Research Article

Identification of Metabolites Derived from the Anti-trypanosomal Drug Megazol

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Abstract

Background: African Trypanosomiasis is an endemic vector-borne parasitic disease in sub-Saharan Africa. It is caused by different parasites of the genus *Trypanosoma* and is transmitted through a tsetse fly (*Glossina sp*.) bite during a blood meal. This neglected tropical disease remains difficult to control due to the complexity of treatment protocols and use of toxic drugs. Over the decades, nitroimidazole compounds have been promising molecules for anti-parasite therapy. One of them, megazol, has proven to be an effective anti-trypanosomal drug, but interest dropped after reports were published concerning its mutagenic properties. Objectived: We therefore decided to characterize and identify megazol metabolites, with the hypothesis that they could be less toxic. Methods: We treated groups of mice with different derivatives and then detected metabolites by high performance liquid chromatography combined with mass spectrometry in urine, feces, and plasma samples from mice. Results: *In vivo* results showed that eleven metabolites were detected in urine (M1 to M11); six metabolites were detected in plasma (M1a/b, M2a/b, M5, M7a/b M9, andM10a/b) and in feces, only two (M1 a/b and M5) were found. Conclusions: The structures of metabolites were deduced using chromatograms and mass spectra data combined with usual metabolic patterns.

Introduction

African Trypanosomiasis is an endemic Neglected Tropical Disease in 36 sub-Saharan Africa countries. It is a zoonosis, called either Human African Trypanosomiasis (HAT) or Animal African Trypanosomiasis (AAT). The disease is transmitted by the bite of a tsetse fly and different *Trypanosoma* species can cause pathology. Both disease forms are fatal if left untreated (Büscher *et al.* 2017). For HAT, 2 subspecies are responsible for 2 different disease forms: (*i*) Gambian sleeping sickness corresponds to an infection by *Trypanosoma brucei (T. b.) gambiense*, characterized by chronic disease with the average infection lasting around 3 years, and (*ii*) Rhodesian sleeping sickness corresponds to an infection by *T. b. rhodesiense*, characterized by an acute disease in which death can occur within weeks of infection. The infection progresses from the haemolymphatic stage where parasites spread to the lymphatics, blood, and peripheral organs, to the late encephalitic stage where parasites invade the central nervous system (CNS) to cause a serious meningo-encephalitis that can result in death. For AAT, there is a diversity of parasites and animals, but the sympto-

matology is the same (Franco *et al.* 2017).

Until 2018, chemotherapy for HAT relied on five drugs including 2 drugs (Suramin, Pentamidine) that are active in the early disease stage and 3 drugs (Melarsoprol, Eflornithine and Nifurtimox–Eflornithine combination therapy (NECT)) that are effective during CNS invasion. These drugs are available for patients through a donation from SANOFI (a global biopharmaceutical company focused on human health), but there are still many drawbacks to their use. The main drawbacks are: (1) high toxicity for the hosts, which is mainly due to their poor selectivity for parasite cells compared to mammalian cells; (2) route of administration (intramuscular or intravenous injections); (3) very narrow anti-trypanosomal spectrum; and (4) high cost of hospitalization (WHO, 2019). Overall, although active, these drugs are not ideal for use in rural settings, drug resistances are spreading and there is a need for an oral form of a drug that could be used for both disease stages. Clearly, improved chemotherapy with better selectivity for trypanosomes is needed to control this disease. Unfortunately, very few new drugs were proposed; only two candidates were selected. Fexinidazole, 2-substituted 5-nitroimidazole, belonging to the nitroimidazole class of drugs, exhibits trypanocidal properties, and has the potential to become a safe, short-course oral treatment for both HAT stages (Torreele *et al.* 2010). Its *in vivo* activity is most likely due to its two major metabolites, oxide and sulfone (Kaiser *et al.* 2011). The other potential candidate, Acoziborole or SCYX-7158 has proven highly effective as a single dose oral treatment for both HAT stages (Jacobs *et al.* 2011) and is in phase 2 of clinical trials (DNDI, 2019). Since November 2018, the European Medicines Agency (EMA) agency recommends the approval of fexinidazole as the first line of treatment by oral administration against *T.b.g* (DNDI, 2019), but nothing has been proposed against *T.b.r*. For AAT, the therapy is old and numerous resistances occur, but no new treatment exists. It is important to develop new medications to treat HAT and AAT to reduce cases of resistance and work towards the elimination of the disease.

Among nitro-heterocyclic compounds known for their anti-trypanosomal activity and their abilities to be selectively activated by parasitic nitroreductases (NTRs) to generate cytotoxic metabolites, megazol, was a promising compound that diffused easily into the CNS and could be used orally. However, its development was discontinued due to reports of *in vitro* mutagenicity (Ferreira & Ferreira 1986), and genotoxicity (Poli *et al.* 2002, Nesslany *et al.* 2004), but its pharmacological mechanism of action still remains. In fact we only know that trypanocidal and mutagenic effects of megazol are due to the parent drug and/or its metabolites (de Morais *et al.* 1998). The few tests done on 3 *Salmonella* strains with murine plasma and urine containing metabolites of megazol showed that two were mutagenic in plasma and three in urine, suggesting that megazol metabolites present in the systemic circulation and in urine might differ and be associated with different effects. Recently, Enanga *et al.* (2000) found 4 different metabolites of megazol in primate urine, but further identification could not be performed.

The aim of the present was to determine megazol metabolites and, notably, characterize these metabolites in biological fluids of mice for futures studies.

Materials & Methods

Animals and protocol administration

Twenty-five female mice (weighing 30g) of 8 weeks old were clinically healthy 1 month prior to experimentation. The first group (n = 10) was treated with a suspension of ^{13}C megazol. It was administered *per os* in a 50 µL final volume of an aqueous solution of 2% low viscosity carboxymethylcellulose (Merck, Germany) at a dose of 60 mg/kg body weight. ^{13}C-megazol was used to control the structure of detected metabolites. The second group (n = 10) was treated by the same dose of unlabeled megazol. The third group (n = 5) only received the suspension of carboxymethylcellulose without megazol. It is used like a control.

Mice were housed and procedures were conducted in agreement with european directive 2010/63/EU on animals used for scientific purposes applied in France as the 'Décret n°2012-118 du 1er février 2013 relatif à la protection des animaux utilisés à des fins scientifiques'. Accordingly, the present project was APAFIS#16655-2018090710063103 authorized by the 'Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche'. Megazol (CAS: 19622-55-0) powder was synthesized as previously described (Chauviere *et al.* 1998, 2003)

Sample collection

Urine and feces were collected from a metabolism cage (Tecniplast® Italy). These samples were

gathered prior to treatment, after megazol administration to animals on the first 12h and then for the last 12 h (a total of 24 h). For each group, urine and feces of all mice were pooled to generate a single sample and were stored at -20°C until analysis. Blood samples (0.5 mL) were collected by sinus retro-orbital puncture prior to treatment and 3h after first and last administration of megazol to animals. Blood was immediately centrifuged at 1630×*g* for 10 min at 5°C to obtain the serum samples.

Sample extraction for megazol metabolites

We optimized an extraction method previously established by Enanga *et al.* (1997), replacing dichloromethane solvent by butan-2-ol in order to obtain better metabolite recovery. Urine (100 µL) was vortexed with 20 µL NaOH (1N, AVS Titrinorm®; Fontenay-sous-Bois, France) then left to stand 5 min. Butan-2-ol (5mL)(Prolabo Rhone Poulenc, Paris, France) were added and the mixture was kept under rotary stirring (25 rpm, 65°C; Rotator SB3, Stuart®, Stone, Staffordshire, United Kingdom) for 20 min and then centrifuged for 10 min at 3 500 rpm. The homogeneous liquid was further collected (alkaline extract) and the precipitate underwent an acid extraction by the addition of 65 µL HCl (1N AVS TITRINORM®, Fontenay -sous-Bois, France) followed by vortexing. Samples were then left to stand for 5 min and 5 mL of butan-2-ol were added, kept under rotary stirring for 20 min, centrifuged 10 min, and the non-precipitated acid extract was transferred. The alkaline and acid extracts were evaporated to dryness using a Speed-Vac® (Thermo-Fisher, Les Ulis, France) and reconstituted in 100 µL methanol/ water/acetic acid (50:50:0.1 v/v/v) by vortex agitation, just before liquid chromatography / mass spectrometry (LC-MS) analysis. For feces, the same protocol was used, with the first step consisting of adding water to dissolve feces and vortexing the product to homogenize the samples.

For the MRM method, we used the same extraction method except that we added 20 µL internal standard (Metronidazole (a nitro-heterocyclic family) at 25 ng/µL in methanol) to 100 µL initial samples.

Detection of megazol metabolites by reverse-phase chromatography MS/MS

The High Performance Liquid Chromatography (HPLC) system consisted of a Shimadzu LC 10 ADvp pump with an SIL-Ht autoinjector and a CTO-10ASvp column oven. Chromatographic separation was performed on a Nucleosil C18 column (125 x 2.1 mm i.d., 100Å-5 µm, Macherey-Nagel; Germany). The flow rate was 0.2 mL/min. The mobile phase was a gradient of 0.1 % acetic acid in water (A) and 0.1 % acetic acid in methanol (B) programmed as follows: initial, 5 % B, increased to 50 % in 19 min, then increased to 100 % B in 1.5 min, maintained at 100 % for 2.5 min, and finally, decreased to 5% B in the last 10.5 min. The injection volume was 20 µL except for samples prepared from urine (3 µL).

Exploration and detection were carried out with a 4 000 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (SCIEX, Framingham, MA, USA) equipped with a Turbo Ion Spray electrospray ionization (ESI) source and controlled by the Analyst software version 1.6.2 (SCIEX). Ionization was achieved with electrospray in the positive ionization mode with the following settings: ion spray voltage, 5 500 V; curtain gas, 15 arbitrary units; ion source gas 1 and 2, 20 and 30 arbitrary units, respectively; collision cell exit potential, 16 V; declustering potential, 46 V. The settings of the GUS (General Unknown Screening) procedure were previously reported in details (Picard *et al.* 2009, Sauvage *et al.* 2006, 2009). Briefly, acquisition was performed in the IDA (Information-Dependent Acquisition) mode, in which MS/MS continuously switched between a survey scan acquired in the enhanced-MS mode and up to three dependent scans obtained in the enhanced product ion (EPI) scan mode.

Detection of the putative metabolites was carried out using the PeakView 2.2 software (SCIEX). The total ion current chromatogram of urine samples collected from mice receiving 60 mg/kg megazol was compared to that of the controls (0 mg/kg megazol) for additional peaks. Potential metabolites were named M1 to M11 according to their retention time and major fragments (n = 2-3) were selected for each metabolite. Collision energy (CE) settings were automatically optimized for megazol and metronidazole (SI) transitions and were set at 35 or 50 V, depending on the metabolite transitions. The MRM transitions of metronidazole (Internal Standard), megazol and potential metabolites were simultaneously monitored in the urine of the megazol (60 mg/kg) and control group in parallel for confirmation.

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Compound no.	t_R(min)	Q1 ions	Q3 ions	CE (V)	DP (V)
Metronidazole SI	9.1	172.0	128.0	21	46
			98.0	50	46
			82.0	35	46
Megazol MGZ	18.0	227.0	181.0	27	60
			197.0	25	60
			154.0	31	60
M1a	6.5	310.0	239.0	35	46
			197.0	35	46
			141.0	35	46
M2a	6.9	403.0	181.0	50	60
			269.0	35	60
			227.0	35	60
M3	8.5	243.0	226.0	35	60
			168.0	35	60
			141.0	35	60
M4	10.6	316.0	229.0	35	60
			196.0	50	60
			141.0	35	60
M1b	11.0	310.0	239.0	35	46
			197.0	35	46
			141.0	35	46
M5	11.2	239.0	197.0	50	60
			141.0	35	60
			123.0	50	60
M6	11.5	301.0	214.0	35	46
			255.0	35	46
			194.0	35	46
M7a	11.7	419.0	157.0	35	60
			243.0	35	60
			170.0	35	60
M2b	11.8	403.0	181.0	50	60
			269.0	35	60
			227.0	35	60
M8	13.2	358.0	141.0	35	60
			229.0	35	60
			196.0	35	60
M9	14.0	213.0	196.0	35	60
			167.0	35	60
			127.0	35	60
M7b	14.3	419.0	157.0	35	60
			243.0	35	60
			170.0	35	60
M10a	15.2	343.0	214.0	35	60
			140.0	35	60
M11	15.4	243.0	228.0	35	60
			213.0	35	60
			157.0	35	60
M10b	17.2	343.0	214.0	35	60
			140.0	35	60

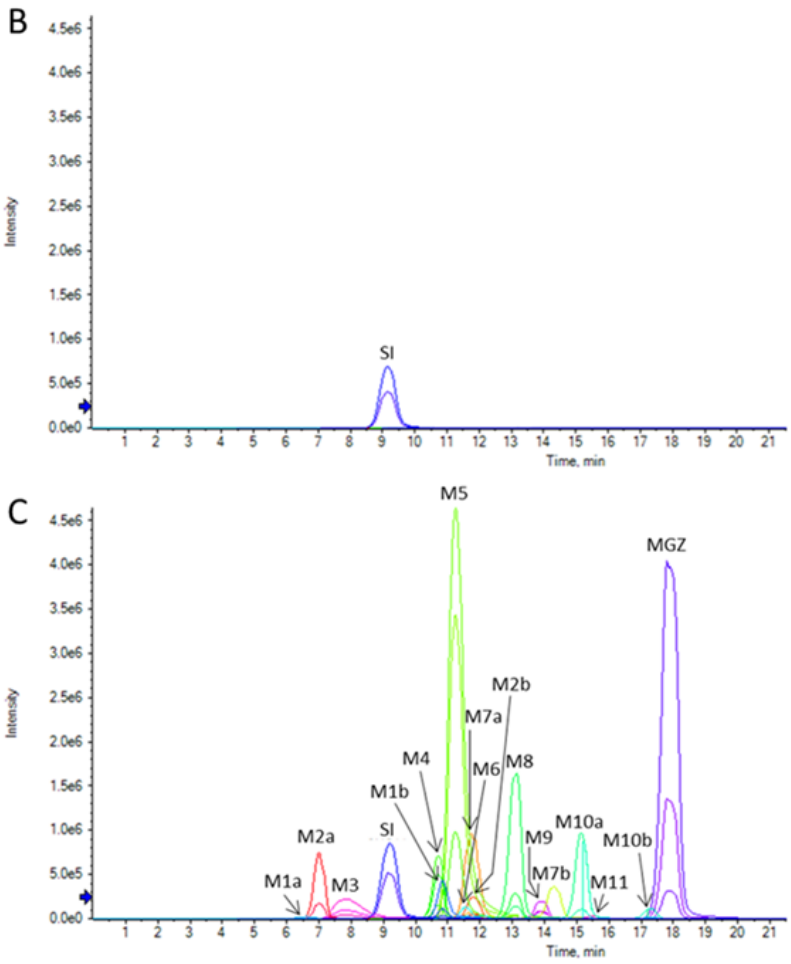

Figure 1. MRM method (A) used to detect the megazol (MGZ), its potential metabolites and the internal standard (IS, metronidazole) in control mice urine (B) and megazol (60mg/kg) mice urine (C)

Confirmation of the raw formula of putative metabolites by micro-LC QTOF

Urine samples were analysed by microLC-MS/MS using a nanoLC 425 in a micro-flow mode (Eksigent, Dublin, CA) system coupled with a TripleTOF 5 600+ (SCIEX, Framingham, MA). The chromatography was performed on a HALOfused C18 column (50 x 0.5 mm i.d., 2.7 µm; SCIEX) at a 20 µL/min flow rate. The mobile phase was water/acetonitrile/formic acid 95:0:0.1 v/v/v (A) and 5:95:0.1 v/v/v (B) gradient programed as follows: initial, 5 % B, increased to 50 % in 16 min, then increased to 95 % B in 2 min, maintained at 95 % for 3 min, and finally, decreased to 5 % B for reequilibration.

The TTOF 5 600+ was operated in an information-dependent acquisition (IDA) mode with Analyst 1.7TF software (SCIEX). MS and MS/MS data were continuously recorded with up to 15 precursors selected for fragmentation from each MS survey scan. Precursor selection was based upon ion intensity and whether or not the precursor had been previously selected for fragmentation (dynamic exclusion). Collision energies were set at 40 ± 15 eV.

Both molecular formula identification and structure elucidation were performed using the latest Sirius software (Version 4.0.1, https://bio.informatik.uni-jena.de/sirius/). First, megazol and metabolite high resolution MS/MS spectra were converted to a text file containing fragment *m/z* and intensities. The top 10 best formulas with a tolerance of 20 ppm and minimum 5 nitrogen atoms were retained for structural elucidation.

Results & Discussion

Identification of megazol metabolites

After extraction by butan-2-ol in either alkaline medium for the majority or acid medium for the rest, fifteen megazol metabolites were detected in

mice urine by the GUS method and confirmed by MRM on control and megazol groups (Figure 1). The same protocol, used with ^{13}C-megazol, had shown all metabolites with a Δ mass+1 corresponding to the presence of one ^{13}C in the megazol structure which confirmed they were related to megazol.

Once the metabolites of interest were identified and confirmed by the shift of mass due to the ^{13}C, Sirius software used MS/MS data to propose elemental composition and fragmentation pathways for the top 10 best results. Structural formula were proposed according to Sirius results and to specific neutral loss on MS/MS spectra and confronted to fragmentation patterns of structural formula generated using Mass Frontier 8.0 (Thermo Electron, San Jose, CA). Comparisons between theoretical and experimental fragmentation spectra further aided in the identification of metabolite structures and site(s) of biotransformation with the parent megazol.

Some fragmentation losses that are characteristic of nitroimidazole compounds are the following: $[M-16]^+$ corresponds to –O loss; $[M+H-30]^+$ to –NO loss; $[M+H-46]^+$ to $–NO_2$ loss; $[M+H-47]^+$ to $–HNO_2$ loss; and $[M+H-73]^+$ to $–NO_2$–HCN loss. According to MS losses and chromatographic mobility obtained with the 4000QTRAP, the metabolites were produced via one to three combined biotransformations targeting four preferred sites -NH_2, -CH_3, and -NO_2 with or without involvement of the imidazolyl-conjugated p-bond.

Megazol [m/z 227]

The fragmentation spectrum of megazol was established by infusing pure compound. The drug at m/z 227 ($[M+H]^+$) with an elemental composition of $C_6H_6N_6O_2S$ H^+ eluted at 17.8 min. The MS/MS spectrum of $[M+H]^+$ megazol displays the signature fragmentation pattern of nitroimidazole compounds ($[M-16]^+$, $[M+H-30]^+$, $[M+H-46]^+$, $[M+H-47]^+$, $[M+H-73]^+$). Sirius computation proposed megazol molecular formula as TOP 1 result with a 90.7% score, explaining 47 over 60 peaks and 93.5% of total ions intensities. After confronting Sirius and Mass Frontier results, we really assigned 57% of global ion intensities of megazol MS/MS spectrum (Figure 2).

Megazol metabolites

Table 1 lists the detailed information of these metabolites, including retention times, proposed elemental compositions, and mass errors. Spectra obtained from low and high-resolution mass spectrometry for each compound can be found in Supplementary Table 1.

Metabolites M3, M9 and M11 were generated by modifications targeting a single site of the parent drug. M9 was characterized by $[M+H]^+$ *m/z* 213, corresponding to a difference of 14 Da with megazol $[M+H]^+$ *m/z* 227, generally associated with a demethylation. Such a structure was in full agreement with the reverse phase HPLC elution order, pointing to a higher mobility for M9 (T_r 13.9 min), more polar than the parent compound (T_r 17.8 min) which was obviously slowed down by the original methyl group. The MS/MS spec-

Table 1. The predicted elemental composition of megazol and metabolites with measured m/z and mass errors. M1 and M4 m/z on HR-MS were too low to be informative. M1 formula was proposed by extrapolation with parent compound (M5) and M4 m/z (*) was reported from M8 HR-MS spectrum by homology with M6/M10.

Compound no.	Mesured *m/z*	Proposed formula	Error (mDa)	Error (ppm)
Megazol MGZ	227.0326	$C_6H_6N_6O_2S$ H+	-2.0	-8.7
M1a/b	N/A	$C_{11}H_{15}N_7O_2S$ H+	N/A	N/A
M2a/b	403.0659	$C_{12}H_{14}N_6O_8S$ H+	-0.8	-1.9
M3	243.0272	$C_6H_6N_6O_3S$ H+	-2.3	-9.4
M4	316.0664 (*)	$C_{12}H_9N_7O_2S$ H+	5.3	16.7
M5	239.0691	$C_8H_{10}N_6OS$ H+	-1.9	-7.8
M6	301.0526	$C_{12}H_8N_6O_2S$ H+	2.4	7.9
M7a/b	419.0610	$C_{12}H_{14}N_6O_9S$ H+	-0.6	-1.4
M8	358.0744	$C_{14}H_{11}N_7O_3S$ H+	2.7	7.6
M9	213.0165	$C_5H_4N_6O_2S$ H+	-2.4	-11.4
M10a/b	343.0633	$C_{14}H_{10}N_6O_3S$ H+	2.5	7.3
M11	243.0668	$C_7H_{10}N_6O_2S$ H+	0.9	3.8

trum of M9 at *m/z* 213 $[M+H]^+$ showed the presence of nitroimidazole-specific product ions ($[M-16]^+$, $[M+H-46]^+$, $[M+H-47]^+$, $[M+H-73]^+$) and like megazol, *m/z* 127. The elemental composition of *m/z* 213 proposed by Sirius was $C_5H_4N_6O_2S$ H^+ with an error of -11.4ppm and 85% of total ion intensities were assigned comparing with Mass Frontier. QTRAP data suggested that M3 and M11 $[M+H]^+$ *m/z* 243 could be structural isomers, but HR-MS data did not corroborate this hypothesis as the exact masses were too different (162.9ppm).

M3 was characterized by $[M+H]^+$ *m/z* 243, corresponding to a difference of 16 Da with megazol $[M+H]^+$ *m/z* 227 induced by an extra hydroxyl group. In comparison with megazol and M9, M3 (T_r 7.6 min) was eluted faster, must have distant OH and NH_2 polar groups to enhance aqueous solvation, so we proposed that M3 was hydroxylated on the methyl group. The MS/MS spectrum of M3 at $[M+H]^+$ *m/z* 243 showed some nitroimidazole-specific fragments ($[M+H-46]^+$, $[M+H-47]^+$ and $[M+H-73]^+$) and highly abundant product ions at *m/z* 226 $[M+H-OH]^+$, *m/z* 168 and low abundance product ions at *m/z* 141. The elemental composition of M3 *m/z* 243 proposed from HR-MS spectrum was $C_6H_6N_6O_3S$ H^+ with an error of -9.4ppm.

M11 which had the same $[M+H]^+$ *m/z* 243 with a T_r 15.5 min, eluted more slowly than M9 and M3, meaning M11 was less polar than the two others. The MS/MS spectrum of M11 at *m/z* 243 $[M+H]^+$ showed product ions at *m/z* 228, *m/z* 213 ($[M+H-30]^+$), *m/z* 186, *m/z* 157. Its elemental composition proposed from HR-MS spectrum was $C_7H_{10}N_6O_2S$ H^+ with an error of 3.8ppm.

Currently, conjugation reactions occurring with glucuronic acid, serine, alanine and acetic acid resulted in eleven metabolites: M1a,b, M2a,b, M4, M5, M6, M7a,b, M8, and M10a,b. M2a,b $[M+H]^+$ *m/z* 403 and M7a,b $[M+H]^+$ *m/z* 419, each exhibited an abundant daughter ion $[M+H-176]^+$ arising from the characteristic neutral loss of anhydroglucuronic acid or equivalent. The *m/z* 227 $[M+H-176]^+$ for glucuronides M2a,b corresponded to a protonated megazol moiety yielding, as did megazol itself, the same lower fragment ion pattern. As the parent drug offers only the outer ami-

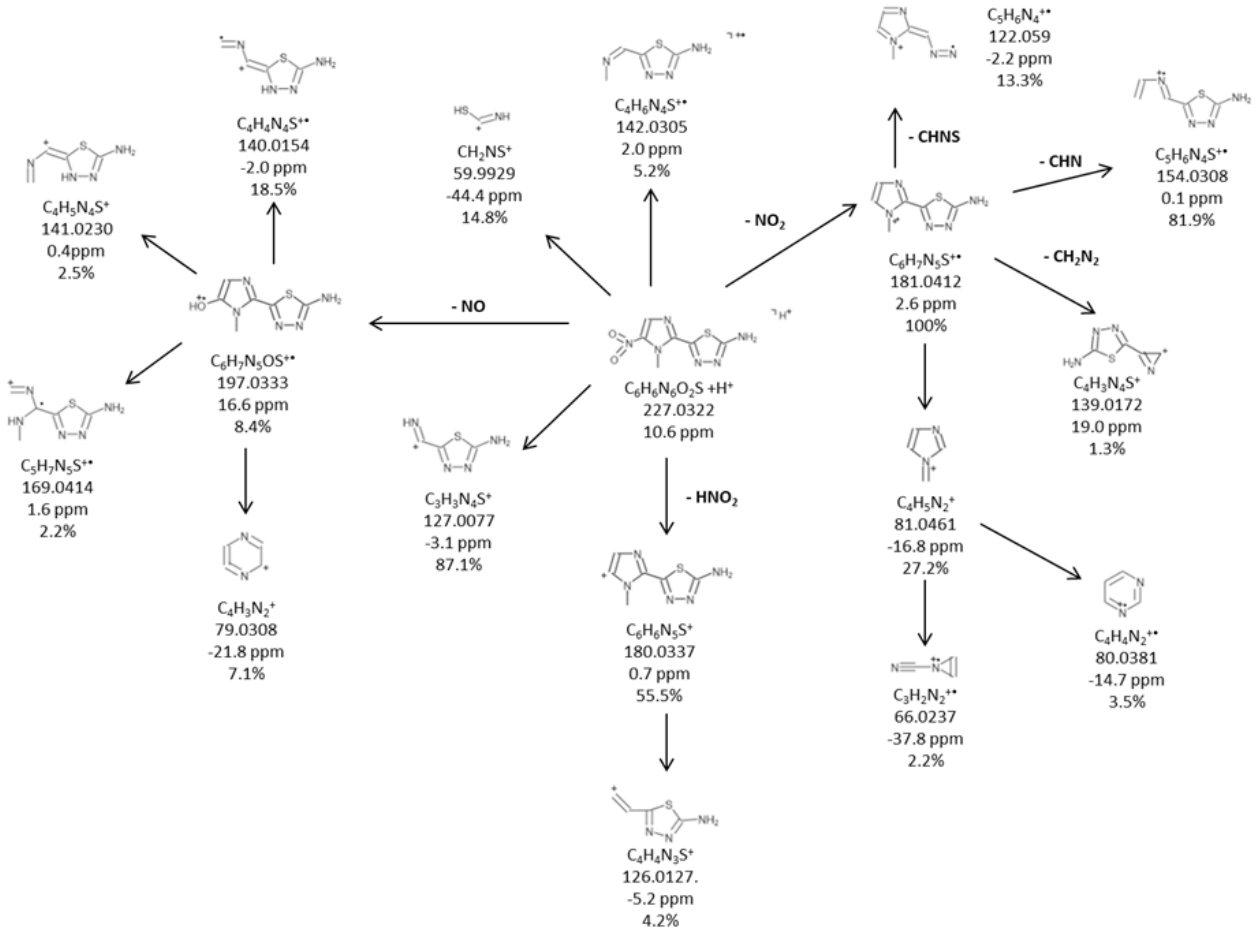

Figure 2. The proposed fragmentation pathway of megazol.

Table 2. The metabolites in different biological mouse samples (+: found / -: not found).

	Formula	Urine	Serum	Feces
Megazol	$C_6H_6N_6O_2S$ H^+	+	+	+
M1 a/b	$C_{11}H_{15}N_7O_2$ S H^+	+	+	+
M2 a/b	$C_{12}H_{14}N_6O_8$ S H^+	+	+	-
M3	$C_6H_6N_6O_3S$ H^+	+	-	+
M4	$C_{12}H_9N_7O_2S$ H^+	+	-	-
M5	$C_8H_{10}N_6OS$ H^+	+	+	+
M6	$C_{12}H_8N_6O_2S$ H^+	+	-	-
M7 a/b	$C_{12}H_{14}N_6O_9$ S H^+	+	+	-
M8	$C_{14}H_{11}N_7O_3$ S H^+	+	-	-
M9	$C_5H_4N_6O_2S$ H^+	+	+	-
M10 a/b	$C_{14}H_{10}N_6O_3$ S H^+	+	+	-
M11	$C_7H_{10}N_6O_2S$ H^+	+	-	-

no group to conjugation, the two isomers –major M2a and minor M2b– would appear to differ through the major pyrano- and the minor furanoglucuronyl parts. With 16 Da more, M7a,b were also deduced to be the pyrano- and the furano- glucuronides derived from one of the two reported metabolites M3 or M11 with $[M+H-176]^+$ *m/z* 243. M3 was selected according to the significant $[M+H-176-17]^+$ *m/z* 226 and the low fragmentation pattern. Attachment of the glucuronyl unit could occur on the amino or hydroxymethyl groups. As M7a,b had a lower mobility (M7a: T_r 11.7 min; M7b: T_r 14.4 min) when compared to that of M2a,b (M2a: T_r 6.9 min; M2b: T_r 11.8 min), attachment to the hydroxymethyl group was discarded because, with an additional free OH group, M7a,b should have been more polar than glucuronides M2a,b. The elementary composition proposed from HR-MS data was $C_{12}H_{14}N_6O_8S$ H^+ with an error of -1.9ppm for M2 and $C_{12}H_{14}N_6O_9S$ H^+ with an error of -1,4ppm for M7.

Metabolites M4 $[M+H]^+$ *m/z* 316 (T_r 10.7 min) and M8 $[M+H]^+$ *m/z* 358 (T_r 13 min) both showed the abundant daughter ion *m/z* 229 giving the same low fragmentation pattern in each case (*m/z* 227, *m/z* 196, *m/z* 168, *m/z* 141) which tended to prove that M4 and M8 belong to the same compound class. The presence of a minor fragment at *m/z* 316 $[M+H-42]^+$ on HR-MS spectrum of M8 could imply that M8 was an acetylated M4. The amino acid residue identified in the conjugation could be respectively serine ($[M+H-87]^+$ *m/z* 229) and acetylserine ($[M+H-42-87]^+$ *m/z* 229) for M4 and M8, but the *m/z* 229 derivative of megazol was not identified. The elemental composition proposed from HR-MS data with the lowest error is $C_{14}H_{11}N_7O_3S$ H^+ (7.6ppm) for M8. The precursor peak on HR-MS spectrum was too low for M4 to be informative for elemental composition. However, using the minor fragment at *m/z* 316 on M8 HR-MS spectrum, we suggested that the elemental composition of M4 corresponded to $C_{12}H_9N_7O_2S$ H^+ (16.7ppm).

Similarly to M4 and M8, isomer conjugates M10a,b $[M+H]^+$ *m/z* 343 (a, T_r 15.1 min and b, T_r 17.3 min) and M6 $[M+H]^+$ *m/z* 301 (T_r 11.6 min) exhibited a loss of acetylserine (-42-87 Da) and serine moiety (- 87 Da) resulting in the same abundant fragment *m/z* 214 (not identified). Both MS/MS spectrum showed product ions at *m/z* 255 $[M6+H-NO_2]^+$, *m/z* 212, *m/z* 140. The elemental composition proposed from HR-MS data with the lowest error was $C_{14}H_{10}N_6O_3S$ H^+ (7.3ppm) for M10 and $C_{12}H_8N_6O_2S$ H^+ (7.9ppm) for M6.

M5 $[M+H]^+$ *m/z* 239 (T_r 11.2 min) did not exhibit nitroimidazole-specific fragments, but a fragment at *m/z* 197 $[M+H-42]^+$, *m/z* 141, *m/z* 123. The elemental composition proposed from HR-MS spectrum with the lowest error (-7.8ppm) was $C_8H_{10}N_6OS$ H^+. This composition with only one oxygen was in accordance with the absence of nitroimidazole-specific fragments. Metabolite M5 was tentatively identified as acetylated reduced megazol.

The metabolites M1a/b $[M+H]^+$ *m/z* 310 were detected at T_r 6.7 and 10.8 min. The MS/MS spectrum of M1 was more informative from the ^{13}C-analysis at *m/z* 311 $[^{13}C\text{-}M+H]^+$ and showed a highly abundant product ion at $[M+H-71]^+$, and low abundant product ions probably corresponding to low mass fragmentation of M5. This could suggest some parenthood between M5 and M1, which differed from a mass of 71 Da, potentially corresponding to alanine loss. The precursor peak on HR-MS spectrum was too low for M1 to be informative for elemental composition, but considering the possible parenthood between M5 and M1, we could propose $C_{11}H_{15}N_7O_2S$ H^+, corresponding to an alanine conjugate of acetylated reduced megazol, but the accurate site of conjugation could not be confirmed

As a result, the reported megazol metabo-

lites found in mouse urine were formed via either a single biotransformation or two combined biotransformations and even three. In parallel with the analysis of megazol metabolites in mouse urine, the same analysis was conducted in feces and serum samples. The results showed fifteen metabolites (M1 to M11) in urine, five metabolites in serum and two metabolites in feces (Table 2).

Conclusion

Although the chemical configuration (imidazole and thidazole cycles) is rigid, the fragmentation of megazol is complex. For the first time, we identified, fifteen metabolites of megazol in Swiss mice urine by using micro LC-MS/MS and some of their structures were elucidated based on the fragmentation patterns. Among these 15 compounds, our goal is to find at least one efficient non-toxic compound. Their interest or qualities cannot be measured without isolation and/or a chemical synthesis and without in vitro and in vivo characterization.

These 15 metabolites are divided into phase 1 and phase 2 metabolites. The first ones seem to be more promising because they can be active (alcohol or aldehyde functions). The metabolites of phase 2 are bigger and have different hydrophilic group (glucuronidate addition) that allow them to be eliminated from the body more quickly.

Our next step is to find a way to purify some of the metabolites. Isolation of a specific metabolite from mice urine is currently not possible because urine is a too complex matrix.

We propose to use microsomal incubation with megazol. This is a quick and simple way to mimic renal or liver enzymatic degradation of a drug to obtain metabolites especially the metabolite of phase 1 which can be the best molecules to be active and less toxic.

After a successful isolation of a metabolite, NMR analysis would provide structural information and propose a chemical structure. Chemical synthesis would confirm or invalidate structural hypothesis by comparing to LC-MS/MS data (retention time and fragmentation patterns). Their role must be elucidated to provide prospects for a new, effective and convenient treatment where megazol has failed in African Trypanosomiasis.

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Conflicts of Interest

All authors declare that they have no conflicts of interest.

Authors' Contributions

Clotilde Boudot: conceptualization, formal analysis, investigation, methodology, roles/writing-original draft; Emilie Pinault: conceptualization, formal analysis, investigation, methodology, roles/writing-original draft; Eden Lebrault: conceptualization, investigation; Julien Bonnet: conceptualization; Vincent Sol: conceptualization, roles/writing-original draft, reviewing; Bertrand Courtioux: conceptualization, roles/writing-original draft, reviewing, funding acquisition.

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