Supplemental Material

A new non-polar *N*-Hydroxy Imidazoline lead compound with improved activity in a murine model of late stage *T. b. brucei* infection is not cross-resistant with diamidines

Carlos H. Ríos Martínez[a], *Florence Miller*[b,c,d], *Kayathiri Ganeshamoorthy*[e,f], *Fabienne Glacial*[e,f], *Marcel Kaiser*[g,h], *Harry P. de Koning*[i], *Anthonius A. Eze*[i,j], *Laura Lagartera*[a], *Tomás Herraiz*[k], *Christophe Dardonville*[a#]

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Background. It has been previously reported that reduction of *N*-hydroxylamines, amidoximes and *N*-hydroxyguanidines could be an important pathway for drug activation *in vivo*. *N*-hydroxylation of amidine groups converts them into amidoximes improving their oral bioavailability. It is assumed that the *N*-hydroxylated amidines are reduced back to amidines by a reductive system present in the microsomal and mitochondrial fractions (1-3). In this study we have investigated whether the *N*-hydroxy bisimidazoline derivative **14d** (i.e. a cyclic *N*-hydroxyguanidine) was metabolized to the corresponding imidazoline by *N*-hydroxylamine reductases occurring in the liver, in a similar way as amidoxime prodrugs generate amidine active drugs.

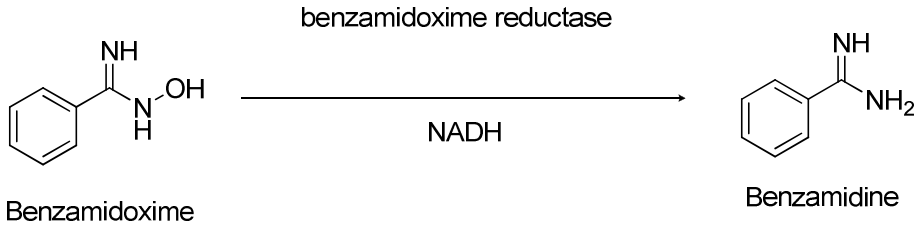
1) Materials and enzymatic fractions

Benzamidine hydrochloride was obtained from Alfa Aesar, benzamidoxime from Thermo Fisher Scientific, and NADH from Sigma-Aldrich. Compound **I** was synthesized as previously reported (4). Human liver microsomes were obtained from Gentech (BD) and human liver mitochondria obtained from Xenotech (Tebu-bio).

2) Hepatic reduction of benzamidoxime as a control (benzamidoxime reduction activity)

Before running the experiments with compound **14d**, we checked the amidoxime reductase activity (*N*-hydroxylamine reductase) of the enzymatic fractions following the reduction of benzamidoxime to benzamidine by RP-HPLC using the analytical method described below. For that, incubations (final volume, 0.2 mL) containing 40 mM phosphate buffer pH 6, human liver microsomes or human liver mitochondria (0.5 mg/mL protein) and 0.5 mM benzamidoxime were pre-incubated at 37 ºC for 3 min. The reaction was started by the addition of 1.2 mM NADH and subsequently incubated

at 37 ºC in a TS 100 thermo shaker (900 rpm) for 60 min. Reaction were terminated with the addition of ice-cold methanol (150 µL), and centrifuged at 10000 rpm for 10 min. Subsequently, aliquots of 20 µL of reaction media were injected into the HPLC or HPLC-MS to determine the content of benzamidine formed in the reaction (Scheme S1).



Scheme S1. Metabolism of benzamidoxime to benzamidine by benzamidoxime reductase

Chromatographic analysis of the reduction of benzamidoxime. The formation of benzamidine from benzamidoxime in the incubation media was assessed by using an HPLC system consisting of a 1050 quaternary pump (Hewlett-Packard, CA) coupled to a DAD detector (Agilent 1100 series). The separation was performed using a Novapak C18 column (150 mm × 3.9 mm i.d., 4 µm) (Waters, Milford, MA). The chromatographic conditions were as follows: Eluent A: 50 mM phosphate buffer containing 20 mM sodium acetate, adjusted to pH 6; eluent B: 50 % A in acetonitrile. Separation was carried out under isocratic conditions at 92% A and 8 % B. Detection of benzamidine was done at 235 nm (retention time was 2.5 min). Column temperature 40 ºC; flow rate: 1 mL/min. A calibration curve of the benzamidine (absorbance at 235 nm) against concentration was used to determine the concentration of benzamidine formed in the incubation media.

Analysis of the enzymatic incubations by RP-HPLC-MS (ESI). RP-HPLC-Mass spectrometry analysis was performed with an Agilent 1200 HPLC equipped with a DAD 1200 series detector coupled to a 6110 MSD quadrupole detector (Agilent Technologies). Chromatographic separation was done on a Zorbax SB-C18 column (2.1 i.d. x 150 mm, 5 μm) (Agilent technologies). Eluent A. 0.1 % formic acid in water and Eluent B: 0.1% formic acid in acetonitrile. Linear gradient from 0 % B to 12 % B in 20 min (**14d** assays) and isocratic (100 % A) (benzamidoxime assays). The flow rate was 0.3 mL/min. Column temperature 40 ºC. Injection volume, 20 μL. The MSD analyzer was set in the electrospray ionization mode (ESI) (positive ionization) at 90 V fragmentator (**14d** assays) and 60 V (benzamidoxime assays) and acquisition was set in the scan mode with a mass range from 50-600 uma.

Results. Benzamidoxime was reduced to benzamidine by microsomal and mitochondrial hepatic fractions (Table S1). The metabolic rate obtained for the formation of benzamidine from benzamidoxime was 0.86 nmoles/min mg prot. for human liver microsomes and 0.26 nmoles/min mg prot. for human liver mitochondria. A chromatogram of the reduction of benzamidoxime and the formation of benzamidine is given in Figure S1. The formation of benzamidine in the reduction of benzamidoxime was confirmed by the corresponding HPLC-MS analysis ($(M+H)^+$ at *m/z* 121) of the incubations with microsomes and mitochondria. The values of benzamidoxime reduction by hepatic microsomes and mitochondria were comparable to those reported by others (5-7).

Table S1. Biotransformation of the *N*-hydroxy imidazoline derivative **14d**, and benzamidoxime used as a reference for amidoxime or *N*-hydroxylamine reductase activities.

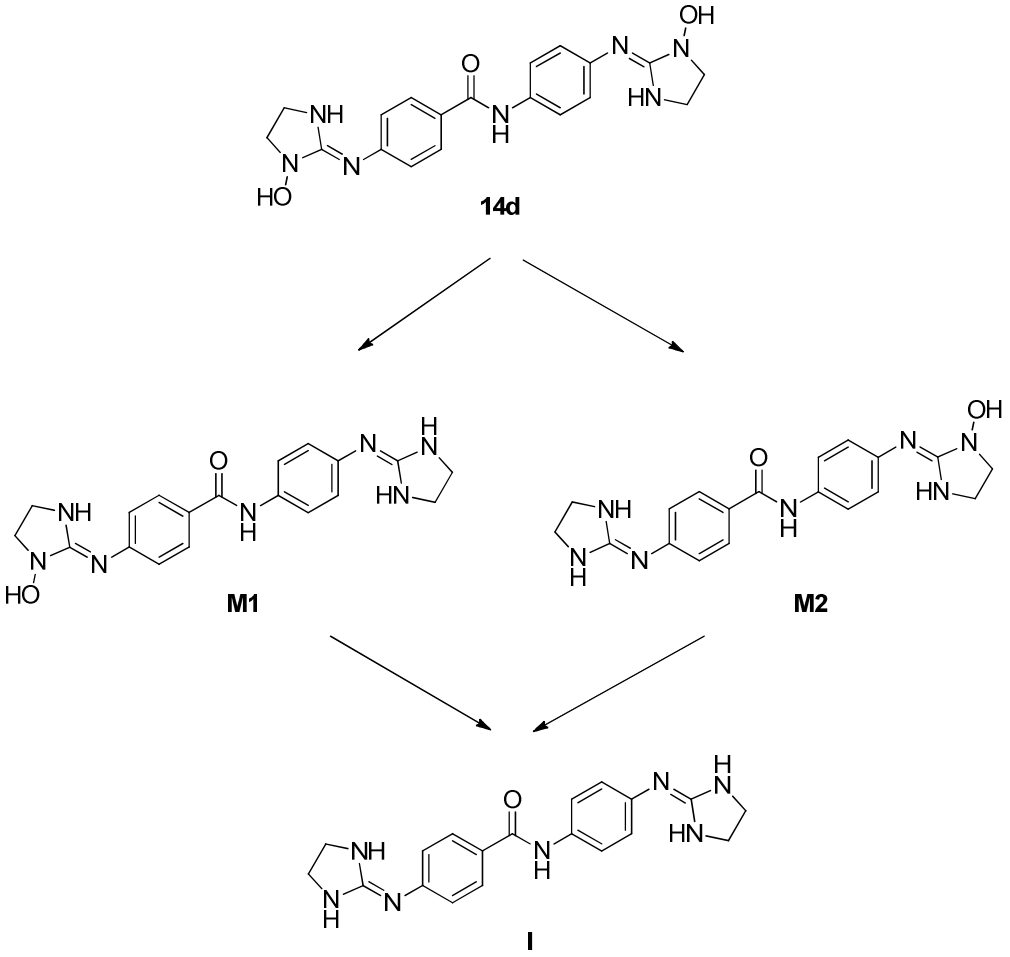
Parent compound	Metabolites	Human liver microsomes	Human liver mitochondria
		V as nmoles/min mg prot.[a]	
Benzamidoxime	Benzamidine	0.86 ± 0.01	0.26 ± 0.04
14d	**I** **M1** **M2**	-[c]	-[c]

[a] Formation of benzamidine was confirmed by HPLC-MS (ESI, positive ionization) and coelution with standard. Controls in presence of NADH but in absence of enzymatic fractions gave only a residual reduction (0.049 ± 0.038). Data are from duplicates.

[b] These putative metabolites are shown in Scheme S2.

[c] Undetectable significant metabolism of **14d** to **I** or **M1** or **M2** by RP-HPLC and HPLC-MS (ESI, positive ionization) when compared with controls without hepatic fractions.

3) Assays of reduction of 14d to I by hepatic microsomes and mitochondria.



Scheme S2. Hypothetical route of metabolism of **14d** by amidoxime reductases studied in this assay.

To assess the enzymatic reduction of **14d**, incubations media (final volume, 0.2 mL) containing 40 mM phosphate buffer (pH 6 or pH 7.4), human liver microsomes or human liver mitochondria (0.5 mg/mL protein), and 0.5 mM **14d** dissolved in milli-Q water were pre-incubated at 37 ºC for 3 min. The reaction was started by the addition of 1.2 mM NADH and incubated at 37 ºC in a TS 100 thermo shaker at 900 rpm for 60 min. Reaction were terminated with the addition of ice cold methanol (150 µL), and centrifuged at 10000 rpm for 10 min. Aliquots of 20 µL of the incubation reaction were

injected into the HPLC or HPLC-MS to assess the reduction of **14d** to give the *N*-dehydroxylated compounds.

Chromatographic analysis of the reduction of 14d. The reduction of **14d** and the formation of *N*-dehydroxylated compounds (e.g. compound **I**) through enzymatic reduction in the incubation media was assessed by using an HPLC system consisting of a 1050 quaternary pump (Hewlett-Packard, CA) coupled to a DAD (Agilent 1100 series) detector. The separation was performed using a Novapak C18 column (150 × 3.9 mm i.d. 4 μm) (Waters, Milford, MA). The chromatographic conditions were as follows: eluent A: 50 mM ammonium phosphate buffer (pH 3); eluent B: 20 % A in acetonitrile. A linear gradient from 0 % B to reach 14.2% of B in 20 min was used. Column temperature 40 ºC; flow rate: 1 mL/min. Detection was performed at 280 nm (retention times of compounds **14d** and **I** were 13.9 min and 14.6 min, respectively).

Results. Following incubations of **14d** with human liver microsomes or mitochondria, no appreciable reduction (taken as double reduction to **I**) was observed by RP-HPLC when compared with the corresponding controls carried under the same conditions but without enzymatic fractions. A chromatogram is presented in Figure S2. Hence, the compound was not apparently reduced to give the free imidazoline compound **I**. To confirm the absence of reduction by these enzymatic fractions, the incubations mixtures were subjected to HPLC-MS (see above). The analysis by HPLC-MS confirmed the absence of significant reduction to give the double *N*-dehydroxylated compound **I** under the conditions investigated. Besides, no intermediate metabolites corresponding to single *N*-dehydroxylation (M1 or M2, Scheme S2) were observed in those incubations when compared with controls.

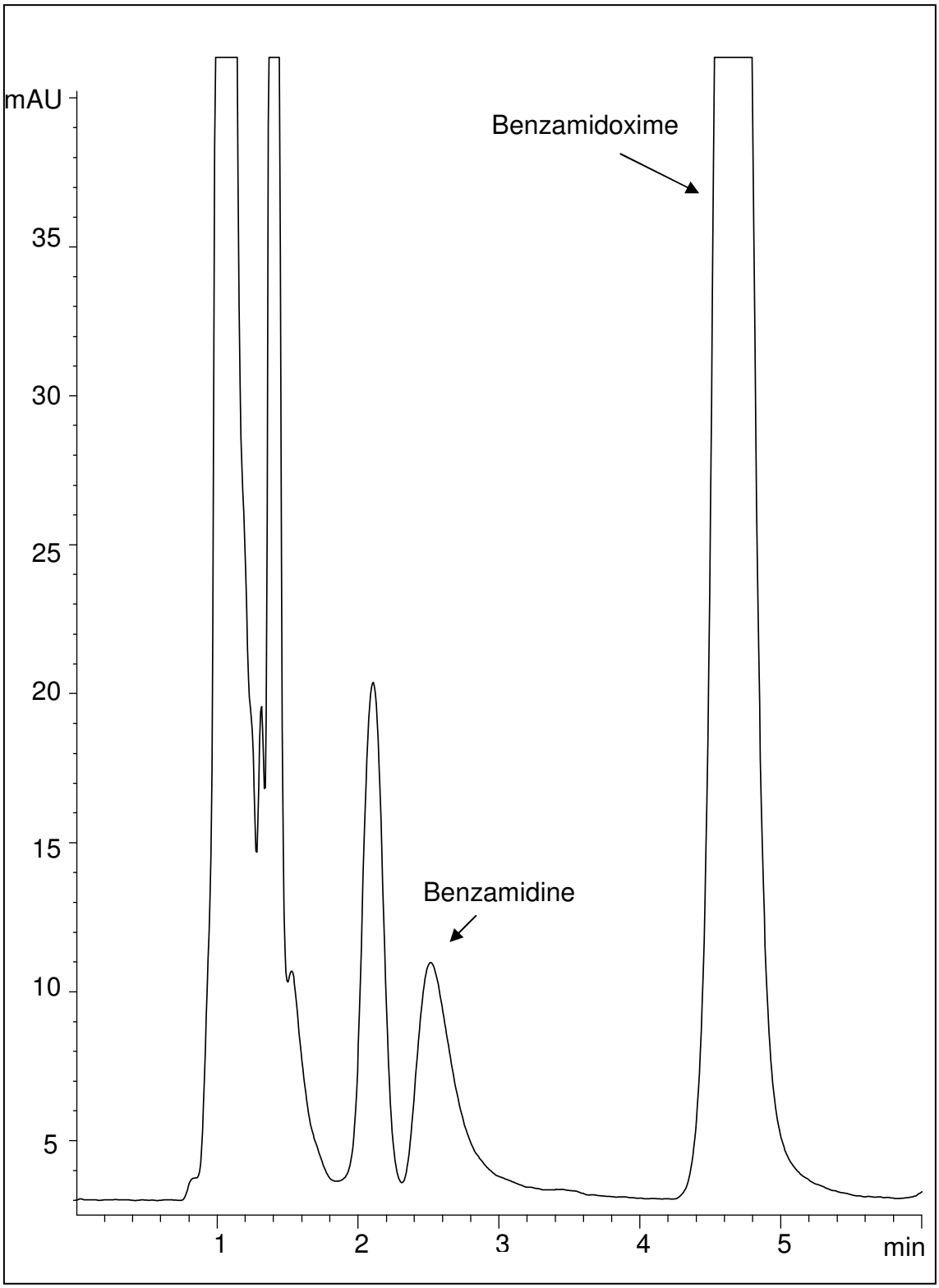


Figure S1. Chromatogram (absorbance 235 nm) of the reduction of benzamidoxime (R_T = 4.6 min) to benzamidine (R_T = 2.5 min) with human liver microsomes in presence of NADH.

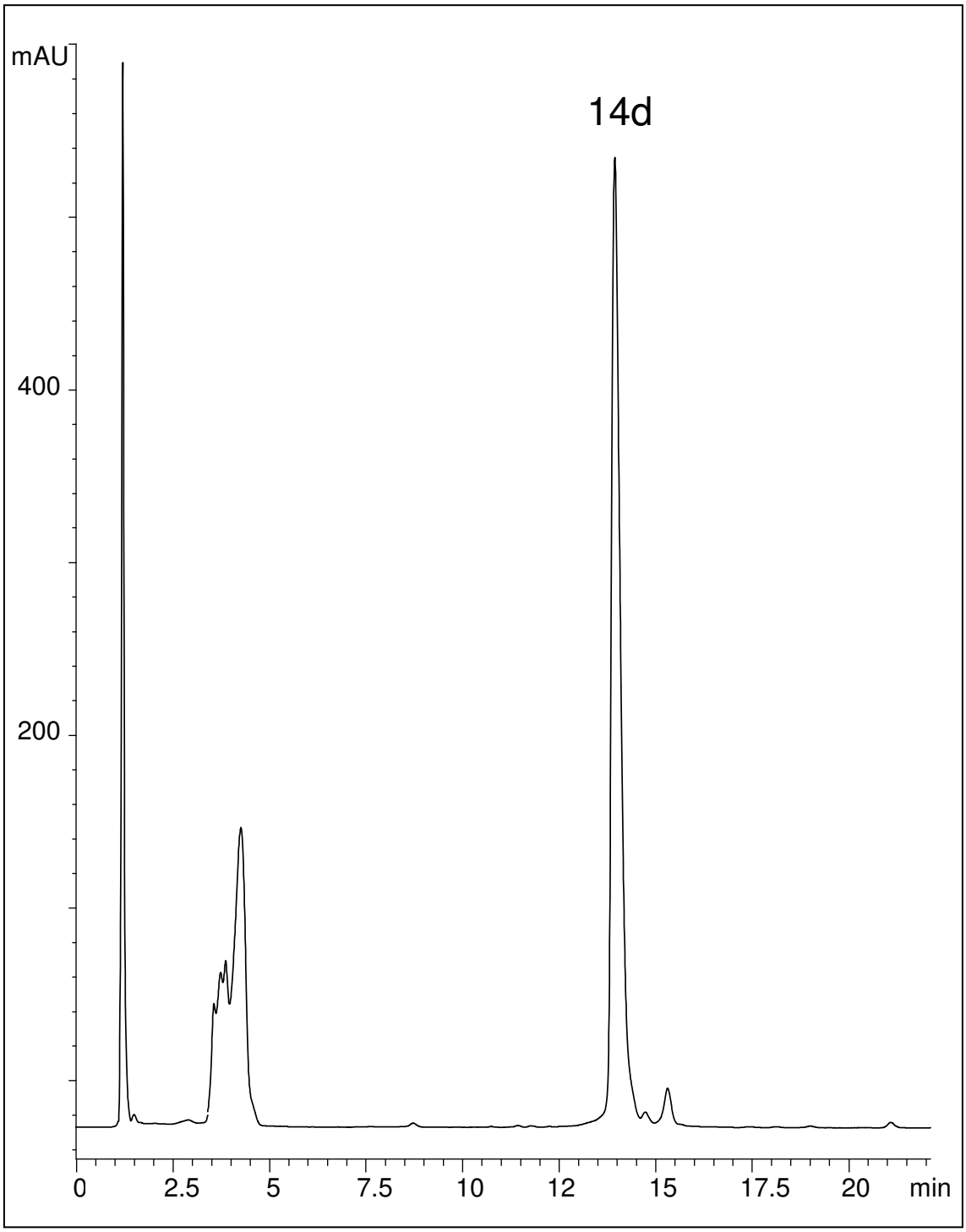
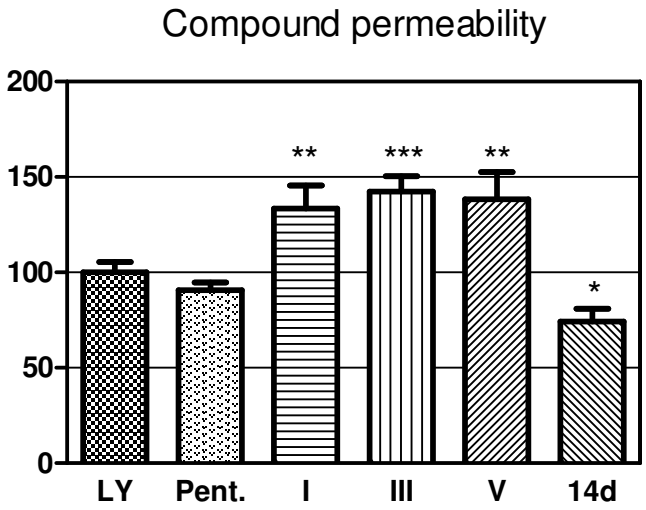
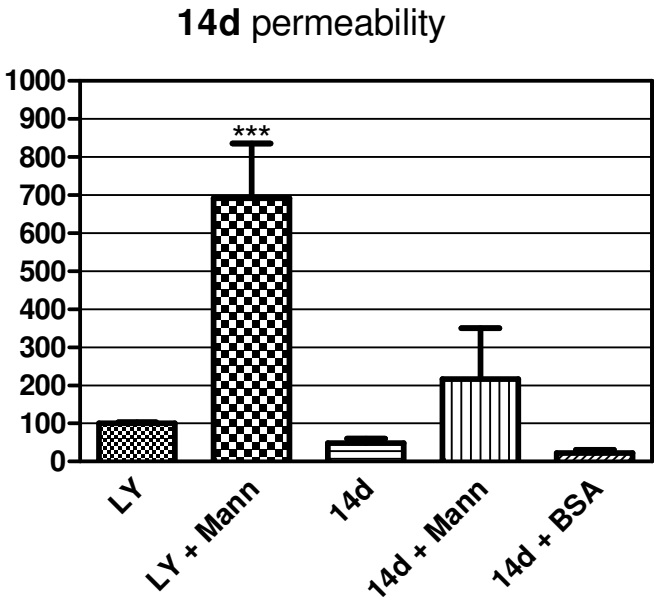


Figure S2. Chromatogram (absorbance 280 nm) of the incubation of **14d** (R_T = 13.9 min) with human liver microsomes in presence of NADH. No reduction to **I** (R_T = 14.6 min) was observed when compared with the corresponding controls carried under the same conditions but without enzymatic fraction.



(a)



(b)

FIG. S3. Permeability values in the human brain endothelial cell line hCMEC/D3 of pentamidine (pent), **I**, **14d**, **III**, and **V** compared to lucifer yellow (LY). Permeability values are represented as a percentage of the P_e value of LY (normalized to 100%). (a) P_e of LY, pentamidine, lead compounds **I**, **III**, **V,** and analogue **14d**. (b) P_e of LY and compound **14d** in absence and presence of mannitol (Mann) or bovine serum albumin (BSA). Data represent means of three independent culture inserts per condition (significant difference versus LY: $P < 0.05$). One-way ANOVA with Dunnett's post test was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com)..

References

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