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EFFECT OF 3-AMINOBENZAMIDE ON ANTIGENIC VARIATION OF *TRYPANOSOMA BRUCEI*

A. W. C. A. CORNELISSEN,* P. A. M. MICHELS,*† P. BORST,* W. SPANJER,‡
J. A. M. VERSLUIJS-BROERS,‡ C. VAN DER MEER,‡ F. FARZANEH§‖ and S. SHALL§

*Division of Molecular Biology, The Netherlands Cancer Institute (Antoni Van Leeuwenhoek Huis), Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands; ‡Department of Pharmacology, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands; §Cell and Molecular Biology Laboratory, Biology Building, University of Sussex, Brighton BN1 9QG, U.K.

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Abstract—African trypanosomes, like *Trypanosoma brucei*, depend on antigenic variation to evade the immune response of the vertebrate host. An antigenic switch corresponds to the activation of a variable surface glycoprotein (VSG) gene from a large silent repertoire. Most switches require the duplicative transposition of a VSG gene, which involves strand breaks in DNA and subsequent repair. The nuclear enzyme adenosine-diphosphoribosyl transferase (ADPRT), which is dependent on the presence of DNA strand breaks for its activity, might be involved in this process because it has a regulatory role in DNA repair in all eukaryotic cells studied so far. In previous work, the presence of ADPRT activity was demonstrated in *T. brucei*. Moreover, it was also shown in isolated trypanosomes the ADPRT activity, which is stimulated by the induction of DNA strand breaks, could be blocked by the competitive inhibitor 3-aminobenzamide. Here we report experiments using rats which were infected with small numbers of *T. brucei* expressing VSG gene 118. After two days, the rats were coupled to a continuous intraperitoneal infusion system administrating 3-aminobenzamide in 0.9% NaCl (81.4 mM) at a rate of 0.65 ml/hr/rat for a period of up to five days. Control rats received only a 0.9% NaCl infusion. At days 1, 3 and 5, 250 μl blood was obtained from a tail artery. Plasma 3-aminobenzamide was determined using a new high performance liquid chromatography method, developed for these experiments. In most rats the plasma concentrations were maintained between 0.8 and 1.2 mM. The rate of antigenic switching was determined by quantitating the fraction of trypanosomes that had lost their VSG 118 coat, using antibody against VSG 118 and a limiting dilution in mice. The average switching rate found was 2.0×10^{-6} in controls and 1.3×10^{-7} in drug-treated rats (15-fold reduction). This suggests that ADPRT is required for completing most antigenic switching events. We discuss the possibility that drug-resistant switching only involves non-duplicative VSG gene activation.

The nuclear enzyme adenosine-diphosphoribosyl transferase (ADPRT), which is dependent on the presence of DNA strand breaks for its activity [1], is involved in a variety of eukaryotic cellular processes which require the ligation of DNA strand breaks (see reviews [2] and [3]), probably because it regulates DNA ligase activity [4]. These processes include DNA excision repair [5], a number of examples of eukaryotic cellular differentiation [6–9], mitogen activation of quiescent lymphocytes [10, 11], sister chromatid exchange [12] and stable integration of transfected DNA into the host genome [13]. The presence of ADPRT activity has been demonstrated in the Kinetoplastid species *Trypanosoma cruzi* [9] and *T. brucei* [14]. *T. brucei* belongs to the African trypanosomes, which depend on antigenic variation to evade the immune response of their vertebrate hosts (reviewed in [15–17]). An antigenic switch involves the activation of a silent variable surface glycoprotein (VSG) gene from a large repertoire (reviewed in [18–22]). Many, but not all, switches require the duplicative transportation of a DNA segment containing a VSG gene to an expression site. This requires DNA strand breaks and subsequent repair, a process in which ADPRT might be involved. The availability of inhibitors of ADPRT [23] makes it possible to test this hypothesis.

We have previously demonstrated the presence of ADPRT activity in isolated *T. brucei*, the stimulation of this activity by DNA strand break formation, and its inhibition by benzamide analogues like 3-aminobenzamide [14]. In the present paper we describe an experimental system in which the effect of 3-aminobenzamide on the antigenic switching process of trypanosomes can be studied in intact rats. We show that the drug significantly inhibits antigenic variation in trypanosomes.

MATERIALS AND METHODS

Trypanosomes. Trypanosomes used belong to *T. brucei* stock 427 clone 60. The isolation of variant 118a (MITat 1.5a) has been described by Cross [24]. Trypanosomes were grown for four days in a mouse infected with a stabilated cloned population (see [25]). The blood was taken from the mouse and

† Present address: International Institute of Cellular and Molecular Pathology, ICP-TROP., Avenue Hippocrate 74, B-1200 Brussels, Belgium

‖ Present address: Harris Birthright Research Centre for Fetal Medicine, Department of Obstetrics and Gynaecology, King's College School of Medicine and Dentistry, Denmark Hill, London SE5 8RX, U.K.

diluted with guinea pig serum. Trypanosomes were counted under a microscope in droplets of diluted blood. A droplet containing 10–15 trypanosomes was used to infect female Pfd: WU (WI) rats of approx. 200 g by the intraperitoneal route (i.p.). The infusion with 3-aminobenzamide was started two days post-infection (p.i.) as described below.

Determination of the switching rate by limiting dilution. After the infusion, the trypanosomes were harvested and purified from blood elements as described by Lanham and Godfrey [26] and Michels *et al.* [27]. Neutralizing tests on these trypanosome populations were performed essentially as described [25, 28]. The unswitched trypanosomes—expressing VSG gene 118—were eliminated *in vitro*, using VSG-specific antiserum and complement from guinea pig serum. Duplicate aliquots containing about 10^5, 10^6, 10^7 or 10^8 trypanosomes were incubated at room temperature for 2–4 hr with appropriate dilutions of antiserum directed against VSG 118. The antiserum was diluted with PSG buffer (Na_2HPO_4, 56.4 mM; NaH_2PO_4, 6.9 mM; NaCl, 43.6 mM; 1% glucose (w/v) [26]) supplemented with 50% guinea pig serum. Final incubation volumes were 300 μl (10^5–10^7) or 500 μl (10^8). After immune lysis, trypanosomes were titrated by inoculation in mice. For each dilution of the parasite treated with antiserum, two female BALB/c mice (strain 11, Animal Breeding Unit, The Netherlands Cancer Institute) were used. Mice were monitored daily for 14 days. Blood films of parasite positive mice were fixed in acetone for 10 min, air-dried and stored at −20° prior to use. In order to verify that no trypanosomes expressing VSG gene 118 had survived the antibody treatment (see [25]) the slides were rechecked by indirect immunoflorescence [25, 27].

Determination of 3-aminobenzamide in blood plasma. Approximately 250 μl blood was obtained by puncturing a tail artery under ether anaesthesia. The blood was collected in a tube containing 25 μl heparin, cooled immediately on ice, centrifuged (2 min at 8800 *g*) and the plasma was taken and stored at −20°. Plasma concentrations of 3-aminobenzamide were determined using high performance liquid chromatography (HPLC), with 2-nitrobenzamide as an internal standard. The extraction mixture, in a total volume of 3.85 ml, comprised 50 μl plasma, 0.1 ml 2-nitrobenzamide (0.5 mM), 0.4 ml distilled water, 0.3 ml saturated Na_3PO_4 solution and 3 ml 1-butanol. The sample was mixed on a Vortex mixer three times for 15 sec. The phases were separated by centrifugation at 1600 *g* for 10 min. About 2.5 ml of the butanol layer was dried in an airstream in a waterbath at 60°. The residue was redissolved in 0.2 ml elution mixture consisting of methanol and 6% acetic acid in a ratio of 65:35. This solution was used for HPLC, using a Lichrosorb RP 18 column of 25 × 0.46 cm. The flow rate was 1 ml/min and the injection volume was 10 μl. Detection of 3-aminobenzamide and 2-nitrobenzamide was performed by an ultraviolet monitor (Perkin–Elmer, LC 75) at 254 nm. The drug concentration was calculated from the ratio of the peak heights. A calibration curve was prepared by adding 0.1, 0.2 and 0.4 ml of 3-aminobenzamide (0.25 mM; made up to a final volume of 0.4 ml with distilled water) and 0.1 ml 2-nitrobenzamide (0.5 mM) to 50 μl plasma and treating these samples as described above.

Drug treatment and infusion experiments. In order to obtain an estimate of the maximum tolerated dose, the absorption rate from the peritoneal cavity, distribution and elimination rate, preliminary experiments were performed using intravenous (i.v.) infusions as well as i.p. and i.v. injections of 3-aminobenzamide as described below. When serial samples were taken an identical volume of fresh rat blood was injected after each sampling. In the final infusion experiments a long PVC catheter (inner diameter 1 mm) was introduced into the peritoneal cavity under ether anaesthesia. The catheter was exteriorized at the back of the rat and passed through a steel spring (inner diameter 5 mm, outer diameter 7 mm, length 30 cm), which was fastened to the skin and passed through the cover of the cage. The canula was connected to an automatic infusion apparatus (Braun, Melsingen, F.R.G.); solutions were infused at different rates for 3–5 days as indicated in the text. Isotonic saline (0.9% NaCl) was given to the rats of the control group. The experimental group was infused with a solution of 81.4 mM 3-aminobenzamide in isotonic saline. Rats had free access to food and water while kept in individual cages at an environmental temperature of 30° during the experiment. Attempts were made to analyse the plasma data given in Fig. 1 (see Results) with the aid of the NONLIN computer program, which is a model-fitting program [29], in which the pharmokinetic parameters giving the best fit to the experimental points are determined.

Preparation of the solution of 3-aminobenzamide. A solution of 81.4 mM in 0.9% NaCl was prepared by stirring the mixture at room temperature for 1 hr in the dark. After filtration, a clear solution is obtained which remains stable for at least five days at room temperature.

Chemicals. 3-Aminobenzamide was initially obtained from Sigma, St. Louis, MO, U.S.A., for the final experiments it was obtained from Tokyo Kasei Kogyo, Tokyo, Japan. 2-Nitrobenzamide was obtained from Janssen Chimica, Beerse, Belgium, Heparin (500 IU/ml) was obtained from Leo, Emmen, The Netherlands. The Lichrosorb RP18 column was obtained from Chrompack, Middelburg, The Netherlands. All other chemicals were of analytical grade and were obtained from Merck, Darmstadt, F.R.G.

RESULTS

Plasma levels in rats after administration of 3-aminobenzamide

A plasma level of about 2 mM 3-aminobenzamide was already present at 5 min following a single i.p. injection with 5 mmoles/kg body wt while a plateau level of approx. 3 mM was reached at 10 min after the injection and maintained for at least 45 min (Table 1). In a second experiment using two rats and i.v. injection of 1 mmole 3-aminobenzamide/kg body wt, it was similarly found that very little change in plasma levels occurred over a time period up to 60 min (data not shown). These results indicate that the uptake of 3-aminobenzamide from the peritoneal

Table 1. Plasma concentrations of 3-aminobenzamide in rats after a single i.p. injection of the drug

t (min)	Plasma concentration (mM) Rat 1	Rat 2
5	2.0–2.1	
10	2.6–2.7	3.0–3.1
20	3.1–3.0	3.0–2.7
30	3.1–2.9	3.4–3.6
45	3.4–3.1	

Rats received one i.p. injection of 5 mmoles 3-aminobenzamide/kg body wt. Blood was taken at the times indicated using either one or two rats at each point in time. Duplicate 3-aminobenzamide determinations were done on each sample.

Table 2. Plasma concentration of 3-aminobenzamide in rats receiving a continuous intraperitoneal infusion of the drug

Rat No.	Plasma concentration (mM) Day 1	Day 2	Day 3
1 (202)	1.5	1.8	1.5 (175)
2 (201)	1.9	1.2	1.3 (167)
3 (200)	1.6	1.3	1.2 (179)

Body weight in parentheses.

Rats were infused for three days with a solution of 75 mM 3-aminobenzamide in 0.9% NaCl, each rat receiving 9 mmoles/kg body wt/24 hr.

cavity is rapid, while the elimination from plasma seems to be relatively slow. Two rats, approx. 200 g, received an i.v. infusion of 20 μmoles 3-aminobenzamide/min for 20 min. Plasma concentrations were determined up to 200 min (Fig. 1). A rough estimate of plasma half-time calculated from these data was 133 min, while the distribution volume was estimated at 198 ml. These estimated parameters were used to calculate the infusion rate that would be required to obtain a steady-state concentration of approx. 1.2 mM (> 100 times the apparent K_i value for 3-aminobenzamide in isolated *T. brucei* [14]). It was calculated that an infusion of 9.2 mmoles/kg body wt/24 hr would establish such a concentration. This was verified in the experiment summarized in Table 2, in which three rats received an i.p. infusion for three days. Plasma concentrations of 3-aminobenzamide were relatively stable over this period and ranged from 1.2 to 1.9 mM. Attempts were made to determine the pharmokinetic parameters by fitting curves to the plasma concentration curve of Fig. 1, using standard computer programs (see Methods). However, neither the assumption of a two-compartment model, nor that of a one-compartment model yielded parameters which simulated the steady-state concentrations observed with sufficient accuracy.

Table 2 also shows that there is a considerable loss in body weight of the rats (mean ± S.E.M.: 28 ± 4 g), indicating that this procedure is relatively toxic. For this reason the dose was reduced to approx. 6.3 mmoles/kg body wt/24 hr in the final experiments.

Effect of 3-aminobenzamide upon the switching rate in T. brucei

Exposure of bloodstream trypanosomes to 3-aminobenzamide had no effect on their multiplication rate, since the trypanosome numbers per μl blood at the day of autopsy did not differ significantly between the control and the treated group (Table 3). Rats infused with 3-aminobenzamide had a weight loss of 25 ± 2 g by the end of the experiment, which is comparable to the loss observed in the experiments described in Table 2 (28 ± 4 g), although the mean steady-state level of 3-aminobenzamide was about 40% higher in the latter experiment (compare with Table 3). Control rats, however, had a significantly lower but still considerable weight loss (12 ± 2 g), indicating that the loss of weight may be caused in part by the toxicity of 3-aminobenzamide and in part by the experimental set-up, such as restriction of mobility by the steel spring. Plasma levels of 3-aminobenzamide in the eight rats used in this experiment reached the expected levels (1.0 mM; see Table 3 and previous section), the mean level over the whole infusion period was 0.9 ± 0.04 mM (minimum value 0.5 mM; maximum value 1.2 mM). The numbers of switched trypanosomes in rats with and without 3-amino-

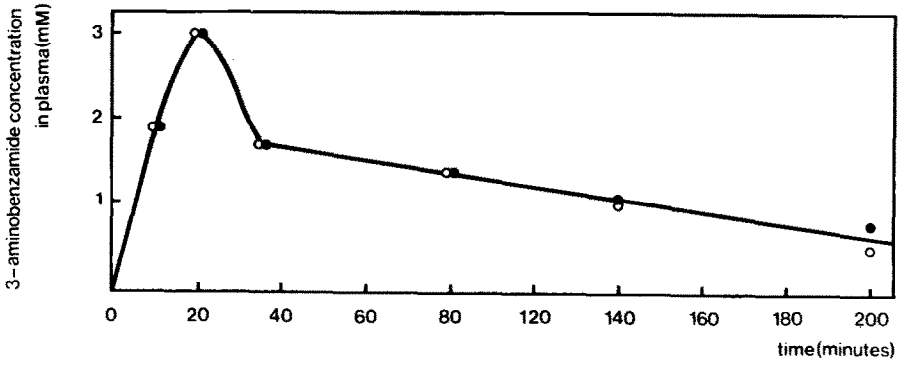

Fig. 1. Time course of plasma concentrations of 3-aminobenzamide in rats receiving a continuous i.v. infusion. Two rats were infused for 20 min with 20 μmoles 3-aminobenzamide/min in 0.9% NaCl. Blood samples of each rat were taken at the times indicated.

Table 3. Response of trypanosomes to 3-aminobenzamide and the effect of the drug on the rate of coat switching

Rats	Mean plasma level of 3-aminobenzamide (mM)	Number of trypanosomes $\times 10^3$ μl blood	Titration of trypanosomes* (% switched trypanosomes in each of two mice) 10^8	10^7	10^6	10^5
Control 1		100	100/100	100/NT	(0)/(0)	(0)/(0)
2		100	20/50	50/70	(0)/(0)	(0)/(0)
3		50	NT	100/(0)	(0)/(0)	(0)/(0)
4		50	100/100	100/100	100/100	100/(0)
5		40	100/0	100/(0)	(0)/(0)	(0)/(0)
6		NT	NT	100/100	100/100	(0)/(0)
7		200	90/95	95/95	100/100	(0)/(0)
8		10	100/100	100/(0)	(0)/(0)	(0)/(0)
Treated 1	0.9 ± 0.1	300	(0)/(0)	(0)/(0)	(0)/(0)	(0)/(0)
2	0.8 ± 0.2	100	50/90	80/90	(0)/(0)	(0)/(0)
3	0.7 ± 0.2	100	100/0	(0)/(0)	(0)/(0)	(0)/(0)
4	1.0 ± 0.1	60	(0)/(0)	(0)/(0)	(0)/(0)	(0)/(0)
5	0.9 ± 0.2	50	100/100	100/100	(0)/(0)	(0)/(0)
6	1.0 ± 0.2	50	90/95	90/95	(0)/(0)	(0)/(0)
7	0.7 ± 0.3	200	100/100	100/100	(0)/(0)	(0)/(0)
8	1.0 ± 0.2	NT	20/95	(0)/(0)	(0)/(0)	(0)/(0)

NT: Not tested.

* The percentage of trypanosomes (rounded off at increments of five) expressing VSG genes other than 118 are given for each individual mouse. Zero values in brackets indicate that no parasitaemia developed in the mouse (= no switched trypanosomes present in the inoculum).

Rats were infected i.p. with 10–15 trypanosomes with a coat composed of VSG 118. Rats 1–4 were infused from days 3–6 p.i.; rats 5–8 from days 3–7 p.i. with 6.3 mmoles/kg body wt/24 hr. At autopsy rats were bled, trypanosomes purified from blood elements and divided into duplicate inocula containing from 10^5 to 10^8 parasites. Trypanosomes with a coat composed of VSG 118 were immunolysed as described in Materials and Methods and the remaining trypanosomes were then injected i.p. into two mice. Mice were checked daily for 14 days. Bloodfilms of positive mice were rechecked with indirect immunofluorescence using VSG 118-specific antiserum. In this way it was possible to differentiate between parasitaemias in which the observed parasitaemia resulted from trypanosomes expressing VSG gene 118, which had survived the antibody treatment and from trypanosomes switched to a new VSG gene.

benzamide are presented in Table 3. The calculated switching rate with and without 3-aminobenzamide differ about 15-fold (Table 4). Statistical analysis (log-rank test) [30] of the data shows that the decrease in the apparent switching rate induced by 3-aminobenzamide is significant ($P = 0.029$). A second experiment, comprising six control and five treated animals, also resulted in a significant reduction (approx. 150-fold) in the switching rate by 3-aminobenzamide ($P = 0.034$; data not shown). In this experiment, however, the control rats did not receive a saline infusion. Hence, the experimental data are not completely comparable with the experiment presented in Table 3.

DISCUSSION

We have developed an experimental system that allows the continuous maintenance of a high blood level of 3-aminobenzamide (approximately the maximum tolerated level) in rats for a period of up to five days. This system was used to demonstrate that 3-

Table 4. Summary of the effect of 3-aminobenzamide on the rate of surface coat switching by *T. brucei* in rats (see Table 3)

Rats	Number of rats with switched trypanosomes per titration point 10^8	10^7	10^6	10^5	Calculated switching rate*
Controls ($n = 8$)	8	8	3	1	$\sim 2.0 \times 10^{-6}$
Treated ($n = 8$)	6	4	0	0	$\sim 1.3 \times 10^{-7}$

* Calculated for each rat from the titration data in Table 3;

$$\frac{1}{N_{k+1}} \ln\left(+\frac{N_{k+1}}{N_k}\right),$$

in which N_k = highest trypanosome titre without switching and N_{k+1} = lowest trypanosome titre where switching was demonstrated.

aminobenzamide inhibits the apparent rate at which trypanosomes switch their surface coat composition. The simplest interpretation of these experiments is that an active ADPRT is required either for the gene switching process or for the repair of the DNA breaks induced by it and that this ADPRT activity is blocked by 3-aminobenzamide. Direct evidence for the presence of ADPRT in *T. brucei* has been obtained by the demonstration of enzyme activity in permeabilized trypanosomes and by the effect of benzamide analogues on this activity [14]. The functional importance of ADPRT in trypanosomes is indicated by the observation that the inhibition of this enzyme blocks the morphological differentiation of *T. cruzi* amastigotes to epimastigotes and trypomastigotes, however, the differentiation-independent proliferation of *T. cruzi* epimastigotes was not affected by inhibitors of ADPRT [9]. Nevertheless, we cannot exclude the possibility that the effects of 3-aminobenzamide on switching are not mediated by ADPRT. Even though 3-aminobenzamide had no significant effect on the multiplication rate of the trypanosomes, other non-specific effects might have affected the switching rate. Moreover, the determination of switching rates is an indirect one and is affected by complex parasite–host interactions, both in the rat and in the mice used for titrating the number of switched trypanosomes. Development of a reliable *in vitro* switching system should allow a more detailed analysis of the specificity of the 3-aminobenzamide effect reported here.

Although the effect of 3-aminobenzamide on apparent switching rates is statistically significant, the inhibition is not complete. This might be due firstly to competition with the relatively high concentrations of intracellular NAD^+. Indeed, during studies of DNA repair, it has been shown that inhibition of ADPRT activity, even at higher inhibitor concentrations than used in this study, only reduces the rate of DNA strand ligation and does not completely block this process [5]. Secondly, although the plasma concentration continuously maintained in our experimental rats was more than 100-fold the apparent K_i value of $4.3 \pm 0.5\ \mu M$ measured in isolated permeabilised trypanosomes [14], it has been shown that bloodstream trypanosomes are found in extravascular sites, including brain tissue (see [31]). It is possible that the concentration of 3-aminobenzamide at these extra-vascular sites is considerably lower than the plasma concentration. An alternative possibility is that not all switching of coat proteins requires the duplicative transposition of a VSG gene. Some VSG genes can be activated *in situ* without duplication (see [18–22]). It is quite possible that this activation process does not involve DNA strand breakage and repair. The switches which have escaped the 3-aminobenzamide effect may therefore involve the activation of VSG genes without duplicative transposition. This remains to be analysed.

To test the effects of 3-aminobenzamide on the apparent switching rate of trypanosome coats, methods had to be developed for the continuous infusion of 3-aminobenzamide in rats and for the determination of the plasma levels of the drug. The HPLC assay reported here allows a determination of plasma 3-aminobenzamide in the range of interest. We have demonstrated that it is possible to maintain a 1 mM concentration of 3-aminobenzamide for five days and rough estimates of pharmacokinetic parameters of this drug in rats were made. These results should be useful to other workers who are studying the function of ADPRT in mammals.

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