98. Importantly, for this work, a major supplementary nitroreductase is absent in TA98 (and also in TA1535) as a result of these deletions (34). Thus, for TA98NR and TA1535NR, these strains lack the two important nitroreductases for *Salmonella*, while the other NR strains used only lack the classical nitroreductase.

To evaluate bacterial mutagenicity, full Ames tests were carried out on fexinidazole and fexinidazole sulfone using the five strains of *Salmonella typhimurium* recommended by the relevant International Conference for Harmonisation (ICH) regulatory guideline, as well as the corresponding nitroreductase-deficient strains, as above. Standard bacterial plate incorporation assays were carried out, essentially as described by Maron and Ames (35).

Tests on fexinidazole were carried out with and without rat liver post-mitochondrial fraction plus co-factors (S9 mix) to provide a mammalian metabolic activation system. The S9 fraction was prepared from Sprague–Dawley rats pretreated with the mixed cytochrome P 450 enzyme inducer Aroclor 1254. A total of 0.5 ml of S9 mix containing 10% S9 fraction was added to 2.5 ml agar overlays.

Tests on fexinidazole sulfone were carried out only in the absence of rat liver S9 (as this is a metabolite). After incubation at 37°C for 3 days, plates were scored for mutant colonies using a Seescan Colony Counter (Seescan plc).

In vitro micronucleus tests using human lymphocytes

Duplicate human lymphocyte cultures were prepared from the pooled blood of two donors in two independent experiments as defined in the OECD Guideline 487 (36). For fexinidazole, treatments covering a broad range of concentrations, separated by narrow intervals, were carried both in the absence and in the presence of metabolic activation rat liver (S9). The final concentration of S9 fraction in the media used for this test was 2%.

The highest concentration of fexinidazole tested was 220 μg/ml. Cells were treated with the drug 24 and 48 h following stimulation by the mitogen phytohaemagglutinin (PHA). In the absence of S9, the exposure period was 20 h plus a 28-h expression period (−S9) or for 3 h with a 45-h expression period (+S9). The test concentrations for analyses of micronuclei were selected by evaluating the effect of fexinidazole on the replication index. In each experiment, micronuclei were analysed at three concentrations. The highest concentrations tested were limited by precipitation observed at the end of the treatment period.

Similar tests were carried out on fexinidazole sulfone. As per the Ames test, this was carried out in the absence of rat liver S9 only. In this case, the cells were treated 48 h after mitogen stimulation with PHA and as there were no tests in the presence of S9, an extended exposure period was included (24 h, no expression time).

Mouse bone marrow micronucleus test

Groups of six Crl:CD-1 (ICR) male mice were treated with fexinidazole using two oral doses of 0 (vehicle control), 500, 1000 or 2000 mg/kg given 24 h apart, and bone marrows were harvested 24 h after the second dose (37). Slides of bone marrow cells were prepared, Giemsa stained and 2000 polychromatic erythrocytes per animal were scored for micronuclei. The percentage of polychromatic erythrocytes of the total of erythrocytes in each bone marrow sample was used to estimate toxicity.

Ex vivo UDS test in rat liver

The standard autoradiographic method was used (38). Groups four male Sprague–Dawley Crl:CD∃ (SD) rats received fexinidazole doses of 500, 1000 or 2000 mg/kg orally (two groups per dose), and the livers were sampled either 2–4 h or 12–14 h after test compound administration. Hepatocyte suspensions were prepared and incubated in the presence of tritiated thymidine. Slides of fixed hepatocytes were coated in photographic emulsion and stored for 14 days at 4°C in the dark. The silver grains that accumulated in the emulsion due to radioactive decay of the tritium were counted above both the nuclei and equivalent areas of cytoplasm. Grain counting was performed using a microscope with a video camera connected to an image analysis system (Perceptive Instruments) and a computer programmed for automatic data capture. A total of 100 cells were analysed per animal (50 from each of two slides). The extent of DNA repair was estimated by calculating the mean of the difference between the nuclear and cytoplasmic counts (nuclear net grain count) per sample.

All genotoxicology studies in animals were carried out by Covance Laboratories Ltd, Harrogate, England, according to their internal Standard Operating Procedures, the applicable ICH guidelines (ICH S2A,B) and in compliance with the UK GLP Regulations 1999, Statutory Instrument No. 3106 as amended by the GLP (Codification Amendments Etc.) Regulations 2004 and the OECD Principles on GLP (January 1998) ENV/MC/CHEM (98) 17. Animals were maintained as required by the 'Code of practice for the housing and care of animals used in scientific procedures' (Home Office, London, 1989).

Toxicokinetic measurements

Blood samples were taken from satellite animals from the bone marrow micronucleus study and from the UDS study, 1 h after dosing with 2000 mg/kg in each case. This is close to the Tmax for fexinidazole. Concentrations of fexinidazole and its two major metabolites were determined in plasma from these samples by a liquid chromatography-mass spectroscopy-mass-spectroscopy (LC–MS–MS) method following plasma protein precipitation in a 96-well plate format.

These analyses were carried out by The Bioanalysis and Analytical Control Department, Neviano Medical Sciences, Accelera, Milan Italy. A preliminary study using the same methodology had also been carried out at CIT, Evreux, France, but in this study, samples were taken 4 h after dosing.

Metabolism of fexinidazole using Aroclor 1254-induced rat liver S9 fraction

Fexinidazole was incubated at a single nominal concentration of 200 μg/ml with Aroclor 1254-induced rat liver S9 (1 mg/ml) at 37°C for 0, 15 and 60 min. Additional incubations were conducted with fexinidazole at a nominal concentration of 200 μg/ml for 0, 2, 3 and 6 h. Aroclor 1254-induced rat liver S9 and [^{14}C]fexinidazole in Tris–HCl buffer, pH 7.4, were warmed to ∼37°C for ∼5 min before initiation of the reaction by the addition of nicotinamide adenine dinucleotide phosphate reduced (2 mM). The total incubation volume was 0.2 ml. All reactions were terminated by the addition of 0.1 ml acetonitrile. Incubations were also conducted in the absence of rat liver S9. All test incubations were conducted in triplicate. Following centrifugation (15 300g, 5 min), protein-free supernatants were transferred to clean vials and stored frozen prior to analysis or injected directly onto the high performance liquid chromatography system. Recovery of radioactivity from each incubation mixture was measured by liquid scintillation counting. Chromatographic data were captured online using a validated data acquisition system (Laura 3.4.7; LabLogic Systems Ltd). All other calculations were carried out using Excel 2003.

Nitroreductase measurements of selected Ames strains

Two new nitroreductase-deficient strains, TA1537NR (YG7167) and TA102NR (YG7168), were kindly constructed for this project by Dr Y. Yamada from the National Institute of Health Sciences Tokyo by disrupting the gene for the supplementary nitroreductase, *nsfB*, using a procedure reported previously (33). To confirm that the procedure had been successful, nitroreductase measurements were made of extracts from these strains, their nitroreductase parental strains and also the strain TA98NR. The procedure used to quantify nitroreductase enzymatic activity was carried out at the University of Swansea under contract and were not carried out under GLP conditions. The nitroreductase activities of cell-free extracts from the selected strains were estimated by the decrease in absorbance at 373 nm resulting from the reduction of the nitrofurantoin substrate (39). Each strain was tested in parallel and the whole procedure repeated on different days up to five times.

Redox potential measurement

One-electron reduction potentials were determined by fast kinetic spectrophotometry (pulse radiolysis) in aqueous solutions using an established procedure (non-GLP) (40).

Results

Nitroreductase activities of selected strains

The nitroreductase activities of TA102/TA102NR (YG7168), TA1537/TA1537NR (YG7167) and TA98NR are shown in

Figure 2. The data support the selection of the new NR strains through the disruption of the *nsfB* gene as being truly nitroreductase deficient. The remaining nitroreductase activity seen in TA1537NR and TA102NR is presumably due at least in part to the activity of the 'classical nitroreductase gene (*cnr*)'. It was surprising to see low levels of nitroreductase activity remaining in TA98NR, which has lost the activity of both *nsfB* and *cnr* genes, which suggests that there is a third nitroreductase gene active in these cells. Although TA100 is not included in these assay of nitroreductase activity, it is known that it retains at least one functional nitroreductase (34) and as shown in its sister strains.

In vitro metabolism of fexinidazole

In vivo studies had shown that fexinidazole is rapidly metabolised to form the sulfoxide and the sulfone (see Table VII from the toxicokinetic studies carried out to support the *in vivo* genotoxicity tests); thus, the exposure is mainly to these two metabolites. To ascertain that these two metabolites are formed under the conditions of the *in vitro* genotoxicity studies, *in vitro* metabolism was studied using rat liver S9. The results show that the sulfoxide is formed rapidly and efficiently under these conditions, but only trace amounts of the sulfone are found (Figure 3). Therefore, the sulfone was synthesised and tested separately, in addition to the parent molecule.

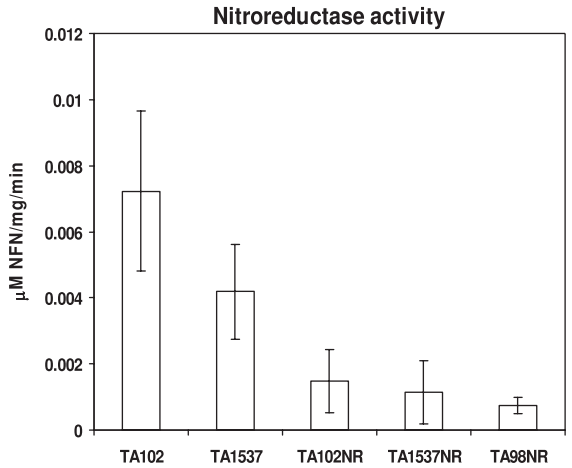

Fig. 2. Nitroreductase activities of various strains used in this study, including TA1537NR (YG7167) and TA102NR (YG7168).

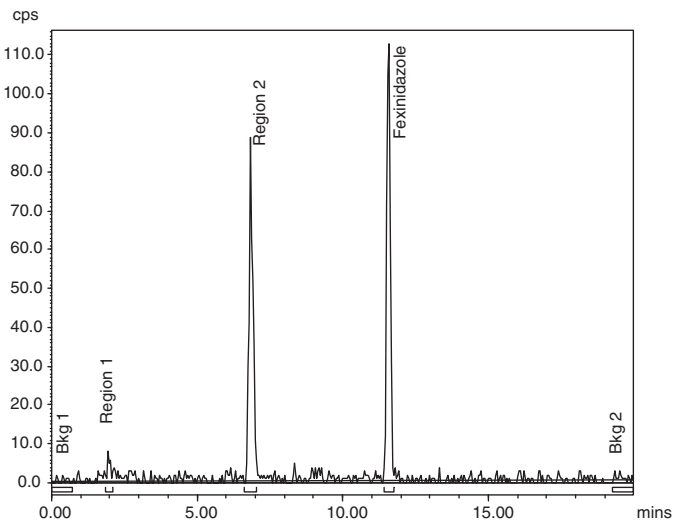

Fig. 3. Metabolism of fexinidazole *in vitro* following incubation with rat liver S9 (HPLC data). Regions 1 and 2 represent the positions of fexinidazole sulfone and fexinidazole sulfoxide, respectively.

Ames tests of fexinidazole

Fexinidazole was tested in the five standard *Salmonella* strains as required by the ICH guideline for genotoxicity testing for pharmaceutical registration. In addition, to determine the contribution of bacterial nitroreductase enzymes to mutagenicity, the compound was also tested in the corresponding strains deficient in one or two nitroreductases. A summary of the results is shown in Table I. Figures 4–7 show the results for TA98 and TA100 plus their nitroreductase-deficient counterparts, with (Figures 4 and 5) and without (Figures 6 and 7–9) metabolic activation (S9). Mutagenicity was observed in all strains to different degrees, except in TA1537 where the original weak positive response was not reproducible. In each case, mutagenicity was either lost (e.g. TA98NR versus TA98)

Table I. Summary of Ames test data on fexinidazole showing the lowest detectable mutagenic response (micrograms per plate)[a]

Ames strain[b]	−S9	+S9
TA1535	20	4
TA1535NR	Negative	350
TA1535 delta NR (YG7127)	350	4–20
TA1537	Negative	100 (negative in a repeat study)
TA1537NR (YG7167)	Negative	Negative
TA98	350	20
TA98NR	Negative	Negative
TA100	20	4
TA100NR	500	100
TA102	Negative	100
TA102NR (YG7168)	Negative	158

[a]Based on a >2-fold increase in concurrent negative control values.
[b]Strains possessing active major nitroreductase (NR) genes—TA100, TA1537 and TA102 ; strains lacking an active classical NR gene—TA100NR; YG7127 (TA1535 derivative), YG7167 (TA1537 derivative) and YG7168 (TA102 derivative); strain lacking an active supplementary NR gene (TA98) and strains lacking both major NR genes—TA98NR and TA1535NR.

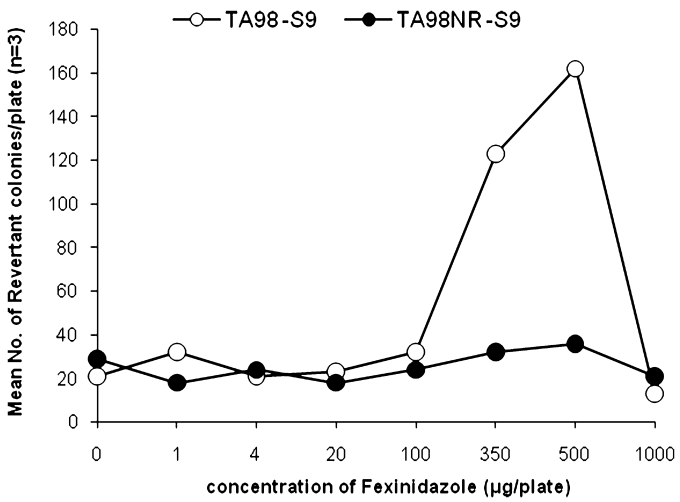

Fig. 4. Ames tests of fexinidazole using strains TA98 and TA98NR (absence of S9).

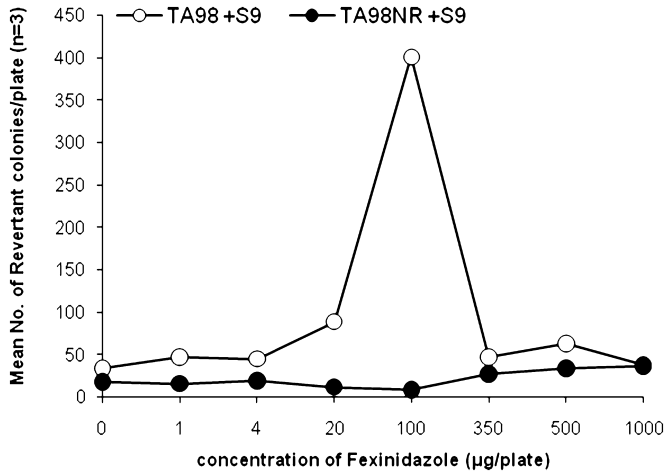

Fig. 5. Ames tests of fexinidazole using strains TA98 and TA98NR (presence of S9).

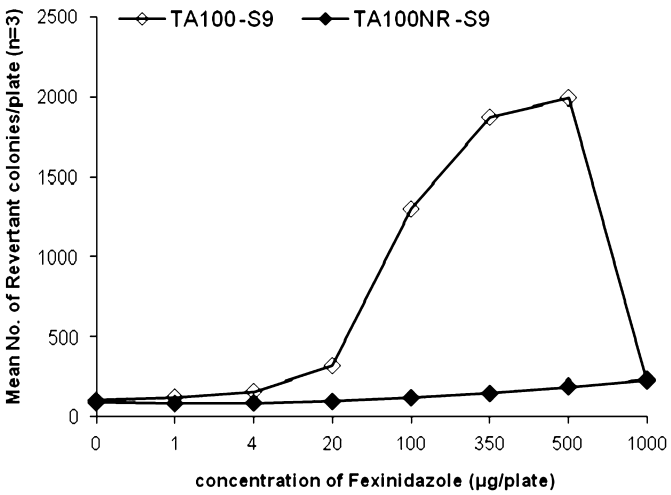

Fig. 6. Ames tests of fexinidazole using strains TA100 and TA100NR (absence of S9).

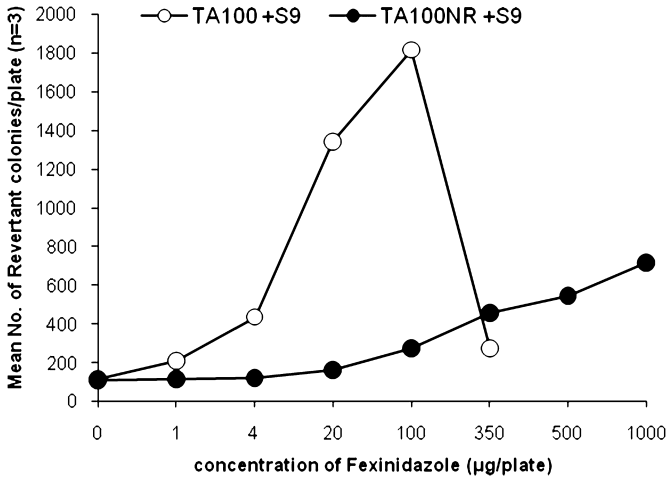

Fig. 7. Ames tests of fexinidazole using strains TA100 and TA100NR (presence of S9).

Table II. Summary of Ames test data on fexinidazole sulfone showing the lowest detectable mutagenic response (micrograms per plate)[a]

Ames strain[b]	−S9
TA1535	8
TA1535NR	200
TA1537	200
TA1537NR (YG7167)	Negative
TA98	40
TA98NR	1000
TA100	8
TA100NR	200
TA102	200
TA102NR (YG7168)	1000

[a]Based on a >2-fold increase in concurrent negative control values.
[b]Strains possessing active major nitroreductase (NR) genes—TA100, TA1537 and TA102; strains lacking an active classical NR gene—TA100NR; YG7167 (TA1537 derivative) and YG7168 (TA102 derivative); strain lacking an active supplementary NR gene (TA98) and strains lacking both major NR genes—TA98NR and TA1535NR.

or significantly attenuated (e.g. TA100NR versus TA100) in the strains deficient in nitroreductase compared to their nitoreductase-proficient counterpart. In the strains lacking both the classical nitroreductase and the supplementary nitroreductase, i.e.TA98NR and TA1535NR, mutagenicity was all but abolished, suggesting that the residual mutagenicity seen in the other strains was due to the activity of the supplementary nitroreductase. These data suggest that the positive mutagenicity signal observed for fexinidazole in the Ames test is due to a reduction of fexinidazole by either or both bacterial nitroreductases.

In most cases where mutagenicity was observed, potency of the signal was increased in the presence of rat liver S9. As fexinidazole is rapidly metabolised to its sulfoxide by rat liver S9 (Figure 2), this suggests that this metabolite is a more active bacterial mutagen than fexinidazole itself. In the absence of rat liver S9, no activity was observed in TA102. However, activity was observed in this strain in the presence of S9. These data suggest that the sulfoxide possesses additional mutagenic activity for this strain. However, again, activity was attenuated in the nitroreductase-deficient derivative showing that nitroreduction plays a part in the mutagenicity observed.

Ames tests of fexinidazole sulfone

As only trace amounts of fexinidazole sulfone are formed by incubation of fexinidazole with rat liver S9, this metabolite was tested in its own right. As this compound is a metabolite, tests were carried out in the absence of rat liver S9 only. The results shown in Table II and Figures 8 and 9 for TA98 and TA100 (plus their nitroreductase-deficient counterparts) demonstrate a broadly similar result to fexinidazole, with mutagenic activity lost or attenuated in the strains deficient in nitroreductase activity.

In vitro micronucleus tests of fexinidazole and fexinidazole sulfone

Both fexinidazole and fexinidazole sulfone were subsequently tested in *in vitro* micronucleus tests using human peripheral lymphocytes. As it was shown that fexinidazole is metabolised to its sulfoxide under the conditions of the assay, the results shown in Table IIIa and b cover fexinidazole and fexinidazole sulfoxide. The results for fexinidazole sulfone are shown in Table IV. Neither compound induces chromosome damage in human lymphocytes under the conditions of these assays. Thus, the mutagenic potential seen in the bacterial Ames test is not realised in these mammalian cells *in vitro*.

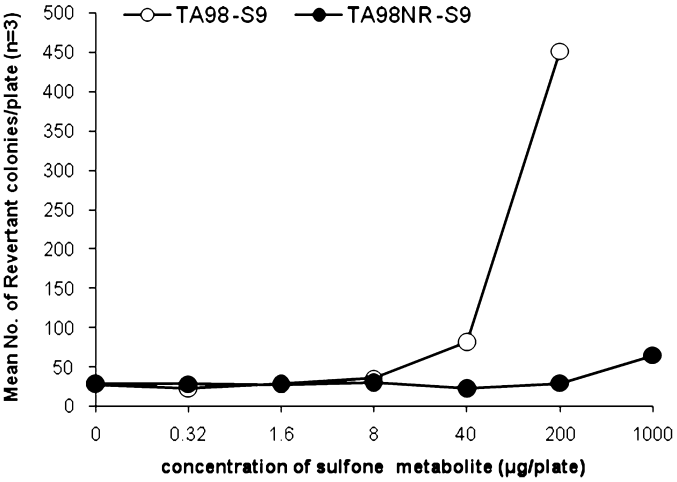

Fig. 8. Ames tests of sulfone metabolite using strains TA98 and TA98NR (absence of S9).

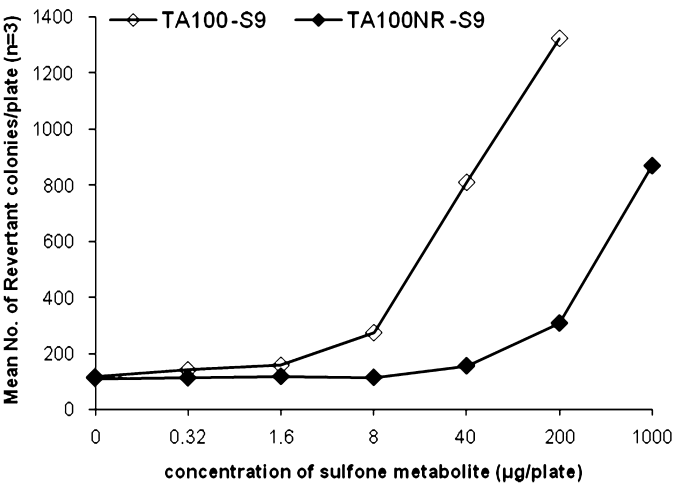

Fig. 9. Ames tests of sulfone metabolite using strains TA100 and TA100NR (absence of S9).

In vivo mouse bone marrow micronucleus test

Fexinidazole was subsequently tested in an *in vivo* bone marrow micronucleus test. As shown in Table V, even when given at doses up to the maximum recognised by international guidelines, fexinidazole does not induce chromosomal damage in this assay, confirming the lack of activity seen in the *in vitro* micronucleus study. A preliminary study had also been carried out at the CIT Laboratories in France with the same outcome (data not shown). The exposure to fexinidazole and the sulfoxide and sulfone metabolites in these assays was confirmed by HPLC analysis (Table VIIa).

Ex vivo UDS test

The rationale for choosing the *ex vivo* UDS test as the second *in vivo* test was as follows:

1. Previous studies carried out by Bayer had shown that UDS was detected at low doses in three of five tests of fexinidazole using A549 cells in vitro. Thus, there was a wish to explore this endpoint in an *in vivo* test.
2. Fexinidazole is relatively non-toxic in rat studies given high doses for 28 days (32). The only target organ in the rat is the liver where increased liver weight and hypertrophy of centrilobular hepatocytes were seen.
3. The liver is the site of primary metabolism of fexinidazole, so the cells in this organ are exposed to the highest concentrations of the major metabolites. It is also likely that the liver is also exposed to high concentrations of the parent fexinidazole as it is rapidly metabolised by the liver, reducing systemic exposure, as per the toxicokinetic profile in this paper and from separate drug metabolism pharmacokinetic studies (32).
4. Some nitro goup containing aromatic compounds have been shown to induce UDS in hepatocytes *in vitro* and *in vivo* (41,42).

As shown in Table VI, there was no indication of any UDS at any dose at either time point. The exposure to fexinidazole and the sulfoxide and sulfone metabolites in this study was confirmed by HPLC analysis (Table VIIb).

Redox potential studies

It is known that the nitroreductase enzymes in bacteria can nitroreduce compounds at low redox potentials, whereas equivalent mammalian enzymes cannot, under normal conditions. Fexinidazole, the sulfoxide and the sulfone were shown to have low single-electron redox potentials being −511, −493 and −488 mV, respectively. The same parameter for metronidazole was −516 mV and megazol was significantly less negative at −422 mV.

Discussion

Substituted nitroimidazoles are widely used as antimicrobials and anti-parasitic compounds, but there are persistent concerns about genetic toxicity and potential carcinogenicity of the drugs in present use. There is a pressing need for an effective, safe and easy to administer therapy for African sleeping sickness (HAT). Fexinidazole, a 5-nitroimidazole dropped from drug development by Hoechst in the 1980's, was rediscovered by DNDi as a potential new treatment for HAT. The potent anti-trypanosomal activity of fexinidazole was confirmed in acute mouse models, including being able to cure a stringent model of second stage HAT and the chronic mouse model involving brain infection (32). As part of the efforts to develop this compound for possible therapeutic use, a full toxicology battery was completed, including a full assessment of genotoxicity.

Because fexinidazole is rapidly metabolised *in vivo* to sulfoxide and sulfone metabolites, the studies were designed to include the assessment of both metabolites. Metabolism studies *in vitro* showed that the sulfoxide metabolite is formed efficiently by rat liver S9 in the conditions of the *in vitro* genotoxicity assays, but only traces of the sulfone metabolite were detected. Therefore, the *in vitro* genotoxicity studies (Ames and *in vitro* micronucleus test) were repeated with this compound. For the *in vivo* studies, the adequate exposure to all three compounds was confirmed by measuring the plasma levels in satellite animals.

As expected, mutagenic activity was observed for fexinidazole and its metabolites in the Ames test. However, this activity was attenuated or lost in Ames strains lacking one or more of the nitroreductases normally present in the *Salmonella* cells. It is known that bacterial nitroreductases can reduce nitro-containing compounds of low redox potentials, whereas the mammalian counterparts cannot. This nitroreduction results in the formation of reactive chemical species with potential to

Table III. *In vitro* micronucleus tests of fexinidazole using human peripheral lymphocytes

Treatment	Compound	Concentration (μg/ml)	Cytotoxicity (%)	Mean MNBN cell frequency	Statistical significance
24-h PHA					
20 + 28 h (–S9)	Vehicle[a]	—	—	0.75	—
	Fexinidazole	20.0	19	0.50	NS
	Fexinidazole	40.0	0	0.40	NS
	Fexinidazole	80.0[b]	32	0.70	NS
	5NQO[c]	5.0	ND	10.80	$P < 0.001$
3 + 45 h (+S9)	Vehicle[a]	—	—	0.40	—
	Fexinidazole	80.0	27	0.50	NS
	Fexinidazole	140.0	43	0.55	NS
	Fexinidazole	180.0[b]	47	0.75	NS
	CPA[c]	6.25	ND	3.60	$P < 0.001$
48-h PHA					
20 + 28h (−S9)	Vehicle[a]	—	—	1.05	—
	Fexinidazole	20.0	4	1.10	NS
	Fexinidazole	40.0	23	0.60	NS
	Fexinidazole	80.0[b]	29	0.85	NS
	5NQO[c]	5.0	ND	8.95	$P < 0.001$
3 + 45 h (+S9)	Vehicle[a]	—	—	0.90	—
	Fexinidazole	80.0	2	0.60	NS
	Fexinidazole	140.0	13	1.20	NS
	Fexinidazole	180.0[b]	8	0.35	NS
	CPA[c]	6.25	ND	12.85	$P < 0.001$

NS, not significant; ND, not determined.
[a]The vehicle was DMSO.
[b]The highest concentrations selected were the maximum practicable concentrations limited by precipitation observed at the end of the treatment period.
[c]The positive controls were 5-nitroquinoline-*N*-oxide (5NQO) –S9 and cyclophosphamide (CPA) +S9.

Table IV. *In vitro* micronucleus tests of fexinidazole sulfone using human peripheral lymphocytes (treatment was –S9 only and with cultures treated 48 h after PHA treatment)

Exposure period + expression time	Compound	Concentration (μg/ml)	Cytotoxicity (%)	Mean MNBN cell frequency	Statistical significance
3 + 24 h	Vehicle[a]	—	—	0.70	—
	Sulfone	300	0	0.45	NS
	Sulfone	400	7	0.40	NS
	Sulfone	500[b]	14	0.35	NS
	MMC[c]	0.8	ND	11.90	$P < 0.001$
24 h + 0	Vehicle[a]	—	—	0.15	NS
	Sulfone	50	21	0.25	NS
	Sulfone	100	25	0.35	NS
	Sulfone	200[b]	59	0.15	NS
	VIN[c]	0.02	ND	17.01	$P < 0.001$

NS, not significant; ND, not determined.
[a]The vehicle was DMSO.
[b]The highest concentrations selected were selected on toxicity, precipitation or a maximum of 500 μg/ml.
[c]The positive controls were mitomycin C (MMC) and vinblastine (VIN).

react with DNA and thus cause genetic damage. The observation that the mutagenic activity is attenuated or lost in *Salmonella* strains deficient in nitroreductases suggests that mutagenicity is not an intrinsic property of these compounds but the result of activation by bacteria-specific nitroreductases.

When tested in the *in vitro* micronucleus test on human lymphocytes, the *in vivo* bone marrow micronucleus test and the *ex vivo* UDS test rat liver cells, no mutagenic activity was seen for fexinidazole and its sulfoxide and sulfone metabolites. Toxicokinetic studies demonstrated significant exposure to all the three compounds in the plasma of the treated animals and thus, the bone marrow and the liver were used as targets for genotoxicity evaluations. This combination of tests meets the ICH (S2B) genotoxicity testing requirements for registration of new pharmaceuticals.

Redox measurements showed that fexinidazole and its sulfoxide and sulfone metabolites possess highly negative single-electron redox potentials of −511, −493 and −488 mV, respectively. This is consistent with the absence of mutagenic activity in mammalian cells as mammalian nitroreductases are expected to be incapable of nitro-reducing compounds with such negative single-electron redox potentials.

The potential of nitrogroup containing compounds to cause mutagenic damage to intestinal cells following nitroreduction by bacteria in the intestinal flora, with possible diffusion of reduced metabolites has been contested in the literature for more than 30 years (review 43). However, this is deemed unlikely to be significant due to the short half-life of reduced intermediates of 5-nitroimidazoles and the failure to detect nitroreduced metabolites of compounds like metronidazole in the intestine of human patients (44). This issue was considered by the Scientific Committee for Animal Nutrition (SCAN) of the European Commission for the 5-nitroimidazole dimetrinidazole (metronidazole was also discussed). Dimetrinidazole

has a similar genotoxicity profile to fexinidazole. The SCAN committee noted that 'when the production within intestinal flora of DMZ reactive metabolites is concerned, it must be noted that the lifetime of these metabolites depends on the reduction potential of the parent molecule the lower the potential the longer is the half-life (~12 sec for 5-nitroimidazoles) and the more limited is the damage (45). The absence in long-term studies with 5-nitroimidazole compounds of intestinal tumours is consistent with these observations (46). In support of this, latter statement for the related nitro-containing compounds (all of whom induce mutations in the Ames test due to bacterial nitroreduction), metronidazole (47,48), benznidazole (49) and nifurtimox (50) have all been tested for carcinogenicity in rodents and none have produced intestinal tumours. In further support of the lack of proven hazard to intestinal cells for nitro-containing compounds, a study of AMP397, a compound with this chemical substructure (see Figure 10) proposed for use as an anti-epilectic, addressed the issue of possible gut bacteria generated genotoxins due to nitroreduction (51). AMP397 has a very similar profile to fexinidazole in genotoxicity tests: mutagenic activity was observed in the standard Ames test strains but loss of that activity in their nitroreductase-deficient counterparts and negative results in the chosen mammalian cell assays. A mutagenicity study was carried out *in vivo* in the MutaMouse model, in which the test animals were given five daily treatments of AMP397 at the maximum tolerated dose. Samples were taken at 3, 7 and 31 days after treatment. No evidence of mutagenicity was seen in the colon or liver. Similarly, an *in vivo* comet assay in the rat (measuring DNA strand breakage) did not detect any genetic damage in the jejunum or liver of treated animals after dosing the animals at a dose six times higher than that possible in the MutaMouse study. A radioactive DNA-binding study of AMP397 also failed to show any DNA binding in rat liver. Thus, if a mutagenic metabolite was formed by intestinal bacteria, it was unable to induce genetic damage in local intestinal tissue.

In summary, there is no evidence to prove that 5-nitroimidazoles (and compounds with related nitro-containing substructures) pose a genotoxic or carcinogenic risk to intestinal cells, whereas there is significant evidence that supports a lack of risk from this theoretical pathway. It is legitimate to consider 5-nitroimidazoles as a class with similar biological properties; thus, there is no compelling reason to regard fexinidazole as separate to the examples given above. However, to provide additional weight of evidence as to its lack of genotoxicity, an additional *in vivo* assay measuring effects on cells of the GI tract (e.g. a Comet assay) would be of value.

Taken together, these studies show that fexinidazole is unlikely to pose a genotoxic hazard for patients. In terms of human hazard assessment, it is important to recognise that any treatment of patients with fexinidazole will be short-term (7–14 days), multiple treatments are unlikely, HAT is 100% lethal if untreated, most existing treatments are either highly toxic and are becoming increasingly ineffective due to resistance of the parasite. Fexinidazole has received regulatory approval for the conduct of studies in humans from the French regulatory authorities (AFSSAPS) and is undergoing clinical trials (http://clinicaltrials.gov/ct2/show/NCT00982904). There are further nitroimidazoles under investigation for possible development

Table V. Bone marrow micronucleus test of fexinidazole in Crl:CD-1 (ICR) mice

Dose (mg/kg)	MN PCE/1000	% PCE
Vehicle[a]	1.0	43.27
500	1.4	44.62
1000	1.1	43.27
2000	1.8	43.33
CPA[b]^ 40	19.7	46.95

[a]The vehicle was Methocel 0.5% (w/v) with 5% (v/v) Tween 80.
[b]The positive control was cyclophosphamide (CPA).

Table VI. *Ex vivo* UDS test of fexinidazole in rat hepatocytes

Compound	Dose (mg/kg)	Net nuclear grain count 2–4 h	Net nuclear grain count 12–14 h
Vehicle[a]	—	0.2	0.6
Fexinidazole	500	0.0	0.4
Fexinidazole	1000	−0.1	0.6
Fexinidazole	2000	−0.4	0.2
DMN[b]	10	8.9	—
2-AAF[b]	75	—	9.8

[a]The vehicle was Methocel 0.5% (w/v) with 5% (v/v) Tween 80.
[b]Positive controls were dimethylnitrosamine (DMN) and 2-acetamidofluorene (2-AAF).

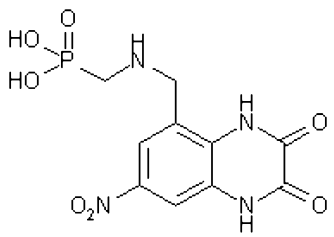

Fig. 10. Chemical structure of AMP397.

Table VII. Plasma levels of fexinidazole, fexinidazole sulfoxide and fexnidazole sulfone in satellite animals from the (a) *in vivo* bone marrow micronucleus studies in the mouse and (b) from the *ex-vivo* UDS study in rat

Testing facility	Species	Oral dose of fexinidazole (mg/kg)	Sampling time (h)	Fexinidazole (ng/ml)	Sulfoxide metabolite (ng/ml)	Sulphone metabolite (ng/ml)
(a)						
Accelera[a]	Mouse	2000	1.0	1080.0	30 666.7	57 333.3
CIT[b]	Mouse	2000	4.0	300.7	20 766.7	107 233.3
(b)						
Accelera[a]	Rat	2000	1.0	988.0	27 667.0	5017.0

[a]Nerviano Medical Science, S.r.l., Accelera, Milan, Italy.
[b]CIT, Evreux, France.

for use in treating HAT and Chagas disease that are being selected on the basis of high efficacy and low genotoxic potential (52).

Funding

This work was supported by DND*i*, who retained the leadership in design, data collection and analysis (even if performed by partners and contract research organisations), decision to publish and preparation of the manuscript. DND*i* received financial support from the following donors for these studies: the Department for International Development (DFID) of the UK, the German Agency for technical Cooperation (GTZ), Médecins Sans Frontières (MSF), Ministry of Foreign and European Affairs of France, the Spanish Agency for International Cooperation and Development and a Swiss foundation. None of these donors had any role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Acknowledgements

We thank George Johnson and Ricardo Del Sol Abascal (University of Swansea, Wales) for discussions and for the nitroreductase measurement studies; Masami Yamada (National Institute of Health, Tokyo) for the construction and kind supply of various nitroreductase-deficient Ames strains for use in this study and R. F. Anderson (University of Aukland, New Zealand) for the redox potential measurements. We thank the DNDi fexinidazole development project team for helpful discussions, to various contract research organisations for conduct of the studies outlined, in particular Covance Ltd (Harrogate, UK) for the various genotoxicity studies; Accelera SpA (Milan, Italy) for the toxicokinetic studies; CIT (Evreux, France) for preliminay micronucleus and toxicokinetic studies and Centipharm (Grasse, France) for the manufacture of fexinidazole and fexinidazole sulfone.

Conflict of interest statement: None declared.

References

1. World Health Organisation Media Centre. WHO Fact Sheet Number 259, January 2012. http://www.who.int/mediacentre/factsheets/fs259/en/.
2. WHO (2006) Human African trypanosomisasis: where do we stand and what comes next? *PLoS Med*., **5**, e55.
3. Legros, D., Ollivier, G., Gastellu-Etchegorry, M., Paquet, C., Burri, C., Jannin, J. and Buscher, P. (2002) Treatment of human African trypanosomiasis: present situation and needs for research and development. *Lancet Infect. Dis*., **2**, 437–440.
4. Robays, J., Nyamowala, G., Sese, C., Kande, V. and Lutumba, P. (2008) High failure rate of melarsoprol for sleeping sickness, Democratic Republic of Congo. *Emerg. Infect. Dis*., **14**, 966–967.
5. Bouteille, B., Marie-Daragon, A., Chauviere, G., de Albuquerque, C., Enanga, B., Darde, M. L., Vallat, J. M., Perie, J. and Dumas, M. (1995) Effect of megazol on *Trypanosoma brucei brucei* acute and subacute infections in Swiss mice. *Acta Trop*., **60**, 73–80.
6. Nesslany, F., Brugier, S., Mouries, M. A., Le Curieux, F. and Marzin, D. (2004) *In vitro* and *in vivo* chromosomal aberrations induced by megazol. *Mutat. Res*., **560**, 147–158.
7. Raether, W. and Hanel, H. (2003) Nitroheterocyclic drugs with broad spectrum activity. *Parasitol. Res*., **90** (Suppl. 2), S19–S39.
8. Friedman, G. D., Jiang, S. F., Udaltsova, N., Quesenberry, C. P., Jr, Chan, J. and Habel, C. A. (2009) Epidemiologic evaluation of pharmaceuticals with limited evidence of carcinogenicity. *Int. J. Cancer*, **125**, 2173–2178.
9. WHO. (2011) *17th list of Essential Medicines, English Version Updated March 2011*. whqlibdoc.who.int/hq/2011/a95053_eng.pdf.
10. Granizo, J. J., pia Rodicio, M., Manso, F. J. and Gimenez, M. J. (2009) Tinidazole, a classical anaerobic drug with multiple potential uses nowadays. *Rev. Esp. Quimioter*., **22**, 106–114.
11. Rassi, A., Jr, Rassi, A. and Marin-Neto, J. A. (2010) Chagas disease. *Lancet*, **375**, 1388–1402.
12. Issa, V. S. and Bocchi, E. A. (2010) Antitrypanosomal agents treatment or threat? *Lancet*, **376**, 768; Author Reply 768–769.
13. Buschini, A., Ferrarini, L., Franzoni, S. *et al*. (2009) Genotoxicity revaluation of three nitroheterocyclic drugs: nifurtimox, benznidazole and metronidazole. *J. Parasitol. Res*., **2009**, 2009article id 463575. Epub 2009 October 21.
14. Lopez Nigro, M. M. and Carballo, M. A. (2008) Genotoxicity and cell death induced by tinidazole (TNZ). *Toxicol. Lett*., **180**, 46–52.
15. Ehlhardt, W. J., Beaulieu, B. B., Jr and Goldman, P. (1988) Mammalian cell cytotoxicity and bacterial mutagenicity of nitrosoimidazoles. *Biochem. Pharmacol*., **37**, 2603–2606.
16. Edwards, D. I. (1993) Nitroimidazole drugs-action and resistance mechanisms. 1 Mechanisms of action. *J. Antimicrob. Chemother*., **31**, 9–20.
17. Mital, A. (2009) Synthetic nitroimidazoles: biological activities and mutagenicity relationships. *Sci. Pharm*., **77**, 497–520.
18. McCann, J., Spingarn, N. E., Kobori, J. and Ames, B. N. (1973) Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. *Proc. Natl. Acad. Sci. USA*, **72**, 979–983.
19. Blumer, J. L., Friedman, A., Meyer, L. W., Fairchild, E., Webster, L. T., Jr and Speck, W. T. (1980) Relative importance of bacterial and mammalian nitroreductases for niridazole mutagenesis. *Cancer Res*., **40**, 4599–4605.
20. Speck, W. T., Carr, H. S. and Rosenkranz, H. S. (1985) Niridazole: evidence for promutagenic events involving guanine-cystosine and adenine-thymine base pairs. *Environ. Mutagen*., **7**, 429–437.
21. Walsh, J. S., Wand, R., Bagan, E., Wand, C. C., Wislocki, P. and Miwa, G. T. (1986) Structural alterations that differentially affect the mutagenic and antitrichomonal activities of 5-nitroimidazoles. *J. Med. Chem*., **30**, 150–156.
22. De Meo, M., Vanelle, P., Bernadini, E., Laget, M., Maldonado, J., Jentzer, O., Crozet, M. P. and Dumenil, G. (1992) Evaluation of the mutagenic and genotoxic activities of 48 nitroimidazoles and related imidazole derivatives by the Ames test and the SOS chromostest. *Environ. Mol. Mutagen*., **19**, 167–181.
23. Buschini, A., Giordani, F., Northfleet de Albuquerque, C., Pellacani, C., Pelosi, G., Rossi, C., Araujo, T. M., Zucchi, D. and Poli, P. (2007) Trypanocidal nitroimidazole derivatives: relationships among chemical structure and genotoxic actiovity. *Biochem. Pharmacol*., **73**, 1537–1547.
24. Crozet, M. C., Botta, C., Gasquet, M., Curti, C., Remusat, V., Hutter, S., Chapelle, O., Azas, N., De Meo, M. and Vanelle, P. (2009) Lowering of 5-nitroimidazole's mutagenicity; towards optimal antiparasitic pharmacophore. *Eur. J. Med. Chem*., **44**, 653–659.
25. Wilkinson, S. R., Taylor, M. C., Horn, D., Kelly, J. M. and Cheeseman, I. (2008) A mechanism of cross-resistance to nifurtimox and benznidazole in trypanosomes. *Proc. Nat. Acad. Sci. USA*, **105**, 5022–5027.
26. Sokolova, A. Y., Wyllie, S., Patterson, S., Oza, S. I., Reid, K. D. and Fairlamb, A. H. (2010) Cross-resistance to nitro drugs and implications for treatment of human African trypanosomiasis. *Antimicrob. Agents Chemother*., **54**, 2893–2900.
27. Stover, C. K., Warrener, P., Van Devanter, D. R. *et al*. (2000) A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis,. *Nature*, **405**, 962–966.
28. Matsumoto, M., Hashizume, H., Tomishige, T., Kawasaki, M., Tsubouchi, H., Sasaki, H., Shimokawa, Y. and Komatsu, M. (2006) OPC-67683 a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis *in vitro* and in mice. *PLoS Med*., **3**, e466.
29. Winkelman, E. and Raether, W. (1978) Chemotherapeutically active nitro compounds. 4,5-nitroimidazoles (Part iii). *Arzneimittelforschung./Drug Res*., **28**, 739–749.
30. Raether, W. and Deutschlander, N. (1979) *Hoe 239 (Fexinidazole), A 5-Nitroimidazole Highly Potent Against Trypanosoma cruzi in NMRI Mice*. International Congress on Chagas Disease, Rio de Janeiro , Abstracts, p. 142.
31. Raether, W. and Seidenath, H. (1983) The activity of fexinidazole (HOE 239) against experimental infections with *Trypanosoma cruzi*, trichomonads and *Entamoeba histolytica*. *Ann. Trop. Med. Parasit*., **77**, 13–26.
32. Torreele, E., Bourdin Trunz, B., Tweats, D., Kaiser, M., Brun, R., Mazue, G., Bray, M. A. and Pecoul, B. (2010) Fexinidazole—a new oral nitroimidazole drug candidate entering clinical development for the treatment of sleeping sickness. *PLoS Negl. Trop. Dis*., **4**, e923doi: 10.1371/journal.pntd.0000923.
33. Yamada, M., Espinosa-Aguirre, J. J., Watanabe, M., Matsui, K., Sofuni, T. and Nohmi, T. (1997) Targeted disruption of the gene encoding the classical nitroreductase enzyme in Salmonella typhimurium Ames test strains TA1535 and TA1538. *Mutat. Res*., **375**, 9–17.
34. Porwollik, S., Wong, R. M., Sims, S. H., Schaaper, R. M., DeMarini, D. M. and McClelland, M. (2001) The DeltauvrB mutations in the Ames strains of Salmonella span 15 to 119 genes. *Mutat. Res*., **483**, 1–11.
35. Maron, D. M. and Ames, B. N. (1983) Revised methods for the Salmonella mutagenicity test. *Mutat. Res*., **113**, 173–215.

36. OECD (2007) *'Genetic Toxicology: OECD Guideline for the Testing of Chemicals. Draft Proposal for a New Guideline 487: In Vitro Micronucleus Test'*. December 13 2007 (version 3) www.oecd.org/dataoecd/38/58/39780112.doc.
37. Krishna, G. and Hayashi, M. (2000) *In vivo* micronucleus assay: protocol, conduct and interpretation. *Mutat. Res*., **455**, 155–166.
38. Kennelly, J. C., Waters, R., Ashby, J., Lefevre, P. A., Burlinson, B., Benford, D. J., Dean, S. W. and Mitchell, I.de G. (1993) *In vivo* rat liver UDS assay. In Kirkland, D. J. and Fox, M. (eds), *Supplementary Mutagenicity Tests, UKEMS Recommended Procedures*. Cambridge University Press, Cambridge, UK, pp. 52–77.
39. Liochev, S. I., Hausladen, A. and Fridovich, I. (1999) Nitroreductase A is regulated as a member of the soxRS regulon of Escherichia coli. *Proc. Natl. Acad. Sci. USA*., **96**, 3537–3539.
40. Wardman, P. (1989) Reduction potentials of one-electron couples involving free radicals in aqueous solution. *J. Phys. Chem. Ref. Data*, **18**, 1637–1756.
41. Parton, J. W. and Yount, D. J. (1995) Improved sensitivity of the unscheduled DNA synthesis assay in primary rat hepatocytes following culture in serum free media. *Environ. Mol. Mutagen*., **26**, 147–154.
42. Mirsalis, J. C. and Butterworth, B. E. (1982) Induction of unscheduled DNA synthesis in rat hepatocytes following in vivo treatment with dinitrotoluene. *Carcinogenesis*, **3**, 241–245.
43. Barry, C. E., III, Boshoff, H. I. M. and Dowd, C. S. (2004) Prospects for clinical introduction of nitroimidazole antibiotics for the treatment of tuberculosis. *Curr. Pharm. Des*., **10**, 3229–3262.
44. Roe, F. J. (1983) Toxicologic evaluation of metronidazole with particular reference to carcinogenic, mutagenic and teratogenic potential. *Surgery*, **93**, 58–64.
45. Edwards, D. I. (1993) Nitroimidazole drugs action and resistance mechanisms. I Mechanisms of action. *J. Antimicrob. Chemother*., **31**, 9–20.
46. SCAN. (2000) *Opinion of the Scientific Committee for Animal Nutrition on the Use of Dimetridazole in Animal Feedstuffs, European Commission Health and Consumer Protection Directorate-General*. ec.europa.eu/food/fs/sc/scan/out51_en.pdf.
47. Cavaliere, A., Bacci, M., Amorosi, A., Del Gaudio, M. and Vitali, R. (1983) Induction of lung tumours and lymphomas in BALB/c mice by metronidazole. *Tumori*, **69**, 379–382.
48. Rustia, M. and Shubik, P. (1979) Experimental induction of hepatomas, mammary tumours and other tumours with metronidazole in noninbred Sas:MRC (WI) BR rats. *J. Natl. Cancer Inst*., **63**, 863–868.
49. Teixeira, A. R., Calixto, M. A. and Teixeira, M. L. (1994) Chagas' disease: carcinogenic activity of the antitrypanosomal nitroarenes in mice. *Mutat. Res*., **305**, 189–196.
50. Iatropoulos, M. J., Wang, C. X., von Keutz, E. and Williams, G. M. (2006) Assessment of chronic toxicity and carcinogenicity in an accelerated cancer bioassay in rats of Nifurtimox, an antitrypanosomiasis drug. *Exp. Toxicol. Pathol*., **57**, 397–404.
51. Suter, W., Hartmann, A., Poetter, F., Sagelsdorff, P. and Hoffmann, P. (2002) Genotoxicity assessment of the antiepileptic drug AMP397, an Ames positive aromatic nitro compound. *Mutat. Res*., **518**, 181–194.
52. Bourdin Trunz, B., Jedrysiak, R., Tweats, D., Brun, R., Kaiser, M., Suwinski, J. and Torreele, E. (2011) 1-Aryl-4-nitro-1H-imidazoles, a new promising series for the treatment of human African trypanosomiasis. *Eur. J. Med. Chem*., **46**, 1524–1535.