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ORIGINAL ARTICLE

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Phase I and pharmacokinetic study of weekly NV06 (Phenoxodiol™), a novel isoflav-3-ene, in patients with advanced cancer

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Abstract *Background*: We wished to define the maximum tolerated dose (MTD), toxicity, and pharmacokinetics of the novel isoflav-3-ene, NV06 (Phenoxodiol™), a compound with a diphenolic structure related chemically and biologically to genistein and flavopiridol. *Patients and Methods*: Twenty-one patients with advanced cancers were treated with weekly intravenous administration of NV06 at escalating dose levels with 1–4 patients at each dose cohort. Plasma sampling was undertaken to characterize the pharmacokinetic (PK) profile of the compound. *Results*: Toxicity was minimal, with asymptomatic Grade 3 lymphocytopenia occurring in nine patients. Nine patients developed Grade 1 nausea, six patients developed Grade 1 increases in alkaline phosphatase, and six patients developed Grade 1 increases in transaminases. Two patients experienced hypersensitivity reactions. The MTD was not reached. Most patients had progressive disease on treatment but eight completed 12 weeks and two completed 24 weeks of treatment. The best response was stable disease of 6 months duration. The plasma half-life (T1/2), clearance (Cl), and volume of distribution (V_D) were 304 (±91) min, 82 (±19) ml/min and 32,663 (±7,199) ml, respectively, for total NV06. *Conclusions*: NV06 is well tolerated and can be given safely as an intravenous infusion over 1–2 h at a dose of at least 30 mg/kg.

Keywords Clinical trial · Isoflavones · Signal transduction

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Introduction

NV06 (Phenoxodiol™) is a synthetic compound based on the diphenolic (*isoflavonoid*) ring structure. Recently, it was granted Fast Track status by the FDA in its development as a chemo-sensitiser for platinums and taxanes for the treatment of recurrent ovarian cancer. The main mechanism of action of NV06 appears to be related to Akt-mediated activation of FAS mediated signaling through inhibition of anti-apoptotic factors [6], resulting in either apoptosis or potential reversal of drug-mediated resistance. Other supplementary mechanisms of action involving down-regulation of sphingosine kinase or toposisomerase II are also possibly relevant [4]. NV06 shows broad activity against human cancer cells, inducing mitotic arrest (G_1 phase of mitosis), terminal differentiation, and apoptosis; NV06 also is a weak oestrogen, but is an inhibitor of the androgen-associated enzymes 5-α-reductase and 17β-hydroxysteroid dehydrogenase [9]. Recently, induction of p21WAF1/CIF1 by NV06 and subsequent specific cdk2 inhibition was shown for HN12 cell lines [1].

We, and others, have previously shown that NV06 has activity against a variety of human cancer cell lines. The concentration required to inhibit by 50% the growth of PC3 and DU145 prostate cancer cells (IC50) in vitro was 3–5 μM [8]. In other cell lines, the IC50 was reported as 1.5, 1.5, 4.5, and 15.0 μM in HL60 leukemia, MCF7 breast, H460 NSCLC, and HT29 colon cancer cell lines, respectively [9]. NV06 administered orally once daily for 18 days or intraperitoneally three times weekly for 21 days to athymic mice bearing xenografts of prostate cancer cells (DU145, PC3) or ovarian cancer cells (A2780) resulted in a significant inhibition of tumor

growth [6, 9]. In mice, intravenously administered NV06 is subject to extensive conjugation (glucuronidation and sulfation) [9]. As with steroidal hormones, bio-activation of the drug follows deconjugation by glucuronidases and sulfatases within target tissues. In rats, no toxicity by way of histological changes in a variety of organs, hematological, or serum biochemical abnormalities was associated with the intravenous dosage form. However, functional toxicity such as subdued behavior, reduced activity, loss of balance, and head tremors, was noted at the highest doses. The maximum tolerated dose (MTD) by repeated (daily for 5 consecutive days) bolus injection in rats was 80 mg/kg [9]. When NV06 was delivered by continuous intravenous injection for 28 days, the MTD was determined to be 20 mg/kg/24-h on the basis of reversible, moderate nephrotoxicity that was also observed in the vehicle (hydroxypropyl-β-cyclodextrin) control group.

Flavonoids, and in particular, flavopiridol [7, 11–16], have attracted much interest recently, as they represent new agents active against specific cellular targets, targeting key signaling pathways such as Akt that are critical to cell survival. Inhibition of Akt has also attracted much interest as Akt is a mediator of the effects of multiple genetic abnormalities including PTEN deletion, BCR—ABL translocation, Her2—neu amplification and EGFR overexpression [18]. Rapamycin is an inhibitor of the downstream effector of Akt, MTOR [3], and analogues are currently undergoing clinical trials.

On the basis of the pre-clinical properties of NV06, we performed a Phase I dose-escalation trial in patients with advanced cancer with the aim of determining a recommended Phase II dose based on a target concentration commensurate with active in vitro doses.

Patients and methods

Patients

Patients were eligible for the study if they had refused standard therapy or had failed at least one standard systemic treatment regimen for their malignancy, were ≥18 years of age, had Eastern Cooperative Group (ECOG) performance status of 0–2, and a life expectancy of at least 3 months. Adequate organ function was required, including ANC $\geq 1.5\times10^9\ l^{-1}$, platelets $\geq 100\times10^9\ l^{-1}$, Hb≥10.0 g/dl for men and ≥9.0 g/dl for women, serum creatinine ≤ 0.12 mmol/l, and AST/ALT levels ≤ 5 times the upper limit of normal. Patients were ineligible if they had more than 25% of hemopoietic bone marrow previously irradiated, active infection, or active, untreated CNS metastases. No investigational agent or chemotherapy was allowed within at least 3 weeks of study entry (6 weeks for nitrosureas or mitomycin C), and patients must have recovered from previous side effects. All patients gave written informed consent, and the protocol was approved by the South Eastern Area Health Service Ethics Committee. The trial was conducted in accordance with the Declaration of Helsinki (2000).

Dose escalation

Acute dose limiting toxicity (DLT) within the first 4 weeks of treatment was defined as (1) Grade 4 neutropenia lasting > 7 days with or without colony stimulating factor support; (2) Febrile neutropenia; (3) Grade 4 thrombocytopenia; (4) Grade 2 or worse CNS neurotoxicity; or (5) Grade 3/4 non-hematologic toxicity, except for alopecia, nausea or vomiting.

The starting dose of NV06, 5 mg/kg weekly as a 1-h intravenous infusion, was one-tenth of the dose (50 mg/kg) that caused subdued behaviour in rats in pre-clinical studies [9]. Previous preliminary pharmacokinetic data in patients with advanced malignancies had established that a single dose of 5 mg/kg had produced NV06 concentrations that were compatible with in vitro activity, and mild nausea was the only reported adverse event (J.B. Howes, P.L. de Souza, L. West, J.L.L. Huang and L.G. Howes, in preparation). In view of the potential for cumulative toxicity, however, lower doses were studied in single patient cohorts (1 and 2.5 mg/kg) to observe toxicity for at least 6 weeks prior to the main starting dose level (5 mg/kg). For this and subsequent dose levels, a minimum of three patients was studied unless DLT was observed. If there was no DLT among the first three patients entered at a given dose level at the end of 3 weeks, three more patients were recruited for the next highest dose level. If a DLT was observed in any of the three patients at any dose level, we planned to enter three more patients at the same dose level. If a second patient experienced DLT then dose escalation stopped. The MTD was defined as the dose level below the level that produced DLT in ≥33% of patients. The recommended Phase II dose was defined as either the MTD or a dose commensurate with maximum plasma concentrations above the IC50. The in vitro IC50 concentrations were thought to be more clinically relevant because pharmacokinetic assessments in vivo had been performed in single dose studies only in rats [9].

Drug administration

NV06 was supplied by Novogen Pty Ltd (Australia) in glass vials at a concentration of 15 mg/ml suspended in hydroxypropyl-β-cyclodextrin (HPBCD) in isotonic saline. The stock solution was diluted in normal saline to approximately 5 mg/ml and the resulting solution was administered via an infusion pump over 20 min through a peripheral intravenous cannula. At dose levels greater than 15 mg/kg, duration of infusion was increased to 2 h to reduce pain at the site of infusion. NV06 was administered weekly, based on pre-clinical data in mice.

We assigned a treatment period of 4 weeks as equivalent to one ''cycle'', in order to assess acute toxicity within a reasonable time frame.

Pretreatment and follow-up investigations

Prior to the start of treatment, a medical history, physical examination, chest X-ray, ECG, vital signs, ECOG performance status, full blood count including differential, serum biochemistry (electrolytes, urea, creatinine, liver function tests, calcium, phosphate, random blood sugar), prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen were performed. Males and females also had testosterone, FSH, LH, and oestradiol, FSH, and LH measured, respectively. Laboratory testing was performed using standard techniques by the South Eastern Area Laboratory Service (SEALS). Glomerular filtration rate (GFR) was estimated using the Cockcroft–Gault method and by isotope scanning. A calculated GFR using the Cockcroft–Gault method was required at weeks 5 and 9 during treatment. Tumor size was assessed by radiological imaging and PSA or other tumor markers (where appropriate) were performed every week for the first cycle, then every 2 weeks thereafter. Male and female sex hormones (as for baseline) were performed monthly, along with fibrinogen, APTT and PT. ECOG performance status was assessed at the beginning of each cycle. For patients who had measurable disease, repeated radiological assessment of measurable and evaluable disease occurred at 6 and 12 weeks during therapy. Patients who responded to treatment without toxicity at the end of 12 weeks were offered continuing treatment for a total of 24 weeks.

Toxicity assessment

Toxicity was graded using the NCI Common Toxicity Criteria, version 2.0. Toxicity assessment, physical examination and vital signs were carried out weekly in the first cycle, then once every cycle thereafter.

Pharmacokinetic studies

During and after the first infusion of NV06, plasma samples were taken at time 0 (0–5 min prior to infusion), at 20 min (just before termination of infusion), then at 30, 40, 60, 90, 120, 150, 180, 240 and 360 min for the measurement of free and conjugated NV06 concentrations. Pharmacokinetic measurements were performed on day 1 of cycle 1. Plasma samples were centrifuged at the bedside and stored at −20°C prior to analysis. Pharmacokinetic parameters (PK) were derived using compartmental methods with Win Nonlin statistical software (Pharsight corporation, NC, USA).

NV06 assay methods

Plasma NV06 assays were performed at Novogen Research Laboratories, Sydney, Australia. The free NV06 was assayed using an HPLC-UV method and the total NV06 was assayed using an LC-MS (ESI-MRM) method, following incubation with ß-glucuronidase. Both methods are validated but are not published. For the HPLC-UV method, the calibration curve was linear in the range of 0.25–10 μg/ml with a correlation coefficient (R^2) >0.994. The lower limit of quantitation (LOQ) for NV06 was 0.25 μg/ml. Recovery of NV06 was >71% at low (0.25 μg/ml), medium (1 μg/ml) and high (10 μg/ml) concentrations. The intra-day accuracy was in the range of 86.2–107% and the inter-day accuracy was in the range of 94.5–107% at all concentrations. For the LC-MS method, the calibration curve was a polynomial curve in the range of 0.025–20 μg/ml with correlation coefficient (R^2) >0.992. The LOQ for NV06 was 0.025 μg/ml by this assay. Recovery of NV06 was >93% at low (0.063 μg/ml), medium (5 μg/ml) and high (15 μg/ml) concentrations. The inter-day accuracy was in the range of 97–108% at all concentrations.

Statistical analysis

All results are expressed as the mean ± standard deviation (SD) except where the median and range are presented.

Results

General

Twenty–one patients were enrolled between November 2000 and January 2002. Patient characteristics are listed in Table 1. The median age was 61 (range 25–84) and there were 16 males. Most patients had performance status of ECOG 1–2, and there were a variety of cancers represented. All patients but two had received prior therapy (Table 1). A total of 185 infusions were administered with a median of 7 and range of 2–24 infusions.

Dose escalation

The dose-escalation scheme is summarized in Table 2. No DLT was seen during the first cycle. However, DLT was later encountered in two patients during cycle 2. Both were considered possibly related to the drug, but did not recur during cycle 3 despite continuation of treatment. The MTD was not reached by the 30 mg/kg dose level and the trial was terminated because difficulty in dissolving higher concentrations of the drug precluded further dose escalation.

Table 1 Patient characteristics

Characteristics	Number of patients
Total	21
Age, years	
Median	61
Range	25–84
Gender	
Male	16
Female	5
Performance status, ECOG	
0	8
1	11
2	2
Tumor type	
Kidney	2
Prostate	8
Melanoma	1
Head and neck	3
Pancreas	3
Breast	1
NSCLC	2
Leiomyosarcoma	1
Number of previous treatments	
0	2
1	10
2	7
3	1
Baseline weight, kg	
Mean	73
Range	46–96

Abbreviations: NSCLC, non-small cell lung cancer

Hematologic toxicities

Lymphocytopenia was the most consistent hematologic toxicity noted (Table. 3, 4), although this did not lead to any apparent clinical problems. Lymphocytopenia did not correlate with increasing dose levels, and did not correspond to increasing duration of therapy. Other hematologic toxicity was very mild, with fewer than 10% of patients experiencing Grade 2 or worse anaemia, thrombocytopenia, or leucopenia.

Non-hematologic toxicities

One patient developed isolated, asymptomatic, Grade 2 hyperbilirubinaemia, peaking during cycle 2 at the 1 mg/kg dose level of NV06. His pre-study bilirubin was slightly elevated and did not increase above 34 μmol/l, despite continued treatment with NV06. After cessation of NV06, the patient's bilirubin levels remained slightly above normal. The only other DLT was the development of a Grade 2 mood disorder (depression) in one patient at the 5 mg/kg dose level during cycle 2 that responded promptly to an antidepressant. Two patients experienced hypersensitivity reactions after several infusions. The first discontinued treatment after sudden onset of flushing, back pain and a transient (48 h) Grade 3 thrombocytopenia. The second was able to continue treatment with steroid and anti-histamine pre-medication. One death due to progressive disease occurred during the study. Other non–hematologic toxicities were mild (Table 5). GFR calculated by the Cockcroft–Gault formula did not change during the study.

Table 3 Overall worst CTC toxicity (any toxicity, any grade, all cycles)

Dose level (mg/kg)	Grade 2	Grade 3	Toxicity
1			Hyperbilirubinaemia
2.5	1/1		Nausea
5	1/3		ALP
10		2/3	Lymphocytopenia
15		3/3	Lymphocytopenia
20		1/3	Lymphocytopenia
25		1/3	Lymphocytopenia
30		2/3	Lymphocytopenia

Table 4 Worst CTC hematologic toxicity per patient, all cycles

Toxicity	Grade 1	Grade 2	Grade 3
Hemoglobin	6/21	1/21	
WBC	3/21	2/21	
Neutrophils	1/21	1/21	
Lymphocytes	1/21	9/21	9/21
Platelets	5/21		

Responses

Eighteen patients were evaluable for response. No objective responses were seen. Ten patients had stable disease of at least 6 weeks duration, and eight patients

Table 2 Dose level cohorts and patient outcome

Dose level	Dose (mg/kg)	Mean dose (mg/m^2) per cycle	Number of patients	Number of patients completing 12 weeks	Number of patients with DLT
−2	1	–	1	1/1	1
−1	2.5	–	1	0/1	0
0	5	197	3	1/3	1
1	10	413	3	1/3	0
2	15	564	3	3/3	0
3	20	767	4	1/4	0
4	25	902	3	0/3	0
5	30	1216	3	1/3	0

Table 5 Worst CTC non-hematologic toxicity per patient, all cycles

Toxicity	Grade 1	Grade 2	Grade 3
Infections	1/21		
Fever (no infection)	2/21		
Nausea/vomiting	9/21	1/21	
Constipation			
Diarrhoea	3/21		
Stomatitis			
Pulmonary	1/21		
Cardiac			
Hypotension	1/21		
Neurosensory	1/21		
Neuro-mood		1/21	
Neuro-headache	2/21		
Alopecia			
Skin	1/21		
Local site reaction	2/21		
Weight gain/loss	1/21		
Allergy	1/21		
Laboratory			
Alkaline phosphatase	6/21	2/21	
Transaminases	6/21		
Creatinine	3/21		
Bilirubin			1/21
Proteinuria			
Hematuria			

had progressive disease. Eight patients were able to complete three cycles of treatment (12 weeks), and two of these completed 24 weeks of treatment (including one patient with renal cell cancer).

Pharmacokinetics

Data from cycle 1 in 15 patients was used to determine pharmacokinetic parameters (Table 6); one patient did not have a baseline value, and is not included in the PK analysis. Only total NV06 was analyzed as "free" NV06 concentrations were only just above the limit of quantitation (data not shown). NV06 reached a mean maximum concentration (Cmax) of 88.7 μg/ml (±25) (=369 μM) in the highest dose cohort (30 mg/kg) at the end of the infusion. The mean volume of distribution (V_D) was 32,663 ml (±7,199). NV06 was rapidly eliminated with a mean clearance of 82 ml/min (±19) and a mean half-life of 304 min (±91). The area under the curve (AUC) for total NV06 increased linearly with dose (Fig. 1) and is described by the equation $y = 5.08x + 2{,}510$ ($R^2 = 0.69$). Figure 2 shows persistent levels of NV06 at least 6 h after a bolus dose for each patient, with a general trend towards higher concentrations in higher dose cohorts.

Discussion

Our Phase I trial demonstrates that NV06 was well tolerated in a weekly treatment schedule. Lymphocytopenia was the main hematologic toxicity seen, although low-grade neutropenia, anaemia and thrombocytopenia occasionally occurred (Table 4). Only three patients developed Grade 1 diarrhoea, whereas flavopiridol, another flavonoid currently under investigation, appears to be associated with dose limiting diarrhoea [12, 16]. On the other hand, lymphocytopenia was relatively uncommon in the Phase I study of flavopiridol [16]. No thromboembolic complications were observed in our study, in contrast to flavopiridol. Two patients in our study developed hypersensitivity reactions, one of whom also developed associated back pain. The cause of these reactions is unclear, but may be similar to the pro-inflammatory phenomena postulated for flavopiridol. Alternatively, they may be a side effect of the hydroxypropyl-β-cyclodextrin carrier, which is known to be a vascular irritant, and can cause vacuolation of renal cells in rabbits [5] and distress and pulmonary oedema in other species [2]. One patient in our study had isolated hyperbilirubinaemia, which we attributed to pre-existing Gilbert's syndrome on the basis of elevated pre-treatment and post-treatment bilirubin levels. Hyperbilirubinaemia had been previously reported with flavopiridol [16], so it is possible that the same proposed mechanism of a shared glucuronidation pathway is also relevant for NV06, at least for this patient. Overall, however, the toxicity profile of NV06 appears to be quite different to flavopiridol. MTD was not reached with this treatment schedule because of difficulty in dissolving the drug at concentrations higher than 30 mg/kg and the practical limitations on the volume of drug able to be delivered.

Pre-clinical studies with NV06 have established that the anti-tumor effect of the drug is proportional to its

Table 6 Pharmacokinetic parameters for Total NV06 (mean and SD for all figures)

Dose level (mg/kg)	*N*	Cmax (μg/ml)	AUC 0-∞ (min μg/ml)	AUC (% extrapolated)	V_D (ml)	CL (ml/min)	T1/2 (min)
5	3	17.5 ± 1.6	7,218 ± 2,923	37	23,621 ± 10,027	58 ± 36	313 ± 79
10	3	29.4 ± 4.5	11,316 ± 3,363	40	36,686 ± 8,295	78 ± 31	366 ± 161
15	1	42.9	16,266	43	33,924	74	317
20	3	46.7 ± 20.2	21,450 ± 12,852	52	42,505 ± 20,219	76 ± 34	419 ± 204
25	3	59.5 ± 14.2	15,256 ± 479	36	34,323 ± 6,731	98 ± 26	254 ± 89
30	2	88.7 ± 25	23,039 ± 2,249	28	24,918 ± 1,692	111 ± 19	156 ± 16
Mean					32,663 ± 7,199	82 ± 19	304 ± 91

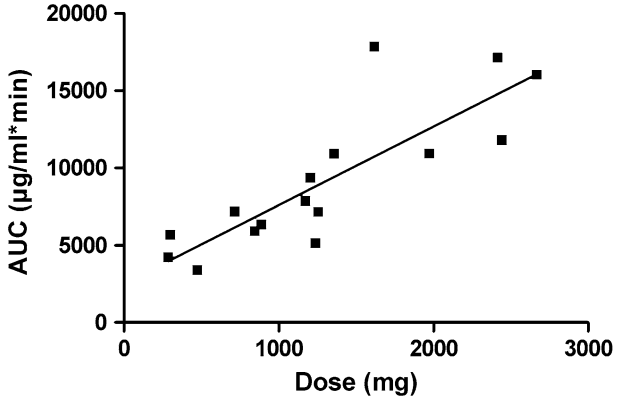

Fig. 1 Dose (mg) plotted against AUC (min µg/ml). Equation for line of best fit is described as $y = 5.08x + 2{,}510$ ($R^2 = 0.69$)

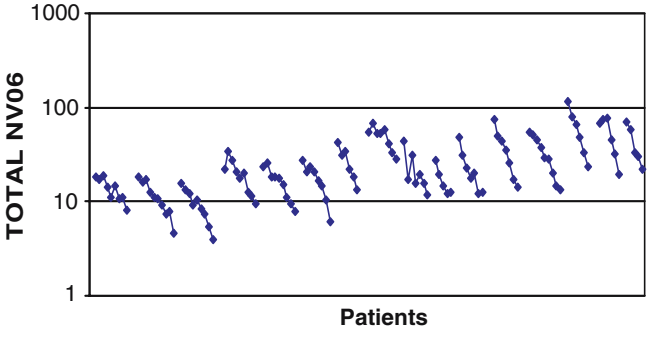

Fig. 2 Total NV06 concentrations (µg/ml) expressed on a logarithmic scale, plotted over at least 6 h for each of 16 patients

duration of contact with the tumor cells [9], an effect consistent with the known biochemical effects of NV06 in down-regulating the phosphorylation of anti-apoptotic proteins [6]. The pulsatile treatment regimen used in this study, therefore, was selected as a starting point for toxicity profiling rather than for efficacy, and, not surprisingly, we did not see any objective tumor responses with this weekly schedule. Nevertheless, several patients did show stable tumor marker levels during treatment, including one man with androgen independent prostate cancer who failed two prior hormone regimens and who had rising PSA levels prior to treatment with NV06. Also, in one patient with oestrogen receptor positive breast cancer treated at the 20 mg/kg dose level, and who previously had been treated with three chemotherapy regimens including an anthracycline-based regimen and docetaxel, was able to complete 12 weeks of treatment with stable CA15.3 levels.

Flavopiridol has been studied in Phase I trials by continuous infusion [13, 17] and by daily 1-h infusions for 3 days [16]. In the bolus study, neutropenia was the DLT, and peak concentrations achieved appeared to approximate peak concentrations in pre-clinical models associated with significant tumor regressions [16]. When flavopiridol was combined with paclitaxel in a Phase I study, however [12], an increase in clearance between the 80 and 94 mg/m^2 dose levels was seen. The authors ruled out an interaction with paclitaxel, and postulated that enterohepatic circulation of the compound was occurring, but did not find evidence of a post-infusion spike in concentrations. In our study, evidence of such a spike with NV06 was not found, though the dominant form of NV06 is conjugated. This may be the more important and bioavailable form of NV06 as "free" NV06 concentrations were only just above the limit of detection (data not shown), and perhaps bound up with the cyclodextrin carrier. Experiments to clarify this possibility are underway. In this study, we were not able to determine the relationship between low concentrations of free NV06, its high protein binding, and what impact this may have had on the lack of clinical activity seen with this schedule compared to in vitro findings. Since relevant pharmacodynamic or surrogate markers were not available at the time of the study, we cannot categorically state that NV06 at concentrations achieved by weekly intravenous bolus infusions is having a significant biological effect. Given the half-life of NV06, we postulate that a more continuous or chronic schedule, such as oral dosing, may be more appropriate to take into the clinic.

The clinical utility of NV06 is most likely to lie with its effect on apoptosis and potential reversal of drug resistance. Subsequent results from a Phase Ib/IIa trial in ovarian cancer have provided preliminary evidence of a striking reversal of paclitaxel resistance in women treated more intensely than a weekly schedule [19]. Further, in vitro evidence suggests that docetaxel resistance in ovarian cancer cell lines may be mediated by XIAP, and that this may be overcome by pretreatment with NV06 [10]. The priority, therefore, is to achieve a biologically effective concentration over a sustained period of time rather than the MTD. The plasma concentrations achieved in this study are well above those required to achieve cytotoxicity in vitro and inhibit Akt [6, 8], though the duration of exposure is perhaps insufficient to mimic in vitro conditions over a 24 h period (Fig. 2) Studies to better characterize its mechanism of action, to correlate PK with pharmacodynamic parameters of Akt inhibition and to explore its combination with standard cytotoxic agents are underway.

In conclusion, NV06 is a novel isoflav-3-ene that is well tolerated in a weekly intravenous schedule. Its main side effects are mild nausea, asymptomatic lymphocytopenia, and mildly raised alkaline phosphatase levels. Concentrations required for cytotoxicity and inhibition of Akt can be achieved without significant toxicity.

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