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# CYTOTOXICITY OF MONENSIC ACID AND ITS BIOMETAL(II) COMPLEXES AGAINST ANAEROBIC BACTERIAL STRAIN *CLOSTRIDIUM PERFRINGENS* SPP.

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## ABSTRACT

*The cytotoxic properties of Monensic acid (MonH) and its biometal(II) complexes [M(Mon)$_2$(H$_2$O)$_2$] (M = Mg, Ca, Mn, Co, Ni, Zn) against the Gram-positive anaerobic bacterium Clostridium perfringens spp. are reported. All the studied Monensin complexes possess similar structures, but their activity varies in the concentration range from 0.17 µmol/L to 11.90 µmol/L. Biometal(II) complexes improved the properties of Monensic acid in in vitro experiments, suggesting their possible practical application as more effective antibacterial agents than the non-coordinated antibiotic.*

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## Introduction

Drug-resistant bacterial strains pose a serious problem due to their resistance towards the antimicrobial mode of action of single antibiotics or their combinations. Polyether ionophores have long been known as effective compounds due to their coccidiostatic activity, as well as to their ability to fight infections caused by mycoplasma, fungi, etc. (6). Although Monensin, Salinomycin, Maduramycin, etc. cannot penetrate the membrane of multidrug-resistant Gram-negative bacteria, they are quite effective against *Clostridium* spp., *Enterococcus* spp. and *Staphylococcus* spp. in the concentration range from 0.25 µg/mL to 16 µg/mL (12). These compounds are usually applied in the form of the corresponding sodium complexes, which possess strong antimicrobial efficacy, but many studies have revealed that their biological activity is sensitive to the local cation(II) environment both in *in vivo* and *in vitro* experiments (1, 2, 3, 4, 5). The potency of antibiotics affected by metal(II) ions could be at least partially explained by the formation of new metal(II) species, e.g. by the ability of ionophores to bind the corresponding divalent cation.

Up to 2008, metal(II) complexes of polyether ionophores with determined structure had not been isolated and characterized. In this respect we have recently studied the ability of these antibiotics, especially Monensin, to coordinate divalent bio- and toxic metal ions. We were able to characterize complexes of various compositions and different structures depending on the antibiotic applied (acidic or sodium form) as well as on the metal(II) ion tested (7, 8, 9, 10). Our studies revealed that the activity of metal(II) complexes of Monensin and Salinomycin is enhanced compared to the non-coordinated antibiotics against Gram-positive aerobic bacteria. The results obtained confirm that complexation of biologically active compounds with ions of essential and bio-metals could be effectively applied as a strategy to improve the properties of the starting drug.

The aim of this study was to further evaluate the ability of metal(II) complexes of Monensic acid (MonH) to inhibit the visible growth of a Gram-positive anaerobic bacterium, *Clostridium perfringes* spp.

## Materials and Methods

Sodium Monensin (MonNa) was supplied from Biovet Ltd. (Bulgaria); metal(II) salts, solvents and $Et_4NOH$, from Merck or Fluka (Bulgaria). Monensic acid (MonH) and its biometal(II) complexes with ions of Mg(II), Ca(II), Co(II), Mn(II), Ni(II) and Zn(II) were prepared as previously reported (8, 9, 10). Their structural characteristics have also been previously described (8, 9, 10).

The antimicrobial properties of Monensic acid, metal(II) complexes and the corresponding metal(II) salts were studied against a strain of the Gram-positive anaerobic bacterium *C. perfringens* spp. isolated from a patient and kindly provided by Assoc. Prof. Dr. M. Marina from the National Reference Laboratory of Anaerobes in the Department of Microbiology, NCIPD, Sofia.

Screening was performed by determining the minimum inhibitory concentration (MIC, µmol/L), which is defined as the lowest concentration of compound inhibiting the visible growth of the given strain. Brucella blood agar (BBA) was prepared by addition of human/sheep blood (25 mL) to autoclaved (120 ºC, 20 min) Brucella agar (24.5 g in 500 mL $H_2O$) containing vitamin K (0.5 mL) and hemin (0.5 mL). A preculture of the bacteria was grown in BBA at 37 ºC for 24 h to 48 h in anaerobic medium. Several morphologically similar colonies were suspended in sterile water and the turbidity

of the inoculum (λ = 650 nm, *b* = 1 cm against water) was adjusted to that one of a 4 McFarland standard. The standard is prepared by mixing 0.4 mL of 1.0 % barium chloride dihydrate ($BaCl_2.2H_2O$) with 9.6 mL of 1 % sulfuric acid ($H_2SO_4$), $12.10^8$ cfu/mL. The inoculum (0.9 mL) was suspended in BBA (20 mL, 24.5 g/L) at 37 ºC ($10^8$–$10^9$ cfu/mL). The sterile agar (10 mL) and the inoculated medium (10 mL) were consecutively poured into Petri dishes. Each tested solution (20 µL) was added into the holes (d = 6 mm) made after solidification of the agar. The diameter of inhibition zones was measured 24 h to 48 h after the inoculation at 37 °C. All measurements were performed in triplicate and confirmed by three separate experiments. All equipment and culture media were sterile.

The tested compounds were dissolved in MeOH/$H_2O$ (50/50 v/v) to obtain working solutions with concentrations ranging from 1 mg/mL to 0.5 µg/mL. Control tests with no active ingredients were also performed.

## Results and Discussion

A series of Monensin metal(II) compounds $[M(Mon)_2(H_2O)_2]$ (M = Mg, **1**, Ca, **2**, Co, **3**, Mn, **4**, Ni, **5**, Zn, **6**) with a similar bidentate coordination mode of monensinate monoanion, where the metal(II) ion is placed in a distorted octahedral environment (8, 9, 10), were used.

These isostructural complexes are a suitable system for evaluation of the influence of the metal(II) ion on the properties of starting monensic acid. Therefore, we performed a preliminary study on their activity against the Gram-positive anaerobic bacterium *C. perfringens* spp. *C. perfringens* causes gas gangrene, which is defined as Clostridial myonecrosis, a severe wound infection with enhanced invasive potential. An important feature of *C. perfringens* is that, unlike other gas-gangrene clostridia, it produces enterotoxin, which contributes to food poisoning and other gastrointestinal illnesses (11).

The experimental results expressed as the minimum inhibitory concentration (MIC, µmol/L) are summarized in **Table 1**. For comparative purposes, data obtained using Gram-positive aerobic bacteria are also included (7, 8, 9, 10). The bacterial assay showed that metal(II) salts are ineffective by themselves and do not affect the growth of aerobic and anaerobic microorganisms.

Monensic acid and its sodium complex (MonNa) were found to possess a similar activity against *B. subtilis* and *B. mycoides*, while MonNa decreased the bacterial growth of *C. perfringens* at a significantly lower concentration, e.g. it is more toxic than Monensic acid.

Generally, all of the biometal(II) complexes enhanced the antimicrobial activity of Monensic acid and sodium Monensin, with the strains of *B. mycoides* and *C. perfringens* being more chemosensitive than *B. subtilis* and *S. lutea*.

The data revealed that complexes 1-3 showed comparable inhibitory effect against Gram-positive microorganisms, which is stronger than that of non-coordinated ligands. The complexes of Ni(II) (**5**) and Zn(II) (**6**) are effective against *B. subtilis*, *B. mycoides* and *C. perfringens*, although they do

**TABLE 1**

Minimum inhibitory concentration of monensins, divalent metal complexes and metal(II) salts against Gram-negative and Gram-positive bacteria

| Compound \ Bacteria | MIC [µmol/L] | | | |
|---|---|---|---|---|
| | ***C. perfringens*** | ***B. subtilis*** [a] Pantcheva et al. (7, 8, 9, 10) | ***S. lutea*** [b] Pantcheva et al. (7, 8, 9, 10) | ***B. mycoides*** [c] Pantcheva et al. (7, 8, 9, 10) |
| MonH | 11.60 | 23.90 | 23.9 | 11.90 |
| MonNa | 2.90 | 23.20 | 23.20 | 11.30 |
| $[Mg(Mon)_2(H_2O)_2]$, **1** | 0.70 | 1.40 | 1.40 | 0.70 |
| $[Ca(Mon)_2(H_2O)_2]$, **2** | 1.40 | 1.40 | 1.40 | 0.70 |
| $[Co(Mon)_2(H_2O)_2]$, **3** | 1.40 | 2.80 | 2.80 | 1.40 |
| $[Mn(Mon)_2(H_2O)_2]$, **4** | 0.17 | 10.30 | 10.30 | 5.10 |
| $[Ni(Mon)_2(H_2O)_2]$, **5** | 0.70 | 1.40 | > 700 | 0.70 |
| $[Zn(Mon)_2(H_2O)_2]$, **6** | 0.17 | 1.40 | > 700 | 0.70 |
| $MgCl_2.6H_2O$ | $5 \times 10^3$ | $5 \times 10^3$ | $5 \times 10^3$ | $1 \times 10^3$ |
| $CaCl_2$ | $9 \times 10^3$ | $5 \times 10^3$ | $5 \times 10^3$ | $1 \times 10^3$ |
| $CoCl_2.6H_2O$ | $4 \times 10^3$ | $2 \times 10^3$ | $2 \times 10^3$ | $2 \times 10^3$ |
| $MnCl_2.4H_2O$ | $5 \times 10^3$ | $5 \times 10^3$ | $5 \times 10^3$ | $5 \times 10^3$ |
| $Ni(NO_3)_2.6H_2O$ | $3 \times 10^3$ | $3 \times 10^3$ | $3 \times 10^3$ | $3 \times 10^3$ |
| $Zn(NO_3)_2.6H_2O$ | $1 \times 10^3$ | $3 \times 10^3$ | $3 \times 10^3$ | $3 \times 10^3$ |

[a] *Bacillus subtilis* (ATCC 6633)

[b] *Sarcina lutea* FDA strain PCI 1000 (ATCC 10054)

[c] *Bacillus mycoides spp*.

not show toxicity against *S. lutea* in the studied concentration range.

Notably, the Mn(II) complex (**4**) was shown to be the least toxic among the metal(II) compounds against Gram-positive aerobic bacteria. On the other hand, the same Mn(II) complex **4** and its Zn(II) analogue (**6**) are most effective against *C. perfringens*, with a toxicity *approx*. 70 and 20 times higher than that of MonH and MonNa, respectively.

The studied compounds can be ordered according to their activity against bacterial strains in the following hierarchy starting from the most effective compound:

*B. subtilis*:**1** = **2** = **5** = **6** > **3** > **4** > MonH = MonNa;

*B. mycoides*: **1** = **2** = **5** = **6** > **3** > **4** > MonH = MonNa;

*S. lutea*: **1** = **2** > **3** > **4** > MonH = MonNa >> **5** = **6**;

*C. perfringens*: **4** = **6** > **1** = **5** > **2** = **3** > MonNa > MonH.

These results suggest that the role of the biometal(II) ion in the corresponding isostructural Monensin complexes is not easy to discuss, since the results are heterogeneous, with MIC values depending to a large extent on the origin of the bacterial strain. To obtain a deeper insight into the antibacterial properties of metal(II) complexes of the polyether ionophores as well as to understand the mechanism(s) by which they inhibit the visible growth of bacterial strains, more studies are required in the future.

## Conclusions

The antibacterial activity of Monensic acid (MonH) and its biometal(II) complexes $[M(Mon)_2(H_2O)_2]$ (M = Mg, Ca, Co, Mn, Ni, Zn) was evaluated against a Gram-positive anaerobic bacterium, *C. perfringens* spp. The results showed that the presence of biometal(II) ions improves the properties of the ligand. The influence of cations of Mn(II) and Zn(II) should be especially taken into account, since the corresponding complexes exert significantly increased cytotoxicity against the tested bacterial strain.


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