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Pharmacokinetics of a Pediatric Tribendimidine Dose-Finding Study To Treat Hookworm Infection in African Children

Noemi Hiroshige,[a,b] Jean Coulibaly,[a,b,c,d] Jörg Huwyler,[e] Peter L. Bonate,[f] Jennifer Keiser[a,b]

[a]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Basel, Switzerland
[b]University of Basel, Basel, Switzerland
[c]Unité de Formation et de Recherche Biosciences, Université Félix Houphouët–Boigny, Abidjan, Côte d'Ivoire
[d]Centre Suisse de Recherches Scientifiques en Côte d'Ivoire, Abidjan, Côte d'Ivoire
[e]Department of Pharmaceutical Sciences, Division of Pharmaceutical Technology, University of Basel, Basel, Switzerland
[f]Astellas, Northbrook, Illinois, USA

ABSTRACT Tribendimidine is a broad-spectrum anthelminthic available in China, which is currently being pursued for U.S. Food and Drug Administration approval for soil-transmitted helminth infections. Pharmacokinetic (PK) studies with tribendimidine in children, the main target group for treatment programs, have not been conducted to date. In the framework of a dose-ranging study in hookworm-infected school-aged children in Côte d'Ivoire, children were treated with either 100, 200, or 400 mg tribendimidine. Dried blood spot samples were collected up to 22 h after treatment. The active metabolite, deacetylated amidantel (dADT) and its metabolite acylated dADT (adADT) were quantified using liquid chromatography tandem mass spectrometry. PK parameters were calculated using a noncompartmental model, and univariate logistic regression was applied using maximal blood concentrations (C_{max}) and area under the blood concentration-time curve for 0 to 22 h (AUC_{0-22}) as predictors of drug efficacy. Dried blood spot samples of 101 children were analyzed. We observed a less than proportional and proportional exposure in dADT's median C_{max} and AUC_{0-22}, respectively, following administration of 100 mg ($C_{max} = 853$ ng/ml; $AUC_{0-22} = 3,019$ h · ng/ml) and 400 mg ($C_{max} = 2,275$ ng/ml; $AUC_{0-22} = 12,530$ h · ng/ml) tribendimidine. There were large, dose-independent variations in the time to C_{max} (T_{max}) and ratios of dADT to adADT. We did not detect an influence of C_{max} or AUC_{0-22} of dADT or adADT on drug efficacy or adverse events. Since our study population was bearing hookworm infection of mainly low intensity, additional studies with heavy intensity infections might be required to confirm this observation.

KEYWORDS hookworm, pharmacokinetics, soil-transmitted helminths, tribendimidine

An estimated 400 to 500 million people are globally infected with hookworms, mainly with the two hookworms *Necator americanus* and *Ancylostoma duodenale*. Hookworms belong to the soil-transmitted helminths and infections are endemic in tropical and subtropical areas, including Southeast Asia, Papua New Guinea, most of Africa, and Central and South America (1, 2). Infection with blood-feeding hookworms is associated with intestinal blood loss leading to iron deficiency and anemia. Chronic infections during childhood and puberty can stunt physical and cognitive development (2). Preventive chemotherapy with a single dose of albendazole or mebendazole is applied to control the disease burden (3). However, infections are not fully cured, and there is a trend of decreasing efficacy of the benzimidazoles, which might be due to emerging drug resistance, as observed in the veterinary field (4, 5). Alternative drugs,

which could be used in addition to these heavily applied drugs, would decrease the probability for the emergence of drug resistance by alleviating drug pressure.

Tribendimidine, developed and approved in China in 2004 for the treatment of soil-transmitted helminth infections, is known for its comparable drug efficacy and safety to albendazole (6–8). Tribendimidine is a prodrug which degrades quickly to deacylated amidantel (dADT) and the terephtaldehyde. dADT is further metabolized to acylated dADT (adADT) probably by arylamine *N*-acetyltransferases (9). dADT is the major compound responsible for drug activity against hookworm *in vitro* and *in vivo* as opposed to adADT (10), acting as a selective B-subtype nicotinic acetylcholine receptor (nAChR) agonist, depolarizing muscular nAChRs of the parasite. This mechanism of action is different from other nAChR agonistic anthelminthics such as levamisole and pyrantel (11).

The long clinical experience has proven excellent efficacy and safety of tribendimidine (8), and efforts to register tribendimidine with the U.S. Food and Drug Administration (FDA) for global access are currently being undertaken. For adults, a single dose of 400 mg tribendimidine was elucidated in dose-response studies (8), while the pediatric dose of 200 mg tribendimidine was arbitrarily chosen. Population pharmacokinetics indicated younger people to be less exposed to dADT due to higher drug clearance (12). These findings question the effectiveness of the 200-mg pediatric dose, and hence further investigations are necessary before recommending tribendimidine for preventive chemotherapy in children (12).

The aim of the present study was to determine for the first time the pharmacokinetic (PK) properties of tribendimidine in African school-aged children, the main target group of preventive chemotherapy. A tight sampling scheme was applied, in the framework of a dose-ranging study (100 to 400 mg tribendimidine), using dried-blood-spot technology. dADT and adADT were quantified using liquid chromatography tandem mass spectrometry. The PK parameters were calculated by noncompartmental modeling. Logistic regression was used to obtain an insight into PK/pharmacodynamic (PD) behavior.

RESULTS

Accuracy, precision, recovery, and matrix effect. After slight modifications made to the dADT and adADT sample preparation, the accuracy and precision of analyte extraction were evaluated and summarized in Table S1 in the supplemental material. The extraction method quantified dADT across the dynamic range within the limits of the FDA guidance. Concentrations were within 94% to 97% of the nominal concentrations within one measurement of standards, showing most variation at the lower limit of quantification (LLOQ) with a relative standard deviation (SD) of 14.4%. The accuracy of two measurements combined was between 98% and 99%, with the highest variation at the LLOQ with 13.6%. Similarly, adADT was within the acceptance range with measured concentrations between 94% and 105% of the nominal values with the LLOQ again showing the highest relative standard deviation of 12.4%. The interassay accuracy was between 95% and 101%, with the highest variation of 14.5% at the LLOQ.

As summarized in Table S2 in the supplemental material, recovery experiments showed dADT to be recovered to approximately 91% from dried blood spots of spiked whole-blood standards, with a slight tendency for higher recovery at low concentrations (97%) than at higher concentrations (84%). However, the overall relative standard deviation was 9.7%, which indicated an acceptable range of deviation. The matrix effect of dADT was 82% with a variation of 8.7%, showing no dependence on analyte concentration. The recovery of adADT was 105% $\pm$ 11.2%, and similarly to dADT, showing a slight tendency for higher recovery at lower concentrations (109%) than at higher concentrations (102%) with a maximum variation of 16.6% at low concentrations. The matrix effect of adADT was independent of concentration with a mean value of 69% and a variation of 9.9%.

Number and characteristics of study participants. A total of 102 eligible participants participated in the PK study. We analyzed the dried blood spots of 101 children,

TABLE 1 Participant characteristics

Characteristic	Tribendimidine dose		
	100 mg	200 mg	400 mg
No. of participants	34	33	34
No. of girls	13	8	10
Mean (SD)			
Age (yr)	9.0 (2.2)	9.2 (2.2)	8.8 (2.4)
Ht (cm)	129 (14)	128 (11)	123 (14)
Wt (kg)	27 (6)	25 (7)	24 (6)
Egg reduction rate (%)	57	81	92
Cure rate (%)	19	42	53

of which all 11 time points were collected ($n = 98$) or missing one time point ($n = 3$), excluding the samples of one child, of which we had an incomplete set of only four time points. The PK analysis was performed on 34 children which had received 100 mg tribendimidine, 33 which had received 200 mg, and 34 children which had obtained 400 mg. Each treatment group involved a similar distribution of sexes and were of similar age, height, and weight (Table 1).

Pharmacokinetic profiles. In 99% of all samples taken between the first (1 h) and the last (22 h) posttreatment, both analytes could be quantified at concentrations above the respective LLOQs. Six percent of the samples between the first and the last sample collected after treatment were diluted and remeasured due to dADT concentrations exceeding the ULOQ.

The PK concentration-over-time profiles of mean dADT and adADT values after oral administration of ascending tribendimidine doses are depicted in Fig. 1 and Fig. 2, respectively. The mean maximal concentrations (C_{max}) of dADT were 666 ng/ml after administration of 100 mg tribendimidine measured 1 h after treatment (T_{max}), 850 ng/ml 3 h after administration of 200 mg tribendimidine, and 1,689 ng/ml 2 h after administration of 400 mg tribendimidine. As for adADT, C_{max} was 108 ng/ml 2 h after the administration of 100 mg tribendimidine, 154 ng/ml 2 h after administration of 200 mg, and 307 ng/ml 3 h after administration of 400 mg tribendimidine. Three children showed an atypical PK profile: the dADT concentrations did not exceed 105 ng/ml during 1 h to 8 h after tribendimidine administration, but C_{max} was measured 22 h after treatment with an average of 342 ng/ml (287 to 422 ng/ml). All three children belonged to the treatment arm, which had received 200 mg tribendimidine.
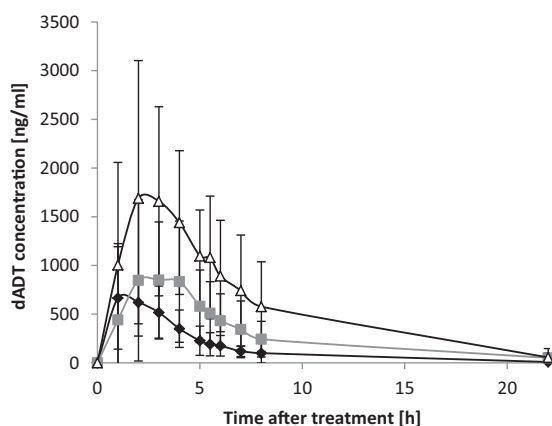

FIG 1 Mean blood concentrations with standard deviations of dADT after a single oral dose of 100 mg tribendimidine (black diamonds with black line, $n = 34$), 200 mg tribendimidine (gray squares with gray line, $n = 33$), or 400 mg tribendimidine (white triangles with black line, $n = 34$).

FIG 2 Mean blood concentrations with the standard deviations of adADT after a single oral dose of 100 mg tribendimidine (black diamonds with black line, $n = 34$), 200 mg tribendimidine (gray squares with gray line, $n = 33$), or 400 mg tribendimidine (white triangles with black line, $n = 34$).
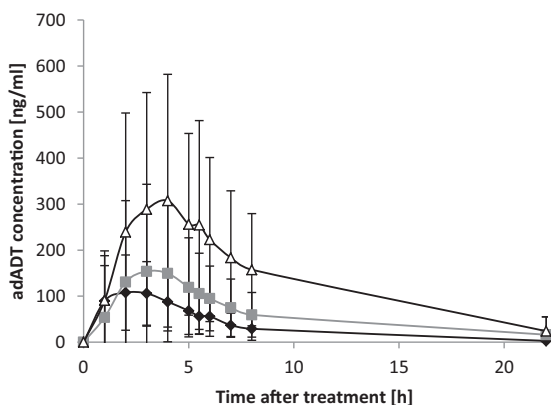
Pharmacokinetic parameters. Pharmacokinetic parameters of dADT and adADT were calculated for each child, and median values with ranges of the 25th and 75th percentiles of each treatment group are presented in Table 2. Median dADT C_{max} values ascended in a less than proportional manner; the slope of the power model was 0.52 (90% confidence interval [CI]: 0.30, 0.72). Values for the area under the blood concentration-time curve for 0 to 22 h (AUC_{0-22}) increased in a dose-proportional manner with a slope under the power model of 0.86 (90% CI = 0.68 to 1.03). Little of the area to calculate AUC_{∞} was extrapolated as AUC_{0-22}, which was approximately equal to AUC_{∞}. As such, dADT was cleared dose-independently at rates of 31 liters/h (200-mg dose) to 33 liters/h (100- and 400-mg doses). dADT's half-life remained comparable for all the dosages with values of 3.7 and 3.8 h. The mean residence time (MRT) of dADT increased from 4.3 h (100-mg dose) to 5.6 h (200-mg dose).

The PK parameters of adADT revealed less systemic exposure than its precursor molecule dADT, as presented in Table 2. Median C_{max} values of adADT were 111 ng/ml after treatment with 100 mg and 124 ng/ml after treatment with 200 mg tribendimidine, whereas treatment with 400 mg tribendimidine resulted in a C_{max} of 297 ng/ml adADT. The T_{max} of adADT was at 2 h after administration of 100 mg tribendimidine, whereas the T_{max} for the 200-mg and the 400-mg treatment arms were at 4 h posttreatment: the adADT values for AUC_{0-22} and AUC_{∞} were 797 and 683 h · ng/ml, respectively, after 100 mg tribendimidine administration, 1,211 and 1,260 h · ng/ml,

TABLE 2 Pharmacokinetic parameters of dADT and adADT after treatment with 100, 200, or 400 mg tribendimidine

Compound	Parameter	Median (25th–75th percentile range)		
		100 mg	200 mg	400 mg
dADT	C_{max} (ng/ml)	853 (693–1,115)	1,530 (1,150–1,960)	2,275 (1,618–2,875)
	T_{max} (h)	1 (1–2)	3 (2–4)	3 (2–5)
	AUC_{0-22} (h · ng/ml)	3,019 (2,666–4,221)	6,467 (5,231–7,457)	12,530 (10,597–14,886)
	AUC_{∞} (h · ng/ml)	3,059 (2,753–4,422)	6,638 (5,610–7,613)	12,530 (11,311–15,558)
	$AUMC_{\infty}$ (h² · ng/ml)	13,249 (10,615–22,175)	35,986 (29,564–53,870)	75,341 (59,252–94,861)
	$t_{1/2}$ (h)	3.7 (3.5–4.2)	3.8 (3.3–4.1)	3.7 (3.2–4.3)
	MRT_{∞} (h)	4.3 (3.8–5.1)	5.6 (4.2–6.7)	6.1 (4.7–6.6)
	CL/F (liters/h)	33 (24–38)	31 (27–38)	33 (27–38)
adADT	C_{max} (ng/ml)	111 (46–216)	124 (71–326)	297 (124–546)
	T_{max} (h)	2 (1–3)	4 (2–5)	4 (3–5.5)
	AUC_{0-22} (h · ng/ml)	683 (267–1,223)	1,211 (488–2,015)	2,791 (970–3,775)
	AUC_{∞} (h · ng/ml)	695 (290–1,293)	1,260 (545–2,238)	3,023 (986–4,583)
	$AUMC_{\infty}$ (h² · ng/ml)	3,914 (1,612–7,045)	8,772 (4,024–15,274)	21,472 (7,029–33,091)
	$t_{1/2}$ (h)	3.8 (3.2–5.0)	3.7 (3.6–4.6)	3.8 (3.5–5.0)
	MRT_{∞} (h)	5.4 (4.8–6.3)	6.7 (5.7–7.8)	7.3 (5.8–8.0)

respectively, after 200 mg, and 2,791 and 3,023 h · ng/ml, respectively, after 400 mg. Area under the first moment curve from 0 h to infinity (AUMC$_\infty$) values increased from 3,914 h^2 · ng/ml (100-mg dose) to 8,772 h^2 · ng/ml (200-mg dose) and to 21,472 h^2 · ng/ml (400-mg dose). adADT's half-life remained comparable for all the dosages with values of 3.7 and 3.8 h. The MRT was prolonged in a linear manner ($R^2 = 0.819$) from 5.4 h (100-mg dose) to 6.7 h (200-mg dose) and to 7.3 h (400-mg dose).

Pharmacokinetic-pharmacodynamic relationship. There was no evidence of an effect of C_{max} or AUC on egg reduction rate or cure status using logistic regression for analysis.

dADT acylation to adADT. There was no indication for metabolism of dADT to adADT to be influenced by dose based on C_{max} and AUC$_{0-22}$ values (see Table S3 in the supplemental material). The dADT C_{max} was observed at 1.4- to 35.4-fold higher concentrations than those of adADT, with a median ratio of 7.2, without a significant dose-dependent trend. As for AUC$_{0-22}$ values, the dADT ratios ranged from 1.5 to 33.1, with a median ratio of 4.6 and no statistical difference between the dosage groups.

Adverse events-PK relationship. Of the 101 children participating in this PK study, 28 children stated ill-being after treatment with one of the three tribendimidine treatment arms: 10 children had received 100 mg tribendimidine, 8 children had received 200 mg tribendimidine, and 10 children had received 400 mg tribendimidine. We could not identify a relationship between adverse events and dADT and adADT exposure, since the children with adverse events displayed the same distribution of drug exposure found in the overall study population.

DISCUSSION

Tribendimidine is a frontrunner in the anthelminthic drug discovery and development pipeline. Alternative anthelminthics are essential to alleviate drug pressure on albendazole. We contributed the efforts to make tribendimidine globally accessible for the treatment of helminth infections and conducted for the first time a PK study in hookworm-infected children treated with tribendimidine. The PK study was embedded in a dose-finding study (J. Coulibaly et al., unpublished data) aiming to support the decision on the accurate dose to treat this population and to further elucidate the PK-pharmacodynamic relationship of tribendimidine treatment against hookworm infection. For the PK sample collection, we used the dried-blood-spot technique and analytical quantification method of dADT (active compound) and adADT (inactive metabolite of dADT) as described earlier by Duthaler et al. (13, 14). The few adaptions made to the protocol did not compromise the quality of the measurements. Our data show a less-than-proportional to proportional increase of systemic dADT exposure when administering doses of 100, 200, or 400 mg tribendimidine. The nonaccumulative behavior of dADT across all three doses assessed was evident by unchanged clearance rates and dose-independent turnover rates of dADT to adADT. Similarly, Duthaler et al. described subproportional to proportional dADT exposure following a single 25- to 400-mg tribendimidine doses in Lao adults infected with *Opisthorchis viverrini*. The dose of 600 mg, however, did not lead to notably higher exposure than the 400-mg dose (13). The PK dose ranging studies of tribendimidine conducted so far imply that the enzymes responsible for dADT degradation are not saturated at a dose of up to 400 mg tribendimidine.

The varying PK profiles of tribendimidine among patients call for caution. We observed large interindividual differences for T_{max} (1 to 22 h postadministration) and variable C_{max}. In particular, three profiles were atypical in that the C_{max} was reached as late as 22 h posttreatment. We do not know whether this is due to biological differences of these participants, for instance due to polymorphism of the acetyltransferase responsible for dADT acetylation (9, 15) or floating of the tablets in the stomach due to the enteric coating (13). We took a food effect into account by regulating the food intake at the day of treatment to minimize variation in stomach emptying, compound liberation, and absorption, and yet obviously floating properties could not be controlled. There is therefore a need to develop an optimal formulation of

tribendimidine for the treatment of soil-transmitted helminthiasis and opisthorchiasis and to investigate in PK studies whether an enteric coating is required. We observed few children chewing the tablets, hence destroying the enteric coating. When analyzing their PK profiles we observed a tendency for a higher and earlier C_{max} and no difference in efficacy and tolerability. However, further studies are required to confirm this finding.

When comparing the drug exposure of this study population to the earlier study with Lao adults diagnosed with an *O. viverrini* infection (13), we observed higher exposure after treatment with 200 or 400 mg tribendimidine, but slightly quicker elimination. In more detail, in Ivorian children we saw on average higher C_{max} (200%) and AUC (120%) values; however, the AUMC values were lower (70%). Also, T_{max} and $t_{1/2}$ were shorter (around 50%). The clearance was the same (400-mg dose) or elevated (140%; 200-mg dose) (13).

Comparing systemic dADT exposure of our study population to those of healthy adult Chinese volunteers, which had been given the recommended dose of 400 mg, we observed lower C_{max} levels in Ivorian children (36%), but higher AUCs (290%). The T_{max} and $t_{1/2}$ were again shorter in our study cohort (around 75%) than in healthy adult Chinese subjects (9). These data demonstrate that Ivorian children show higher dADT exposure than other populations studied so far. This is an unexpected result, since population PK analysis of the data set of the Lao population predicted less systemic drug exposure in children due to higher drug clearance the younger the treated patients (12). However, these variations cannot be fully attributed to differences in age or ethnicity since differences in the provided meals might induce flotation of the tablets and thus have a strong influence on tribendimidine's PK properties. Population PK modeling has been launched with our data set as was done with the studies from Laos (12).

No evidence of an effect of C_{max} and AUC on efficacy was observed. A higher systemic dADT exposure did not correlate with cure of hookworm infection or decreasing egg expulsion. It might be worth highlighting that in our study most hookworm infections were of light intensity, which might lead to a slight bias due to diagnostic limitations as cure rates might have been overestimated. Nonetheless, it is likely that intestinal concentrations of tribendimidine are responsible for activity on intestine-residing helminths rather than the absorbed fraction. Few data are presently available to aid in understanding the PK/PD relationship. PK studies in rodents have not been conducted to date. Moreover, the *in vitro* activity of dADT against *Necator americanus* (the hookworm species at the study site; unpublished observations) has not been elucidated. The 50% inhibitory concentration of dADT against adult *Ancylostoma ceylanicum in vitro* is high (>100 μg/ml) (16). Hence, relevant data are not yet available to conclude on a PK/PD relationship.

On the other hand, in Laotians infected with *O. viverrini*, which resides in the host's bile duct, dADT exposure correlated with cure (13). There is a strong need to develop a better understanding of the relationship between PK parameters and efficacy for anthelminthics used to treat gastrointestinal nematodes (17). To our knowledge, our study is the first to provide knowledge on the PK and PD for the treatment of hookworm infections in humans. Similar studies should be conducted with standard treatments (i.e., albendazole and mebendazole) to better understand the pharmacology of these drugs and to identify PK markers correlating with toxicity. In the present study, the adverse events reported by the study participants could not be correlated to high doses or elevated pharmacokinetic parameters of dADT or adADT. Nevertheless, we should continue to carefully assess side effects after treatment with tribendimidine and examine the drug exposure of these individuals for a more representative statistical evaluation.

In summary, our PK study in school-aged children showed no relationship between dADT exposure and drug efficacy (cure and egg reduction rate) and toxicity. Higher systemic dADT exposure compared to adult populations and a less than proportional as well as a proportional exposure in dADT's C_{max} and AUC was observed. Future studies would benefit from including patients with high infection intensities to rule out

a potential influence of disease burden on the pharmacokinetics and pharmacodynamics of tribendimidine. Moreover, an optimization of the tribendimidine formulation might be envisaged to minimize the risk of food effects.

MATERIALS AND METHODS

Ethical approval, participant selection, and treatment. This PK study was embedded in a dose-finding study of tribendimidine for pediatric use to treat hookworm infection, which is fully described elsewhere (Coulibaly et al., unpublished). The clinical trial including the PK study was approved by the ethical authorities of Switzerland (Ethikkommission Nordwest- und Zentralschweiz, project ID 2017-00139) and the Republic of Côte d'Ivoire (Comité National D'Éthique de la Recherche, reference number 053//MSHP/CNER-kp). Eligible children to participate in the PK study had provided assent and informed consent of their legal guardian, were hookworm positive, were aged between 6 and 12 years, and no clinical concerns regarding study participation were present. Eligible study participants were randomized into three treatment arms of equal size and with an equal distribution of infection intensities. The active treatment arms were single dosages of 100, 200, or 400 mg tribendimidine. Tribendimidine tablets of 50 mg were used for the 100-mg dose. Tablets (200 mg) were used for the 200- and 400-mg doses. All tribendimidine tablets were obtained from Shandong Xinhua Pharmaceutical Corporation, China, and had an enteric coating. The participants were asked to eat no breakfast at the day of treatment. After swallowing the tablets, the children received a small snack (biscuits) and lunch (rice, fish, and vegetable oil) approximately 4 h after treatment.

Collection of capillary blood using died blood spots. Fingers of participants were sanitized and pricked using single-use lancets (e.g., Accu-chek Safe-T-Pro Plus; Roche Diagnostics, Rotkreuz, Switzerland). Blood was collected with heparinized glass capillaries (75 μl; Carl Roth GmbH, Arlesheim, Switzerland), dropped onto filter paper cards (Whatman 903 Protein Saver Snap Apart Card; GE Healthcare, United Kingdom), and air dried for at least 5 h before storing in plastic bags containing desiccant, as described by Duthaler et al. (13). Samples were taken before treatment (T_0), and 1, 2, 3, 4, 5. 5.5, 6, 7, 8, and 22 h after treatment. The samples were stored at ambient temperature (26°C) in Côte d'Ivoire for 4 days and then at −20°C after transport to Switzerland, which have been proven to be stable storage conditions (12, 14), until sample preparation for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

LC-MS/MS equipment and conditions. For high-performance liquid chromatography, a system (Shimadzu, Kyoto, Japan) was used consisting of two LC-AD pumps, an online DG-3310 degasser (Sanwa Thusho, Tokyo, Japan), a CTC HTS PAL autosampler for cooling to 10°C (CTC Analytics, Zwingen, Switzerland), a diverter-valve (Vici Valco Instruments, Schenkon, Switzerland), and a loop for a 10-μl injection volume. Chromatographic separation was performed with a Kinetex core shell pentafluorophenyl column (50 by 4.6 mm; 2.6 μm, 100 Å; Phenomenex, Basel, Switzerland) at 38°C at a flow rate of 0.75 to 1 ml/min. The separation was achieved as described by Duthaler et al. (14), using gradients of an aqueous phase (phase A; 10 mM ammonium acetate and 0.15% formic acid in Milli-Q water) and an organic phase (phase B; 10 mM ammonium acetate and 0.15% formic acid in acetonitrile). All eluents and additives were purchased from Merck Millipore (Schaffhausen, Switzerland) and were of LC-MS-grade. The following elution profile was applied: 90% B between 0 and 2.5 min, linear decrease to 5% B within 0.5 min and continued isocratic elution for 1 min, and linear increase to 90% B within 0.5 min and continued isocratic elution for 1.5 min.

For mass spectrometry, an API 3200 instrument (AB Sciex, Framingham, MA) was used. The compounds were ionized in positive mode and detected by multiple-reaction monitoring at m/z of 178.1/133.2 for dADT, m/z of 220.2/175.0 for adADT, m/z of 184.2/for deuterated dADT (dADT-d6), and m/z of 226.2/175.00 for deuterated adADT (adADT-d6).

Preparation of standards. Standards of dADT and adADT were prepared similar to the protocol described by Duthaler et al. (13, 14). dADT and adADT were donated by Shandong Xinhua Pharmaceutical Company (Zibo, People's Republic of China). dADT-d6 and adADT-d6 were purchased from Toronto Research Chemicals (Ontario, Canada). Stock solutions of 1 mg/ml adADT and dADT dissolved in methanol were diluted in acetonitrile and Milli-Q H_2O (50:50) to serial dilutions, which were further diluted 30-fold in fresh blood from six different donors (donated by the blood bank of Canton Basel-Stadt, Switzerland), which had been adjusted to six different hematocrits (30, 35, 40, 45, 50, and 55%). The reason for not using the blood of only one donor or pooled blood is the concern of the dried-blood-spot technique to be patient and hematocrit dependent (18). The final concentrations of the calibration curves were 2,000, 1,000, 750, 500, 250, 100, 75, 50, 25, 10, and 3 ng/ml for dADT and 2,000, 1,000, 500, 250, 100, 50, 25, 10, 5, 2.5, and 1 ng/ml for adADT, using blood with a hematocrit of 40%. The concentrations of the quality controls included high, middle, and low concentrations within the dynamic range, as well as an LLOQ sample. The concentrations of the quality controls were 1,500, 150, 9, and 3 ng/ml for dADT and 1,500, 150, 3, and 1 ng/ml for adADT. All quality control samples were prepared in the six blood samples from the six donors that had been adjusted to the six different hematocrit values. Blank blood was used for double-blank samples (extraction of analyte-free blood without addition of internal standards) and blank samples (extraction of analyte-free blood following the normal procedure).

Sample extraction. The extraction method was adapted from Duthaler et al. (13, 14). Two disks 3 mm in diameter were punched out of the DBS filter paper and placed in a 1.5-ml Eppendorf tube. The internal standards dADT-d6 and adADT-d6 were prepared from 500-μg/ml stock solutions in methanol. The aqueous extraction solvent (50 μl of H_2O containing 0.2% formic acid and 10 ng/ml internal standard) was added to the dried blood spots, the samples were vortex mixed to reliably moisten them

and left at room temperature for 30 min. Then, the tubes were agitated for 20 min at 1,400 rpm and 21°C (Thermomixer R; Eppendorf, Hamburg, Germany). Organic extraction solvent (150 μl of acetonitrile) was added, vortex mixed, and ultrasonicated for 10 min. Double-blank samples were processed in the same manner, except for using extraction solvents not containing internal standards. The samples were centrifuged at 14,000 rpm (5415R centrifuge; Eppendorf) for 10 min at 21°C, and the supernatant was placed in a 96-deep well plate (deep well, 500 μl; Eppendorf), which was sealed with a sealing mat (Eppendorf) and kept at room temperature until analyzed.

Quantification. Analyst 1.6.2 software (AB Sciex) was used for peak integration and calculation of the calibration curves from at least eight calibrators using weighted linear regression ($y = 1/x^2$). Samples exceeding the upper limit of quantification were diluted with double-blank samples.

Method validation. The extraction method for both analytes, dADT and adADT, underwent a partial validation, since the extraction method was slightly adapted from the fully validated method (14). For this study, we tested the intra- and interassay accuracy and precision, as well as the recovery and matrix effect. To determine the intra- and interassay accuracy and precision, we processed two sets of standards as described above. The accuracy was calculated as the percentage of the measured concentration compared to the nominal concentration. The precision was calculated as the percentage of the standard deviation of multiples compared to their average value. For intra-assay accuracy and precision, the averages of six replicates were used; for interassay accuracy and precision, the average of 12 replicates was used. We followed the US FDA-guidance for industry for bioanalytical method validation (19). The guidance recommends limits for quantification of analytes of the high, middle, and low concentrations to be within ±15% of their nominal concentration or ±20% for the LLOQ. The recovery was tested by comparing the area under the curves of the analytes after their extraction from blood (normal procedure) to the area under the curves of the analytes when added after extraction of blank blood. The matrix effect was tested by comparing the area under the curves of the analytes when added after extraction of blank blood to the area under the curves of the analytes, which were added to extraction solvent.

Pharmacokinetic and statistical analyses. The PK parameters of dADT and adADT were determined for each patient, with a noncompartmental analysis using the software WonNonlin (version 5.2; Certara, Princeton, NJ). Maximal concentration (C_{max} [nanograms per milliliter]) values and the time at C_{max} (T_{max} [hours posttreatment]) were observed values. The area under the blood concentration-time curves (AUC [hours multiplied by nanograms per milliliter]) and the area under the first moment curve (AUMC [hours squared multiplied by nanograms per milliliter]) were calculated using the linear trapezoidal rule. We calculate the AUC values of the all observed analyte concentrations between sampling time point 0 h and time point 22 h after treatment (AUC_{0-22}), as well as the AUC and AUMC values of the extrapolated total drug exposure from time point 0 h to infinity (AUC_∞ and $AUMC_\infty$) for curves with at least three observations in the elimination phase. Also the analytes' half-life ($t_{1/2}$ [hours posttreatment]) was calculated if at least three observations were available in the elimination phase. The mean residence time (MRT [hours]) was calculated as the ratio of $AUMC_\infty$ to AUC_∞. Drug clearance (CL/F [liters per hour]) was determined by dividing the dose by the AUC_{0-22}. Dose proportionality was assessed using the power model using a confidence interval of 90% (SAS for Windows, version 9.4). For each treatment arm, the 50th percentile was calculated as the representative value plus the 25th and 75th percentiles as indicators of the range of values using Microsoft Excel.

The egg reduction rate was calculated as the decreased percentage of geometric mean egg counts found at follow-up compared to the geometric mean egg count found at baseline using the quadruple Kato-Katz method. The cure rate was defined as the percentage of patients that were hookworm negative at follow-up. To evaluate relationship between pharmacokinetics and pharmacodynamics, the cure status was analyzed using univariate logistic regression using C_{max} and AUC as predictors (SAS for Windows, version 9.4). The egg reduction rate was modeled using univariate beta regression using the same predictors as for cure status. Statistical significance was declared if the P value for the predictor was <0.05.

Dose dependence of metabolic turnover of dADT to adADT was assessed by comparing dADT to adADT ratios among treatment groups and determining their differences using the Mann-Whitney U test (StatsDirect software, version 2.7.7).

We furthermore evaluated the systemic drug exposure as a cause for adverse events. In brief, all study participants were asked about their well-being prior to treatment, 3 h after treatment, and 24 h after treatment to monitor acute drug-related effects. The participants were asked about the presence (on a scale) or absence of headache, stomach ache, itching, thrill, nausea, vomiting, and diarrhea, as well as sensation of fever and allergic reactions. To evaluate the effect of dADT and adADT's pharmacokinetic parameters (C_{max}, AUC, AUMC, and MRT) as a cause for ill-being, we compared their magnitude (25th, 50th, and 75th percentiles) for the individuals who had stated the presence of ill-being at any time after treatment to the overall values of their respective treatment group.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC .00959-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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