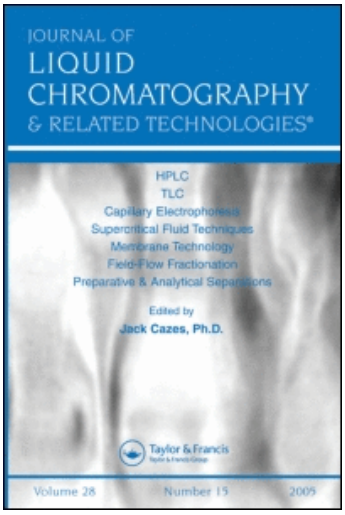

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

DEVELOPMENT AND VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR THE DETERMINATION OF A FLUORINATED ANALOGUE OF THALIDOMIDE, N-(2,6-DIOXOPIPERIDIN-3-YL)-3,4,5,6-TETRAFLUOROPHTHALAMIC ACID, AND LENALIDOMIDE

Kaveshree Pather[a]; Nuala A. Helsby[b]; Brian D. Palmer[a]; Lai-Ming Ching[a]
[a] Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, School of Medicine, University of Auckland, Auckland, New Zealand [b] Department of Molecular Medicine and Pathology, Faculty of Medical and Health Sciences, School of Medicine, University of Auckland, Auckland, New Zealand

Online publication date: 07 January 2011

To cite this Article Pather, Kaveshree , Helsby, Nuala A. , Palmer, Brian D. and Ching, Lai-Ming(2011) 'DEVELOPMENT AND VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR THE DETERMINATION OF A FLUORINATED ANALOGUE OF THALIDOMIDE, N-(2,6-DIOXOPIPERIDIN-3-YL)-3,4,5,6-TETRAFLUOROPHTHALAMIC ACID, AND LENALIDOMIDE', Journal of Liquid Chromatography & Related Technologies, 34: 2, 83 — 92
To link to this Article: DOI: 10.1080/10826076.2010.526853
URL: http://dx.doi.org/10.1080/10826076.2010.526853

Journal of Liquid Chromatography & Related Technologies, 34:83–92, 2011

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826076.2010.526853

Taylor & Francis
Taylor & Francis Group

DEVELOPMENT AND VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR THE DETERMINATION OF A FLUORINATED ANALOGUE OF THALIDOMIDE, N-(2,6-DIOXOPIPERIDIN-3-YL)-3,4,5,6-TETRAFLUOROPHTHALAMIC ACID, AND LENALIDOMIDE

Kaveshree Pather,[1] Nuala A. Helsby,[2] Brian D. Palmer,[1] and Lai-Ming Ching[1]

[1]*Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, School of Medicine, University of Auckland, Auckland, New Zealand*
[2]*Department of Molecular Medicine and Pathology, Faculty of Medical and Health Sciences, School of Medicine, University of Auckland, Auckland, New Zealand*

□ *Fluorinated derivatives of a hydrolysis metabolite of thalidomide are being investigated for their anti-cancer potential. In this communication, we report the development and validation of an HPLC assay for the determination of N-(2,6-dioxopiperidin-3-yl)-3,4,5,6-tetrafluorophthalamic acid (SN 29900) for use in pharmacokinetic measurements of the compound. The intra- and inter-assay accuracy (98–102%) and precision (relative standard deviation <4%) are well within limits of acceptability over the concentration range of 3.12–500.00 μM. The lower limit of quantification is 3.12 μM. Solutions of SN 29900 in dimethylsulfoxide and plasma were stable during short-term (24 hr) and long-term (3 mon) storage, and following three cycles of freezing and thawing. The pharmacokinetics of SN 29900 in C57Bl/6 mice were determined after intraperitoneal administration (100 and 500 mg/kg; n = 3 mice per time-point). The maximum plasma concentration detected at 100 mg/kg for SN 29900 was 72.5 μM and at 500 mg/kg was 1669.9 μM. The area under the curve ($AUC_{0-\infty}$) at 100 and 500 mg/kg was calculated to be 46.1 μM.h and 843.8 μM.h, respectively. The results indicated that in mice, SN 29900 was cleared faster than lenalidomide, a second generation thalidomide analogue that is in clinical use. The maximum plasma concentration of lenalidomide was 337 μM and the $AUC_{0-\infty}$ was calculated to be 338 μM.h following an intraperitoneal dose at 100 mg/kg measured using the same assay validated here for SN29900.*

Abbreviations: acetonitrile (MeCN), area under the concentration time curve (AUC), dimethylsulfoxide (DMSO), high performance liquid chromatography (HPLC), internal standard (I.S.), intraperitoneal (i.p.), lower limit of quantification (LLOQ), maximum concentration (C_{max}), peak area ratio (PAR), quality control (QC), relative standard deviation (R.S.D., time to maximum concentration (T_{max})

Address correspondence to Lai-Ming Ching, Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, School of Medicine, University of Auckland, Private Bag 92019, Auckland, New Zealand. E-mail: l.ching@auckland.ac.nz

Keywords anti-angiogenic, lenalidomide, multiple myeloma, pharmacokinetics, phthalamic acid

INTRODUCTION

With the registration of thalidomide (Figure 1A) for the treatment for multiple myeloma,[1–3] there has been resurgence in the development of novel and more potent analogues of the drug. Lenalidomide (Figure 1B) is the first of the second generation thalidomide analogues to be approved by the United States Federal Drug Agency for use in patients with myelodysplastic syndromes and relapsed multiple myeloma.[4,5] While lenalidomide has a similar profile of biological activities to thalidomide: induction of apoptosis, enhancement of the immune response, inhibition of angiogenesis, and modulation of cytokine production,[6–8] it has a different toxicity profile to that of thalidomide [8]. Perfluorination is a commonly used modification in medicinal chemistry to provide derivatives with improved potency and/or metabolic stability.[9,10] A number of halogen-substituted derivatives of a hydrolysis metabolite of thalidomide were synthesized at the Auckland Cancer Society Research Centre as part of a program to obtain more potent analogues that are less teratogenic than thalidomide. *N*-(2,6-dioxopiperidin-3-yl)-3,4,5,6-tetrafluorophthalamic acid (SN 29900, Figure 1C), was indeed more potent than the parent compound in inhibiting angiogenesis *in vitro* and was selected for further evaluation. Initial attempts to develop a quantitative assay for SN 29900 using high performance liquid chromatography (HPLC)[4] with triple quadrupole mass spectrometry detection were not successful as the high temperatures involved in the ionization of the compound resulted in the formation of insoluble precipitates in the mass spectrometer. HPLC with UV detection is also widely used for pharmacokinetic determinations of novel drugs under development. Although these methods may not have the sensitivity of triple quadrupole spectrometry, they operate under much milder conditions, and the problem of fragmentation and precipitation associated with mass spectrometry can be avoided. We report here the development and validation of

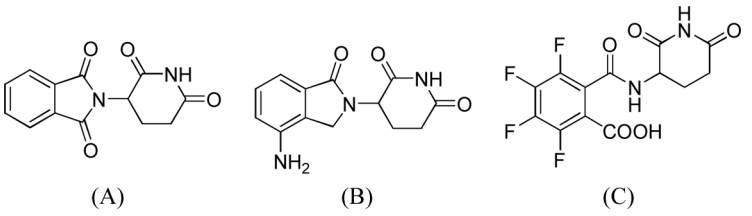

FIGURE 1 The chemical structure of thalidomide (A), lenalidomide (B) and *N*-(2,6-dioxopiperidin-3-yl)-3,4,5,6 tetrafluorophthalamic acid (SN 29900) (C).

an HPLC method for the assay of SN 29900. The assay was successfully applied to the determination of the pharmacokinetics SN 29900 in mice, as well as that of lenalidomide.

EXPERIMENTAL

Chemicals and Reagents

SN 29900 and lenalidomide were synthesized as previously described[11,12] at the Auckland Cancer Society Research Centre, University of Auckland, New Zealand. All other chemicals were commercially available and of analytical grade. Acetonitrile (MeCN) and formic acid were purchased from Merck, KGaA, Darmstadt, Germany. Water used in all experiments was purified by filtering through ion exchange columns and a 0.22 μM filter (Milli-Q purification system, Millipore Corporation, Bedford, MA, USA).

Collection and Preparation of Plasma

Mice were obtained from the Vernon Jansen Unit, The University of Auckland, and all animal procedures had been approved by the University of Auckland Animal Ethics Committee. Mouse plasma was prepared from blood collected through the ocular sinus of healthy C57Bl/6 mice during isofluorane anesthesia into heparinized tubes (Becton-Dickson, Franklin Lakes, NJ, USA). Mice were killed by cervical dislocation immediately after bleeding, and the blood was centrifuged at 3000 g for 10 min and the plasma was transferred into clean micro-centrifuge tubes and stored at −80°C until used.

Analytical Method for the Detection and Quantification of SN 29900 and Lenalidomide

The chromatographic system comprised of a Hewlett-Packard Agilent 1100 HPLC (Hewlett-Packard, Waldbronn, Germany), with a temperature controlled 100 well autosampler (Waters Associates, Milford, MA, USA). HPLC separation was performed using Column Luna 5 μ phenylhexyl 100 × 4.6 mm (Phenomenex, Torrance, CA, USA) with a guard column of the same material. The mobile phase comprised a mixture of 80% MeCN in Milli Q water (A) and 0.45 M NH_4 formate in Milli Q water (pH 3.5) (B).

The initial conditions were 5% solvent A and 95% solvent B. Solvent A was increased linearly to 100% over 25 min and held constant for 5 min at 30 min after which it returned to 5% at a flow-rate of 0.6 ml/min and a total

runtime of 35 min. Determination was at 240 and 270 nm (bandwidth 4 nm) and reference at 550 nm (bandwidth 50 nm). Peak areas were quantified using the Agilent Chemstation Software version B.01.01 (2004).

Preparation of SN 29900, Lenalidomide, and Internal Standard

A stock solution (10 mM) of SN 29900 and lenalidomide was prepared in dimethylsulfoxide (DMSO) and stored at −80°C. These solutions were stable for 30 days. Working solutions (1 mM and 0.1 mM) were prepared by dilution in DMSO. Phenacetin was used as I.S. (1 mM in water) and stored at 4°C.

Calibration Curve

Standard plasma solutions of SN 29900 and lenalidomide were prepared by diluting 0.1 or 1 mM of the working solution with drug free mouse plasma to achieve concentrations ranging from 3.12 μM to 1000 μM. Duplicate samples of the plasma calibrants were prepared by adding 50 μM I.S. (5 μL of a 1 mM stock). To precipitate the plasma proteins, 100 μL of ice-cold trichloroacetic acid solution (10% w/v in Milli Q water) was added and vortexed mixed (30 sec) followed by solvent extraction with ethyl acetate (0.5 mL). The sample was vortexed mixed (1–2 min) and centrifuged (3000 g; 10 min). The organic phase was then carefully removed and placed into a clean microcentrifuge tube. The ethyl acetate extraction step was repeated to ensure maximal recovery and the organic phases were combined and evaporated to dryness using a centrifugal evaporator (Savant Instruments, Farmingdale, NY, USA). The residues were reconstituted in 90 μL of 0.45 M NH_4 formate buffer pH 3.5, and 10 μL MeCN (80%). Aliquots (80 μL) were injected onto the HPLC column. The peak area ratios (PAR) of SN 29900 or lenalidomide and the I.S. were plotted against the known concentrations. The line of best-fit was then obtained by using linear regression using SigmaPlot® (Systat Software INC., San Jose, CA).

SN 29900 quality control (QC) samples were prepared for intra- ($n=8$) and inter-day ($n=8$) assay validations at concentrations of 3.12, 50, 200, and 500 μM. QC samples were stored at −80°C for up to 30 days and concentrations determined from the calibration curve.

Validation Procedures

Analytical specificity was tested by inspection of chromatograms of extracted drug-free mouse plasma for interfering peaks. Absolute recoveries were assessed by comparing peak areas of SN 29900 and I.S. from

extracted plasma QC samples, to standards prepared in the mobile phase. All recovery studies were performed at four different concentrations and in triplicate. To determine intra-day reproducibility, eight replicates of the QC samples were analyzed, including the lower limit of quantitation (LLOQ). Inter-day precision was calculated from QC samples analyzed on eight different days. At each concentration, precision was calculated as the relative standard deviation (RSD) and accuracy as the percentage of the true value. Acceptable precision was defined by a RSD within 15.0%, and accuracy within ±15.0% of the true value. The LOQ was defined to be the lowest concentration that could be measured with adequate accuracy (i.e., ±20.0% of the true value), and precision (RSD within 20.0%). Matrix effects on each analyte were assessed in triplicate by spiking different batches of extracted mouse plasma. Each analytical run consisted of a single calibration curve, triplicate QC samples at three concentrations, one reagent blank, one plasma blank, and one zero-level standard.

The stability of SN 29900 in plasma was measured in duplicate at room temperature over 24 hr. At each time point, plasma samples containing SN 29900 were removed, and then extracted as described previously. In addition, the stability of SN 29900 in plasma was assessed at three different concentrations in triplicate when left on ice or at room temperature up to 24 hr. Similarly, stability during storage in the autosampler was determined at three different concentrations in triplicate over 24 hr at 4°C. The effect of freeze-thaw on the stability of SN 29900 was determined over 3 cycles over 3 days. For short-term, long-term and freeze-thaw stability, mean concentrations of triplicate samples were compared to the initial values.

Determination of Plasma Pharmacokinetics

C57Bl/6 mice were administered SN 29900 at a dose of 100 and 500 mg/kg or lenalidomide at 100 mg/kg in DMSO by intraperitoneal (i.p.) injection and blood was collected at 10, 20, 30, 60, 120 and 240 min ($n = 3$ mice per timepoint). The mean concentration ±SEM of SN 29900 and lenalidomide in the plasma was determined. The area under the concentration time curve (AUC) was calculated using WinNonLin version 4.0.1 (WinNonlin Professional Software, Mountain View, CA, USA).

The optimal system was selected visually from the predicted curves determined by the Akaike Information Criteria and Schwartz Criteria. Data were fitted using a non-compartmental model with first-order absorption and elimination. The maximum concentration (C_{max}) expressed as μM, and the time to maximum concentration (T_{max}) in h was determined

from the time-concentration profile. The elimination rate constant (λ) was determined as the slope of the terminal phase of the log-linear concentration-time curve. The terminal $t_{1/2}$ was calculated as $\ln(0.5)/\lambda$. The AUC from time zero to the last quantifiable concentration (C_t) ($AUC_{0\text{-}t}$) was calculated by trapezoidal rule. AUC extrapolated to infinity was determined from the formula $AUC_{0\text{-}\infty} = AUC_{0\text{-}t} + C_t/\lambda$.

The presence of SN 29900 in bile and urine from mice used for the pharmacokinetic studies following a dose of 500 mg/kg were also investigated.

RESULTS AND DISCUSSION

Validation of the Assay

Linearity, Specificity, Accuracy, and Precision

The calibration curves ($n = 5$) were linear between 3.12–1000 μM, with r^2 values ranging from 0.991–0.999. A representative calibration curve for SN 29900 and lenalidomide is shown in Figure 2A and 2B respectively. The LLOQ, the lowest concentration 3 times greater than the background level for SN 29900, was 3.12 μM. The recovery, calculated as the percentage of the measured concentration relative to the true concentration of SN 29900 across the linear range varied from 97–102%.

Comparison of the HPLC chromatogram of control mouse plasma (Figure 3A) and mouse plasma spiked with of SN 29900 (Figure 3B) or

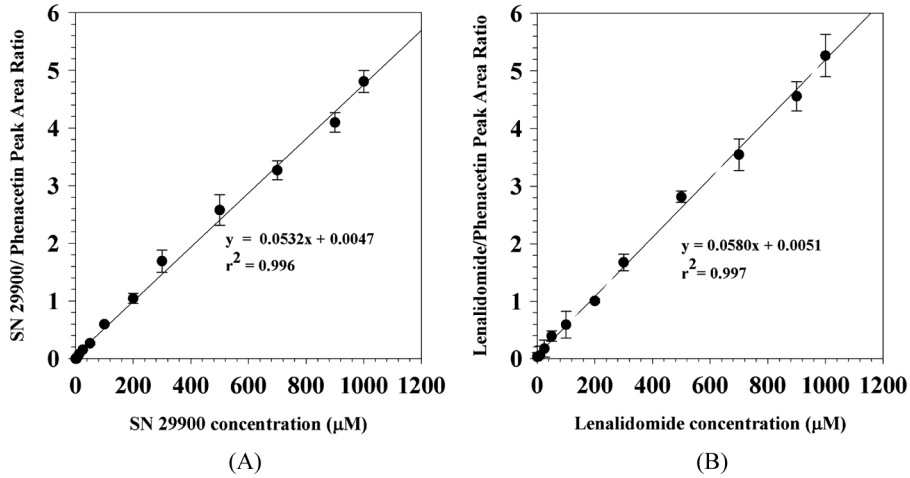

FIGURE 2 A representative standard curve for SN 29900 (A) and lenalidomide (B) in C57Bl/6 mouse plasma over the concentration range of 3.12 to 1000 μM.

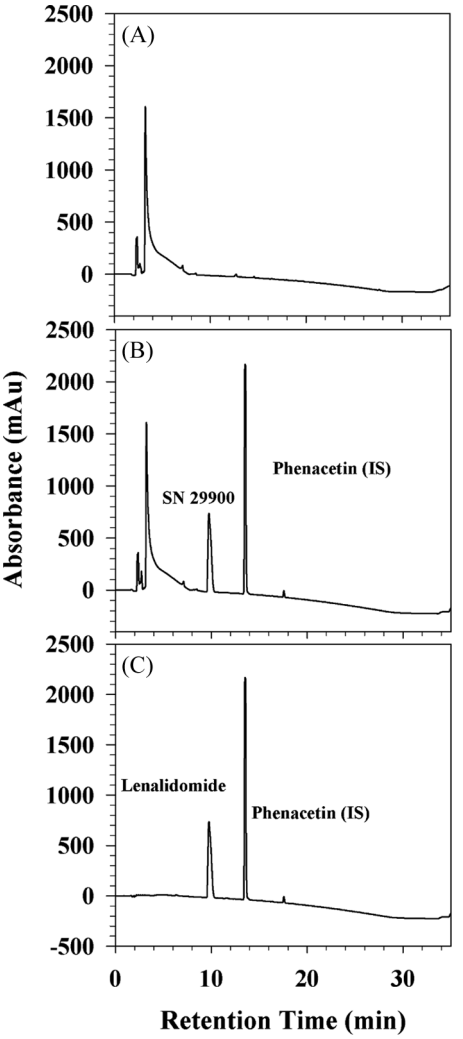

FIGURE 3 HPLC chromatogram of control plasma from untreated mice (A); plasma containing 200 μM SN 29900 (Rt of 9.5 min) compared to Rt of 13.4 min for 50 μM as the I.S. (B); and plasma containing 200 μM lenalidomide (Rt 9.8 min) compared to Rt of 13.4 min for 50 μM of phenacetin (C).

lenalidomide (Figure 3C) showed that there were no interfering endogenous peaks.

Intra- ($n = 8$) and inter-day ($n = 8$) accuracy and precision was determined by measuring the concentration of SN 29900 in the QC samples. The accuracy over the concentration range of 3.12–500 μM of SN 29900 was between 97.5–102%, and within the acceptance criteria (Table 1). The intra-day and inter-day precision did not exceed the co-efficient of variation of 15% at all the concentrations examined (Table 1).

TABLE 1 Intra and Inter-day Accuracy and Precision of SN 29900 Assay

SN 29900 (μM)	Intra-day mean ±SD ($n=8$)	Intra-day CV (%)	Intra-day Accuracy (%)	Inter-day Mean ±SD ($n=8$)	Inter-day CV (%)	Inter-day Accuracy (%)
3.12	3.05 ± 0.07	2.57	97.50	2.99 ± 0.08	2.86	95.80
50.00	53.44 ± 1.80	3.38	106.00	48.81 ± 1.77	3.62	97.61
200.00	206.20 ± 1.20	0.60	103.00	205.71 ± 0.52	0.25	102.83
500.00	509.90 ± 4.60	0.90	102.00	497.92 ± 2.92	0.59	99.60

Validated Assay Determination: The Plasma Pharmacokinetic Profile of SN 29900 and Lenalidomide in Mice

Plasma pharmacokinetic profile of SN 29900 in mice (Figure 4) showed that in comparison to the plasma pharmacokinetic profile of lenalidomide (Figure 4), the clearance of SN 29900 from plasma at a dose 100 mg/kg i.p. was rapid. Detection above the LLOQ was not observed after 1 hr. The C_{max} for SN 29900 (72.55 μM) and lenalidomide (337 μM) was reached at 30 min and the $AUC_{0\text{-}\infty}$ for SN 29900 and lenalidomide was calculated at 46.12 μM.h and 338 μM.h, respectively. We measured the concentration of SN 29900 in plasma at 30 min (T_{max} for the 100 mg/kg dose) following i.p. administration of increasing (100, 250, 500, 750, and 1000 mg/kg), well-tolerated doses of SN 29900. Plasma concentrations increased linearly with doses between 100–500 mg/kg, but were non-linear above 500 mg/kg. We, therefore, also measured the plasma pharmacokinetic profile of SN 29900 at a dose 500 mg/kg. Even at this higher dose, 2 hours after treatment, concentrations of SN 29900 were below the LLOQ of 3.12 μM and

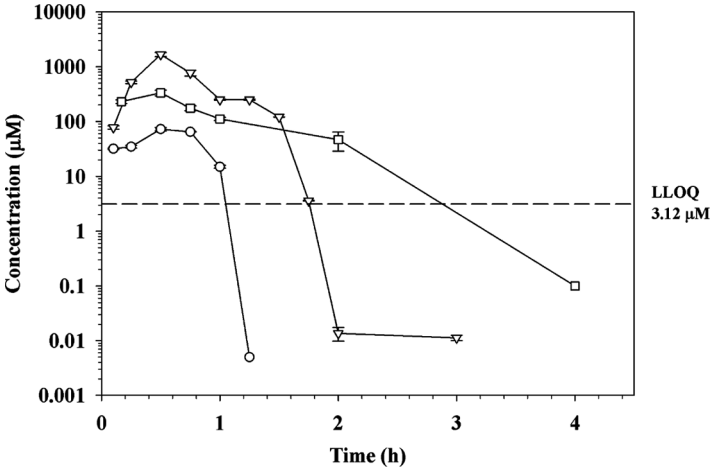

FIGURE 4 Plasma pharmacokinetic profiles following i.p. administration of SN 29900 at 100 mg/kg (circles) and 500 mg/kg (triangles); or lenalidomide at 100 mg/kg (squares) in C57Bl/6 mice.

SN 29900 was undetectable in the plasma 3 hr after treatment (Figure 4A). However, a 5-fold increase in the dose produced a 23-fold increase in the C_{max} (1669.98 μM) with the same T_{max} and an 18.3 fold increase in the $AUC_{0-\infty}$ (843.84 μM.h) over those obtained for 100 mg/kg.

Traces of SN 29900 were not detected in the bile, but large amounts of unchanged SN 29900 were observed in the urine suggesting that it is rapidly eliminated through urinary excretion.

CONCLUSIONS

A robust, reproducible, and specific assay for the quantification of SN 29900 in nonbiological solvents as well as in mouse plasma has been developed. This assay allowed for the measurement of SN 29900 and lenalidomide at concentrations in plasma as low as 3.12 μM with acceptable accuracy and precision. SN 29900 was shown to be stable during three cycles of freezing and thawing as well as at −80°C over 30 days and 4°C for 48 hr. The pharmacokinetics of SN 29900 in comparison to lenalidomide, in particular, its rapid rate of clearance, may preclude its efficacy at low doses. Increasing the dose of SN 29900 5-fold provided a greater than 18-fold increase in AUC, raising the possibility that dose-escalation would greatly improve the chances of obtaining drug exposures that would result in detectable anti-tumor effects. The assay developed here can also be used to measure the pharmacokinetics of related analogues to identify those with slower rates of clearance.

ACKNOWLEDGMENTS

This study was supported by Cancer Society New Zealand, Auckland Division.

REFERENCES

1. Singhal, S.; Mehta, J.; Desikan, R.; Ayers, D.; Roberson, P.; Eddlemon, P.; Munshi, N.; Anaissie, E.; Wilson, C., Dhodapkar, M.; Zeddis, J.; Barlogie, B. Antitumour Activity of Thalidomide in Refractory Multiple Myeloma. *N. Engl. J. Med.* **1999**, *341*, 1565–1571.
2. Barlogie, B.; Shaughnessy, J.; Tricot, G.; Jacobson, J.; Zangari, M.; Anaissie, E.; Walker, R.; Crowley, J. Treatment of Multiple Myeloma. *Blood.* **2004**, *103*, 20–32.
3. Rajkumar, S. V.; Hayman, S.; Gertz, M. A.; Dispenzieri, A.; Lacy, M. Q.; Greipp, P. R.; Geyer, S.; Iturria, N.; Fonseca, R.; Lust, J. A.; Kyle, R. A.; Witzig, T. E. Combination Therapy with Thalidomide Plus Dexamethasone for Newly Diagnosed Myeloma. *J. Clin. Oncol.* **2002**, *20*, 4319–4323.
4. Richardson, P. G.; Schlossman, R. L.; Weller, E.; Hideshima, T.; Mitsiades, C.; Davies, F. et al. Immunomodulatory Derivative of Thalidomide CC-5013 Overcomes Drug Resistance and is Well Tolerated in Patients with Relapsed Multiple Myeloma. *Blood.* **2002**,*100*, 3063–3067.

5. Bartlett, J. B.; Dredge, K.; Dalgleish, A. G. The Evolution of Thalidomide and Its IMiD Derivatives as Anticancer Agents. *Nat. Rev. Cancer.* **2004**, *4*, 314–322.
6. Dredge, K.; Marriot, J. B.; MacDonald, C. D.; Man, H. W.; Chen, R.; Muller, G. W.; Stirling, D.; Dalgleish, A. G. Novel Thalidomide Analogues Display Anti-Angiogenic Activity Independently of Immunomodulatory Effects. *Br. J. Cancer.* **2002**, *87*, 1166– 1172.
7. Mitsiades, C. S.; Hayden, P. J.; Anderson, K. C.; Richardson, P. G. Apoptotic Signaling Induced by Immunomodulatory Thalidomide Analogs in Human Multiple Myeloma Cells: Therapeutic Implications. *Best Pract. Res. Clin. Haematol.* **2007**, *20*, 797–816.
8. Mitsiades, N.; Mitsiades, C. S.; Poulaki, V.; Chauhan, D.; Richardson, P. G.; Hideshima, T. et al. Apoptotic Signaling Induced By Immunomodulatory Thalidomide Analogs in Human Multiple Myeloma Cells: Therapeutic Implications. *Blood*, **2002**, *99*, 4525–4530.
9. Niwayama, S.; Turk, B. E.; Liu, J. O. Potent Inhibition of Tumor Necrosis Factor-α Production by Tetrafluorothalidomide and Tetrafluorophthalimides. *J. Med. Chem.* **1996**, *39*, 3044–3045.
10. Niwayama, S.; Loh, C.; Turk, B. E.; Jun, L. O.; Miyachi, H.; Hashimoto, Y. Enhanced Potency of Perfluorinated Thalidomide Derivatives for Inhibition of LPS-induced Tumor Necrosis Factor-α Production is Associated with a Change of Mechanism of Action. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1071–1076.
11. Muller, G. W.; Stirling, D. I.; Chen, R. US patent 5635517, 1997.
12. Palmer, B. D.; Ching, L.-M. Patent Application WO 2008007979, 2008.