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Short Communication

# In vitro anti-acanthamoeba action by thioureidic derivatives

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**Abstract**

The anti-amoebic power of a series of bis-thioureidic derivatives against amoeba, responsible for a serious form of keratitis of the cornea, has been analysed. The synthesis of these products is also described.


*Keywords:* Anti-amoebic; bis-Thioureidic; Keratitis

## 1. Introduction

In previous researches [1], some of us had studied the amoebicidal power of superior homologues of propamidine (Brolene®) and hesamidine (Desomedine®), present in two collyria used for a serious, even if rare, form of keratitis, due to the invasion of the cornea by amoeba of the Acanthamoeba genus. These studies were carried out on the amoeba cysts, which are the resistant forms originating the infection persistence. These molecules belonged to the to 4-4′-diamidino-difenoxyalkane series (see Fig. 1). The results [2], showed the superiority of action of the hepta, octa and nonamidine that make inactive all the amoeba cysts studied (in 24 h) included the cysts of the resistant forms. From these interesting results we have synthesized a series of bis-thioureidic derivatives (see Fig. 2), in which the phenoxyamidinic function was replaced by a simple thioureidic function. We have studied the influence on the biological activity of the length of the alkyl chain separating the two thioureidic functions for these bis- thioureidic derivatives too, by preparing some derivatives with $n = 4$–9 methylenic groups. The influence of the substituent (aliphatic or aromatic) present on one of the nitrogen atoms of the thiourea has been studied too. The synthesis of the products is reported in Scheme 1. The amoebicidal power against the three strains of the Acanthamoeba genus has been extimated for all the compounds **1a**–**f**; **2a**–**f**; **3a**–**f**.

## 2. Material and methods

### 2.1. Chemistry

Melting points were determined by a Kofler apparatus and are reported in Tables 1–3. IR spectra were recorded on a Shimatzu 8000 spectrometer. $^1$H NMR spectra were recorded on a Brucker DRX 600 spectrometer in DMSO-$d_6$ solvent. Chemical shifts, expressed as $\delta$ (ppm), are shown in Tables 4–6. The purity of the compounds was checked by TLC (thin layer chromatography), using ethyl acetate as eluent. The structural attributions were performed also by the support of the elemental analysis, in which found and calculated values atom percentages were identical within $\pm 0.4\%$. The

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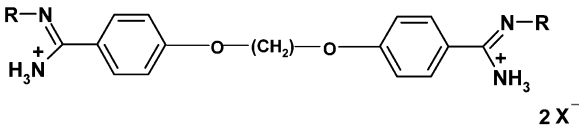


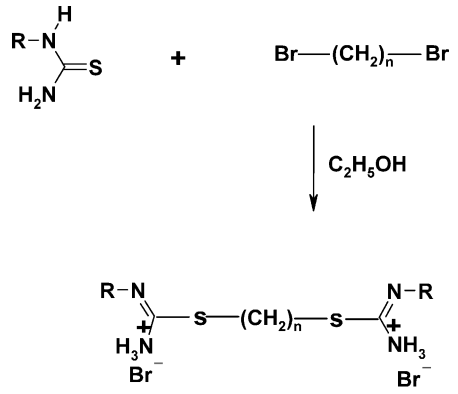

n= 3-9
n= 3 Brolene[R] 4,4'-trimetylenedioxydibenzamidine
n= 6 Desomedine[R] 4,4'-diamidino-1,6-diphenoxyhexane

Fig. 1.

synthesis were carried out in the laboratories of C.E.R.M.N. Caen (France) and in the University of Pharmacy in Salerno (Italy); the biological tests were executed by C.E.R.M.N.

### 2.1.1. Preparation of *1a–f, 2a–f, 3a–f* compounds

The compounds **1a**–**f**, **2a**–**f** and **3a**–**f** (see Fig. 2) have been synthesized as follows. Two grams of the suitable thiourea (1 equiv.) and dibromoalkane (0.5 equiv.) was refluxed in anhydrous ethanol for 4–5 h. The reaction mixture was evaporated to dryness under vacuum. The residue was crystallised from suitable solvent to give the bis-thioureidic salts (Scheme 1).




R= $CH_3$; $C_6H_5$; p$Cl$-$C_6H_4$
n= 4-9

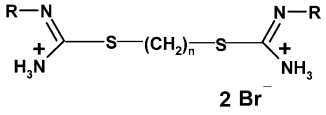
Scheme 1.

## 2.2. Pharmacology

### 2.2.1. Biological assays

The Amoeba strains used in this study were isolated from lenses, lenses care systems or corneal scraping of patients affected by keratitis. Cysts were used as challenge organisms, being the resistant forms of the Acanthamoeba.

The strains were grown at 30 °C on a monoexenic nutrient agar (MNA) medium made of 1.5% agar (Bacto Agar, Difco) aseptically spread over with a live Escherichia Coli suspension.



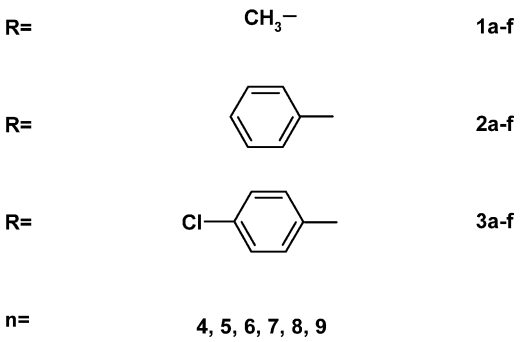



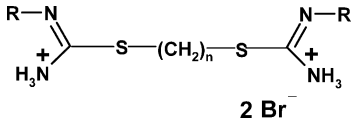

Fig. 2.

The criteria to assign the protozoas to the Acanthamoeba genus were: presence of acanthopolia in slowly moving trophozoites, the use of metamitosis for nuclear division and the presence of double-walled cysts with polygonal or stellate endocysts. After silver staining and microscopic examination, cysts exhibited the features of group II defined by Pussard and Pons [3], [4] and Acanthamoeba strains were identified as *Acanthamoeba polyphaga*, and *Hartmannella varinii* and *Vahlkampfia avara*.

### 2.2.2. Trophozoite and cyst harvesting

The Acanthamoeba strains were grown at 30 °C on MNA medium. Trofozoites were collected from 24 to 48 h cultures and cysts were collected from 3-week cultures. The agar surfaces were flooded with 5 ml of PBS and were gently scraped with an inoculating loop. Trophozoites and cysts were harvested from the suspension by

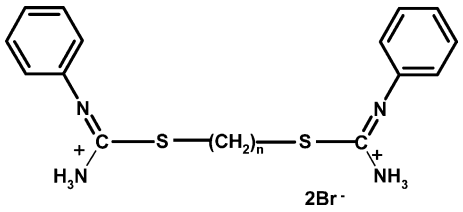
Table 1
Physical and analytical data of compounds **1a**–**f**




| Comp. no. | *n* | Melting point (°C) | Yield (%) |
|---|---|---|---|
| **1a** [a] | 4 | 238 | 60 |
| **1b** [b] | 5 | 170 | 84 |
| **1c** [b] | 6 | 80 | 37 |
| **1d** [a] | 7 | 133 | 72 |
| **1e** [b] | 8 | 180 | 90 |
| **1f** [b] | 9 | 130 | 84 |

[a] Crystallisation solvent: diethyl ether.
[b] Crystallisation solvent: acetonitrile.

Table 2
Physical and analytical data of compounds **2a**–**f**




| Comp. no. | *n* | Melting point (°C) | Yield (%) |
|---|---|---|---|
| **2a** [a] | 4 | 226 | 48 |
| **2b** [a] | 5 | 192 | 43 |
| **2c** [a] | 6 | 198 | 53 |
| **2d** | 7 | Oil | 43 |
| **2e** [a] | 8 | 190 | 20 |
| **2f** | 9 | Oil | 88 |

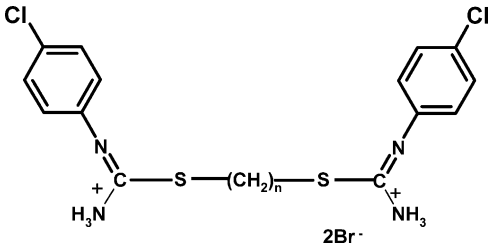
[a] Crystallisation solvent: acetonitrile.

centrifugation at 350 μg for 10 min in a C 400-S4 centrifuge (Jouan, France). The supernatant was aspirated and the sediment was washed twice in PBS in order to eliminate most of the bacteria. The trophozoites or cysts in the resultant suspension were optically counted with a Malassez hemacytometer with a phase-contrast microscope, and the suspension was standardised to $10^5$ trophozoites or cysts for 100 μl.

### 2.2.3. Drug sensitivity tests

Drug solutions were prepared at a concentration ten times the desired one by dissolution in sterile distilled water and were stored at 4 °C until they were tested. The concentrations were adjusted to 1000 μg/ml, corresponding to a range of $1.52 \times 10^3$–$1.92 \times 10^3$ mol/l (when testing on the cysts) and to 100 μg/ml, corresponding to $1.52 \times 10^2$–$1.92 \times 10^2$ mol/l (when testing on the trophozoites), by dilution in a suitable volume of sterile distilled water. Experiments and their controls were carried out in well plates. Sixteen sets of experimental plates containing each 100 μl of thioureidic derivatives were used. Control plates containing 100 μg of phosphate-buffered saline (PBS, pH 7.2) were also used. A 100 μl amount of standardised trophozoites or cysts suspension was inoculated into each plate. All plates were sealed with Parafilm (American Can Co.) to prevent evaporation, incubated at 30 °C, and examined daily with an inverted microscope to determine the presence of active trophozoites. Each plate was evaluated and scored as either positive or negative. From the number of positive cultures observed in each set, the most probable number of viable cysts or trophozoites was read on statistical tables corresponding to the plated volumes (American Public Health Association. Standard method for the Examination of Water and Wastewater. American Public Health Association, Washington DC, 1971). Experiments and their control were repeated five times. The minimum concentration of

Table 3
Physical and analytical data of compounds **3a**–**f**




| Comp. no. | *n* | Melting point (°C) | Yield (%) |
|---|---|---|---|
| **3a** [a] | 4 | 210 | 47 |
| **3b** | 5 | Oil | 55 |
| **3c** [b] | 6 | 182 | 36 |
| **3d** | 7 | Oil | 68 |
| **3e** | 8 | Oil | 81 |
| **3f** | 9 | Oil | 69 |

[a] Crystallisation solvent: chloroform.
[b] Crystallisation solvent: carbon–tetrachloride.

Table 4
$^1$H NMR data ($\delta$ ppm) of compounds **1a**–**f** (DMSO-$d_6$) and signal attribution

| | |
|---|---|
| **1a** | 3.35–3.25 (4H, m, 2S–$CH_2$); 1.75–1.68 (4H, m, 2-$CH_2$); 1.08 (6H, s, 2$CH_3$) |
| **1b** | 3.33–3.23 (4H, m, 2S–$CH_2$); 1.70–1.32 (6H, m, 3-$CH_2$); 1.03 (4H, s, 2$CH_3$) |
| **1c** | 3.33–3.23 (4H, m, 2S–$CH_2$); 1.76–1.52 (8H, m, 4-$CH_2$); 1.06 (6H, s, 2$CH_3$) |
| **1d** | 3.40–3.35 (4H, m, 2S–$CH_2$); 1.90–1.35 (10H, m, 5-$CH_2$); 1.09 (6H, s, $CH_3$) |
| **1e** | 3.35–3.23 (4H, m, 2S–$CH_2$); 1.78–1.30 (12H, m, 6-$CH_2$); 1.02 (6H, s, $CH_3$) |
| **1f** | 3.32–3.20 (4H, m, 2S–$CH_2$); 1.70–1.27 (14H, m, 7-$CH_2$); 1.00 (6H, s, 2$CH_3$) |

Table 5
$^1$H NMR data ($\delta$ ppm) of compounds **2a**–**f** (DMSO-$d_6$) and signal attribution

| | |
|---|---|
| **2a** | 7.52–7.40 (10H, m, HAr), 3.42–3.38 (4H, m, 2S–$CH_2$), 1.80–1.75 (4H, m, 2$CH_2$) |
| **2b** | 7.50–7.39 (10H, m, HAr), 3.30–3.20 (4H, m, 2S–$CH_2$), 1.71–1.34 (6H, m, 3$CH_2$) |
| **2c** | 7.51–7.47 (10H, m, HAr), 3.33–3.25 (4H, m, 2S–$CH_2$), 1.79–1.56 (8H, m, 4$CH_2$) |
| **2d** | 7.56–7.35 (10H, m, HAr), 3.33–3.23 (4H, m, 2S–$CH_2$), 2.20–1.35 (10H, m, 5$CH_2$) |
| **2e** | 7.40–7.35 (10H, m, HAr), 3.34–3.20 (4H, 2S-$CH_2$), 1.66–1.26 (12H, m, 6$CH_2$) |
| **2f** | 7.44–7.36 (10H, m, HAr), 3.35–3.20 (4H, m, 2S-$CH_2$), 3.12–1.25 (14H, m, 7$CH_2$) |

Table 6
$^1$H NMR data ($\delta$ ppm) of compounds **3a**–**f** (DMSO-$d_6$) and signal attribution

| | |
|---|---|
| **3a** | 7.57 and 7.38 (8H, $J_{a,b} = 8$ Hz, 8H–Ar), 3.40–3.35 (4H, m, 2S–$CH_2$), 1.78–1.75 (4H, m, 2$CH_2$) |
| **3b** | 7.56 and 7.38 (8H, $J_{a,b} = 8$ Hz, 8H–Ar), 3.32–3.25 (4H, m, 2S–$CH_2$),1.68–1.03 (6H, m, 3$CH_2$) |
| **3c** | 7.65 and 7.48 (8H, $J_{a,b} = 8$ Hz, 8H–Ar), 3.43–3.32 (4H, m, 2S–$CH_2$), 1.91–1.68 (8H, m, 4$CH_2$) |
| **3d** | 7.55 and 7.38 (8H, $J_{a,b} = 8$ Hz, 8H–Ar), 3.32–3.20 (4H, m, 2S–$CH_2$), 2.06–1.36 (10H, m, 5$CH_2$) |
| **3e** | 7.44 and 7.38 (8H, $J_{a,b} = 8$ Hz, 8H–Ar), 3.34–3.23 (4H, m, 2S–$CH_2$), 1.62–1.27 (12H, m, 6$CH_2$) |
| **3f** | 7.47 and 7.37 (8H, $J_{a,b} = 8$ Hz, 8H–Ar), 3.35–3.25 (4H, m, 2S–$CH_2$), 3.27–1.27 (14H, m, 7$CH_2$) |

Table 7
Acanthamoeba polyphaga

| Comp. no. | CMA (μg/ml) | | | CMC (μg/ml) | |
|---|---|---|---|---|---|
| | 24 h | 48 h | 78 h | 24 h | 48 h |
| **2c** | 50 | 50 | 20 | >1000 | >1000 |
| **2e** | 25 | 20 | 20 | >1000 | >1000 |
| **3c** | 10 | 10 | 10 | | |

the drugs required to kill 100% of trophozoites is defined as minimal amoebicidal concentration (MAC). The minimum concentration of the drug required to kill 100% of cysts is defined as minimal cysticidal concentration (MCC).

## 3. Results and discussion

All the products **1a**–**f**, **2a**–**f**, **3a**–**f** were tested on three different type of amoebae, but here we report only the most interesting data regarding compounds **2c**, **2e** and **3c**. The results of these products are shown in Tables 7–9. All compounds, especially **2e** and **3c**, were effective on the *A. polyphaga*, *H. varinii* and *V. avara*. These compounds might be assumed as leads for developing new drugs actives against Acanthamoeba. However the length of alkyl chain may be playing an important role. Finally when an electron-withdrawing group (the chlorine atom in our case), is on the benzene ring in para position, a big increase in the activity is observed.

Table 8
Hartmannella varini

| Comp. no. | CMA (μg/ml) | | | CMC (μg/ml) | |
|---|---|---|---|---|---|
| | 24 h | 48 h | 78 h | 24 h | 48 h |
| **2c** | 50 | 50 | 50 | >1000 | >1000 |
| **2e** | 10 | 10 | 10 | >1000 | >1000 |
| **3c** | 10 | 10 | 10 | >100 | >100 |

Table 9
Vahlkampfia avara

| Comp. no. | CMA (μg/ml) | | | CMC (μg/ml) | |
|---|---|---|---|---|---|
| | 24 h | 48 h | 78 h | 24 h | 48 h |
| **2c** | 50 | 50 | 20 | >1000 | >1000 |
| **2e** | 20 | 10 | 10 | >1000 | >1000 |
| **3c** | 5 | 5 | 5 | | |

## 4. Conclusions

The following conclusions can be drawn out from these first results:

- when R = $CH_3$, the minimal amoebicidal concentration (MAC) is always >di 100 μg/ml whatever the length of the alkyl chain separating the two thioureas;
- when R = $C_6H_5$, the minimal amoebicidal concentration (MAC) is of 20 μg/ml if $n = 6$ and is halved (10 μg/ml) when $n = 8$. This means that the amoebicidal power is linked both to the general lipophily of the molecule and to the alkyl chain separating the two thioureas;
- the best products **2c**, **2e** and **3c**, have resulted to be very good antiamoebics as able to destroy the amoebae completely at the concentration between 50 and 5 μg/ml.

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