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Research Paper

Arsthinol nanosuspensions: pharmacokinetics and anti-leukaemic activity on NB4 promyelocytic leukaemia cells

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Abstract

Objectives The organoarsenical arsthinol was used in the 1950s in the treatment of amoebiasis and yaws and was considered as 'highly tolerated'. The aim of this work was to study its anti-leukaemic activity and to develop nanosuspensions of the drug, thereby limiting brain concentrations and the risk of encephalopathy.

Methods Arsthinol nanosuspensions were produced by high-pressure homogenization. The anti-leukaemic activity was assessed on NB4 acute promyelocytic leukaemia cells (vs solutions of arsthinol, As_2O_3 and melarsoprol). In addition, a pharmacokinetics study was performed to compare the nanosuspensions and the solution of arsthinol.

Key findings Arsthinol induced growth inhibition of NB4 cells at lower concentration (IC50 (concentration inhibiting growth by 50%) = 0.78 ± 0.08 μmol/l after 24 h) than As_2O_3 (IC50 = 1.60 ± 0.23 μmol/l after 24 h) or melarsoprol (IC50 = 1.44 ± 0.08 μmol/l after 24 h). When formulated as nanosuspension, arsthinol remained cytotoxic (IC50 = 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).

Conclusions Nanosuspensions of arsthinol could be proposed for further studies in the treatment of acute promyelocytic leukaemia.

Keywords anti-leukaemic activity; arsthinol; nanosuspensions; pharmacokinetics

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 1700s until the introduction of modern chemotherapy and radiation therapy in the mid 1900s, arsenic was a mainstay in the treatment of leukaemia. The discovery in the 1980s that arsenic trioxide induces complete remission in a high percentage of patients with acute promyelocytic leukaemia (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 the 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 2–10% of melarsoprol treated patients. The syndrome is characterized by increased mental excitement, twitching and choreoatherosis, followed by confusion, hyperkinesis, seizures and death in up to 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 pain, burns and necrosis).

The lesser-known arsthinol (Figure 1), which was used in the treatment of amoebiasis[6] and in dermatology,[7] has shown a very good effectiveness on U937 myelomonocytic cells and on K562 erythroleukaemic cells as compared with arsenic trioxide (As_2O_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 1950s.[11]

Compared with contemporary formulations, the administration of intravenous nanosuspensions of arsthinol 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

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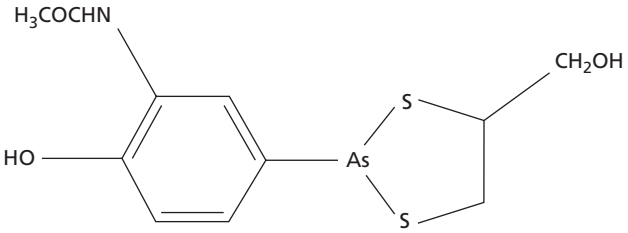

Arsthinol
Chemical formula: $C_{11}H_{14}AsNO_3S_2$
Molecular weight: 347.29

Figure 1 Chemical structure of arsthinol

in the bone marrow.[13–15] Therefore, formulation of arsthinol as a nanosuspension could increase the suitability of the drug for the treatment of leukaemia.

In this work, arsthinol has been tested in NB4 APL cells, which are known to be very sensitive to trivalent arsenicals.[16,17] A comparison between a nanosuspension of arsthinol (NS-ARL), a solution of arsthinol (dissolved in propylene glycol) and As_2O_3 was carried out. Pharmacokinetics studies were used to confirm the distribution of the drug and consequently assess the value of the nanosuspension formulation.

Materials and Methods

Materials

Arsthinol and melarsoprol were synthesized according to the method described by Friedheim.[3,18] As_2O_3, Pluronic F-127 (poloxamer 407) and mannitol were purchased from Sigma-Aldrich (St Louis, USA). RPMI 1640 with Glutamax-I and antibiotic/antimycotic were purchased from Gibco (Gibco Invitrogen, Cergy Pontoise, France).

HPLC for determination of arsthinol

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

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.

Plasma protein binding of arsthinol

Plasma was obtained from five 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.

Preparation of nanosuspension

To produce the arsthinol nanosuspension (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, Staufen, Germany) for 1 min at 9500 rev/min. This predispersion was homogenized using an Avestin Emulsiflex-B3 (Avestin, Ottowa, Canada); 2 cycles at 2×10^4 kPa and 2 cycles at 5×10^4 kPa were applied as pre-milling, then 30 homogenization cycles at 15×10^4 kPa were run to obtain the nanosuspensions.

For lyophilization, the nanosuspensions were immediately stored at −20°C for 24 h, and freeze-dried in an 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.

Particle size analysis

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

Dissolution studies of lyophilized nanosuspensions

Dissolution studies were performed using a shaking water bath (WB 14, Memmert GmbH + Co.KG, Schwabach, Germany; 60 strokes/min). 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 samples 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.

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 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 anaesthetized 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 (5000*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), weighed and stored at −20°C until analysis. Bone marrow was flushed from femur shafts with 0.9% NaCl. Three mice were used for each determination.

Quantification of arsenic in plasma and tissue samples

The amount of total arsenic in the samples was determined using a colorimetric method[20] after digestion with nitric acid (HNO_3; 65%) and hydrogen peroxide (H_2O_2; 30%). In brief, each sample (tissues or plasma) was placed in a digestion tube with 5 ml HNO_3 (65%) and 5 ml H_2O_2 (30%). The tubes were heated with a digester apparatus DK-20 (Velp Scientifica, Milan, Italy), 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 M) and introduced into an arsine generator apparatus (European Pharmacopoeia). The reaction was initiated by zinc powder after reduction to trivalent arsenic (As^{III}) with tin chloride ($SnCl_2$; 40%) and potassium iodide (KI; 15%). After 30 min, the pentavalent arsenic (As^{V}) was completely reduced to arsine (AsH_3) 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, Palo Alto, USA). A calibration curve was obtained with increasing amounts of arsenic (As_2O_3, 0–0.09 μmol, $n = 3$).

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. As_2O_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 arsthinol, NS-ARL, As_2O_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]

Statistical analysis

All results are expressed as a mean ± standard deviation. Curves and pharmacokinetics parameters were compared using the 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 the Kruskal–Wallis test (significance: $P < 0.05$). A post test (Dunn's test) was used to compare individual differences between the treatments.

Results

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.

Characteristics of arsthinol nanosuspensions

A prerequisite for the intravenous 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 intravenous injection.[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]

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 was 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.

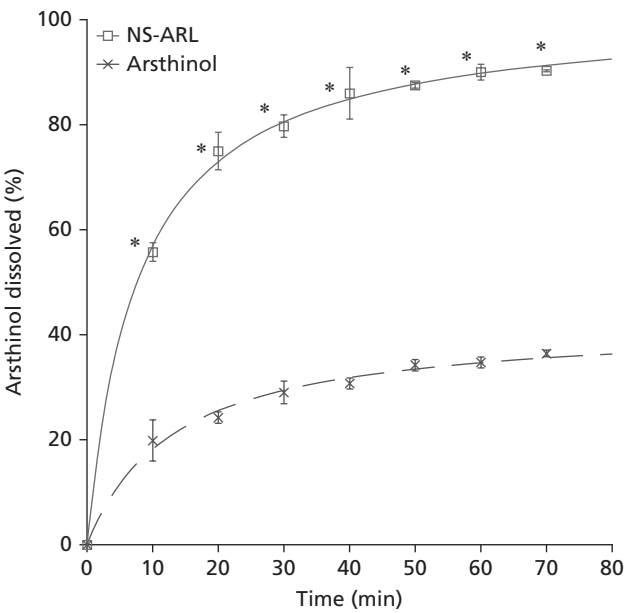

Figure 2 Dissolution profiles for arsthinol nanosuspension and rough powder of arsthinol. Data are presented as mean values ± SD, $n = 3$. NS-ARL, arsthinol nanosuspension. *$P < 0.05$, NS-ARL vs arsthinol (Mann–Whitney test).

Pharmacokinetic 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 pharmacokinetic parameters were practically identical for NS-ARL and arsthinol (Figure 3, Table 1; $P > 0.05$). The volume of distribution was 221 ± 22 ml for arsthinol and 219 ± 35 ml for NS-ARL. Even though these values are close to one another, each formulation did not spread equally in all the tissues.

Both formulations (arsthinol and NS-ARL) concentrated efficiently in the bone marrow ($C_{max} > 2$ μmol/g; Figure 4a) whereas only NS-ARL concentrations remained low in the brain ($C_{max} = 0.03$ μmol/g; Figure 4b). We consistently found a secondary peak that was assumed to be a consequence of the enterohepatic recycling (Figure 4b, c).

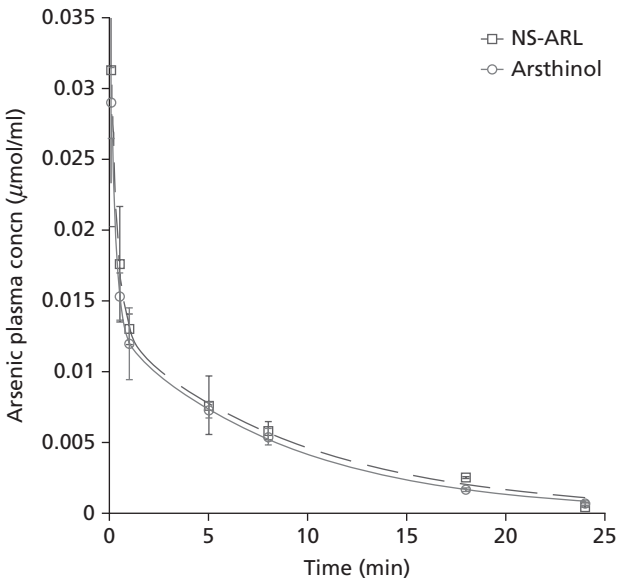

Figure 3 Plasma concentration–time curve of arsenic in mice after intravenous administration of 0.2 mmol arsthinol/kg. Data are presented as mean values ± SD, $n = 3$. NS-ARL, arsthinol nanosuspension. $P > 0.05$, NS-ARL vs arsthinol at each time (Mann–Whitney test).

Table 1 Pharmacokinetics parameters after intravenous administration of arsthinol and arsthinol nanosuspension to mice

	Arsthinol	NS-ARL
AUC (μmol h/ml)	0.22 ± 0.03	0.23 ± 0.04
C_0 (μmol/ml)	0.036 ± 0.003	0.037 ± 0.007
Cl_{tot} (ml/h)	25 ± 9	22 ± 5
K_{el} (1/h)	0.113 ± 0.029	0.103 ± 0.114
Vd_{β} (ml)	221 ± 22	219 ± 35
t½ (β)	6.1 ± 1.1	6.7 ± 1.3

Arsthinol and arsthinol nanosuspension were administered intravenously to mice at a dose of 0.2 mmol/kg. Data are presented as mean ± SD, $n = 3$. AUC, area under plasma concentration–time curve. NS-ARL, arsthinol nanosuspension. $P > 0.05$ for all parameters (arsthinol vs NS-ARL; Mann–Whitney test).

Cytotoxic activity on the NB4 cell line

We tested the effect of arsenic compounds (arsthinol, NS-ARL, melarsoprol and As_2O_3) *in vitro*, on the growth of NB4 promyelocytic leukaemia cells (Table 2). Arsthinol was found to be significantly more effective (IC50 (concentration inhibiting cell growth by 50%) = 0.78 ± 0.08 μmol/l) than As_2O_3 (IC50 = 1.60 ± 0.24 μmol/l). Moreover, the LD50 of arsthinol, previously determined on CD1 mice (22–24 g),[18] is significantly higher than that of As_2O_3, (402 ± 12 μmol/kg and 57 μmol/kg, respectively).

Discussion

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 epidermally localized causative bacteria. Arsthinol was marketed as Balarsen and a few years later it was tested *in vitro* and *in vivo* in the treatment of amoebiasis.[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 intravenous administration) is less toxic than melarsoprol (LD50 = 112 ± 1 μmol/kg) and As_2O_3 (LD50 = 57 μmol/kg).[18]

During the last decade, the mechanism of the anti-leukaemic 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 with 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_2O_3 acts on numerous intracellular targets, including several signal transduction pathways, and that this appears to be dependent or independent of PML-RARα. 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_2O_2), compared

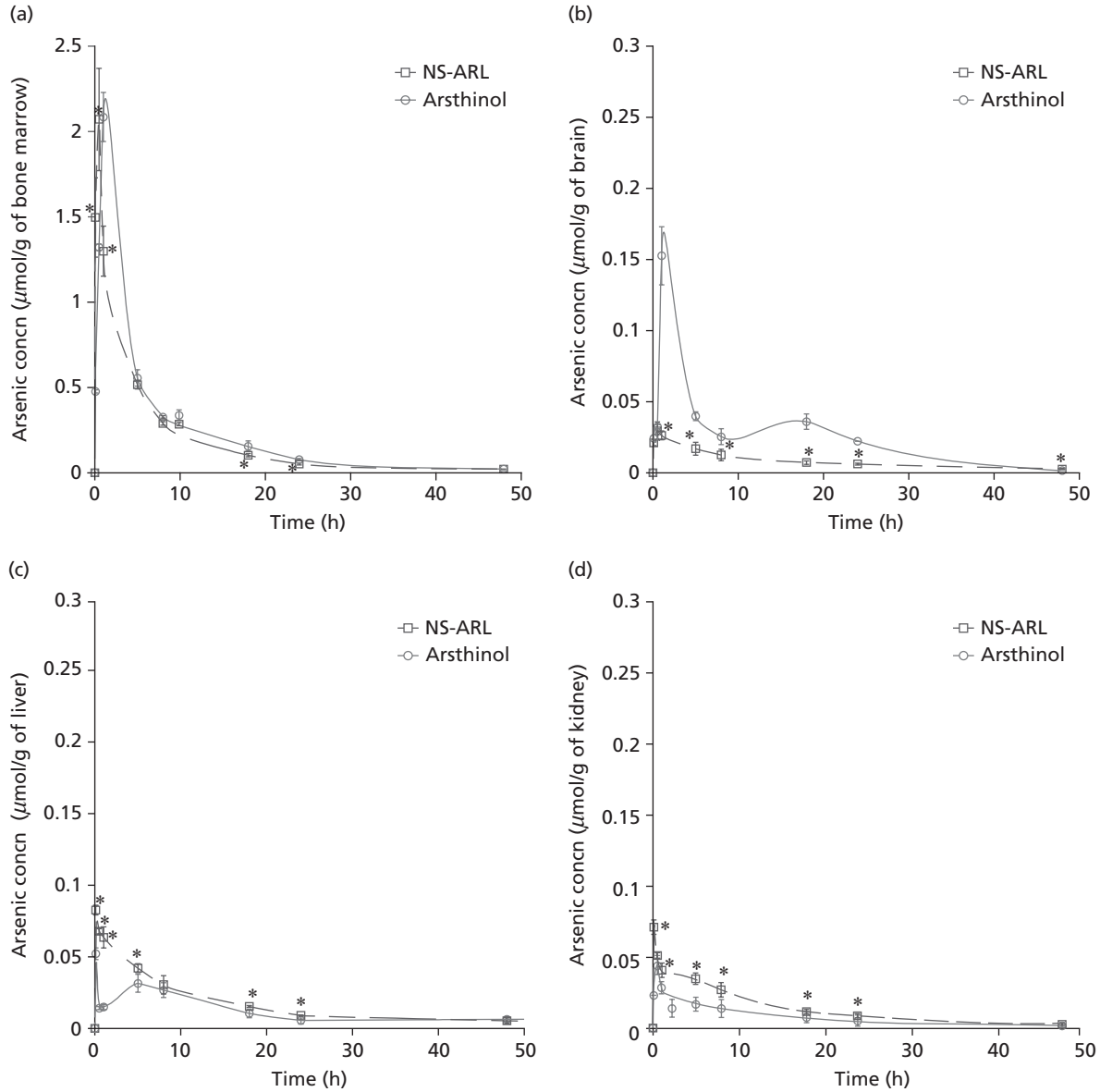

Figure 4 Concentration–time curve of arsenic in mice after intravenous administration of 0.2 mmol/kg of arsthinol. (a) Bone marrow, (b) brain, (c) liver, (d) kidney. Data are presented as mean values ± SD, $n = 3$. NS-ARL, arsthinol nanosuspension. *$P < 0.05$, NS-ARL vs arsthinol (Mann–Whitney test).

Table 2 Cytotoxicity parameters of free arsthinol, arsthinol nanosuspension, As_2O_3 and melarsoprol on NB4 cells

	Time	IC50 (μM)
Arsthinol	24 h	0.78 ± 0.08*
	48 h	0.69 ± 0.07
NS-ARL	24 h	1.33 ± 0.30
	48 h	0.77 ± 0.21
As_2O_3	24 h	1.60 ± 0.23
	48 h	0.84 ± 0.11
Melarsoprol	24 h	1.44 ± 0.08
	48 h	0.88 ± 0.05

Data are presented as mean ± SD, $n = 3$. NS-ARL, arsthinol nanosuspension. $P < 0.05$ at 24 h, $P > 0.05$ at 48 h (Kruskal–Wallis test). *Dunn's test shows significant differences vs As_2O_3.

with other leukaemia cells that are less sensitive to As_2O_3, suggesting that NB4 cells detoxify As_2O_3 and catabolize H_2O_2 less efficiently.[17]

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

In this study, arsthinol induced growth inhibition in NB4 cells at lower concentration (IC50 = 0.78 ± 0.08 μmol/l after 24 h) than either As_2O_3 (IC50 = 1.60 ± 0.23 μmol/l after 24 h) or melarsoprol (IC50 = 1.44 ± 0.08 μmol/l after 24 h). In addition, bone marrow concentrations of arsenic were higher than brain concentrations ($C_{max(bone\ marrow)}$/ $Cmax_{(brain)}$ = 13). Similar results were obtained after injection of melarsoprol.[12]

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 injection 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 (arsthinol 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 nanosuspension in bone-marrow macrophages and granulocytes[14] and by the very high lipophilicity of this organoarsenical drug. Interestingly, NS-ARL concentrates more rapidly in the bone marrow (T_{max} = 1 h) than arsthinol (T_{max} > 2 h), indicating two different mechanisms of drug accumulation.

As anticipated, arsthinol can cross the blood–brain barrier 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 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 analysed. In our study, kidney and liver concentrations are slightly increased (Figure 4c and 4d).

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

Conclusions

Treatment for APL includes 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_2O_3, arsthinol, which was frequently used in the 1950s, has a better anti-leukaemic 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.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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