International Journal of Pharmaceutics 426 (2012) 67–75

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical development of the novel arsenical based cancer therapeutic GSAO for Phase I clinical trial

M.A. Elliott[a,*], S.J. Ford[a], E. Prasad[a], L.J. Dick[a], H. Farmer[b], P.J. Hogg[c], G.W. Halbert[a]

[a] Cancer Research UK Formulation Unit, Strathclyde Institute for Pharmacy and Biomedical Sciences, Robertson Wing, University of Strathclyde, 161 Cathedral Street, Glasgow, G4 0RE, Scotland, UK
[b] Cancer Research UK Drug Development Office, Angel Building, 407 St. John Street, London, EC1V 4AD, England, UK
[c] Adult Cancer Program, Lowy Cancer Research Centre and Prince of Wales Clinical School, University of New South Wales, Sydney, NSW 2052, Australia

ARTICLE INFO

Article history:
Received 29 August 2011
Received in revised form 9 January 2012
Accepted 10 January 2012
Available online 21 January 2012

Keywords:
GSAO
Arsenical
Cancer
Anti-neovascular

ABSTRACT

The novel organoarsenical GSAO, 4-(N-(S-glutathionylacetyl)amino) phenylarsonous acid, has potential anti-angiogenic capability with application in cancer where tumour metastasis relies on neo-vascularisation. As GSAO arsenic is trivalent, the arsenoxide moiety reacts with appropriately spaced cysteine residues on adenine nucleotide translocase (ANT) mitochondrial membrane protein. Molecular oxidation of the arsenic to the pentavalent structure, as in the degradant GSAA (4-(N-(S-glutathionylacetyl)amino) phenylarsonic acid), prevents sulphydryl interaction and risks abolition of activity. We report here on formulation studies aiming to produce a parenteral product with the primary objective of restricting GSAA transformation from GSAO to protect maximal potency of the molecule. Successful anti-oxidant strategy primarily came from pH control. The presence of glycine was proposed to form a stabilising five-membered oxazarsolidinone ring with arsenoxide and this was investigated using potentiometric assays. We report on these tritration studies identifying a pK_a of 8.2 associated with an As-OH, but not confirming ring presence. An original clinical trial pharmaceutical was successfully realised by lyophilisation of 50 mg/mL GSAO in 100 mM glycine solution, pH 7 to obtain a 48-month shelf life for the freeze-dried vials. The Phase I clinical study is ongoing in patients with solid tumours refractory to standard therapy.

1. Introduction

GSAO (4-(N-(S-glutathionylacetyl)amino) phenylarsonous acid, Fig. 1) is a novel, trivalent arsenical compound known to interact with redox active, closely spaced, endothelial cell mitochondrial protein dithiols (Donoghue et al., 2000). Inhibition of angiogenesis in murine solid tumours, without apparent toxicity, was brought about by action on proliferating endothelial cells (Don et al., 2003). Dilda et al. (2005a) determined that GSAO had preferential activity in proliferating endothelial cells over tumour cells owing to the tumour's ability to pump out GSAO via higher levels of the multidrug resistance protein (MRP) 1 and MRP 2. As the need for angiogenesis for tumour sustainability and metastatic potential is known (Carmeliet and Jain, 2000), the use of GSAO as a novel anti-neovascular agent could have promise in human oncology therapeutics with the prospect of its endothelial selectivity and limited toxicity.

Dilda et al. (2008) reported that cell surface γ-glutamyl transpeptidase cleaves GSAO to GCAO (4-(N-(S-cysteinylglycyl-acetyl)amino)phenylarsonous acid). Membrane transport by organic anion transporting polypeptide (OATP) and thereafter action by intracellular dipeptidases cleaves GCAO to its final metabolite, CAO (4-(N-(S-cysteinylacetyl)amino)phenylarsonous acid). CAO bound to cysteine residues 160 and 257 will inhibit adenine nucleotide translocase (ANT) an abundant mitochondrial membrane protein, stop cell proliferation, and induce apoptosis. Quiescent cells were reportedly unperturbed.

In the described cellular metabolism steps, a trivalent arsenoxide (phenylarsenous acid) was conserved, maintaining a binding capacity to appropriately spaced membrane thiols from which mitochondrial toxicity is derived. Therefore, GSAO oxidation to GSAA (4-(N-(S-glutathionylacetyl)amino) phenylarsonic acid Fig. 1), in which arsenic is pentavalent, would be expected to restrict or eliminate drug activity.

A formulation and manufacturing strategy was required for GSAO, and this was to be the aim of this GSAO Phase I pharmaceutical study. The strategy was to be guided by clinical requirements for a stable dosing solution, ideally at 50 mg/mL, achieving a physiologically acceptable pH and osmotic pressure fitting for parenteral

* Corresponding author. Tel.: +44 0 141 548 2454; fax: +44 0 141 548 4903.
E-mail address: moira.elliott@strath.ac.uk (M.A. Elliott).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2012.01.024

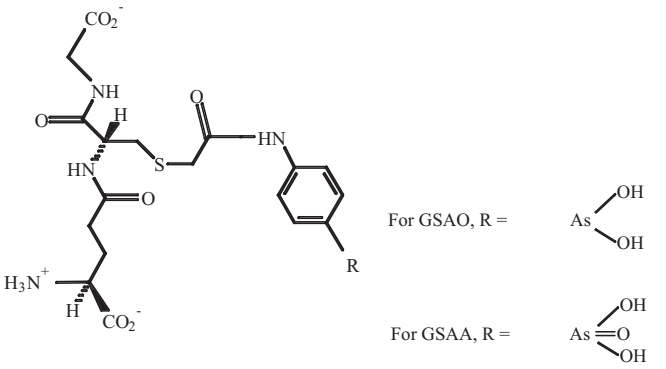

Fig. 1. Molecular structure of GSAO and GSAA.

administration, and equally to attain at least a two-year shelf life for the investigational medicinal product (IMP).

From the mechanistic data presented by Dilda et al. (2008) one of the key contributors to the clinical trial formulation strategy was clear; GSAO would have to be formulated for patient treatment in its active trivalent arsenic configuration. To achieve an effective clinical trial pharmaceutical, evidence had to be sought as to the extent of GSAO oxidation susceptibility, if at all. If susceptible to oxygen, the level of oxidative degradant formation would therefore have to be controlled to within acceptable limits for both IMP production and patient administration.

Seeking a formulation context from the literature confirmed that modern medicine does not make extensive use of organic arsenicals (Dilda and Hogg, 2007). Nonetheless, inorganic arsenic, in the form of arsenic trioxide, is available for the treatment of Acute Promyelocytic Leukaemia after US Food and Drug Administration approval of Trisenox® in 2000 (Zhu et al., 2002). For organoarsenicals, it is informative to look back to the anti-syphilitic work carried out by Ehrlich who knew that only certain arsenical compounds interacted with parasitic thiols, and that pentavalent arsenic had no effect unless reduced to the trivalent arsenoxide. However, by considering arsenoxide drugs too toxic for human treatment, this led to decades of anti-syphilitic therapy with the less potent molecule, Arsphenamine (3,3′-Diamino-4,4′-Dihydroxyarsenobenzene), a drug more toxic to the host owing to the higher doses required for efficacy (Williams, 1995). However, given what was understood of the pharmacology of GSAO, toxicity was not anticipated within what would be its therapeutic range.

To safeguard GSAO arsenic oxidation status and molecular configuration, the Phase I clinical trial would require formulation and manufacturing strategies to present this novel organoarsenical for intravenous delivery in oncology patients ideally achieving maximal potency and on target activity. The molecule's originators had suggested that inclusion of a molar excess of glycine in solution could stabilise GSAO arsenic conformation over several days. Stabilisation was proposed to occur through 5-membered cyclic 1,3,2-oxazarsolidin-5-one ring formation with arsenoxide (Donoghue et al., 2000). Nonetheless, ring formation was only theorised. We considered that ring formation would abolish the arsenic-OH pK_a, and that by following GSAO pK_a abolition with pH variance in the presence of glycine, this would represent a novel approach to examining GSAO-glycine interaction. Using this technique, results are presented that do not confirm the ring stabilisation theory.

A successful clinical trial pharmaceutical was realised in the form of 2 mL fill GSAO lyophilised vial at 50 mg/mL of the active in 100 mM glycine solution pH 7. The material was found to have shelf life of 4 years when presented in this form.

2. Materials and methods

2.1. Chemicals and materials

GSAO raw material (as di-sodium salt) was manufactured according to current Good Manufacturing Practice (cGMP) by Dr Reddy's Laboratories, Hyderabad, India. For molecular analysis, potassium bromide and deuterium oxide NMR grade (99.96%, v/v) were purchased from the Sigma–Aldrich Chemical Company Limited, Gillingham, UK. For HPLC, potassium dihydrogen orthophosphate and 85% orthophosphoric acid (AnalaR grade) were purchased from VWR International, Lutterworth, UK, and acetonitrile HPLC grade was purchased from Rathburn Chemicals Limited, Walkerburn, UK. For pre-formulation studies, glycine, L(+) ascorbic acid, sodium hydrogensulphite, citric acid and sodium citrate dihydrate (all general purpose reagents) were purchased from the Sigma–Aldrich Chemical Company Limited. Di-sodium hydrogen orthophosphate di-hydrate (general purpose reagent grade) was purchased from VWR International. For the manufacture of stability and clinical products, excipients were purchased as follows: glycine (Ph. Eur) and NaOH (extra pure Ph. Eur, BP, JP, NF) were sourced from VWR International; Water for Irrigation (WFI, Ph. Eur) in bulk was from Baxter's Healthcare Ltd., Norfolk, UK. Pharmaceutical product primary packaging was sourced as follows: Type 1 clear glass 10 mL vials (manufacturer Schott AG Pharmaceutical Packaging, Müllheim) with siliconised, ready to sterilise, 20 mm butyl rubber freeze drying stoppers (Manufacturer: West Pharmaceutical Services, Singapore Pte. Ltd.), crimped with 20 mm centre tear off aluminium overseals (Manufacturer: West Pharmaceutical Services, Deutschland GmbH), all purchased from Adelphi Healthcare Packaging, Haywards Heath, UK. Pharmaceutical product secondary packaging was sourced as 10 × 20 mL white card vial boxes, (Catalogue number BO031) from Adelphi (Manufacturing) Limited, UK.

2.2. Confirmation of the molecular structure of GSAO

Drug substance elemental analysis for carbon, hydrogen, nitrogen and sulphur content was performed by the University of Strathclyde Department of Pure and Applied Chemistry Microanalysis service. Results were expressed as percent actual versus percent theoretical, calculated from the molecular formula.

The Strathclyde Institute for Pharmacy and Biomedical Sciences NMR service (University of Strathclyde) carried out 400 MHz ^{1}H and ^{13}C NMR of 50 mg/mL GSAO in deuterium oxide at room temperature.

Mass spectral analysis was performed at the Strathclyde Institute of Pharmacy and Biomedical Sciences Mass Spectrometry service. GSAO was dissolved in 50:50 0.1% formic acid:methanol and assayed using positive ion electrospray ionisation.

For UV analysis of the drug substance, GSAO was dissolved in WFI at 0.05 mg/mL and scanned from 200 nm to 400 nm to establish peak maxima using a Unicam UV4-100 UV–visible spectrophotometer (v5.03). Both molar absorption co-efficient λ_{max} ($mol^{-1}\ dm^{-3}\ cm^{-1}$) and A (1%, 1 cm) were determined.

2.3. High performance liquid chromatography

HPLC of GSAO, as both raw material and pharmaceutical product, was carried out using a Surveyor HPLC system (Thermo Fisher Scientific, Hemel Hempstead, UK). Analysis was performed using a Luna C18(2) 150 mm column (Phenomenex, Macclesfield, UK). Detection was by UV using dual wavelength at 210 nm and 260 nm, with injection volume at 5 μL, flow rate at 1 mL/min and column oven at 25 °C. The analytical gradient was as follows; 0 min = 100% A: 0% B; 20 min = 89% A: 11% B; 25 min = 67% A: 33%

B; 26 min = 0% A: 100% B; 29 min = 0% A: 100% B; 30 min = 100% A: 0% B; 45 min = 100% A: 0% B where Mobile phase A is 95:5 0.02 M phosphate buffer:acetonitrile, pH 2.5 and Mobile phase B is 50:50 0.02 M phosphate buffer:acetonitrile. For raw material analysis an HPLC specification was set as follows; purity >90% by peak area (at 260 nm), complex B <3% peak area/peak area, any other single impurity <1% pa/pa, total impurities limited to not more than (NMT) 7.5%. GSAA (4-(N-(S-glutathionylacetyl)amino) phenylarsonic acid, the main oxidative degradant), had specification of <1%w/w. As only small quantities of GSAA were available, there was insufficient material to prepare a GSAA calibrant solution for every analysis to be performed throughout the full stability study. This drove additional studies on the GSAA material that showed that the chromatographic response factors for GSAA and GSAO were equivalent, and that the peak area ratio of GSAA:GSAO was therefore a suitably accurate surrogate for the w/w ratio of the same analytes.

The GSAO batch used for reference standard purposes was designated as Dr. Reddy's API Batch Number 6 against which all batch analyses were made.

2.4. Formulation studies

2.4.1. Basic solubility in aqueous solution

The basic solubility of GSAO in water, and in a separate experiment in 25 mM citrate buffer (pH 6.5), 25 mM phosphate buffer (pH 6, 7 and 8) and 25 mM Glycine–NaOH buffer (pH 8), were investigated by dissolving fixed amounts of GSAO in the test solvent. Solubility was determined visually, that is test solutions had to be visually clear without precipitation to be determined as 'soluble.' If precipitation was observed, no further powder additions would be made. The solubility level was then approximated as equivalent to the total powder mass added by the last powder addition in the total test solvent volume.

2.4.2. Extended solubility studies

GSAO solubility measurements were performed using a Sirius T3 (Sirius Analytical Instruments Ltd., Forest Row, East Sussex, UK) at $25 \pm 1\,^{\circ}C$ fitted with a Ag/AgCl, double junction reference electrode, an ultra mini immersion probe attached to an MMS UV/VIS Carl Zeiss Microimaging spectrophotometer and a stirrer, controlled by Sirius CheqSol method software. Titrations were carried out in ion strength adjusted water (0.15 M KCl) titrating with 0.5 M KOH and 0.5 M HCl under argon in the direction pH 12 down to pH 2.

2.4.3. pK_a analysis

Measurements were performed using a Sirius T3 as for the extended solubility studies. Potentiometric pK_a titrations were also carried out in ion strength adjusted water (0.15 M KCl) titrating with 0.5 M KOH and 0.5 M HCl under argon.

2.4.4. Use of antioxidants and glycine in solution

The effect of antioxidants was briefly investigated using GSAO aqueous solutions with water-soluble antioxidants, specifically 0.1% w/v sodium hydrogen sulphite solution and 1% w/v ascorbic acid. Solutions were stored at 40 °C for 48 h in closed containers (without oxygen removal), and the contents were analysed after storage by HPLC for the presence of GSAA, presented as percent peak area with respect to main GSAO peak area.

For glycine studies, GSAO was dissolved at 50 mg/mL in 50 mM, 100 mM and 200 mM glycine solution, each glycine strength at pH 7, pH 8 and pH 9. Initial GSAA contribution was determined by HPLC (as percent peak area). After 6 days storage under either standard laboratory ambient conditions (controlled at 15–25 °C), or at 40 °C incubation, the solutions were re-analysed by HPLC for the presence of GSAA and results reported as percent increase with respect to initial GSAA value.

2.4.5. Differential scanning calorimetry (DSC)

Thermal analysis to guide lyophilisation conditions of test formulations was carried out using DSC. Liquid solutions were transferred to 40 μL aluminium pans and sealed. Analysis was performed against a sealed empty reference pan using nitrogen as purge gas. The test program was as follows; cooling gradient from 20 °C to −40 °C at 2 °C/min, isothermal phase holding at −40 °C for 5 min, followed by heating at 2 °C/min from −40 °C to 20 °C.

2.4.6. Pilot scale lyophilisation

Small-scale test lyophilisations were carried out manually using a Lyostar tray drier Model Number LSACC4, with test programs guided by DSC results.

2.5. Batch production

2.5.1. Production scale lyophilisation

Lyophilisation to produce stability batches and clinical pharmaceutical product were carried out using an LSL Secfroid FCFV600S freeze drier (LSL Secfroid, Aclens, Switzerland). The final program was as follows; Freezing: +20 °C to −40 °C over 3 h, hold for 2 h, Primary drying: ramp from −40 °C to −25 °C over 1 h, hold at −25 °C for 34 h, Secondary drying; ramp from −25 °C to +10 °C over 2 h, hold at +10 °C for 3 h, ramp from +10 °C to +20 °C over 1 h, hold at +20 °C for 2 h. Primary drying chamber pressure was 0.1 mbar. Secondary drying progress was followed by means of pressure rise testing. Solutions for lyophilisation were aseptically prepared to the following formula: for 2 mL per vial at 50 mg/mL GSAO (as di-sodium salt) pH 7; GSAO 100 mg, glycine 15 mg, NaOH 0.08 mL, WFI in quantity sufficient to 2 mL. Bulk solutions were sterile filtered using Millipak 20 filters (Millipore (UK) Limited, Watford) into washed, depyrogenated and sterilised 10 mL Type 1 clear glass vials. To conclude the lyophilisation run, the chamber was backfilled to 95% atmospheric pressure with filtered nitrogen, after which pneumatic stoppering was carried out. Vials were manually crimped within a Grade A environment using washed and steam sterilised overseals.

2.5.2. Stability testing

Lyophilised stability batches were analysed according to a stability program with storage at +5 °C ±3 °C, +25 °C/60% Relative Humidity (RH), +40 °C/75% RH and +55 °C over 1, 3, 6, 9, 12, 18, 24, 30, 36 and 48 months (note that not all temperatures were studied at all time points). Analysis was by HPLC for GSAO percent content and GSAA % w/w degradant levels.

3. Results

3.1. Confirmation of the molecular structure of GSAO

For elemental analysis, comparison was made between percent actual and percent theoretical elemental content of GSAO raw material as follows; carbon 34.64% (actual by analysis) versus 34.68% (theoretical), hydrogen 4.27% versus 4.57%, nitrogen 9.09% versus 8.98% and sulphur 5.39% versus 5.14%. For NMR analysis, proton assignments and carbon assignments from Jmod experiments are given in Table 1. With molecular formula $C_{18}H_{25}AsN_4O_9S$, mass spectral analysis confirmed a signal at 571 m/z consistent with the protonated monosodium GSAO $[M+H]^+$ ion. A single absorption maximum under UV analysis was observed at 257 nm, for which GSAO λ_{max} was calculated as 17,460 $mol^{-1}\,dm^{-3}\,cm^{-1}$ and A (1%, 1 cm) as 305.

Table 1
^{1}H NMR proton and ^{13}C NMR (Jmod) assignments for GSAO.

Chemical shift ^{1}H NMR	Proton equivalence	Assignment (proton number)	Chemical shift, ^{13}C NMR (Jmod)	Assignment
7.722–7.676 (d)	1.00	1, 2, 3, 4	174.77	
7.585–7.515 (d)	1.02		173.44	
4.900–4.801 (m)	–	Water	172.50	Carbonyl groups
4.609–4.596 (dd)	0.56	16	170.36	7, 11, 13, 14 and 18
3.760–3.746 (m)	1.73	10, 11	169.63	
3.725–3.625 (m)		9	142.87	Aromatic ring
3.519–3.437 (t)	1.05	5, 6	137.53	3 and 6
3.206–3.159 (m)	0.54	7, 8	128.71	Aromatic ring
3.113–2.878 (m)	0.54		120.04	1, 2, 4 and 5[a]
2.590–2.404 (m)	1.13	12,13	52.69	Alpha carbons
2.21–2.05 (m)	1.17	14, 15	51.58	(CH) 10 and 17
			41.95	8
			34.77	9
			32.31	Alpha carbons (CH_2)
			29.97	12 and 15
			24.69	16 (beta carbon)

d, doublet; t, triplet; m, multiplet.
[a] Carbons 1 and 5, and 2 and 4 are equivalent.

3.2. HPLC analysis of drug substance and pharmaceutical product

The GSAO main peak retention time was 10.6 min, with GSAA retention time at 5 min (Fig. 2). Further impurities were identified in the raw material. Results of drug substance analysis were observed to meet specification as follows (see Section 2.3); purity 91.93% by peak area (at 260 nm), complex B 2.7% peak area/peak area, any other single impurity passed specification, and total impurities were calculated at 5.8%. GSAA was measured as 0.86%w/w.

3.3. Formulation studies

3.3.1. Basic solubility in aqueous solution

GSAO solubility in water was observed up to 200 mg/mL (final solution pH 8.8). When this test solution pH was titrated with acid down to pH 5, some evidence of precipitation was observed at the lower pH. From this first simple solubility result, GSAO could be considered as potentially freely soluble in water over the range >pH 5 and <pH 9. With this water solubility result, and with further consideration to physiological pH, GSAO solubility at 100 mg/mL in phosphate buffer pH 7 was examined, but resulted in precipitation at the first addition of the powder to the solvent. Two new phosphate buffers at pH 6 and pH 8 were tested in an identical manner, with the same observation as for pH 7. GSAO was therefore initially considered to be less soluble in phosphate buffer than in water, and possibly also incompatible with phosphate buffer. A non-phosphate citrate buffer was next selected, albeit at the upper range of citrate's buffering capacity (a test of pH 6.5 was chosen where the true buffering range can be expected from pH 3 to 6.2). To examine a higher pH solution, a glycine–NaOH buffer at pH 8 was also included. Using this basic methodology, GSAO solubility to at least 100 mg/mL was observed for both citrate and glycine–NaOH buffer tests. Results are presented in Table 2.

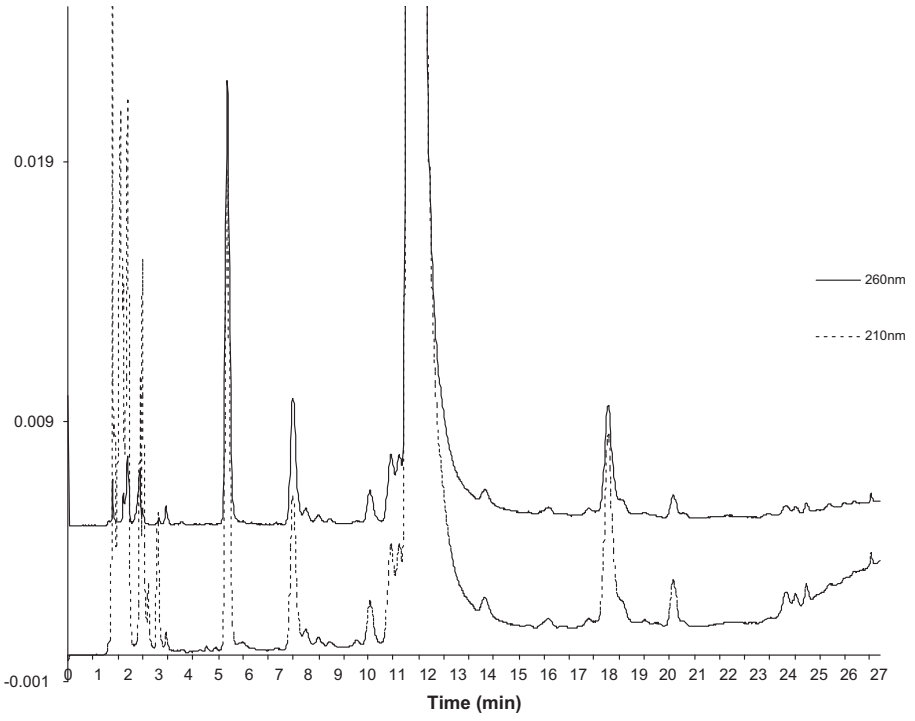

Fig. 2. Representative chromatogram of GSAO analysed by HPLC with UV detection at 210 nm and 260 nm.

Table 2
Basic solubility studies with GSAO.

Test solvent	Initial pH (on dissolution)	Final pH (after titration or adjustment)	GSAO solubility observation
Water	8.8	–	Soluble to at least 200 mg/mL
Citrate buffer pH 6.5	8.9	6.3	Soluble to at least 100 mg/mL
Phosphate buffer pH 6	Insoluble	–	Precipitation at first powder addition Solubility <100 mg/mL
Phosphate buffer pH 7	Insoluble	–	Precipitation at first powder addition Solubility <100 mg/mL
Phosphate buffer pH 8	Insoluble	–	Precipitation at first powder addition Solubility <100 mg/mL
Glycine–NaOH buffer pH 8	7.9	7.6	Soluble to at least 100 mg/mL
T3 solubility test (CheqSol method)	2	12	Soluble to at least 182 mg/mL across whole pH range

3.3.2. Extended solubility studies

Using the Sirius T3 instrument and their proprietary CheqSol method, GSAO intrinsic or kinetic solubility could not be determined as precipitation did not occur at the test concentration over the full test pH range (precipitation must occur for these solubility values to be determined using this method). Therefore, the conclusion of the extended solubility study was that GSAO is at least soluble to 182 mg/mL over the range pH 2–12. This was in concordance with the general observation of 'freely water soluble' made under the basic solubility studies (Table 2).

3.3.3. Use of antioxidants and glycine in solution

Aqueous unbuffered solutions of 100 mg/mL GSAO were prepared with 0.1%w/v sodium hydrogen sulphite pH 4.2 and 1% w/v ascorbic acid pH 2.3, with two comparator solutions of GSAO in 100 mM glycine and GSAO in water only. Analysis by HPLC after 48 h storage determined the amount of GSAA with respect to the GSAO main peak area. For 0.1% w/v sodium hydrogen sulphite solution, GSAA was measured at 10.4% w.r.t. GSAO after incubation, and for the 1% ascorbic acid test, 13.8% GSAA was present after storage. In the comparator glycine and water only solutions, tests contained only 4.2% and 3.9% GSAA respectively under the same analysis.

Further studies examined GSAO in 50 mM, 100 mM or 200 mM glycine solution after storage under laboratory ambient (15–25 °C) and 40 °C conditions after which the percent change in GSAA content from the initial value was determined. Results are presented graphically in Fig. 3.

3.3.4. pK_a analysis

Potentiometric pK_a analysis of GSAO identified a pK_a value of 8.2 associated with one of the As-OH protons in the arsenoxide moiety. The UV-metric assay results are presented in Fig. 4.

3.3.5. DSC

Thermal analysis of 50 mg/mL GSAO in 100 mM glycine showed a freezing exotherm onset at −14.4 °C, with an onset of melting endotherm at −10 °C.

3.3.6. Pilot scale lyophilisation

After 48 h of laboratory ambient storage (15–25 °C), lyophilised vials along with the drug solution were assayed by HPLC for %GSAA content with respect to GSAO. In the input drug solution, GSAA initial content was 1.4%, whereas after 48 h of storage the same solution assayed with an increased GSAA content of 1.58%. Assay of lyophilised vials under the same conditions confirmed no GSAA content increase (1.41% after storage).

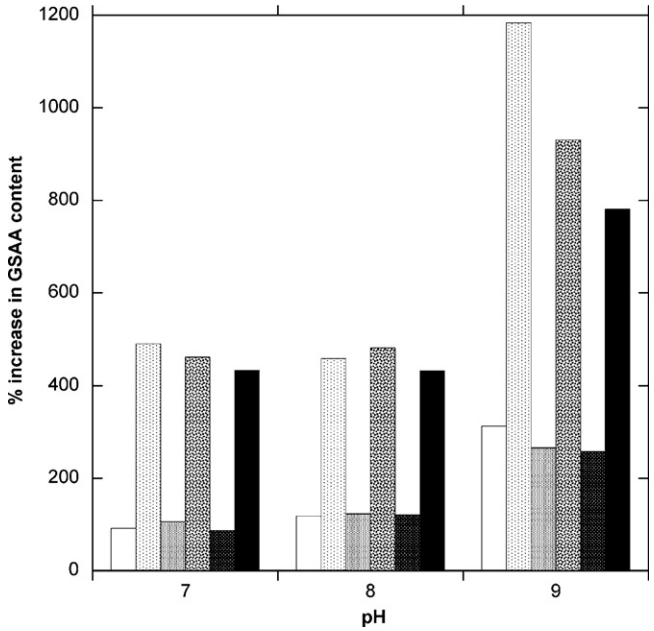

Fig. 3. The effect of temperature, pH and glycine concentration on the development of GSAA after 6 days storage of GSAO in solution under ambient and +40 °C storage conditions; 50 mM glycine □; 50 mM glycine (40 °C) ⬚; 100 mM glycine ▩; 100 mM glycine (40 °C) ■; 200 mM glycine ■; 200 mM glycine (40 °C) ■.

3.4. Batch production

3.4.1. Production scale lyophilisation

Stability study, toxicology study and clinical use batches of lyophilised GSAO were manufactured according to the program in Section 2.5.1.

3.4.2. Stability testing

Results from the programmed stability study for GSAO and GSAA content are presented in Figs. 5 and 6. Analysis for GSAO content confirmed compliance to a 90% to 110% content specification (with respect to stated amount) throughout the test for all temperatures studied. Equally, no failures were noted at any temperature when analysed for GSAA % w/w content, limited by a not greater than 1% w/w content of the oxidised degradant (the result for GSAA at 36 months is not related to variability in the validated analytical method and is out of trend in the context of the surrounding results).

4. Discussion

Based on clinical predictions, a GSAO solution of around of 50 mg/mL for patient dosing was preferred, and in this context,

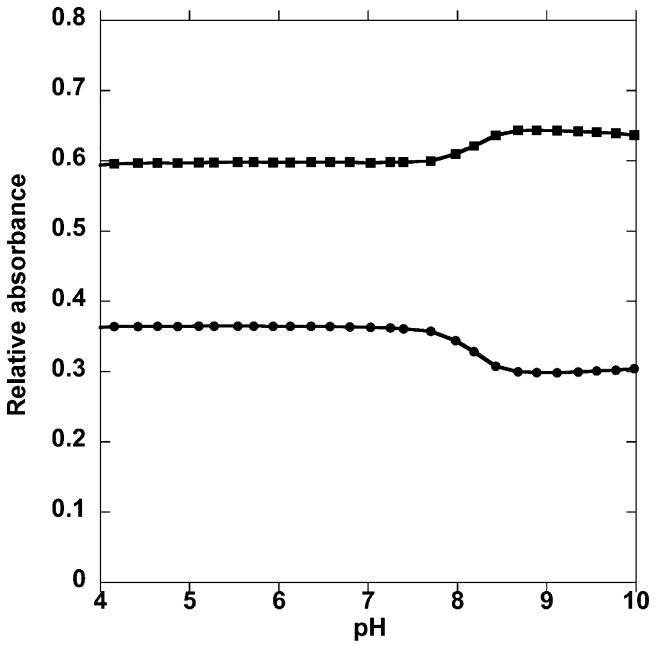

Fig. 4. Results from a UV-metric Sirius T3® titration of GSAO with pH; GSAO alone at 266 nm ■, GSAO alone at 235 nm ●.

basic GSAO solubility was of primary interest. Indeed GSAO showed good aqueous solubility, as predicted by its glutathione adduct structural chemistry (glutathione is freely soluble in water, Merck Index 2006). A potentiometric UV study indicated solubility to at least 182 mg/mL in plain water across the pH range 2–12, a significant and welcome property for any oncology therapeutic. Unbuffered in water and at 200 mg/mL, GSAO solution developed a pH of 8.8, considered at this level of alkalinity as unsuitable for parenteral administration. Therefore buffered solutions in the physiologically acceptable pH range of 6–8 were examined, in the first instance at half the water solubility level, but in excess of clinical need, that is at a target of 100 mg/mL GSAO (in common with many Phase I units, sample availability can be limited in early stages, and as such experimental design could not be extended or complex in these basic studies). Despite demonstrating a satisfactory water solubility result, GSAO failed to dissolve in phosphate buffer at the target concentration at pH 6, 7 or 8 showing visually determined evidence of precipitation in each case. Using non-phosphate buffer solutions at pH 6.5 and 8 reconfirmed that GSAO solubility at 100 mg/mL could be achieved. A first conclusion against the 50 mg/mL dosing solution objective was therefore made; at 182 mg/mL GSAO was water soluble in excess of clinical needs across a pH range of 2–12, although a pH independent phosphate buffer incompatibility was also suggested by these

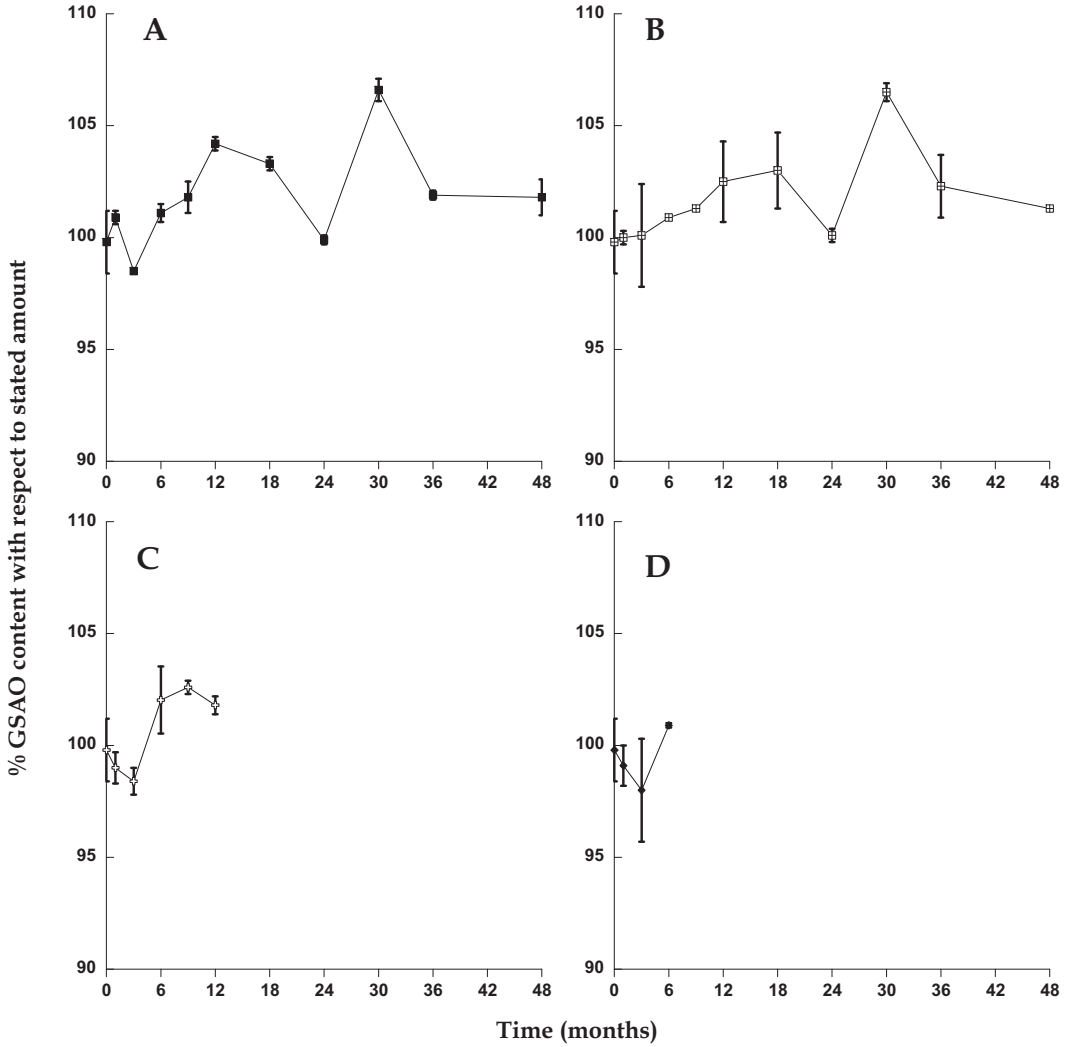

Fig. 5. Results from 48-month stability study presented as percent GSAO content for all temperatures (specification: 90% to 110% of stated content); Panel A = +5 °C, Panel B = +25 °C, Panel C = +40 °C and Panel D = +55 °C.

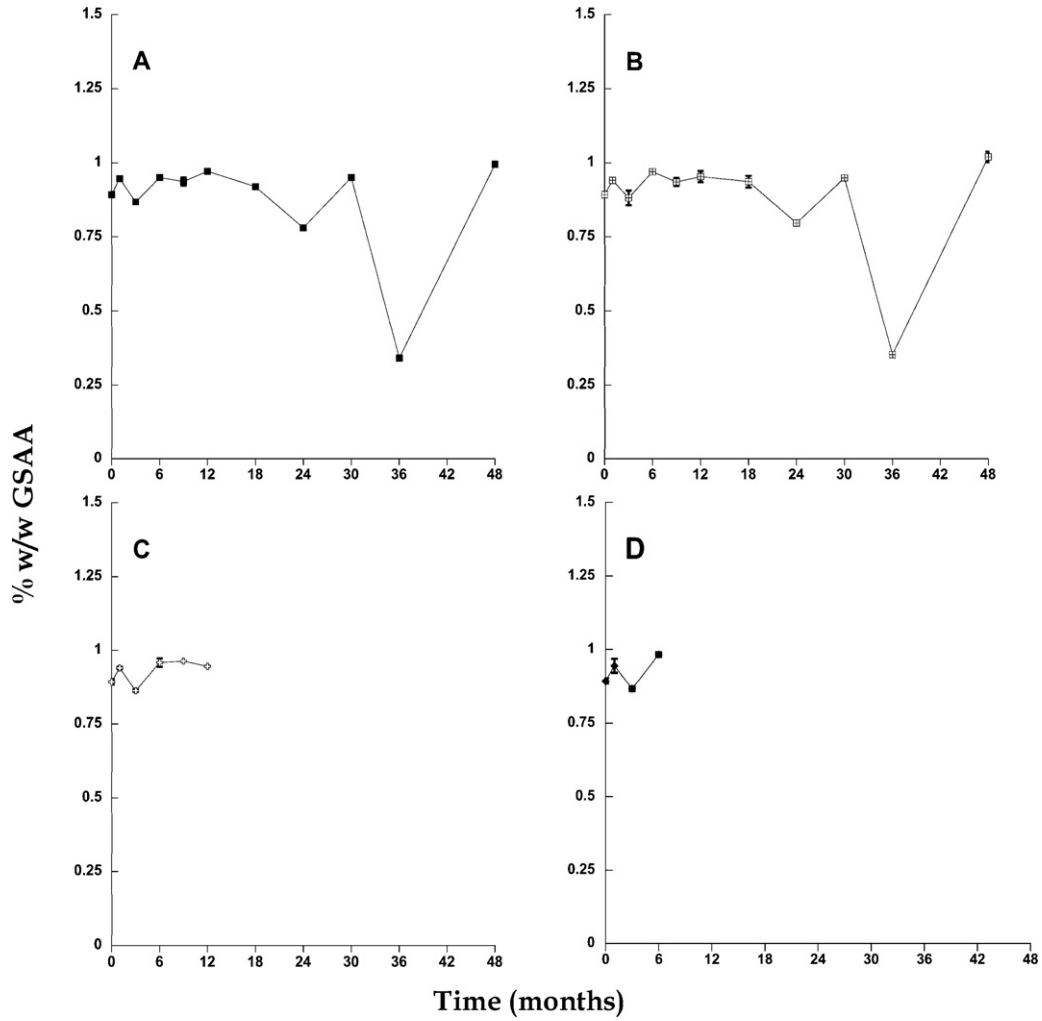

Fig. 6. Results from 48-month stability study presented as percent GSAA content for all temperatures (specification: not more than 1% w/w); Panel A = +5 °C, Panel B = +25 °C, Panel C = +40 °C and Panel D = +55 °C.

early results. It was clearly recognised that selection of a buffering agent to maintain a physiologically acceptable pH was required. Nonetheless, a solution formulation for clinic was highly unlikely, as inactivation in solution to GSAO's pentavalent degradant GSAA had been previously reported (Donoghue et al., 2000). Control of GSAO arsenic oxidation status had to be addressed in order to achieve the goal of a stable dosing solution for both manufacture and patient administration.

In their report on the possible in vivo metabolism of GSAO, Dilda et al. (2008) proposed that sequential metabolism cleaves the parent molecule to the final active agent, CAO. Notably the trivalent arsenoxide moiety is continually conserved during the metabolic sequence. In work in which the arsenoxide position in GSAO was modified from the para- to ortho-position on the benzene ring, although 50 times more potent as an endothelial cell proliferation inhibitor (attributed to faster cell accumulation largely owing to lower cell export by MRP1), the ortho-analogue was more toxic in vivo and therefore was not selected for further development (Dilda et al., 2005b). A cyclic complex is believed to form between GSAO arsenic and appropriately closely spaced protein dithiols. This reaction does not occur for protein monothiols (Donoghue et al., 2000). Whereas molecular spatial considerations to ensure potent target site interaction can be designed by medicinal chemistry, arsenoxide conservation can be addressed by pharmaceutical formulation strategy. Indeed, the development of a formulation that maintained the trivalent arsenic speciation of the arsenoxide moiety was essential.

In their work with organoarsenical solutions, Hogg's group showed time dependent inactivation, specifically in the transformation of the trivalent arsenic in GSAO to the pentavalent conformation in GSAA (Donoghue et al., 2000). Reaction restriction was achieved by oxygen removal from solution, lowering pH, but in particular by introducing glycine in molar excess. Glycine was believed to form a stabilising 1,3,2-oxazarsolidin-5-one ring, although these authors had not proven this experimentally.

Stabilisation of an arsenic moiety by five-membered ring formation is not a new aspect of arsenic chemistry. Indeed, an antidote to arsenic and heavy metal poisoning is the widely recognised chelating agent, 2,3 dimercaptopropanol, or British Anti Lewisite (BAL). Formation of a stabilising ring between arsenic and BAL protects against systemic poisoning derived from arsenic's action on pyruvate dehydrogenase interfering with glucose metabolism (Vilensky and Redman, 2003).

To confirm the need for inclusion of glycine, we used a novel experimental approach incorporating Sirius T3® UV-metric titrations to establish evidence of the theorised stabilising ring formation between glycine and the arsenoxide portion of GSAO. If glycine was to be associated with GSAO-arsenic five membered ring formation, electron re-organisation would occur around both rings, altering molecular spectrophotometric properties relative to

the uncomplexed structure. This re-arrangement would become apparent from a change in relative UV absorbance. Using this titration approach, for the first time we were able to identify a pK_a value of 8.2 associated with one of the GSAO As-OH groups (Fig. 4). For GSAO alone in the absence of glycine, the observation was of a variation in the relative UV absorbance in the pH region where the experimentally determined pK_a of 8.2 predicted that As-OH ionisation would be occurring. In this same region, and in the presence of glycine, flatness of the relative UV absorbance spectrum would either indicate a pK_a shift, or an abolition of ionisation consequent with the relative 'loss' of the As-OH through the proposed ring formation. However, this effect was not observed. Titrations with glycine gave a negative result in that abolition of the pK_a did not occur, and GSAO spectra in the presence of glycine were identical to those without. We proposed, therefore, that ring formation may not be the stabilising mechanism in GSAO–glycine interaction.

The question remained as to whether glycine was to be usefully employed within the desired Phase I pharmaceutical. A head-to-head short solution stability examination was carried out with GSAO in glycine, GSAO in water, and with two common anti-oxidants, and results are reported in Section 3.3.3. It was clear that introduction of the anti-oxidants accelerated transformation to GSAA when compared to both GSAO in water alone and to GSAO in glycine solution. All four test solutions by the nature of their storage (sealed containers, but not oxygen starved) were considered as similarly challenged by atmospheric oxygen, and yet only the solutions with anti-oxidant additives were most affected causing GSAA augmentation. Moreover, comparing the least affected solutions in this test, that is GSAO in water and GSAO in glycine, GSAA development was not substantially different for either (3.9% w/w compared to 4.2% w/w respectively). The acidic nature of the anti-oxidant solutions was confirmed; 0.1%w/v sodium hydrogen sulphite measured at pH 4.2 and 1% w/v ascorbic acid at pH 2.3, and in this experiment, GSAA developed to the greatest extent in the ascorbic acid test. Considering pH alone, these results would seemingly counter Donoghue et al., 2000 in their selection of a low pH to stabilise GSAO.

The data were now presenting several points of information. Firstly, the presence of oxygen alone could not be considered as the only driver to GSAA formation as all solutions examined in the previously discussed test were effectively equally oxygen exposed. Secondly, certain acidic additives if not carefully selected could accelerate the effect of GSAA production from GSAO, even those that are noted as effective oxygen scavengers, a fact that precluded continued investigation of these excipient compounds in our study. Thirdly, and given the acidic nature of the antioxidant solutions used and the effect recorded, pH should be considered and controlled for GSAO. Lastly, for glycine in comparison to a GSAO in plain water solution, the amino acid had neither increased nor reduced GSAA transformation. In addition, in UV-metric experiments the proposed glycine stabilising ring formation had not been proven.

Our study had neither proven nor disproven GSAO–glycine utility, indeed its presence could be seen as no better or worse than using water alone. However, further studies were warranted to examine this excipient in more detail as Donoghue et al. (2000) reported routinely choosing "glycine to prevent oxidation of stock solutions of trivalent organoarsenicals."

For GSAO and an experimentally derived pK_a of 8.2, the Henderson–Hasselbalch equation would predict 50% As-OH ionisation at pH 8.2, augmenting proton dissociation at all pH values above this level. Selection of pH was firstly at pH 7 as the preferred and physiologically acceptable formulation pH, along with pH 8 and pH 9 to examine the effect of increasing molecular ionisation. Further, we elected to study GSAO at each of these pH values at 50 mM, 100 mM and 200 mM glycine concentration representing values from less through to molar excess over GSAO, where GSAO at 50 mg/mL is calculated at 88 mM. Experimentally, pH 9 solutions at all glycine concentrations were observed to cause chemical instability with evidence of increased formation of GSAA, although 200 mM glycine appeared to offer some resistance with respect to the other test glycine concentrations (Fig. 3). Restricting observations to pH 7 and pH 8, variation in glycine concentration through 50 mM, 100 mM and 200 mM seemingly did not favour one solution over another in respect of limiting GSAA formation, despite a molar excess of glycine to GSAO in the 200 mM glycine test solutions. In the pH 9 solution environment, only in the presence of 200 mM glycine was GSAO relatively more protected from oxidative attack. Nonetheless, transformation to GSAA was considerable under these pH conditions.

Feldmann et al. (1999) reported that As(V) formation is thermodynamically favoured in alkaline media. Consequently, but especially in the context of our observations, selection of a pH 7 solution over pH 8 or pH 9 would be pharmaceutically favoured in an effort to maintain the active trivalent arsenic speciation.

From this work, several conclusions regarding potential formulation strategies were made; GSAO solubility decreases with pH lowering in the acidic range, chemical instability manifest as arsenic oxidation increases with alkaline pH values at pH 9, solutions suffer from thermal instability, and although the presence of glycine has been suggested as protective against organoarsenical oxidation in solution, we found no evidence that this occurred as proposed through formation of a stabilising ring complex with the arsenoxide moiety.

The lead solution for GSAO test lyophilisation became 25–50 mg/mL of the active in 100 mM glycine solution adjusted to pH 7. DSC results were used to guide test lyophilisation parameters, and small scale test runs with GSAO at 25 mg/mL, 1 mL fill in 5 mL vials, and at 50 mg/mL, 2 mL fill in 10 mL vials were successful; that is the product dried within 48 h, reconstituted completely in water in 45–60 s, with physiologically acceptable osmotic pressure at 320–380 mOsm (suitable for small volume parenteral administration or administration by infusion), and GSAA levels were restricted or maintained to those measured in the initial pre-lyophilisation solution. Lyophiles were pharmaceutically elegant forming a full white cake in appearance. The final pharmaceutical presentation for production scale up and use in toxicology, stability and Phase I clinical study was a 2 mL fill in 10 mL vial of GSAO at 50 mg/mL (as di-sodium salt) in 100 mM glycine, pH 7.

A programmed 48-month study confirmed acceptable stability of this formulation throughout the time frame of study, with all tests within specification for the entire duration at the recommended storage temperature of refrigeration (+5 °C ±3 °C). Indeed, once lyophilised and under an inert nitrogen vial atmosphere, GSAO chemical analysis over time demonstrated that the formulation was thermally highly robust with little if any evidence of the molecular degradation so easily observed after only days of storage in solution phase.

The Phase I clinical study is ongoing (EudraCT Number 2006-002326-34). The original dose escalation protocol followed a modified Fibonacci sequence increasing by 100%, then 67%, 50%, 40% and 33%, continuing with 33% until a maximum tolerated dose was reached. A subsequent protocol amendment changed the dose escalation scheme to allow dose increases of 10–100% based on plasma arsenic and toxicity data. At the time of writing, the trial has progressed through eight dose escalations from a starting dose of 1.3 mg/m^2/day, with the current dose being 44.0 mg/m^2/day. One dose limiting toxicity (DLT) has been seen in the 12.4 mg/m^2/day dose. To date, eight patients have demonstrated stable disease following their second cycle of GSAO and have therefore gone on to receive further treatment, although all of these patients have now

been withdrawn from the study due to progressive disease after 3–16 cycles of GSAO treatment.

5. Conclusion

GSAO, a novel organoarsenical cancer therapeutic, was formulated for intravenous delivery in a Phase I clinical trial by lyophilisation from glycine–NaOH buffer pH 7. Stability of the IMP for up to 4 years was experimentally confirmed. In this group of chemotherapeutics, arsenic oxidation has been a problem, often observed in analytical solutions and from the experience of early arsenic based therapies. Using neutral pH and lyophilisation (with vial backfill under nitrogen), oxidation was controlled in the pharmaceutical developed for clinical trial. Glycine was incorporated as the sole excipient in the IMP. Glycine stabilisation of the GSAO trivalent arsenic moiety by formation of an oxazarsolidinone ring had been previously theorised. In this work, a novel experimental approach using UV pontentiometric titration was used in an effort to present for the first time experimental evidence of ring formation. However, this mechanism of molecular stabilisation was not proven. Nonetheless, a suitable pharmaceutical GSAO formulation was still realised.

Acknowledgements

This work was funded by Cancer Research UK. The work of the clinical trial investigators is gratefully acknowledged; Professor Gordon Jayson, Cancer Research UK Department of Medical Oncology, Christie Hospital NHS Trust, Manchester, UK, and Professor Adrian Harris and Professor Mark Middleton, both at Oxford Cancer Centre, Churchill Hospital, Oxford, UK.

References

Carmeliet, P., Jain, R.K., 2000. Angiogenesis in cancer and other diseases. Nature 407, 249–257.

Dilda, P.J., Don, A.S., Tanabe, K.M., Higgins, V.J., Allen, J.D., Dawes, I.W., Hogg, P.J., 2005a. Mechanism of selectivity of an angiogenesis inhibitor from screening a genome-wide set of *Saccharomyces cerevisiae* deletion strains. J. Natl. Cancer Inst. 97, 1539–1547.

Dilda, P.J., Decollogne, S., Rossiter-Thornton, M., Hogg, P.J., 2005b. Para to ortho repositioning of the arsenical moiety of the angiogenesis inhibitor 4-(N-(S-glutathionylacetyl)amino)phenylarsenoxide results in markedly increased cellular accumulation and anti-proliferative activity. Cancer Res. 65, 11729–11734.

Dilda, P.J., Hogg, P.J., 2007. Arsenical based cancer drugs. Cancer Treat. Rev. 33, 542–564.

Dilda, P.J., Ramsay, E.E., Corti, A., Pompella, A., Hogg, P.J., 2008. Metabolism of the tumour angiogenesis inhibitor 4-(N-(S-glutathionylacetyl) amino)phenylarsonous acid. J. Biol. Chem. 283, 35428–35434.

Don, A.S., Kisker, O., Dilda, P., Donoghue, N., Zhao, X., Decollogne, S., Creighton, B., Flynn, E., Folkman, J., Hogg, P.J., 2003. A peptide trivalent arsenical inhibits tumour angiogenesis by perturbing mitochondrial function in angiogenic endothelial cells. Cancer Cell 3, 497–509.

Donoghue, N., Yam, T.W., Jiang, X-M., Hogg, P.J., 2000. Presence of closely spaced protein thiols on the surface of mammalian cells. Protein Sci. 9, 2436–2445.

Feldmann, J., Lai, V., Cullen, W-M., Ma, W.R., Lu, M., Le, X.C., 1999. Sample preparation and storage can change arsenic speciation in human urine. Clin. Chem. 45, 1988–1997.

Vilensky, J.A., Redman, K., 2003. British anti-lewisite (dimercaprol): an amazing history. Ann. Emerg. Med. 41, 378–383.

Williams, J.D., 1995. Selective toxicity and concordant pharmacodynamics of antibiotics and other drugs. J. Antimicrob. Chemother. 35, 721–737.

Zhu, J., Chen, Z., Lallemand-Breitenbach, V., de Thé, H., 2002. How acute promyelocytic leukaemia revived arsenic. Nature Rev. Canc. 2, 705–714.