Full Paper

Synthesis and Antitubercular Activity Evaluation of Novel Unsymmetrical Cyclohexane-1,2-diamine Derivatives

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A library of unsymmetrical cyclohexane-1,2-diamine derivatives were synthesized and evaluated for their activity against *Mycobacterium tuberculosis* H37Rv *in vitro*. Out of the 46 compounds synthesized, eight compounds (**11h**, **13a**, **13e**, **13f**, **14a**, **14c**, **14d**, and **15d**) were found to be active at or below 6.25 μM concentration, with negligible toxicity to human red blood cells at a concentration much higher than the MIC_{99}. Compound **13a** was the best active compound showing inhibition at 3.125–6.25 μM, and was found to be non-hemolytic up to 500 μg/mL concentration.

Keywords: Bromhexine / Cyclohexane-1,2-diamine / Hemolysis / *Mycobacterium tuberculosis* / Tuberculosis

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Supporting information available online

Introduction

According to the World Health Organization (WHO), six infectious diseases (pneumonia, tuberculosis, diarrhoea, malaria, measles, and HIV/AIDS) account for half of all premature deaths worldwide, killing mostly children and young adults [1]. Tuberculosis or TB (tubercle bacillus) is one of the bacterial diseases with the highest disease burden, mainly caused by the bacterium *Mycobacterium tuberculosis* (*M. tb*) leading to 8.8 million new TB cases and 1.4 million deaths in the year 2010 (WHO). From the statistics gathered each year it is estimated that around 225 million new cases and 79 million deaths may occur due to tuberculosis between 1998 and 2030. With the emergence of MDR-XDR-TB and HIV-TB nexus, there is always a need for the identification of novel scaffolds with antitubercular activity having newer drug targets [2]. One of the diamine based compounds, namely ethambutol [(2*S*,2′*S*)-2,2′-(ethane-1,2-diyldiimino)dibutan-1-ol], has been used as a drug of choice for the treatment of TB (Fig. 1) in combination with other anti-TB drugs [3, 4]. A library of 63 238 compounds based on the ethambutol structural motif have been synthesized by Sequella Inc., out of which compound SQ109 (*N*-geranyl-*N*′-(2-adamantyl)ethane-1,2-diamine), was found to be the most potent with MIC 0.7–1.56 μM (Fig. 1) and is currently undergoing phase II clinical trials [5, 6]. In a recent study, it has been shown that SQ-109 targets the MmpL3 protein, an enzyme involved in the transport of lipids into the cell wall core of *M. tuberculosis* [7].

Cyclohexane moiety has always drawn the attention of chemists due to its role in many organic transformations [8–12], and that of biologists [13] as it has been part of many biologically active compounds such as oxaliplatin (**1**) [14, 15], bromhexine (**2**) [16], and ambroxol (**3**) [17] (Fig. 2). Oxaliplatin, {(1*R*,2*R*)-cyclohexanediamine}oxalatoplatinum(II) (**1**), is a cyclohexane-1,2-diamine based platinum complex (Fig. 2) which has been approved by the FDA for the first line treatment of colorectal carcinoma in 2004 [18].

Bromhexine [**2**, *N*-2-amino-3,5-dibromobenzyl)-*N*-cyclohexylmethylamine] is another compound based on the cyclohexane motif, a semi-synthetic derivative of the alkaloid vasicine, obtained from the Indian shrub *Adhatoda vasica* which has been used as mucolytic agent [19]. The flowers, leaves, and roots of *A. vasica* have been used for the treatment of asthma and tuberculosis. Ambroxol [**3**, *N*-(*trans*-4-hydroxy-cyclohexyl)-(2-amino-3,5-dibromo-benzyl)-amine], a metabolite of bromhexine, has also shown secretalogic and mucolytic properties [20–22]. In a pilot study by the Medical Research Council of Ireland,

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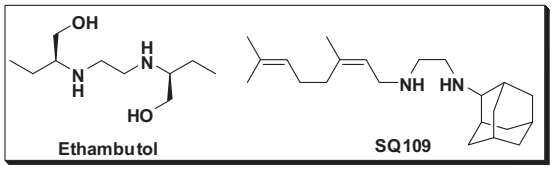


Figure 1. Diamine based antitubercular drugs.

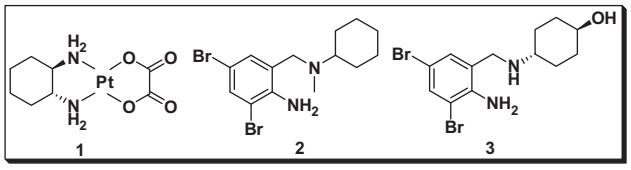


Figure 2. Cyclohexane based biologically active molecules.

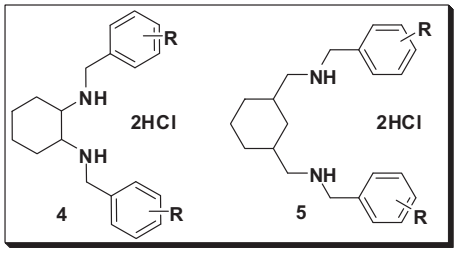


Figure 3. Prototype structure of antibacterial agents.

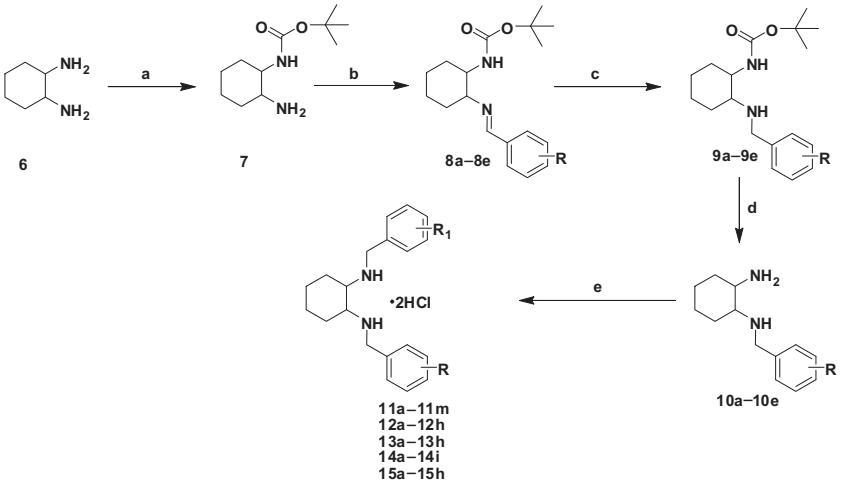
bromhexine was found to inhibit the growth of a strain of *M. tb* at concentrations of 33.3 μg/mL and above, however this effect was pH dependent [19].

We recently reported the synthesis of a series of symmetrical cyclohexane-1,2-diamine [23, 24] and cyclohexane-1,3-diyldimethanamine derivatives [25] (**4** and **5**) and most of these compounds have shown excellent antibacterial activity against Gram-positive and Gram-negative bacteria with low toxicity (Fig. 3). The antimycobacterial activity of the best active symmetrical derivatives against *M. tb* H37Rv lied in the range 3.125–12.5 μM [26]. Encouraged by these observations and as a part of our ongoing work toward the development of novel antimicrobials [27–30], we synthesized a series of unsymmetrical cyclohexane-1,2-diamine compounds with +2 charge and hydrophobic bulk furnished by the substituted aromatic ring and screened them for their antitubercular activity. These compounds belong to the diamine class of antitubercular drugs amongst which ethambutol and SQ109 are the representative examples. In the present study, we also evaluated the toxicity of these compounds toward human red blood cells (hRBCs).

Results and discussion

Chemistry

Mono-functionalized symmetrical or unsymmetrical diamines are important intermediates for the synthesis of many biologically important pharmacophores [31–33]. Often, selective and efficient protecting reagents and mild reaction conditions are required for sequential protection and deprotection of polyfunctional molecules [34–36]. In order to synthesize unsymmetrical cyclohexane-diamine based compounds, one of the primary amine groups of racemic cyclohexane-1,2-diamine (**6**) was protected using $(Boc)_2O$ (Scheme 1) by literature methods [37]. The other NH_2 group of the Boc-protected compound **7** was treated with substituted benzaldehydes to give imines (**8a**, R = H; **8b**, R = 4-Me; **8c**, R = 4-Et, **8d**, R = 4-*i*-Pr; **8e**, R = 4-Br) in quantitative yields. These Schiff bases were reduced using $NaBH_4$ in dry MeOH to give substituted amines (**9a–9e**) containing one NH_2 group protected with Boc, which on reaction with 35% aq. H_3PO_4 in dichloromethane leads to deprotection of the Boc group (**10a–10e**) [38]. Compounds with free NH_2 group (**10a**, R = H; **10b**, R = 4-Me; **10c**, R = 4-Et, **10d**, R = 4-*i*-Pr;



Scheme 1. Synthesis of unsymmetrical cyclohexane-1,2-diamine derivatives. (a) $(Boc)_2O$, dioxane, r.t., 22 h; 92%; (b) substituted benzaldehydes, MeOH, r.t., 4 h; 85–95%. (c) $NaBH_4$, MeOH, r.t., 3 h; 80–93%; (d) 35% aq H_3PO_4, CH_2Cl_2, r.t., 4 h; 75–85%; (e) (i) substituted benzaldehydes, MeOH, r.t., 3–4 h, $NaBH_4$, r.t., 2–3 h; 60–85% (ii) $CHCl_3$, dry HCl gas, 0.5 h.

10e, R = 4-Br) were then treated with different benzaldehydes in dry MeOH and the resulting imines were reduced by $NaBH_4$ *in situ*. The resulting amines were purified by column chromatography using 2% MeOH–$CHCl_3$ as eluent. All these unsymmetrically substituted amines were converted to their respective hydrochloride salts by passing dry HCl gas to the solution of diamines in chloroform. A group of peaks at 2704–2962 cm^{-1} in the IR spectra of the amine salts (**11a–15h**) and broad signals at δ 9.35–10.3 in the ^1H NMR spectra confirmed the formation of salt. The salt formation was also confirmed by conductivity measurements and all the compounds were characterized by standard analytical and spectroscopic methods.

In vitro antitubercular activity

The significant role of diamine based compounds in anti-TB research prompted us to screen these compounds against the *M. tb* H37Rv strain. Rifampicin and ethambutol, used as reference drugs, inhibited mycobacterial growth at a concentration of <0.05 and 1.56 μM respectively. Table 1 clearly shows that most of the synthesized compounds are moderate to less active against *M. tb*. MIC_{99} values >50 μM, indicate low antitubercular activity. Seventeen compounds (**11d–11g**, **11i**, **11j**, **12b–12e**, **13c**, **13d**, **14e**, **14g**, **15c**, **15e**, and **15f**) exhibit moderate activity with MIC_{99} 12.5–25 μM. Thirteen compounds showed good activity with MIC_{99} <12.5 μM. Compound **13a** with an ethyl group at the *para* position of one of the aromatic rings, and a methyl group at the *ortho* position of the other aromatic ring was the most potent anti-TB compound in this series with a MIC value of 3.125–6.25 μM. Along with compound **13a**, compounds **14c**, **14d**, and **15d** can also be considered as leads for further exploration as these compounds possess considerably good MIC (6.25–12.5 μM) against H37Rv. When 4-Et of **13a** was replaced with 4-*i*-Pr keeping the methyl group at *ortho* of the other phenyl ring intact (**14a**, MIC_{99} = 6.25–12.5 μM) the activity of the compound slightly decreased. But a dramatic loss in activity was observed when there was no substituent present (**11a**) or groups such as Br (**15a**) or Me (**12a**) were present at the *para* position of the analogues of **13a**. Compounds **13e** and **13f** also exhibit promising activity with MIC in the range of 6.25–12.5 μM. Amongst the compounds **11a–11m** in which one of the aromatic ring is unsubstituted and the other has various substitutions, **11h** with *t*-butyl at the *para* position showed very good activity (MIC_{99} = 6.25 μM). In this series, when the chain length was increased at the *para* position there was no change in the activity and compounds **11d–11f** exhibited the same level of activity with MIC of 12.5 μM. When one of the aromatic ring contained a 4-Me group and the alkyl substitution at the *para* position of the other ring was varied (**12a–12e**) it was observed that the activity increased with increase in ClogP **12b** < **12c** < **12e** (6.71, 7.24, and 7.76, respectively). Similar trend was observed with compounds having the 4-Et group in one of the phenyl rings and 4-Me, 4-*n*-Pr, 4-*n*-Bu in the

Table 1. Antimycobacterial activity of unsymmetrical cyclohexane-1,2-diamine derivatives.

Entry	R	R_1	H37Rv (MIC_{99}/μM)	ClogP	Entry	R	R_1	H37Rv (MIC_{99}/μM)	ClogP
11a	H	2-Me	>50	5.633	**13c**	4-Et	4-*n*-Pr	12.5	7.769
11b	H	3-Me	50	5.683	**13d**	4-Et	4-*i*-Pr	25	7.639
11c	H	4-Me	50	5.683	**13e**	4-Et	4-*n*-Bu	6.25	8.298
11d	H	4-Et	12.5	6.212	**13f**	4-Et	4-*t*-Bu	6.25–12.5	8.038
11e	H	4-*n*-Pr	12.5	6.741	**13g**	4-Et	3,4-Me	25–50	7.160
11f	H	4-*i*-Pr	12.5	6.611	**13h**	4-Et	4-Cl	50	6.925
11g	H	4-*n*-Bu	25	7.270	**14a**	4-*i*-Pr	2-Me	6.25–12.5	7.060
11h	H	4-*t*-Bu	6.25	7.010	**14b**	4-*i*-Pr	3-Me	12.5–25	7.110
11i	H	3,4-Me	25	6.132	**14c**	4-*i*-Pr	4-*n*-Pr	6.25	8.168
11j	H	4-Cl	12.5	5.897	**14d**	4-*i*-Pr	4-*n*-Bu	6.25–12.5	8.697
11k	H	4-Br	50	6.047	**14e**	4-*i*-Pr	3,4-Me	12.5–25	7.559
11l	H	2-F	50	5.327	**14f**	4-*i*-Pr	4-Cl	25–50	7.324
11m	H	3-F	>50	5.327	**14g**	4-*i*-Pr	4-Br	12.5–25	7.474
12a	4-Me	2-Me	>50	6.132	**14h**	4-*i*-Pr	2-F	25–50	6.754
12b	4-Me	4-Et	25	6.711	**14i**	4-*i*-Pr	3-F	50	6.754
12c	4-Me	4-*n*-Pr	25	7.240	**15a**	4-Br	2-Me	25–50	6.496
12d	4-Me	4-*i*-Pr	12.5–25	7.110	**15b**	4-Br	3-Me	50	6.546
12e	4-Me	4-*n*-Bu	12.5–25	7.769	**15c**	4-Br	4-*n*-Pr	12.5–25	7.604
12f	4-Me	3-Cl	>50	6.396	**15d**	4-Br	4-*n*-Bu	6.25	8.133
12g	4-Me	4-Cl	>50	6.396	**15e**	4-Br	4-*t*-Bu	25	7.873
12h	4-Me	4-Br	50	6.546	**15f**	4-Br	3,4-Me	25	6.995
13a	4-Et	2-Me	3.125–6.25	6.661	**15g**	4-Br	2-F	50	6.190
13b	4-Et	3-Me	25–50	6.711	**15h**	4-Br	3-F	50	6.190

Ref: Rifampicin: <0.05 μM, ethambutol: 1.56 μM.

other **12b** < **13c** < **13e** (ClogP values 6.71, 7.76, and 8.29, respectively). Compound having 4-*n*-butyl group in one of the aromatic rings and the other ring unsubstituted, the resulting compound **11g** showed poor activity. But, presence of substituents like 4-Me (**12e**, MIC_{99} = 12.5–25 μM), 4-Et (**13e**, MIC_{99} = 6.25 μM), 4-*i*-Pr (**14d**, MIC_{99} = 6.25–12.5 μM), and 4-Br (**15d**, MIC_{99} = 6.25 μM) showed better anti-TB activity. Compounds **14a–14e** with 4-*i*-Pr in one of the aromatic rings showed considerably good activity with MIC_{99} = 6.25–12.5 μM. Presence of a fluoro group at the *ortho* or *meta* position (**11l**, **11m**, **14h**, **14i**, **15g**, and **15h**) of one of the aromatic rings does not contribute to the activity, irrespective of any group present in the other ring. Compounds **14c** and **14d** have comparable activity though the lipophilicity of **14d** is greater than that of **14c**. The activity of **15d** is much better than that of **15c** and also the ClogP of **15d** is greater than that of **15c** (8.13 and 7.60).

Hemolytic activity

Toxicity of 16 compounds (**11j**, **12f**, **13a–13c**, **13f**, **13g**, **14b–14d**, **14f**, **14h**, **14h**, **15d**, **15g**, and **15h**) was investigated using hRBCs at up to 500 μg/mL concentration (Fig. 4). Eight compounds **11j**, **12f**, **13a**, **14f**, **15a**, **15d**, **15g**, and **15h** were found to be non-hemolytic with only 6–14% hemolysis at the highest concentration tested. Compounds **13b**, **13c**, **13f**, **13g**, **14b**, **14c**, **14d**, and **15a** were 84, 48, 87, 77, 62, 20, 23, and 52%, hemolytic, respectively, at 500 μg/mL. At half the concentration (250 μg/mL) these compounds were either non-hemolytic or showed a maximum of 23% hemolysis. Compounds **13c** and **14c** showed 4 and 0% hemolysis at 250 μg/mL concentration, which is much higher than their MIC values. Interestingly, the most active compound **13a** was non-toxic to hRBcs and can be considered as a safe molecule.

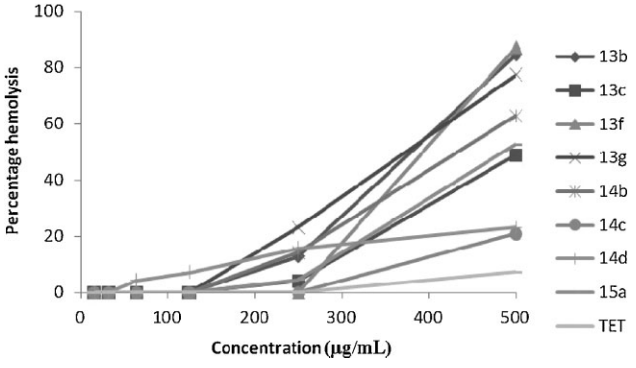


Figure 4. Dose–response curves of hemolytic activity of the designed compounds against human erythrocytes. Different concentrations of compounds were incubated with 4% hRBC suspension for 1 h at 37°C. After 1 h of incubation, hemoglobin release from the ruptured hRBC was studied by measuring the absorbance at 414 nm. For complete lysis, 1% Triton X-100 was taken as positive control, and for no lysis, PBS was taken as negative control.

Conclusion

Herein we report the synthesis and antimycobacterial activity evaluation of a library of 46 unsymmetrically substituted cyclohexane-1,2-diamines. Some compounds showed moderate activity against *M. tb* H37Rv. Toxicity evaluation of these compounds against hRBCs showed no toxicity of these compounds up to a concentration higher than the MIC, therefore making them safe as potent leads.

Experimental

General

All of the chemicals used in the syntheses were purchased from Sigma–Aldrich and were used as such. Thin layer chromatography (Merck TLC silica gel 60 F254) was used to monitor the progress of the reactions. The compounds were purified by silica gel column (60–120 mesh). Melting points were determined on an EZ-Melt automated melting point apparatus, Stanford Research Systems, and are uncorrected. IR (KBr) spectra were recorded using a Perkin-Elmer FT-IR spectrophotometer and the values are expressed as ν_{max} cm^{-1}. Mass spectral data were recorded in a Thermo Finnigan LCQ Advantage max ion trap mass spectrometer. ^1H NMR spectra were recorded on a Bruker Spectrospin spectrometer at 300 MHz and a Jeol ECX Spectrospin 400 MHz instrument while the ^{13}C NMR spectra were recorded at 75.5 and 100 MHz, respectively, using TMS as an internal standard. The chemical shift values were recorded on the δ scale and the coupling constants (J) are in Hz. Elemental analysis was performed on a Carlo Erba Model EA-1108 elemental analyzer and data of C, H, and N is within ±0.4% of calculated values. The data characterizing the unsymmetrical cyclohexane-1,2-diamine derivatives **11a–15h** can be found in the Supplementary Material provided online. ClogP values were calculated using CambridgeSoft ChemOffice Ultra 10.0 software.

*Synthesis of tert-butyl-2-aminocyclohexylcarbamate (**7**) [37]*

To a stirred solution of 1,2-diaminocyclohexane (**6**; 4.0 g, 0.035 mol) in 70 mL dioxane, a solution of di-*tert*-butyldicarbonate (1.0 g, 0.0046 mmol) in dioxane (30 mL) was added dropwise (Scheme 1). The mixture was stirred for 22 h and the solvent was evaporated under reduced pressure. The solid was suspended in H_2O (50 mL) and the disubstituted product was removed by filtration. The filtrate was extracted with CH_2Cl_2 (3 × 50 mL) and the organic fractions were washed with water (3 × 30 mL). The organic layer was dried over Na_2SO_4 and the solvent was then removed to give an oily yellow liquid (**7**), which was dried under vacuum (0.9 g, 92%).

*General procedure for the preparation of compounds **9a–9e** [38]*

To a stirred solution of **7** (0.93 mmol, 200 mg) in dry MeOH (10 mL) the corresponding substituted benzaldehyde (1.1 equiv) was added and the reaction mixture was stirred at room temperature for 4 h (Scheme 1). After completion of the reaction the solvent was removed and product was extracted with $CHCl_3$ (3 × 15 mL). The combined organic layer was dried over sodium sulfate and the solvent was removed under pressure to get an oily

liquid. The Schiff's base obtained (**8a–8e)** was dissolved in dry MeOH (15 mL) and $NaBH_4$ (3.0 equiv) was added and progress of the reaction was monitored by TLC. After 3 h the solvent was removed and the product was extracted with $CHCl_3$ (3 × 15 mL). The reduced product was purified by column chromatography using $CHCl_3$–MeOH as eluent to obtain compounds **9a–9e** in good yield.

*General procedure for the preparation of compounds **10a–10e** [38]*

An aqueous solution of H_3PO_4 (35%, 0.2 mL) was added to a solution of the corresponding aminocarbamate (**9a–9e)** (200 mg, 1.0 equiv) in CH_2Cl_2 (3 mL) and the reaction was stirred for 4 h (Scheme 1). Water (4 mL) was added to the reaction mixture, and the aqueous layer was washed with CH_2Cl_2 (3 × 10 mL), neutralized with NaOH solution and extracted with CH_2Cl_2 (3 × 15 mL). The combined basic organic layer was washed with brine solution (10 mL), dried over Na_2SO_4 and evaporated under reduced pressure to yield the corresponding amines (**10a–10e)** in good yield.

*General procedure for the preparation of compounds **11–15***

To a solution of the amine **10a–10e** (200 mg, 1 equiv) in anhydrous methanol (5 mL), the corresponding aromatic aldehyde (1.1 equiv) was added under nitrogen atmosphere and the reaction mixture was stirred for 3–4 h at room temperature (Scheme 1). To the reaction mixture sodium borohydride (2.0 equiv) was added. After 2–3 h, the solvent was evaporated and water was added to the reaction mixture and extracted with $CHCl_3$ (3 × 15 mL). The combined organic layer was dried over sodium sulfate and the solvent was removed under pressure. Purification by column chromatography using MeOH–$CHCl_3$ as eluent yielded pure amine derivatives which were then converted to their respective hydrochloride salts (**11–15**) by passing dry HCl gas to their solution in $CHCl_3$.

In vitro* assay for *M. tb

A stock culture of *M. tb* H37Rv (ATCC 27294) was grown to Abs_{600nm} 0.2 in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80, 0.2% glycerol, and albumin/NaCl/glucose (ADS) complex. The culture was diluted 1:1000 in 7H9-based medium before aliquoting 50 μL into each well of a 96-well plate. Drugs were dissolved in DMSO (Sigma) to make stock solutions of 50 mM. Drugs (100 μL solution) were added to the first row of the 96-well plate at a final concentration 100 μM. Twofold serial dilutions were made and five dilutions of each drug (50–3.125 μM) were tested for antimycobacterial activity. The drugs were diluted 1:1 by addition of 50 μL of 1:1000 diluted cultures. Rows 6 and 12 of the 96-well plates were no-drug controls. The plates were incubated at 37°C and the MIC_{99} values were read macroscopically using an inverted plate reader after 14 days. Each measurement was made three independent times.

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