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*RESEARCH ARTICLE*

# Pharmaceutical Development of a Parenteral Lyophilized Formulation of the Novel Antitumor Agent Aplidine

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*ABSTRACT:* Aplidine is a naturally occurring cyclic depsipeptide isolated from the Mediterranean tunicate *Aplidium albicans*. Aplidine displays promising *in vitro* and *in vivo* antitumor activities against various solid human tumor xenografts and is therefore developed now for clinical testing. The aim of this study was to develop a stable parenteral pharmaceutical dosage form for clinical Phase I testing. Aplidine raw material was characterized by using several chromatographic and spectrometric techniques. These experiments showed that aplidine exists as two isomers. A stability-indicating HPLC assay was developed. Solubility testing showed that aplidine exhibits very poor aqueous solubility. Because solubilized aplidine showed substantial degradation under heat and light stress testing conditions, it was decided to develop a lyophilized dosage form. Freeze-drying was carried out with a 500 µg/mL solution of aplidine in 40% (v/v) *tert*-butanol in Water for Injection (WfI) containing 25 mg/mL D-mannitol as a bulking agent. Differential scanning calorimetry was applied to determine the optimal freeze-drying cycle parameters. The prototype, containing 500 µg aplidine and 25 mg D-mannitol per vial, was found to be the optimal formulation in terms of solubility, length of lyophilization cycle, and dosage requirements in the forthcoming Phase I clinical studies. Quality control of the freeze-dried formulation demonstrates that the manufacturing process does not affect the integrity of aplidine. The optimal reconstitution solution was found to be 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI (CEW). Both reconstituted product and dilutions of the reconstituted product with normal saline (up to 1:100 v/v) appeared to be stable for at least 24 hours after preparation. Shelf-life data, available thus far, show that the lyophilized formulation is stable for at least 1 year when stored at +2–8°C in the dark.

## Introduction

Aplidine (dehydrodidemnin B (DDB), MW 1109, Fig. 1) is a novel representative of an evolving group of anticancer agents derived from marine sources (1, 2). This naturally occurring cyclic depsipeptide is isolated from the Mediterranean tunicate *Aplidium albicans* and belongs to the didemnin family, a class of marine-derived compounds which exhibit antiviral, antitumor, and immunosuppressive activity. All didemnins share a common macrocyclic peptide structure and differ only in the side-chain attached to the backbone by the amino group of threonine (3). Aplidine exists in two conformers or rotamers referring to the cis and trans isomers of the pyruvoyl-proline amide bond (Fig. 2) (4).

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Didemnin B (DB), the most potent representative of the didemnin class up to now, was the first marine-derived anticancer compound to enter clinical trials in the early 1980's. Although interesting results were seen in this phase I/II programme sponsored by the National Cancer Institute (NCI), the occurrence of dose-limiting neuromuscular and cardiac toxicity hindered further dose-escalation and repeated cycles of therapy (2). The dehydro-derivative of DB, dehydrodidemnin B (DDB) or aplidine, displays even more potent *in vitro* and *in vivo* antitumor activity against various solid human tumor xenografts. As its parent compound, the antitumor effect of aplidine is believed to be primarily mediated through inhibition of the cell cycle progression in the G1 phase by binding to elongation factor 1α in the presence of GTP, thus interfering with protein synthesis (5, 6). *In vitro* experiments revealed that aplidine exerts less neurotoxicity and cardiotoxicity at antitumor concentrations than didemnin B. On the basis of these results, aplidine has been identified as a new generation of the didemnin class, with a possibly significantly higher therapeutic index (2, 7, 8, 9).

In animal toxicology studies a maximum tolerated dose (MTD) of 1250 µg/kg body weight in mice and a MTD of 570 µg/kg body weight in rats were determined, respectively. Taking the 1/10 MTD mouse-equivalent, the initial dosage level in Phase I clinical trials was therefore defined at 350 $\mu g/m^2$ (10, 11). A suitable parenteral formulation containing 500 µg aplidine per dosage unit was required to start early clinical studies. Because of its very poor aqueous solubility (< 0.1 mg/mL), an adequate vehicle had to be found to administer aplidine to the patient. The work described here was directed towards developing a suitable parenteral formulation for toxicological and clinical evaluation according to the EORTC/CRC/NCI Joint Formulation Working Party (JFWP) guidelines (12, 13, 14). Aplidine bulk drug substance was fully structurally and analytically characterized. The formulation approach involved the use of a cosolvent-surfactant system to enhance the solubility, and lyophilization to improve the stability of the compound.

## Materials and Methods

### *Chemicals and Materials*

Aplidine was obtained from natural didemnin A by a three-step synthesis under the responsibility of Pharma Mar s.a. (Tres Cantos, Madrid, Spain) and provided through the New Drug Development Office-Oncology (NDDO-Oncology, Amsterdam, The Netherlands). All chemicals used were of analytical grade and were used without further purification. Distilled water was used throughout. Excipients, including *tert*-butanol, and primary packaging materials used in the manufacturing of aplidine lyophilized product and reconstitution solution were of European Pharmacopeia III (Ph. Eur. III) or United States Pharmacopeia 24 (USP24) grade and provided by the supplier with a Certificate of Analysis. Substances were approved on the basis of in-house quality controls carried out according to monographs in the mentioned pharmacopeias. Normal saline (0.9% w/v sodium chloride in WfI) was manufactured in-house at the Department of Pharmacy of the Slotervaart Hospital (Amsterdam, The Netherlands).

### *Characterization of Aplidine Bulk Drug*

An interim reference standard of the aplidine bulk drug material, i.e., batch of highest purity available, was defined (Lot APL-297) and structurally characterized by fast-atom bombardment mass spectrometry (FAB-MS), nuclear magnetic resonance (NMR), and infra-red (IR) spectroscopy, and analytically characterized by high performance liquid chromatography (HPLC) and ultraviolet/visible (UV/VIS) spectrophotometry. On the basis of these results, specifications were drawn up for the aplidine raw material.

*FAB-MS*: The FAB mass spectrum was obtained with a Model JMS-SX/SX 102A tandem mass spectrometer (BEBE; JEOL, Tokyo, Japan). The acceleration voltage was 10 kV. A xenon source with an energy of approximately 6 keV was used, and the matrix was glycerol. Positive ion spectra were recorded over a mass range of 10-1500 D.

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*NMR-spectroscopy*: $^1$H NMR spectra were recorded with a Gemini 300 BB instrument (Varian Assoc., Palo Alto, CA USA) at 300.1 MHz. The sample (1 mg) was dissolved in deuterochloroform ($CDCl_3$) or hexadeuterodimethylsulfoxide (DMSO-$d_6$). In $CDCl_3$, TMS was used as an internal reference; in DMSO-$d_6$ the central DMSO line was set at 2.50 ppm.

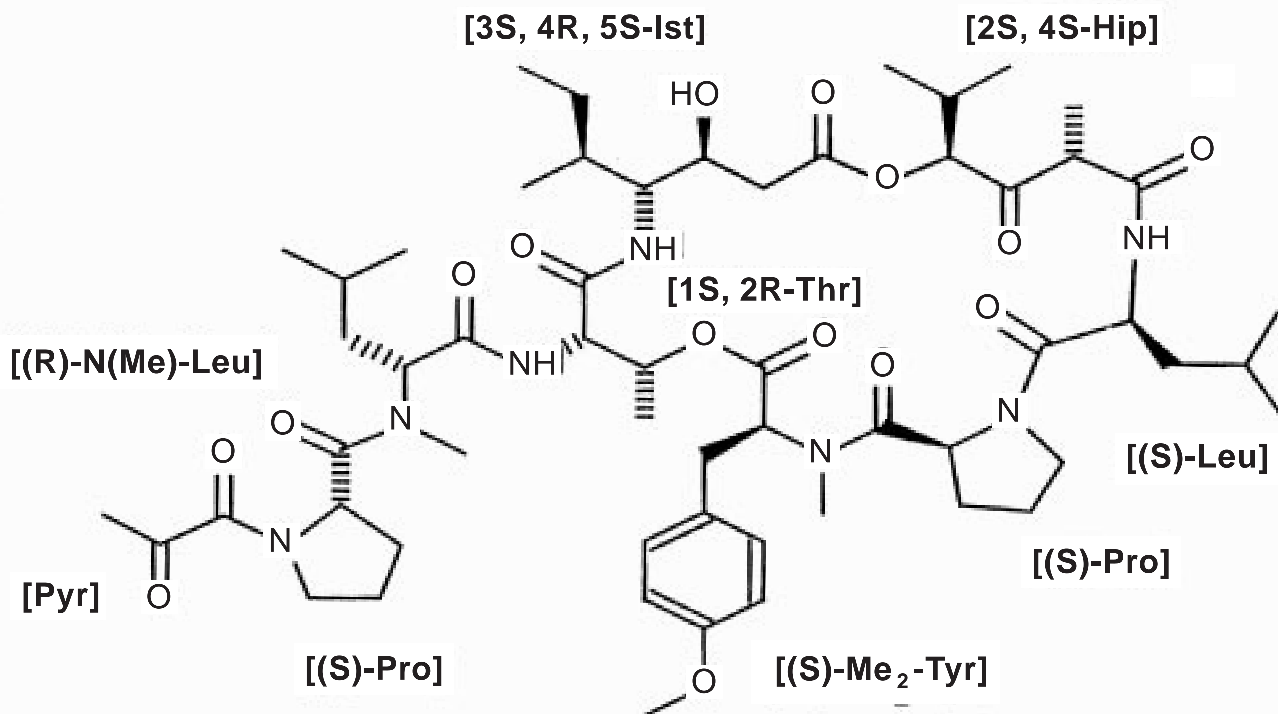
*Infra-red spectroscopy*: Infra-red (IR) spectra (4000–600 $cm^{-1}$) were recorded on a Model PU 9706 IR spectrophotometer (Philips Nederland B.V., Eindhoven, The Netherlands) with the potassium bromide (KBr) pellet technique. The pellet consisted of 1 mg of aplidine bulk drug and 200 mg of KBr. The ratio recording mode was set on autosmooth and the scan time was 8 minutes.

*UV/VIS spectroscopy*: UV/VIS spectra (800–200 nm) of aplidine bulk drug (50 µg/mL in 79% (v/v) methanol/$H_2O$) were recorded with a Model UV/VIS 918 spectrophotometer (GBC Scientific Equipment, Victoria, Australia) equipped with an LEO personal computer and an Epson LX-400 plotter.

*Formulation of Aplidine*

*Solubility studies*: The solubility of aplidine in various solvents at ambient temperature (20–25°C) was examined by accurately weighing approximately 1 mg of aplidine in a glass test tube and adding subsequent solvent volumina of 100 µL, 1 mL, and 10 mL to the bulk drug. After each addition the mixture was vigorously shaken for 30 seconds, placed in an ultrasonic bath for 15 minutes and examined visually under polarized light for complete dissolution of the aplidine drug substance. In this way, the solubilities of aplidine in the various solvents were selectively distributed over four solubility ranges ($s < 0.1$ mg/mL, $0.1$ mg/mL $\leq s <$

**Figure 1: Chemical structure of aplidine (Hip: hydroxyisovalerylproprionyl; Ist: isostatine; Leu: leucine; Pro: proline; Pyr: pyruvoyl; Thr: threonine; Tyr: tyrosine).**

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1 mg/mL, 1 mg/mL ≤ s < 10 mg/mL and s > 10 mg/mL, respectively). In addition, solvents in which aplidine dissolved were examined by diluting the solutions 1:1, 1:5, 1:10, 1:50, and 1:100 (v/v) with normal saline for infusion in glass test tubes. After gentle agitation, each of the dilutions was examined visually under polarized light over a one day period for any sign of precipitation.

Reconstitution of aplidine lyophilized product with solutions composed of 40/10/50% (v/v/v) propylene glycol 400/ethanol/polysorbate 80 (PET), 40/10/50% (v/v/v) propylene glycol 400/ethanol/WfI (PEW), and 5/5/90% (v/v/v), 15/15/70% (v/v/v), and 30/30/40% (v/v/v) Cremophor EL/ethanol/WfI was studied by adding increasing volumes of the reconstitution solutions to 500 µg aplidine lyophilized cake. After each addition, the resulting mixture was agitated and subsequently examined visually under polarized light. Quantitative analysis was carried out by diluting 50 µL samples of reconstituted product with 950 µL of acetonitrile and subsequent injection onto the HPLC system. Furthermore, in case of complete dissolution, the reconstituted solution was diluted 1:10 or 1:100 (v/v) with normal saline for infusion in glass test tubes. Stability of the reconstituted solution and the infusion solutions were examined visually under polarized light and by HPLC without further dilution, over a 24-hour period at room temperature (20–25°C) and a normal day-night light cycle.

*Differential scanning calorimetry*: Transition temperatures and freezing characteristics of *tert*-butanol, D-mannitol, and aplidine solutions were examined by differential scanning calorimetry (DSC). The DSC experiments were carried out with a TA Instruments DSC 2920 (TA Instruments, New Castle (DE), USA) equipped with an LNCA for low temperatures. Samples were sealed in a closed aluminium pan with an empty pan as reference. Temperature scale and heat flux were calibrated with indium. Samples were cooled to -50°C at a rate of 5°C/min. The DSC heating rate was 2.5°C/min. Analyses were performed under a helium purge.

*Formulation process*: Aplidine lyophilized product was aseptically prepared from a 500 µg/mL aplidine solution in 40% (v/v) *tert*-butanol in WfI containing 25 mg/mL D-mannitol as a bulking agent. The formulation solution was prepared by weighing aplidine and D-mannitol and dissolving the substances by magnetic stirring in 40% (v/v) *tert*-butanol in WfI. To make a final concentration of 500 µg/mL aplidine, 40% (v/v) *tert*-butanol in WfI was added. The formulation solution was sterile filtered through a 0.2 µm Midisart 2000 filter (Sartorius, Nieuwegein, The Netherlands). Subsequently, 1 mL aliquots of the formulation solution were filled into 10 mL type 1 glass vials with a Model 501Dz peristaltic pump (Watson Marlow, UK). After filling, vials were partially closed with grey butyl rubber stoppers, placed in a Model Lyovac GT4 freeze-dryer (AMSCO/FinnAqua, Germany), and lyophilized. The freeze-dryer was equipped with a cold trap filled with liquid nitrogen attached to the condensor to ensure complete condensation of *tert*-butanol. After completion of the freeze-drying cycle, sterile filtered medical grade nitrogen gas was leaked into the freeze-drying chamber to reach a final vacuum of 100 mbar. Subsequently, the vials were pneumatically closed, capped, and labeled. In-process-controls consisted of integrity testing of the filter unit, weight variation of the filling volume, and determination of the aplidine concentration of the formulation solution before and after filtration. Only clean, sterile inert materials and glassware were used throughout the manufacturing process. All critical manipulations took place under a class 100 (A) down-flow condition with a class 100 (B) background (Interflow, Wieringerwerf, The Netherlands). Air particle counts in the critical areas as well as microbiological contamination of the area and personnel were monitored at operating state.

Reconstitution solution 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI (CEW) was prepared by mixing the appropriate volumes of excipients by magnetic stirring. The solution obtained was filtered through a 0.2 µm Midisart 2000 filter unit (Sartorius, Goettingen, Germany) and subsequently 2 mL

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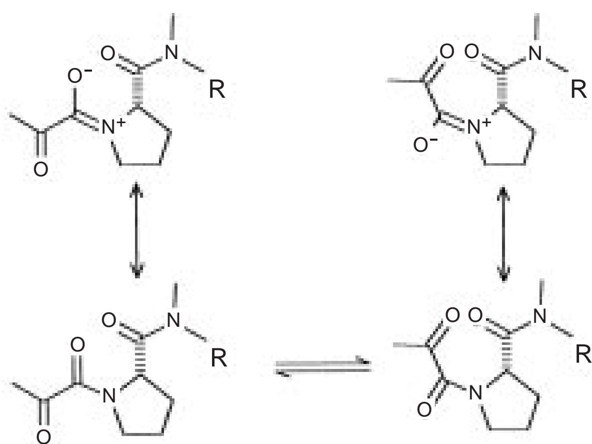
aliquots were filled into type 1 glass ampoules with a Model R910 ampoule fill-and-seal machine (Rota den Boer B.V., The Netherlands). After filling, the solution was sterilized by autoclaving (Model 6.6.15 autoclave, Koninklijke Ad Linden B.V., The Netherlands) for 20 minutes at 120°C. All manipulations involving an "open" solution or excipient were conducted under class 100 (A) conditions. The manufacturing of both aplidine lyophilized product and its reconstituted solution were performed according to the Good Manufacturing Practice (GMP) guidelines (15).

**Figure 2: cis/trans isomerism of the pyruvoyl-proline amide bond.**




*Quality Control of Aplidine Lyophilized Product*

Quality control of aplidine lyophilized product consisted of identification by visual inspection of appearance and colour of the pharmaceutical product; determination of reconstitution characteristics and pH of the reconstituted product; content, content uniformity, and purity determination by HPLC analysis; residual moisture determination with the Karl-Fischer titration method; and residual *tert*-butanol content by gas chromatographic (GC) analysis. Furthermore, sterility of the pharmaceutical product was checked by the filtration method and the presence of bacterial endotoxins with the limulus amoebocyte lysate (LAL) test, both carried out according to the European Pharmacopeia III.

*HPLC analysis*: Aplidine was assayed by a validated, stability-indicating reversed phase-HPLC method. The HPLC system consisted of a Model SP8800 pump (Thermo Separation Products, USA), a Model Spectra 200 UV-VIS detector (Spectra-Physics, San Jose, USA), and a Model SP8880 autosampler (Thermo Separation Products, USA). Analyses were carried out with a Zorbax SB-C18 analytical column (4.6 mm ID x 150 mm, particle size 3.5 mm, Waters, USA) held at a constant temperature of 80°C with a Model 7971 column heater (Jones Chromatography, USA). The mobile phase at a flow of 0.6 mL/min consisted of a linear gradient of acetonitrile (ACN) containing 0.04% trifluoroacetic acid (TFA) 35% to 70% in 15 minutes and water containing 0.04% TFA. An injection volume of 20 µL and a total run time of 30 minutes were used. UV detection was performed at 225 nm. Under these conditions the chromatogram of aplidine consisted of a single peak eluting at 21 minutes. A series of standard solutions of aplidine in ACN in the concentration range of 5 µg/mL to 300 µg/mL were prepared in duplicate from a stock solution of 1 mg/mL aplidine in ACN and injected into the HPLC system. Quality control samples at concentrations of 7.5, 100, 250, and 275 µg/mL of aplidine in ACN were prepared in quadruplicate from another stock solution with separate weighing of aplidine and injected into the HPLC system. Aplidine lyophilized product was diluted to a test concentration of 250 µg/mL by dilution with a solution of 1:1 (v/v) ACN/water. Least squares regression analysis was used to calculate the slope and intercept for the standard calibration curve from measured peak areas versus concentration. Sample concentrations were calculated from the corresponding peak areas using the regression equation. All aplidine chromatograms were electronically stored in the computer system LABNET (Spectra-Physics, San Jose, USA). Reprocessing of aplidine chromatograms was performed using PC1000 software (Thermo Separation Products, USA).

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