**Trypanosoma brucei**: trypanocidal and cell swelling activities of lasalocid acid

Dietmar Steverding¹ · Adam Huczyński²

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**Abstract** Chemotherapeutic treatment of human and animal trypanosomiasis is unsatisfactory because only a few drugs are available. As these drugs have poor efficacy and cause adverse reactions, more effective and tolerable medications are needed. As the polyether ionophore antibiotic lasalocid acid is used as medicated feed additive in cattle, the compound was tested for its trypanocidal and cytotoxic activity against bloodstream forms of *Trypanosoma brucei* and human myeloid HL-60 cells. The concentrations required of lasalocid acid to reduce the growth rate of trypanosomes by 50% and to kill the parasites were 1.75 and 10 μM, respectively. The ionophore displayed also cytotoxic activity against HL-60 cells but the human cells were about 10 to 14 times less sensitive indicating moderate selectivity. As the trypanocidal mechanism of action of polyether ionophore antibiotics is due to a sodium influx-induced cell swelling, the effect of lasalocid acid on cell volume change in bloodstream-form trypanosomes was investigated. Interestingly, lasalocid acid induced a much faster cell swelling in trypanosomes than the more trypanocidal related ionophore salinomycin. These results support further investigations of lasalocid acid and derivatives thereof as potential agents against African trypanosomiasis.

**Keywords** African trypanosomiasis · *Trypanosoma brucei* · Lasalocid acid · Polyether ionophores

**Introduction**

African trypanosomiasis is an infectious parasitic disease of humans (sleeping sickness) and livestock (nagana disease) of similar aetiology and epidemiology. The causative agents of the diseases are flagellated protozoans of the genus *Trypanosoma*. The parasites are transmitted by the bite of infected tsetse flies (*Glossina* sp.) and live and multiply in the blood and tissue fluids of their mammalian host. The distribution of trypanosomiasis in Africa corresponds to the range of tsetse flies and comprises an area of 8 million km² between 14°N and 20°S latitude, a region known as the tsetse belt (Steverding 2017). African trypanosomiasis has severely repressed the economic and cultural development of central Africa in the past (Steverding 2008) and still continues to cause morbidity, mortality and economic deprivation in sub-Saharan Africa (Steverding 2017).

Only a few drugs are currently available for chemotherapy of African trypanosomiasis (Holmes et al. 2004; Steverding 2010). All these drugs have major drawbacks including poor efficacy, significant toxicity and requirement for parenteral administration, and are being increasingly subject to drug resistance (Matovu et al. 2001; Fairlamb 2003; Delespaux and de Koning 2007). Hence, effective and better tolerated chemotherapies are urgently needed for treatment of African trypanosomiasis.

Lasalocid acid (Fig. 1) is a polyether ionophore antibiotic produced by strains of the bacterium *Streptomyces lasaliensis*. It is used in cattle as medicated feed additive (Bovatec®) to improve feed efficiency and to increase the rate of weight gain, and to control coccidiosis caused by *Eimeria bovis* and
Lasalomycin has been shown in clinical pilot studies to be able to eliminate cancer stem cells and to induce partial clinical regression of heavily pretreated and therapy-resistant cancers in patients demonstrating the in vivo activity of polyether ionophore antibiotics (Naujokat and Steinhart 2012). These facts in connection with previous findings that other polyether ionophore antibiotics (salinomycin and monensin) display promising trypanocidal activities (Steverding and Sexton 2013; Steverding et al. 2016) prompted us to investigate the antitrypanosomal action of lasalocid acid and to provide a proof of concept of the potential use of this compound as trypanocide.

**Materials and methods**

**Lasalocid acid**

Lasalocid acid was purified from its sodium salt that was isolated from Avatec Premix as previously described (Huczyński et al. 2013).

**Cell culture**

Bloodstream forms of *T. brucei* (clone 427-221a; Hirumi et al. 1980) and human myeloid leukaemia HL-60 cells (Collins et al. 1977) were maintained in Baltz medium (Baltz et al. 1985) supplemented with 16.7% heat-inactivated foetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37 °C.

**In vitro toxicity assay**

Toxicity assays were carried out as previously described (Merschjohann et al. 2001) with some modifications. In brief, cells (trypanosomes and HL-60 cells) were seeded in 96 well plates in a final volume of 200 µl of the Baltz medium containing various concentrations of test compounds (tenfold dilution from 100 µM to 10 nM) and 1% DMSO. Wells just containing medium and 1% DMSO served as controls. The initial cell densities were 1 × 10⁷/ml for trypanosomes and 5 × 10⁴/ml for HL-60 cells. After 24 h incubation, 20 µl of a 0.5 mM resazurin solution prepared in PBS was added and the cells were incubated for a further 48 h. Thereafter, the absorbance of wells was read on a BioTek ELx808 microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. The 50% growth inhibition (GI₅₀) value, i.e. the concentration of a compound necessary to reduce the growth rate of cells by 50% compared to the control was determined by linear interpolation (Huber and Koella 1993). The minimum inhibitory concentration (MIC) values, i.e. the concentration of the drug at which all trypanosomes and human cells were killed, were determined microscopically.

**Results and discussion**

Lasalocid acid showed a dose-dependent inhibitory effect on the growth of bloodstream forms of *T. brucei* with a MIC value of 10 µM and a GI₅₀ value of 1.75 µM (Fig. 2). Compared with salinomycin, lasalocid acid was 7.6 and 10 times less trypanocidal (MIC and GI₅₀ values for salinomycin were determined to be 1 and 0.23 µM, respectively (Fig. 2)). On the other hand, lasalocid acid was less cytotoxic towards HL-60 cells than salinomycin, the corresponding MIC and GI₅₀ values being 100 and 24.7 µM for lasalocid acid and 1 and 0.32 µM for salinomycin, respectively (Fig. 2). Thus, while salinomycin showed no selectivity (cytotoxic to
trypanocidal activity ratio) with MIC and GI50 ratios of 1 and 1.4, lasalocid acid exhibited moderated selectivity with indices of ≥ 10. The unfavourable selectivity of salinomycin can be attributed to its high cytotoxicity towards HL-60 cells, with our determined GI50 value of 0.32 μM being in good agreement with previously reported values of 0.29–0.44 μM (Huczyński et al. 2012, 2015; Steverding and Sexton 2013; Antoszczak et al. 2014; Steverding et al. 2016).

The biological activity of polyether ionophore antibiotics is due to initiation of an increase in the intracellular concentration of Na+ cations by their ability to transport these ions across biological membranes (Pressman et al. 1980). In cancer cells, this influx of Na+ cations seems to be responsible for the induction of apoptosis (Huczyński 2012) while in bloodstream-form trypanosomes, it leads to swelling of the cell by subsequent entry of water (Steverding and Sexton 2013). In addition, the rate of swelling in trypanosomes seems to be depending on the trypanocidal activity of the ionophore: the higher the trypanocidal activity, the faster the swelling (Steverding et al. 2016). Given that lasalocid acid is less trypanocidal than salinomycin, it was interesting to test whether lasalocid acid would cause a slower swelling rate compared to salinomycin. To be able to record measurable changes in absorbance, a high cell density (5 × 10⁷ cells/ml) and a high ionophore concentration (100 μM) are required (Steverding et al. 2016). Surprisingly, trypanosomes incubated with lasalocid acid swelled much faster than parasites treated with salinomycin (Fig. 3a). Already, after 20 min incubation, no further swelling was recorded indicating that the endpoint of the swelling process was already reached by which the trypanosomes started to die. In contrast, trypanosomes exposed to salinomycin continued to swell until the end of the experiment and started to die after 50 min of incubation. An explanation for the fast swelling activity of lasalocid acid may be the ability of the compound to transport Ca²⁺ ions across membranes that other polyether ionophore antibiotics lack (Pressman et al. 1980). In particular, the large Ca²⁺ concentration gradient of 20,000 (extracellular ~ 2 mM, intracellular ~ 100 nM; Ruben et al. 1991) would be more than sufficient to induce an ionophore-driven influx of Ca²⁺-ions that could cause a rapid swelling of trypanosomes. For comparison, the Na⁺ concentration gradient is just about 10 (extracellular 144 mM, intracellular 13.7 mM; Nolan and Voorheis 2000). In order to test whether the Ca²⁺ transport activity of
lasalocid acid is indeed, the reason for the observed prompt swelling of trypanosomes, a swelling experiment was carried out in the presence of 6 mM of EDTA. This chelating agent has a much higher binding affinity for Ca²⁺-ions than lasalocid acid (K_S values for the Ca²⁺-complexes of EDTA and lasalocid acid are 10⁷.9 (estimated for pH 7.5) and 10².57 (in methanol; Degani and Friedman 1974), respectively). As EDTA binds Mg²⁺ cations as well and as the combined concentration of Ca²⁺ and Mg²⁺ in the Baltz medium is approximately 3 mM, the employed concentration of the chelating agent of 6 mM was determined to be sufficient to reduce the extracellular Ca²⁺ concentration below 10 nM, and thus significantly below the intracellular Ca²⁺ concentration of bloodstream forms of T. brucei. Under these conditions, any ionophore-driven Ca²⁺-transport would be in the efflux direction. However, no difference in the swelling rate of the parasites upon addition of lasalocid acid in the presence or absence of 6 mM EDTA was observed (Fig. 3b). Hence, any Ca²⁺-transport across the plasma membrane seems not to play any role in the lasalocid acid-induced swelling of bloodstream forms of T. brucei, and that the observed fast swelling is solely due to the influx of Na⁺ cations. The reason why lasalocid acid and salinomycin differ in their swelling rates and trypanocidal activities may be just due to their affinity for Na⁺. While the lower K_S value of lasalocid acid for Na⁺ cations (10².57; Degani and Friedman 1974) favours an easier transport of the ion across membranes, the higher K_S value of salinomycin for Na⁺ cations (10³.31; Pressman et al. 1980) facilitates a higher trypanocidal activity.

As chemical modification can increase the trypanocidal activity of polyether ionophore antibiotics (Steverding et al. 2016), we also studied the antitrypanosomal effect of seven Mannich base derivatives of lasalocid acid. The synthesis of the Mannich base derivatives tested is described elsewhere (Huczyński et al. 2013). However, none of the derivatives displayed better trypanocidal activity and selectivity than the parent compound lasalocid acid (Supplementary Table S1). This observation indicates that modification of the carboxyl group of lasalocid acid by Mannich base alkyl/aryl substituents is not the right approach to improve the trypanocidal activity of the ionophore. Perhaps, other modifications of the carboxyl group like esterification or amidation could afford derivatives of lasalocid acid with enhanced antitrypanosomal activity as has recently been shown for salinomycin (Steverding et al. 2016).

This study confirms previous findings that polyether ionophore antibiotics are promising antitrypanosomal agents (Steverding and Sexton 2013, Steverding et al. 2016). Although lasalocid acid, studied here, was found to be less trypanocidal than salinomycin, it had a better selectivity and induced faster swelling than other ionophores. Lasalocid acid may be directly applicable for treatment of nagana disease particularly as the ionophore is used in cattle as medicated feed additive (Bovatec®). As no published data are available, it remains to be shown whether lasalocid acid supplementation can generate high enough plasma levels of the ionophore within its effective concentration range in cattle. However, when chickens were fed with 75 mg sodium lasalocid per kilogram of feed for 1 week, the mean concentration of the antibiotic in serum was 1.36 μg/ml (= 2.3 μM) (Stipkovits and Juhász 1987) which is above the GI₅₀ value of 1.75 μM for the trypanocidal activity of the ionophore (see above). Even if lasalocid acid as feed additive does not provide high enough plasma levels in cattle to affect substantially trypanosomes, the use of the ionophore in food could have a positive impact on the efficiency of the drugs currently employed to treat nagana disease. On the other hand, higher blood levels of lasalocid acid can be achieved by intravenous administration. For instance, intravenous injection of 5 mg of sodium lasalocid per kilogram body weight in a dog resulted in blood levels of the ionophore of > 7.3 μg/ml (> 12 μM) for the following 30 min (Brooks et al. 1975), a concentration that killed trypanosomes in our in vitro assay (MIC = 10 μM, see above). In addition, at normal dosage, lasalocid acid is of low toxicity and usually causes no adverse side effects in cattle. Only in cases of overdosage, cattle show signs of acute intoxication which include anorexia, dyspnoea, tachycardia, ataxia and diarrhoea.Taken together, this information warrants investigations into the in vivo trypanocidal efficacy of lasalocid acid.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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