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Arsthinol nanosuspensions: pharmacokinetics and antileukemic activity on

NB4 promyelocytic leukemia cells

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Abstract

The organoarsenical arsthinol was used in the 1950’s in the treatment of amebiasis and yaws and was considered as “highly tolerated”. The aim of the present work was to study its antileukemic activity and to develop nanosuspensions of the drug, thereby limiting brain concentrations and the risk of encephalopathy. Arsthinol nanosuspensions were produced by high-pressure homogenization. The antileukemic activity was assessed on NB4 acute promyelocytic leukemia cells (vs. solutions of arsthinol, As$_2$O$_3$, and melarsoprol). In addition, a pharmacokinetics study was performed to compare the nanosuspensions and the solution of arsthinol.

Arsthinol induces growth inhibition of NB4 cells at lower concentration (IC$_{50}$ = 0.78 ± 0.08 µmol/l after 24 h) than As$_2$O$_3$ (IC$_{50}$ = 1.60 ± 0.23 µmol/l after 24 h) or melarsoprol (IC$_{50}$ = 1.44 ± 0.08 µmol/l after 24 h). When formulated as nanosuspension, arsthinol remained cytotoxic (IC$_{50}$ = 1.33 ± 0.30 µmol/l after 24 h). This formulation also reduced the drug’s access to the brain (C$_{max}$ = 0.03 µmol/g) whereas bone marrow concentrations remained very high (C$_{max}$ = 2 µmol/g). Consequently, nanosuspensions of arsthinol could be proposed for further studies in the treatment of the acute promyelocytic leukemia.

Keywords: Arsthinol, nanosuspensions, pharmacokinetics, antileukemic activity.
1. Introduction

Arsenic compounds have been used as medicinal agents for many centuries for the treatment of diseases such as psoriasis, syphilis and rheumatosis. From the 1700’s until the introduction of modern chemotherapy and radiation therapy in the mid 1900’s, arsenic was a mainstay in the treatment of leukemia. The discovery in the 1980’s that arsenic trioxide induces complete remission in a high percentage of patients with acute promyelocytic leukemia (APL) has re-awakened interest in this metalloid for the treatment of human diseases [1].

The only arsenical currently formulated for human use in Western countries is melarsoprol, which is synthesized by complexing melarsen oxide with the metal-chelating drug dimercaprol [2,3]. Administration of melarsoprol is indicated in the chemotherapy of second stage of African trypanosomiasis. However, the treatment is complicated [4] and is hampered by severe adverse reactions. A “post-treatment reactive encephalopathy” can occur in 2% to 10% of melarsoprol treated patients. The syndrome is characterized by increased mental excitement, twitching and choreoatherosis, followed by confusion, hyperkinesis, seizures, and death in upto 50% of those affected [5]. Moreover, this drug must be administered by intravenous injection as a 3.6% solution in propylene glycol and exhibits a local intolerability (severe pains, burns and necrosis).

The less-known arsthinol (Figure 1) which was used in the treatment of amebiasis [6] and in dermatology [7] has shown a very good effectiveness on the U937 myelomonocytic cells and on the K562 erythroleukemic cells as compared with As$_2$O$_3$ and other dithiarsolanes [8,9]. Like other dithiarsolanes, arsthinol is very poorly soluble in water [10] and has only been marketed as an oral formulation in the 1950’s [11].
Compared with contemporary formulations, the administration of i.v. nanosuspensions (NS) of arstinol could, modify biodistribution of the drug, limit its access to the central nervous system, and thus, decrease the acute toxicity of organoarsenical compounds [12]. Additionally, colloidal systems have been reported to concentrate the drug in the bone marrow [13-15]. Therefore, formulation of arstinol as a nanosuspension could be increase the suitability of the drug for the treatment of leukemia.

In this work, arstinol has been tested in NB4 acute promyelocytic leukemia (APL) cells, which are known to be very sensitive to trivalent arsenicals [16,17]. A comparison between a nanosuspension of arstinol (NS-ARL), a solution of arstinol (dissolved in propylene glycol; ARL) and As$_2$O$_3$ was carried out. Pharmacokinetics studies were used to confirm the distribution of the drug and consequently assess the value of the NS formulation.
2. Materials and methods

2.1. Materials

Arsthinol and melarsoprol were synthesized according to the method described by Friedheim [3,18]. Arsenic trioxide (As$_2$O$_3$), Pluronic F-127® (poloxamer 407) and mannitol were purchased from Sigma-Aldrich (St Louis, USA). RPMI 1640 with Glutamax$^\text{TM}$-I and antibiotic/antimycotic were purchased from GIBCO (GIBCO Invitrogen, Cergy Pontoise, France).

2.2. HPLC for determination of arsthinol

Concentrations of arsthinol were determined by isocratic reversed-phase HPLC using a Nucleosil C18 column, [4.6 mm x 250 mm, 5 µm (Macherey-Nagel, Eckbolsheim, France)]. The mobile phase of acetonitrile-water (55:45) containing 0.6 % acetic acid, was eluted at flow rate of 1 ml/min. The injection volume was 20 µl and the detection wavelength was 254 nm.

2.3. Solubility of arsthinol in water

An excess of arsthinol (0.28 mmol) was added to phosphate buffer (pH 7.4, 0.1 mol/l, 2 ml). The suspension was stirred in screw-capped vials on a rock and roller agitator (25°C, 6 h) and centrifuged at 7000 g for 5 min. The supernatant was filtered (0.22 µm, MF-Millipore) and arsthinol was quantified in the supernatant by HPLC. The test was performed in triplicate.
2.4. Plasma protein binding of arsthinol

Plasma was obtained from 5 mice, by centrifugation of heparinized blood samples. Arsthinol (0.28 mmol/l = 0.1 mg/ml) was incubated in blank plasma for 30 min or 5 h at 37°C. The binding of the drug to plasma proteins was studied based on a separation by ultrafiltration using a Solvent-Resistant stirred cell (Millipore) and a cellulose membrane of 10,000 Da (ultrafiltration membrane, Millipore, USA). This experiment was performed in triplicate and the concentration of the free drug in the ultrafiltrate was determined by HPLC.

2.5. Preparation of nanosuspension

In order to produce the arsthinol nanosuspensions (NS-ARL), the method previously described was applied [19]. Briefly, the arsthinol powder (0.2 %, w/v, granulometry: 5.7 ± 1.6 µm) was dispersed in an aqueous solution containing 0.8 % Pluronic-127® (Poloxamer 407) and 0.5 % mannitol (w/v) by using an Ultra Turrax stirrer T25® (Janke and Kunkel, IKA® - Labor Technik) for 1 min at 9500 rpm. This pre-dispersion was homogenized using an Avestin Emulsiflex-B3 (Avestin, Canada); 2 cycles at 2.10⁴ kPa and 2 cycles at 5.10⁴ kPa were applied as pre-milling, then 30 homogenization cycles at 15.10⁴ kPa were run to obtain the nanosuspensions.

For lyophilization, the nanosuspensions were immediately stored at -20°C for 24 h, and freeze-dried in SMH15 freeze-drier (Usifroid, Maurepas-France); the temperature of sample, coming from the cold chamber, was first equilibrated with the cooling plate at -56°C for 1 h. Then the total pressure was kept at 5 Pa at 12 °C for 24 h.
2.6. Particle size analysis

The size and zeta potential of nanosuspensions were measured by Zetasizer (3000HS, Malvern instuments, UK) after their dispersion in a drug-saturated solution. Three independent samples produced under identical production conditions were analyzed.

2.7. Dissolution studies of lyophilized nanosuspensions

Dissolution studies were performed using a shaking water bath (WB 14, Memmert GmbH + Co.KG, Schwabach, Germany; 60 strokes per minute). Arsthinol (3.16 µmol of NS-ARL or rough powder) was suspended into 100 ml of phosphate buffer (0.1 mol/l; pH 7.4; 37 °C) under sink conditions. At predetermined time intervals, 2 ml aliquots were withdrawn, filtered (0.22 µm) and centrifuged at 7000 g for 5 min. This experiment was done in triplicate and the amount of dissolved arsthinol was determined by HPLC.

2.8. Pharmacokinetics and tissue distribution studies

Animal handling procedures were performed in accordance with the recommendations of the EEC (86/609/CEE) and French National Committee (décret 87/848) for the care and use of laboratory animals.

CD1 female mice (Charles River Laboratories, France, 24 -26 g) were equally divided into two groups. The first group received a solution of arsthinol (ARL) in a mixture of propylene glycol and sodium chloride 0.9 % (60:40). The second group received a nanosuspension of arsthinol (NS-ARL). Both formulations were administered intravenously at a dose of 0.2 mmol/kg via the caudal vein. Blood samples were obtained from anesthetized mice via cardiac puncture at various time points (5, 30 min, 1, 5, 8, 18, 24 h) post-administration, and collected into heparinized tubes. The samples
were centrifuged (5 000 g, 15 min) and plasma was collected and stored at -20°C until analysis. Tissue samples (liver, kidney and brain) were removed (at 5, 30 min, 1, 5, 8, 18, 24, 48 h), weighted and stored at -20°C until analysis. Bone marrow was flushed from femur shafts with NaCl 0.9%. Three mice were used for each determination.

2.9. Quantification of arsenic in plasma and tissue samples
The amounts of total arsenic in the samples were determined using a colorimetric method [20] after digestion with nitric acid (HNO₃; 65%) and hydrogen peroxide (H₂O₂; 30%). In brief, each sample (tissues or plasma) was placed in a digestion tube with 5 ml of HNO₃ (65%) and 5 ml H₂O₂ (30%). The tubes were heated with a digester apparatus DK-20 (Velp Scientifica), by slowly increasing the temperature from 100°C to 200°C. The clear solution was evaporated to dryness; the residue was taken up with 10 ml of HCl (2 N) and introduced into an arsine generator apparatus (European Pharmacopeia). The reaction was initiated by zinc powder after reduction to trivalent arsenic (As³⁺) with tin chloride (SnCl₂; 40%) and potassium iodide (KI; 15%). After 30 min, the pentavalent arsenic (As⁵⁺) was completely reduced to arsine (AsH₃) and the gas bubbled through a solution of the silver salt of diethyldithiocarbamate in pyridine. The absorbance of the brown complex was measured at 525 nm (Cary-50 Spectrophotometer, Varian). A calibration curve was obtained with increasing amounts of arsenic (As₂O₃, 0-0.09 µmol, n=3).

2.10. Cytotoxic activity on the NB4 cells line
Cell growth inhibition and cytotoxic activity of each arsthinol formulation was determined by using NB4 cells. These cells were cultured in RPMI 1640 + Glutamax™-I medium
supplemented with 10 % fetal bovine serum and 1 % (v/v) antibiotic/antimycotic. Arsenic trioxide (As$_2$O$_3$) was dissolved in sodium hydroxide (1 mol/l), neutralized and diluted further to 10 mmol/l in phosphate-buffered saline (PBS). Arsthinol and melarsoprol were dissolved in propylene glycol and NS-ARL was dispersed in the culture medium. Exponentially growing cells were seeded into a 96-well plate at a concentration of 10$^5$ cells/ml and incubated with ARL, NS-ARL As$_2$O$_3$ and melarsoprol at different concentrations (0.01 µmol/l to 1 mmol/l, n = 3) for 24 h or 48 h, at 37°C in a humidified incubator and 5 % CO$_2$ in air. Viability was assessed using the classical MTT test [21].

2.11. Statistical analysis

All results are expressed as a mean ± standard deviation. Curves and pharmacokinetics parameters were compared using Mann-Whitney test. Significance was tested at the p<0.05 level of probability. A comparison between the four treatments applied on NB4 cells was performed using Kruskal-Wallis test (significance: p<0.05). A post test (Dunn’s test) was used to compare individual differences between the treatments.
3. Results

3.1. Physicochemical characteristics of arsthinol

Arsthinol is a weak organic acid (pKa = 9.5). Its solubility is pH-dependent, and less than 1% of the dissolved arsthinol is ionized at pH 7.4 [10].

In our study, we have clarified some additional physicochemical characteristics of arsthinol. As predicted, its solubility was very low (70 mg/L). In accordance with the lipophilic characteristics of the drug [log P = 2.34 [18]], protein binding was 85 ± 0.8 % after 30 min and 88 ± 1 % after 5 h.

3.2. Characteristics of arsthinol nanosuspensions

A prerequisite for the i.v. injection of suspensions is a small particle size, preferentially in the nanometer range [22-24]. The Emulsiflex-B3 allowed us to produce nanosuspensions of arsthinol suitable for i.v. injections [19]. After 15 homogenization cycles, the particle size was about 500 nm and this was further reduced to 391 ± 21 nm (polydispersity index: 0.285 ± 0.097) after 30 cycles. No further decrease of size was obtained after 30 cycles at 15.10^4 kPa. The zeta potential of about – 20.4 mV can be considered as sufficient to obtain a physically stable suspension [25].

3.3. Dissolution studies of lyophilized nanosuspensions

The dissolution profiles of the freeze-dried NS-ARL (391 ± 21 nm), in comparison with the rough powder (5.7 ± 1.6 µm) are shown in Figure 2. The dissolution rate is significantly increased in the NS-ARL system (p < 0.05): 56 % was dissolved in 10 min as opposed to only 20 % of the rough powder.
3.4. Pharmacokinetics parameters and tissue distribution

The arsenic concentration-time curves for the two formulations were fitted with a two compartment model (Figure 3). In our study, blood pharmacokinetics parameters are practically identical for NS-ARL and ARL (Figure 3, Table 1, p > 0.05). The volume of distribution was 221 ± 22 ml for ARL and 219 ± 35 ml for NS-ARL. Even though these values are close to one another, each formulation does not spread equally in all the tissues.

Figure 4 shows that both formulations (ARL and NS-ARL) concentrate efficiently in the bone marrow ($C_{\text{max}} > 2 \, \mu\text{mol/g}$; Figure 4a) whereas only NS-ARL concentrations remain low in the brain ($C_{\text{max}} = 0.03 \, \mu\text{mol/g}$; Figure 4b). We consistently found a secondary peak that was assumed to be a consequence of the enterohepatic recycling (Figures 4b and 4c).

3.5. Cytotoxic activities on the NB4 cells line

We tested the effects of arsenic compounds (ARL, NS-ARL, melarsoprol and As$_2$O$_3$) in vitro, on the growth of NB4 promyelocytic leukemia cells (Table 2). ARL was found to be significantly more effective ($IC_{50} = 0.78 \pm 0.08 \, \mu\text{mol/l}$) than As$_2$O$_3$ ($IC_{50} = 1.60 \pm 0.24 \, \mu\text{mol/l}$). Moreover, LD$_{50}$ of ARL, previously determined on CD1 mice (22-24 g) [18], is significantly higher than that of As$_2$O$_3$, (402 ± 12 \, \mu\text{mol/kg} and 57 \, \mu\text{mol/kg} respectively).
4. Discussion

4.1. Arsthinol

There is sparse information available in the literature concerning arsthinol. This derivative of arcetarsol (Stovarsol®) was introduced for the treatment of yaws [26] and named STB (complexation of Stovarsol® and British Anti Lewisite). *Treponema Pallidum subspecies pertune*, the bacterial agent responsible for yaws is found predominantly in the granular and basal cell layers of the resulting epidermal lesions. The ability of arsthinol to produce rapid clinical cures in this skin disease suggests that the drug is systemically absorbed and acts on the causative epidermally localized bacteria. Arsthinol was marketed as arsthinol (Balarsen®) and few years latter it was tested in vitro and in vivo in the treatment of amebiasis [27].

Although the neurotoxicity of arsenic has been reported in many studies [28,29], the tissue concentrations of arsenic-based drugs were sparsely reported in the literature. Concerning melarsoprol, pharmacokinetics studies are usually limited to the cerebrospinal fluid (CSF) and authors report very low concentrations [30] but some studies have emphasized high concentrations of arsenic in the spinal cord [28] and in the brain [12]. In contrast, arsthinol was considered as “highly tolerated” [27] in both human and veterinary applications. Recent studies have confirmed that arsthinol (LD₅₀ = 402 ± 12 µmol/kg on female CD1 mice after i.v. administration) is less toxic than melarsoprol (LD₅₀ = 112 ± 1 µmol/kg) and As₂O₃ (LD₅₀ = 57 µmol/kg) [8].

During the last decade, the mechanism of the antileukemic properties of trivalent arsenical derivatives has been partially elucidated. This property was attributed to the linkage between the arsenical compounds and the thiol moieties present on numerous proteins [31-33]. Moreover, trivalent arsenic bonded at a phenyl ring (i.e. arsthinol) is
able to form much more stable covalent cross-links to cysteine residues compared to arsenic in small molecules such as arsenious acid or arsenite [34]. Although the biological targets of the trivalent arsenicals are probably similar, some differences have been pointed out [35]. Some investigations have shown that As$_2$O$_3$ acts on numerous intracellular targets including several signal transduction pathways, which appears to be dependant or independent of PML-RAR$\alpha$. One of the key targets of these compounds could be the intracellular glutathione redox system [36]. NB4 cells contain lower levels of glutathione peroxidase (GPx), glutathione-S-transferase and catalase and relatively higher levels of intracellular hydrogen peroxide (H$_2$O$_2$), compared with other leukemia cells that are less sensitive to As$_2$O$_3$, suggesting that NB4 cells detoxify As$_2$O$_3$ and catabolize H$_2$O$_2$ less efficiently [17].

Although the mechanism of the antileukemic properties of dithiarsolanes (i.e. melarsoprol and arsthinol) is probably quite similar, it does not affect PML-RAR$\alpha$ nuclear localization [16]. Nevertheless it displays a wide cytotoxic spectrum [37].

In this study, arsthinol induces growth inhibition in NB4 cells at lower concentration (IC$_{50}$ = 0.78 ± 0.08 µmol/l after 24 h) than either As$_2$O$_3$ (IC$_{50}$ = 1.60 ± 0.23 µmol/l after 24 h) or melarsoprol (IC$_{50}$ = 1.44 ± 0.08 µmol/l after 24 h). In addition, bone marrow concentrations of arsenic are higher than brain concentrations [C$_{\text{max}}$ (bone marrow) /C$_{\text{max}}$ (brain) = 13]. Similar results were obtained after injection of melarsoprol [12].
4.2. Nanosuspensions of arsthinol

Very few problems were encountered, during the formulation process. The particle size of NS-ARL (391 ± 21 nm) was adapted for intravenous injections and since arsthinol is very poorly soluble in water (70 mg/l), the drug dissolves slowly. Such suspensions may prolong in vivo release and significantly modify the tissue distribution [38]. Similar results were found by Peters et al. [39] for clofazimine nanosuspension with a mean diameter of 385 nm injected intravenously to mice.

Either formulation (ARL and NS-ARL) concentrates very significantly in the bone marrow (Figure 4a). This can be explained, immediately after NS-ARL injection, by the retention of the NS in bone marrow macrophages and granulocytes [14] and by the very high lipophilicity of this organoarsenical drug. Interestingly, NS-ARL concentrate more rapidly in the bone marrow ($T_{\text{max}} = 1$ h) than ARL ($T_{\text{max}} > 2$ h), indicating two different mechanisms of drug accumulation.

As anticipated, ARL can cross the blood-brain barrier (BBB) and enter the brain (Figure 4b). In contrast, particles of NS-ARL covered with poloxamer 407 give much lower concentration in this organ, confirming that nanosized-systems cannot freely diffuse through the blood-brain barrier (BBB) without receptor-mediated transport [40]. Since chronic arsenic exposure can produce damage to the liver and kidney [41], the concentrations of arsthinol in both organs have to be analyzed. In our study, NS-ARL give lower concentration in kidney (Figure 4d) but liver concentrations are slightly increased (Figure 4c).

Finally, in vitro experiments showed that the cytotoxicity of NS-ARL was not significantly different from that of ARL (Table 2). Therefore, our result indicates that NS-ARL has a potent cytotoxic activity on APL.
5. Conclusion

Treatments of APL include simultaneous administration of all-trans retinoic acid (ATRA) and anthracycline-based chemotherapy for induction and consolidation, as well as ATRA-based maintenance. Moreover, an increasing role of arsenic trioxide is emerging in patients relapsing after ATRA-containing regimens; this agent is currently regarded as the best treatment option in this setting.

Compared with As$_2$O$_3$, arsthinol, which was frequently used in the 1950's has a better antileukemic activity when tested on NB4 promyelocytic cells. Moreover, although this drug was considered as "highly tolerated", the use of nanosuspensions (NS-ARL) has allowed us to reduce the cerebral concentration of the arsenical which may decrease the risk of encephalopathy. In contrast, bone marrow concentrations remained very high suggesting that NS-ARL could be proposed for further studies in the treatment of APL.

Acknowledgments

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Table 1  Pharmacokinetics parameters (mean ± S.D., n=3) after intravenous administration of ARL and NS-ARL to mice with dose of 0.2 mmol / kg.

<table>
<thead>
<tr>
<th></th>
<th>ARL</th>
<th>NS - ARL</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (µmol.h/ml)</td>
<td>0.22 ± 0.03</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>C₀ (µmol/ml)</td>
<td>0.036 ± 0.003</td>
<td>0.037 ± 0.007</td>
</tr>
<tr>
<td>Cl_{tot} (ml/h)</td>
<td>25 ± 9</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>K_{el} (h^{-1})</td>
<td>0.113 ± 0.029</td>
<td>0.103 ± 0.114</td>
</tr>
<tr>
<td>Vdβ (ml)</td>
<td>221 ± 22</td>
<td>219 ± 35</td>
</tr>
<tr>
<td>T₁/₂ (β)</td>
<td>6.1 ± 1.1</td>
<td>6.7 ± 1.3</td>
</tr>
</tbody>
</table>

AUC: area under plasma concentration-time curve
Mann-Whitney: p > 0.05 for all parameters (ARL vs. NS-ARL)
Table 2  Cytotoxicity parameters of free arsthinol (ARL), arsthinol nanosuspensions (NS-ARL), As$_2$O$_3$ and melarsoprol on NB4 cells. (mean ± SD, n = 3)

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>IC50 (µM)</th>
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<tbody>
<tr>
<td>ARL</td>
<td>24 h</td>
<td>0.78 ± 0.08 *</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.69 ± 0.07</td>
</tr>
<tr>
<td>NS-ARL</td>
<td>24 h</td>
<td>1.33 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.77 ± 0.21</td>
</tr>
<tr>
<td>As$_2$O$_3$</td>
<td>24 h</td>
<td>1.60 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.84 ± 0.11</td>
</tr>
<tr>
<td>Melarsoprol</td>
<td>24 h</td>
<td>1.44 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.88 ± 0.05</td>
</tr>
</tbody>
</table>

Kruskal-Wallis test: p < 0.05 at 24h, p > 0.05 at 48 h.
* Dunn's test shown significant differences vs. As$_2$O$_3$
Figure 1 Chemical structure of arsthinol.
Figure 2 Dissolution profiles for NS-ARL (-□-) and rough powder of arsthinol (-X-). Mean values ± SD, n=3. * Values are significantly different (Mann-Whitney, NS-ARL vs. ARL, p < 0.05)
Figure 3 Plasma concentration-time curve of arsenic in mice after intravenous administration of 0.2 mmol of arsthibol/kg [ARL (-O-), NS-ARL (-□-), mean values ± SD, n = 3]. Mann-Whitney: p > 0.05 (NS-ARL vs. ARL, at each time)
Figure 4 Concentration-time curve of arsenic in mice after intravenous administration of 0.2 mmol of arsthinol/kg [ARL (-O-), NS-ARL (-□-), mean values ± SD, n = 3]. (a) bone marrow, (b) brain, (c) liver, (d) kidney. * Concentrations are significantly different (Mann-Whitney, NS-ARL vs. ARL, p < 0.05)