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ORIGINAL ARTICLE

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Evaluation of the cassette dosing approach for assessing the pharmacokinetics of geldanamycin analogues in mice

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Abstract *Purpose*: There is currently much interest in developing analogues of the benzoquinone ansamycin geldanamycin that may overcome the limitations of 17-(allylamino)-17-demethoxygeldanamycin (17AAG), which is the first known inhibitor of heat shock protein 90 (Hsp90) to enter clinical trials. Studies were performed to assess whether cassette dosing, the coadministration of several compounds to a single animal, is a suitable approach to evaluate the preclinical pharmacokinetics of geldanamycin analogues in high throughput. *Methods*: Five geldanamycin analogues (17AAG, NSC 255110, NSC 682300, NSC 683661, NSC 683663) were administered intravenously to mice in combination at 5 mg/kg each and as single agents at 5 mg/kg and 50 mg/kg, or 12.5 mg/kg for NSC 682300. The compounds were also incubated with mouse liver microsomes individually and in combination at 15 μM each. Quantitative analysis was performed by LC/MS/MS. Plasma and tissue pharmacokinetic parameters were evaluated by non-compartmental analysis. In vitro metabolic stability was assessed by monitoring disappearance of the parent compound. *Results*: Of the compounds that were detectable following individual administration at 5 mg/kg, 17AAG and NSC 683661 exhibited nonlinear pharmacokinetics. In addition, the plasma area under the curve (AUC) and the half-life of these compounds was greater following cassette dosing at 5 mg/kg compared to single administration at the same dose. When pharmacokinetic parameters were calculated up to the same time point following cassette and individual administration at the higher dose, three of the compounds displayed non-linear increases in AUC and slower clearances following cassette compared to single compound dosing. When all measurable concentrations at the higher dose were included, the half-life of NSC 683663 was nine-fold longer following individual compared to cassette administration. 17AAG displayed the highest AUC following cassette dosing, whereas NSC 683663 displayed the highest AUC following single-compound dosing. Excluding NSC 683663, the rank order from the highest to the lowest AUC was the same; however, NSC 682300, which ranked fifth, was administered at a four-fold lower individual dose than the other compounds. Exposure of the liver and kidneys to the compounds was greater than that of plasma. Despite being administered at a lower dose, NSC 682300 displayed the highest kidney AUC of the five compounds. The same ranking was maintained between cassette and single compound dosing in the kidney. With the exception of NSC 682300, in vitro metabolic stability was predictive of in vivo pharmacokinetics in the plasma and liver. The extent of metabolism of four of the five compounds was lower following microsomal incubation in combination compared to incubation alone, suggestive of likely drug-drug interaction in the cassette. However, for 17AAG this may be partly due to metabolism of NSC 683661 and NSC 683663 to this compound. *Conclusions*: Whilst cassette dosing has advantages for use in drug discovery, it is probably unsuitable to evaluate the pharmacokinetics of geldanamycin analogues due to non-linear pharmacokinetics and drug-drug interaction. The issues identified for this compound series should also be considered in assessing the suitability of cassette dosing for other chemotypes.

Keywords 17AAG · Cassette dosing · Geldanamycin analogues · Hsp90 molecular chaperone · Pharmacokinetics

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Introduction

The benzoquinone ansamycin geldanamycin (Fig. 1) binds to the molecular chaperone Hsp90 and inhibits its

intrinsic and essential ATPase activity [17, 24]. Hsp90 is responsible for chaperoning many oncogenic client proteins involved in cell signalling, proliferation, survival, immortalization, invasion, metastasis and angiogenesis, including ErbB2, Raf-1, Akt and mutant p53 [13]. Inhibition of Hsp90 results in the proteasome-mediated degradation of these proteins, which in turn leads to cell cycle arrest and apoptosis [11].

Geldanamycin displays potent antitumour activity in vitro [6, 22] but has shown unacceptable hepatotoxicity in preclinical studies [22]. The geldanamycin analogue 17AAG (Fig. 1) exerts similar antitumour activity by the same mechanism [12, 20] but has a more favourable toxicity profile [16], and was selected for clinical development [3, 19]. Although preliminary results of the phase I clinical studies of this agent are encouraging [2, 10, 14, 25], 17AAG has several limitations including poor solubility, low oral bioavailability in mice [8] and metabolism by the polymorphic cytochrome P450 CYP3A4 and DT-diaphorase [7, 12]. Consequently, there is an ongoing effort to develop further structural analogues of geldanamycin with improved pharmaceutical properties. Indeed, it was recently demonstrated that 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17DMAG; Fig. 1) has greater in vitro antitumour activity, higher oral bioavailability and limited hepatic metabolism compared to 17AAG [9] and this agent is undergoing preclinical development [19].

In vitro studies have shown that time of exposure is an important factor in the cellular effects of the geldanamycins and thus new analogues will require high exposure levels in animal models and patients. Indeed for certain schedules, such as once a week dosing, more prolonged exposures may be required [2]. A large number of geldanamycin analogues are either already available or could be generated. However, the pharmacokinetic assessment of many analogues in animals by conventional single compound dosing and analysis would be both resource-intensive and low in throughput. Indeed, as the earlier elements of the drug discovery process have become more efficient because of high throughput methodologies, pharmacokinetic analysis has become a serious generic bottleneck [26].

Cassette dosing was developed to accelerate the process of in vivo pharmacokinetic screening whilst reducing animal usage [5]. This approach involves the coadministration of several compounds to a single animal followed by their simultaneous analysis by liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) [4]. In addition to the benefits of cassette dosing there are limitations, such as the potential for drug-drug interaction [23]. Although widely used in industry, the academic literature on cassette dosing is small and a recent paper was the first to report its use in oncology drug development [18]. It should be emphasized that cassette dosing is not a technique for determining the detailed pharmacokinetic parameters for individual agents; rather, it is a rapid and efficient technique with which to prioritize research compounds for further evaluation in a test cascade.

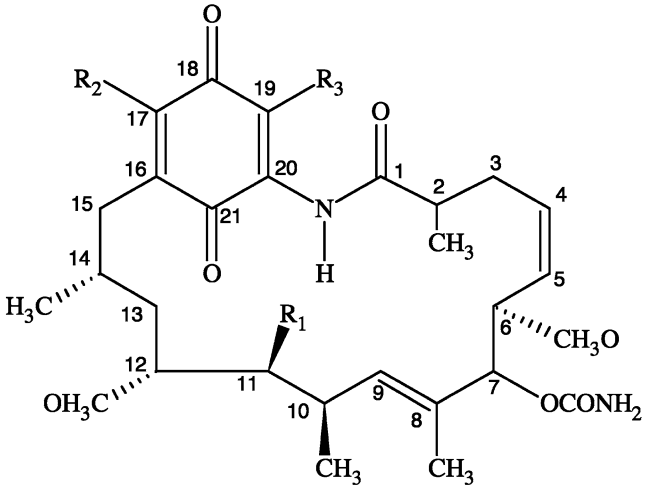

Fig. 1 Chemical structures of geldanamycin analogues

Compound	R_1	R_2	R_3
Geldanamycin	OH	OCH_3	H
17AAG	OH	$NHCH_2CH{=}CH_2$	H
17DMAG	OH	$NHCH_2CHNH_2(CH_3)_2$	H
NSC 255110	OH	OCH_3	$CH{=}NN(CH_3)_2$
NSC 682300	$OCO(CH_2)_3NH_2$	$NH(CH_2)_2CH_3$	H
NSC 683661	$OCOCH_2NH_2$	$NHCH_2CH{=}CH_2$	H
NSC 683663	$OCO(CH_2)_2NH_2$	$NHCH_2CH{=}CH_2$	H

The aim of the current study was to critically assess whether cassette dosing in mice is suitable to evaluate the pharmacokinetics of a series of geldanamycin analogues differing at positions 11, 17 and 19 of the benzoquinone ansamycin ring. This involved a detailed comparison of the pharmacokinetics of 17AAG and four other analogues (Fig. 1) following their administration to mice either in combination or as single agents. In addition to the more common plasma determinations, compound levels were also determined in liver and kidney after single versus cassette administration. Furthermore, to assess the potential role of compound interactions on metabolism, microsomal incubations were performed with the compounds either alone or in combination.

Materials and methods

Chemicals and reagents

Geldanamycin analogues (Fig. 1) and egg phospholipid (EPL) diluent (NSC 704057) were kindly supplied by Dr. Ed Sausville and colleagues at the Developmental Therapeutics Program at the US National Cancer Institute (Bethesda, Md.). DMSO was purchased from Fisher Scientific UK (Loughborough, UK) and HPLC grade methanol was from Laserchrom Analytical (Rochester, UK). All other reagents were from Sigma-Aldrich Company (Gillingham, UK).

Pharmacokinetic studies

Animal experimentation complied with local and national requirements, including the UKCCCR guidelines for the welfare of animals in experimental neoplasia [27]. Female BALB/c mice (6 weeks old) obtained from Charles River UK (Margate, UK) were allowed to acclimatize for 1 week and weighed around 20 g at the time of experimentation. Food and water were available ad libitum. Dosing solutions were prepared by dissolving the compounds in 10% DMSO and 5% Tween 20 in EPL. Administration was by a single bolus injection into a lateral tail vein. 17AAG, NSC 255110, NSC 682300, NSC 683661 and NSC 683663 were administered in combination at 5 mg/kg each and as single agents at 5 mg/kg and 50 mg/kg with the exception of NSC 682300, which was administered at the maximum tolerated dose of 12.5 mg/kg. Control animals received the vehicle alone. Three mice were injected per time point. Blood, liver and kidneys were sampled at 0.083, 0.25, 0.5, 1, 2, 4, 6 and 24 h following administration. Blood was collected by cardiac puncture under anaesthesia and centrifuged at 15,000 *g* for 2 min to obtain plasma. Tissues were rapidly dissected, weighed and snap-frozen in liquid nitrogen. Samples were stored at −20°C until analysis.

Standard and sample preparation

Tissue samples were thawed on ice and homogenized in 3 ml PBS per gram of tissue. A standard curve ranging from 0.5 to 50 μ*M* and quality control standards at 1, 15 and 30 μ*M* were prepared in 100 μl blank mouse plasma and 300 μl blank tissue homogenate. Geldanamycin was added to all standards and samples as an internal standard. Solid-phase extraction was performed on 1 ml C18 Bond Elut cartridges (Varian, Walton-on-Thames, UK) using an Aspec XL4 (Gilson, Villiers-le-Bel, France). The cartridges were conditioned with 1 ml methanol followed by 1 ml distilled water. Tissue homogenates were centrifuged at 2800 *g* for 5 min prior to loading 300 μl of the supernatants onto the cartridges to avoid clogging. The corresponding volume of plasma was 100 μl. Following sample loading, the cartridges were washed with 2 ml distilled water. The compounds were eluted with 1 ml methanol containing 10% formic acid and the samples were evaporated to dryness prior to reconstitution in 80 μl methanol.

Microsomal incubations

Compounds were incubated individually and in combination at 15 μ*M* each with male CD1 mouse liver microsomes (Xenotech XXL, Lenexa, Kan.) in duplicate. Incubations contained 1 mg microsomal protein/ml, 10 m*M* $MgCl_2$, 1 m*M* NADPH, test compound(s) and 50 m*M* PBS (pH 7.4) in a total volume of 500 μl and were performed for 20 min at 37°C. Termination was by the addition of 1 ml methanol and 30 μl geldanamycin as an internal standard. $T_{0\ min}$ incubations were prepared in the same way except that the test compound was added immediately after the addition of methanol. Samples were centrifuged at 2800 *g* for 10 min at 4°C and the supernatants analysed.

Sample analysis

Chromatography was performed using a Supelcosil LC-18 column, 5 cm × 4.6 mm ID, 5 μm particle size (Supelco, Gillingham, UK) and a mobile phase consisting of methanol and 10 m*M* ammonium acetate. The methanol content was increased from 65% to 100% over 5 min, held at 100% for 4 min, then decreased to 65% over 1 min. The total run time was 16 min, the flow rate 0.6 ml/min and the sample injection volume 20 μl. Quantitative analysis was performed on a TSQ 700 triple quadrupole mass spectrometer (ThermoFinnigan, Hemel Hempstead, UK) coupled to a 600 MS pump and 717 autosampler (Waters, Elstree, UK). The mass spectrometer was equipped with an atmospheric pressure chemical ionization (APCI) source and operated in negative mode. The vaporizer, heated capillary and corona were set at 500°C, 250°C and 5 μA, respectively. Detection was by multiple reaction monitoring (MRM)

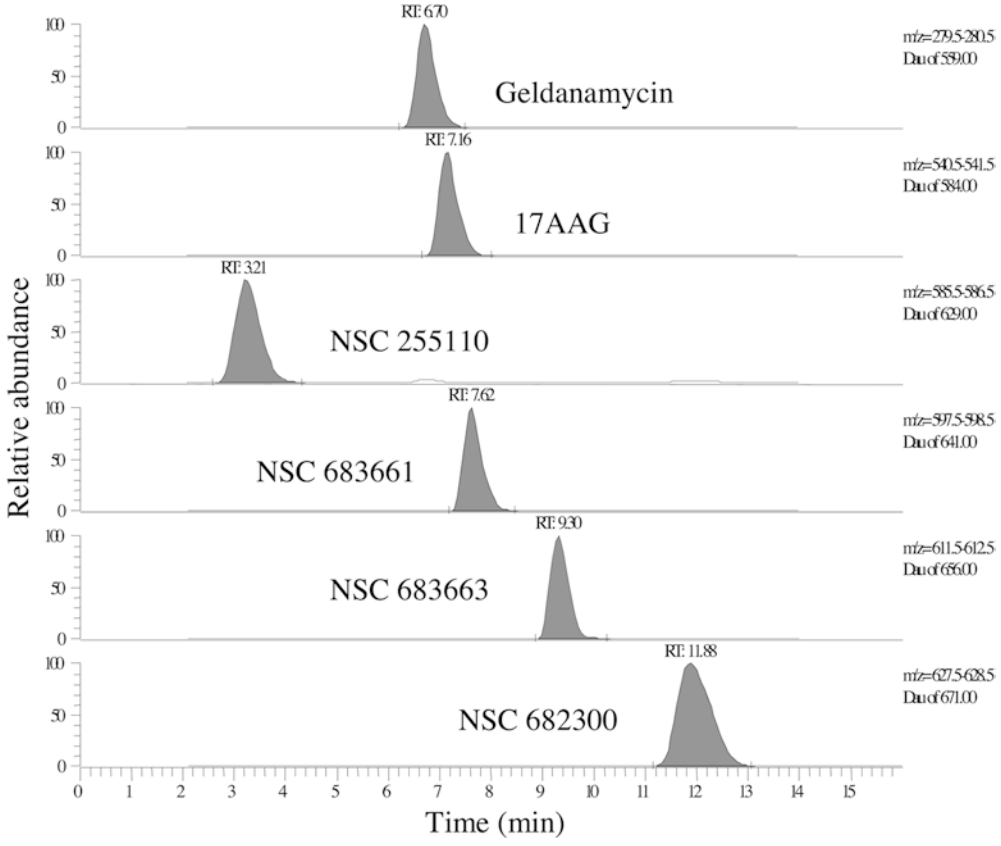

Fig. 2 Multiple reaction monitoring chromatogram of 17AAG, NSC 255110, NSC 682300, NSC 683661, NSC 683663 and the internal standard geldanamycin in blank mouse plasma

of the following transitions: geldanamycin, 559 → 280; 17AAG, 584 → 541; NSC 255110, 629 → 586; NSC 682300, 671 → 628; NSC 683661, 641 → 598; NSC 683663, 655 → 612. Qualitative analysis was performed on an LCQ ion trap mass spectrometer coupled to an AS3000 autosampler and a P4000 pump (ThermoFinnigan). The vaporizer and heated capillary were set at 450°C and 150°C, respectively. Spectra were acquired in full scan mode over the m/z range 50–1000.

Data analysis

Peak area ratios of the analytes to the internal standard were calculated and sample concentrations were interpolated from standard curves constructed by linear regression. Mean concentrations for each time point were calculated. Pharmacokinetic parameters were evaluated by non-compartmental analysis using WinNonlin Professional Version 3.2 (Pharsight Corporation, Mountain View, Calif.). Because of the destructive sampling procedure, the method of Bailer was used to estimate the variance of the area under the curve (AUC) calculated to the last observation (AUC_{last}) [1]. A Z-test was used for the pairwise comparison of AUCs and an observed value of Z greater than the critical value of 1.96 indicated a significant difference [28]. To compare AUCs of the same compound following cassette and single compound administration, cassette plasma concentrations were normalized to the single compound dose and AUCs were calculated up to the same time point. Metabolic stability in mouse liver microsomes was assessed by monitoring disappearance of parent compound as shown below:

$$\%\ \text{Parent loss} = \left[1 - \frac{T_{20\ \text{min}}}{T_{0\ \text{min}}}\right] \times 100$$

where $T_{20\ min}$ and $T_{0\ min}$ represent the peak area ratios of the parent compound to the internal standard at 20 min and 0 min, respectively. Metabolites were tentatively identified by mass difference from the parent compound. Full scan spectra were manually inspected for peaks that were present in $T_{20\ min}$ microsomal incubations and 1 h plasma samples from treated animals, but absent in control samples.

Results

LC/MS/MS method for quantitative analysis

Chromatographic separation of the five compounds plus the internal standard geldanamycin was achieved (Fig. 2). The compounds did not ionize well in the mass spectrometer. For all compounds, the limit of detection in plasma and tissues was 0.5 μM and a linear response was observed up to 50 μM. The quality control samples were within 15% of nominal concentrations.

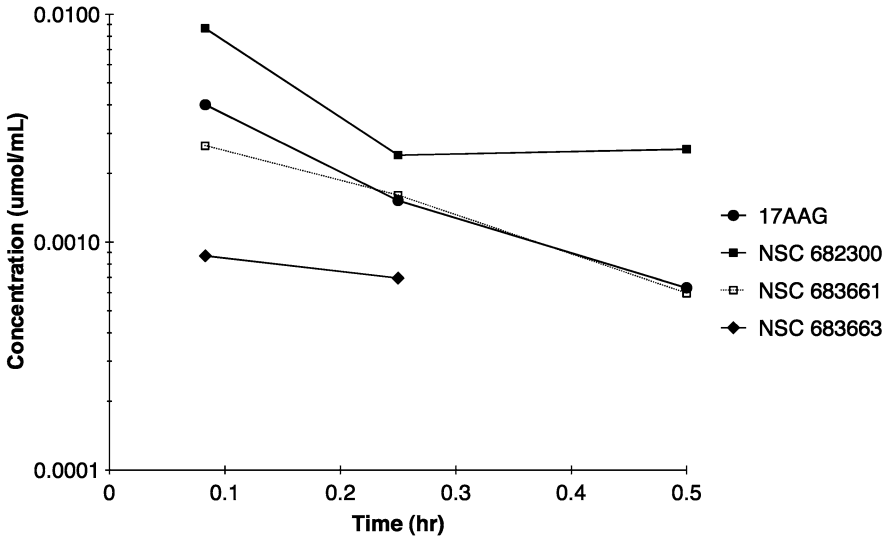

Fig. 3 Plasma concentration-time curves of 17AAG, NSC 682300, NSC 683661 and NSC 683663 following individual intravenous administration to mice at 5 mg/kg. The mean ($n=3$) plasma concentration at each time point is plotted

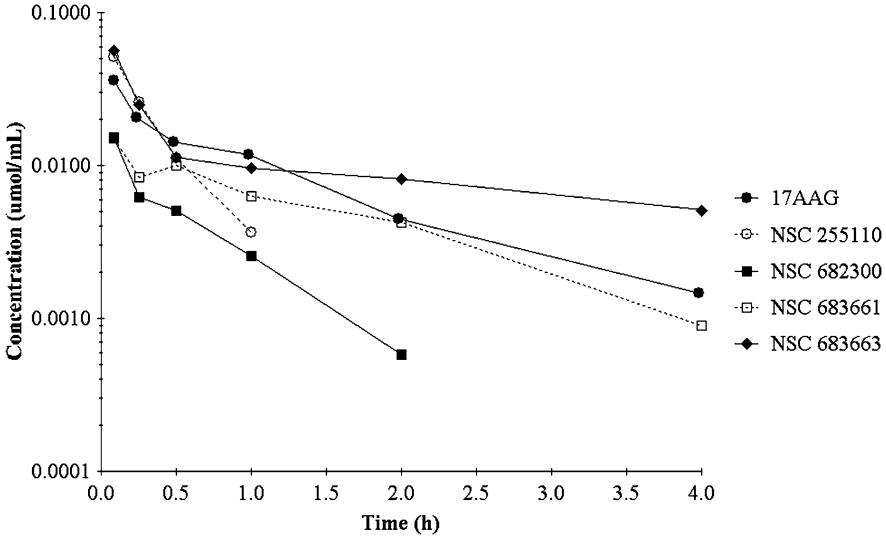

Fig. 4 Plasma concentration-time curves of 17AAG, NSC 255110, NSC 682300, NSC 683661 and NSC 683663 following individual intravenous administration to mice. Compounds were administered at 50 mg/kg with the exception of NSC 682300, which was administered at 12.5 mg/kg. The mean ($n=3$) plasma concentration at each time point is plotted

Plasma pharmacokinetics

Following individual compound administration at 5 mg/kg, the time of the last measurable concentration was 30 min or less (Fig. 3), whereas three of the compounds were detectable for up to 4 h following administration at a higher dose (Fig. 4). The corresponding time following cassette dosing of the compounds at 5 mg/kg each ranged from 30 min to 2 h (Fig. 5). Therefore, pharmacokinetic parameters following individual administration at the highest dose were calculated using all measurable plasma concentrations and also using only those values up to the time of the last measurable concentration following individual or cassette dosing at 5 mg/kg to allow direct comparison (Table 1).

Pharmacokinetic linearity following individual administration could be assessed for only three of the five compounds as NSC 683663 was detectable up to 15 min only and NSC 255110 was undetectable when dosed at 5 mg/kg. For 17AAG and NSC 683661, the AUC increased linearly with a ten-fold increase in dose from 5 mg/kg to 50 mg/kg, when it was calculated up to the same time point (Table 1, values with superscript a). However, inclusion of all measurable concentrations at the higher dose resulted in a disproportionate increase in AUC for both compounds. When calculated up to 30 min, the AUC of 17AAG, for example, increased from 1.17 h nmol/ml at 5 mg/kg to 12.2 h nmol/ml at 50 mg/kg, yet was 32.7 h nmol/ml when all measurable concentrations at the higher dose were included. Furthermore, regardless of whether parameters were calculated up to the same time point or not, plasma clearance decreased and half-life increased with increasing dose, indicative of nonlinear pharmacokinetics. For example, the clearance of NSC 683661 was 141 ml/h and 84.4 ml/h following administration at 5 mg/kg and 50 mg/kg, respectively. The increase in the AUC of NSC 682300 was approximately linear with increasing dose and the

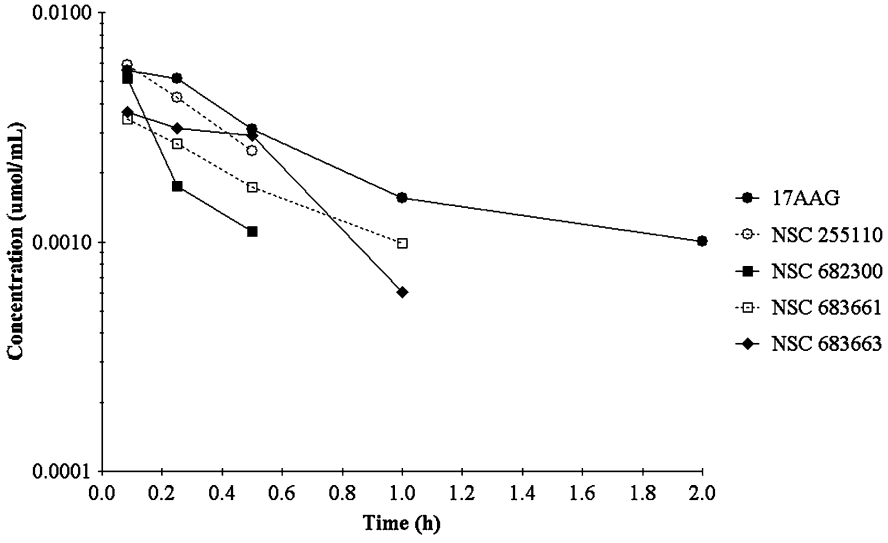

Fig. 5 Plasma concentration-time curves of 17AAG, NSC 255110, NSC 682300, NSC 683661 and NSC 683663 following intravenous administration to mice in combination at 5 mg/kg each. The mean ($n=3$) plasma concentration at each time point is plotted

Table 1 Comparison of the plasma pharmacokinetic parameters of 17AAG, NSC 255110, NSC 682300, NSC 683661 and NSC 683663 following cassette and single compound intravenous administration to mice. Compounds were administered in the cassette at 5 mg/kg each and individually at 5 mg/kg and 50 mg/kg with the exception of NSC 682300, which was administered at 12.5 mg/kg. Pharmacokinetic parameters were derived using non-compartmental analysis (T_{last} time of last measurable positive concentration, AUC_{last} area under a curve computed to the last observation, AUC_{INF} area under a curve from the time of dosing extrapolated to infinity, C_{max} maximum concentration, *CL* total body clearance, *HL Lambda z* terminal half-life, V_z volume of distribution based on the terminal phase)

Compound	Administration	Dose (mg/kg)	T_{last} (h)	AUC_{last} (h·nmol/ml)	AUC_{INF} (h·nmol/ml)	C_{max} (nmol/ml)	$CL_{(obs)}$ (ml/h)	HL Lambda *z* (h)	$V_{z(obs)}$ (ml)
17AAG	Cassette[a]	5	0.5	2.41	4.53	5.85	37.8	0.47	25.7
17AAG	Cassette[b]	5	2	4.86	5.93	5.85	28.8	0.74	30.9
17AAG	Individual[b]	5	0.5	1.17	1.31	6.48	131	0.16	29.9
17AAG	Individual[a]	50	0.5	12.2	18.6	49.3	92.0	0.31	41.2
17AAG	Individual[c]	50	2	26.8	32.1	49.3	53.2	0.83	63.6
17AAG	Individual[b]	50	4	32.7	34.9	49.3	49.1	0.99	70.6
NSC 255110	Cassette[b]	5	0.5	2.23	3.42	6.98	46.2	0.33	22.2
NSC 255110	Individual[c]	50	0.5	16.3	19.4	72.3	81.5	0.19	22.4
NSC 255110	Individual[b]	50	1	20.0	21.5	72.3	73.7	0.27	28.7
NSC 682300	Cassette[b]	5	0.5	1.51	1.82	8.77	77.3	0.20	21.9
NSC 682300	Individual[b]	5	0.5	2.58	3.54	16.4	39.9	0.26	14.9
NSC 682300	Individual[a]	12.5	0.5	4.80	6.90	24.1	51.3	0.28	20.5
NSC 682300	Individual[b]	12.5	2	8.30	8.70	24.1	40.5	0.48	27.9
NSC 683661	Cassette[a]	5	0.5	1.36	2.43	3.87	60.5	0.42	37.1
NSC 683661	Cassette[b]	5	1	2.05	2.78	3.87	53.0	0.51	39.1
NSC 683661	Individual[b]	5	0.5	0.88	1.04	3.39	141	0.19	39.0
NSC 683661	Individual[a]	50	0.5	5.75	17.4	20.3	84.4	0.81	98.3
NSC 683661	Individual[c]	50	1	9.83	18.1	20.3	81.5	0.90	106
NSC 683661	Individual[b]	50	4	20.3	21.6	20.3	68.0	1.03	101
NSC 683663	Cassette[b]	5	1	2.52	2.82	4.01	51.0	0.35	25.6
NSC 683663	Individual[c]	50	1	22.5	27.8	85.0	51.8	0.39	28.9
NSC 683663	Individual[b]	50	4	44.6	67.5	85.0	21.3	3.12	96.1

[a]Parameters calculated up to the time of the last measurable concentration following individual compound administration at 5 mg/kg.

[b]Parameters calculated using all measurable plasma concentrations.
[c]Parameters calculated up to the time of the last measurable concentration following cassette administration at 5 mg/kg

clearance and half-life were similar at both dose levels. However, this compound was dosed at 5 mg/kg and 12.5 mg/kg, unlike the others.

To assess the suitability of cassette dosing as a screening tool to rank the compounds, the rank order from the highest to the lowest plasma AUC was compared following cassette and individual administration (Table 3). Ranking of the compounds following cassette dosing was more similar to that observed following single agent administration at 50 mg/kg than at the equivalent dose of 5 mg/kg. Thus, data obtained at the higher dose was used for statistical comparison of cassette and single pharmacokinetic parameters (see below). When the AUCs were calculated up to the same time point following cassette dosing and individual administration at the highest dose, the rank order of the compounds was the same (Table 3). However, it should be noted that NSC 682300, which ranked fifth, was

administered at a four-fold lower individual dose than the other compounds and ranked second when the AUC value was dose-adjusted to 50 mg/kg, assuming linear pharmacokinetics. When all measurable concentrations following individual administration of the compounds were included in the calculation of the AUCs, there were differences in ranking between cassette and single compound dosing. The AUC of 17AAG was significantly higher than that of NSC 683663 ($Z_{obs} = 5.27$) and the other compounds following cassette administration, whereas NSC 683663 displayed a significantly higher AUC than 17AAG ($Z_{obs} = 3.39$) following single compound dosing.

Statistical analysis revealed that the dose-normalized AUCs differed significantly ($P < 0.05$) whether the compounds were administered alone or in combination, with the exception of NSC 683663 ($Z_{obs} = 1.80$). However, inclusion of all measurable concentrations of NSC 683663 following single agent administration resulted in a disproportionate increase in AUC (from 2.52 h nmol/ml to 44.6 h nmol/ml), a 3.8-fold increase in volume of distribution (from 26.5 ml to 96.1 ml), and a nine-fold increase in half-life (from 0.35 h to 3.12 h) between cassette and single compound dosing. Of the compounds that were detectable following individual dosing at 5 mg/kg, 17AAG and NSC 683661 displayed a higher AUC, a slower clearance and a longer half-life compared to cassette administration at the same dose, with up to 3.6-fold differences observed (Table 1).

Tissue distribution

Exposure of the tissues investigated to the compounds was greater than that of plasma. With the exception of NSC 682300, the compounds distributed better to the liver than to the kidneys (Table 2). Following single compound administration at 50 mg/kg for all but NSC 682300, which was administered at 12.5 mg/kg, liver to plasma AUC ratios ranged four-fold from 2.8 for 17AAG to 10.7 for NSC 682300. At 23.8, the kidney to plasma AUC ratio of NSC 682300 was 17-fold higher than that of the other four compounds, which gave an average ratio of 1.4. Figure 6 shows the liver levels achieved following single agent administration at 50 mg/kg and cassette administration at 5 mg/kg each. NSC 255110 was undetectable in the liver and kidneys following cassette administration.

When calculated up to the same time point, the AUC in the liver increased linearly between cassette and individual dosing at 5 mg/kg and 50 mg/kg, respectively, for 17AAG, NSC 683661 and NSC 683663. However, as observed in plasma, inclusion of all measurable concentrations following individual administration of NSC 683663 resulted in a 20-fold increase in the AUC, from 20.6 h nmol/g to 409 h nmol/g, with the ten-fold increase in dose. The liver AUC of NSC 682300 increased 5.4-fold between cassette dosing at 5 mg/kg and individual dosing at 12.5 mg/kg. The kidney AUCs of 17AAG, NSC 683661 and NSC 683663 increased less than proportionally between cassette and single compound administration. With the 2.5-fold increase in dose between cassette and individual administration of NSC 682300, the kidney AUC increased 4.2-fold when calculated up to the same time point of 30 min and 13.6-fold when all measurable concentrations at the higher dose were included.

Following individual administration, the rank order of the compounds in terms of exposure was similar in the plasma and tissues, with the exception of NSC 682300 (Table 3). Despite being administered at a four-fold lower dose than the other compounds, NSC 682300 displayed the highest kidney AUC, yet ranked fifth and fourth in the plasma and liver, respectively. With the exception of NSC 682300, the rank order in the liver was similar whether the compounds were administered individually or in combination. In the kidney, ranking was maintained between cassette and single compound dosing. The overall coefficient of variation in plasma and tissue remained below 40%.

Metabolism studies

Although formal plasma stability studies were not carried out, there was no evidence of rapid compound degradation in the standard curves prepared in mouse plasma. In contrast, the extent of parent compound loss following individual incubation of the compounds with mouse liver microsomes ranged from 31% for NSC 682300 to 94% for NSC 255110 (Fig. 7). The rank order of the compounds from the least to the most metabolized was the same as that from the highest to the lowest plasma and liver AUCs following individual administration to mice at 50 mg/kg (Table 3) excluding NSC 682300, which was administered at a lower dose. With the exception of NSC 255110, the compounds were metabolized to a lesser extent when incubated with the other compounds in the cassette than when incubated individually at the same concentration. This was most evident for 17AAG, which underwent 84% parent loss when incubated alone, yet did not appear to be metabolized when co-incubated with the other compounds.

In addition to the parent compounds, several peaks were detected in the individual $T_{20\ min}$ incubations for 17AAG, NSC 683661 and NSC 683663 but not in the $T_{0\ min}$ control incubations (Fig. 8). A peak of m/z 584, corresponding to 17AAG, was detected in the NSC 683661 and NSC 683663 samples. A peak of m/z 544, proposed to be 17-(amino)-17-demethoxygeldanamycin (17AG), was detected in the 17AAG and NSC 683661 samples, but not in that for NSC 683663. A peak of m/z 600, thought to represent an epoxide metabolite of 17AAG, was present in the 17AAG sample only. Subsequent studies revealed that the same proposed metabolites of 17AAG, NSC 683661 and NSC 683663

Table 2 AUCs of geldanamycin analogues in the liver and kidney following single compound and cassette intravenous administration to mice. Compounds were administered in the cassette at 5 mg/kg each and individually at 50 mg/kg with the exception of NSC 682300, which was administered at 12.5 mg/kg. Units are hr·nmol/g (*ND* not detected)

	Liver			Kidney		
	Cassette	Individual[a]	Individual[b]	Cassette	Individual[a]	Individual[b]
17AAG	16.6	113	151	11.8	28.9	42.1
NSC 255110	ND	–	56.1	ND	–	25.4
NSC 682300	12.5	66.9	88.7	14.5	61.2	198
NSC 683661	10.6	110	155	4.05	11.1	26.6
NSC 683663	20.6	210	409	11.9	14.1	73.2

[a]AUC_{last} calculated using all measurable concentrations
[b]AUC_{last} calculated up to the time of the last measurable concentration following cassette dosing.

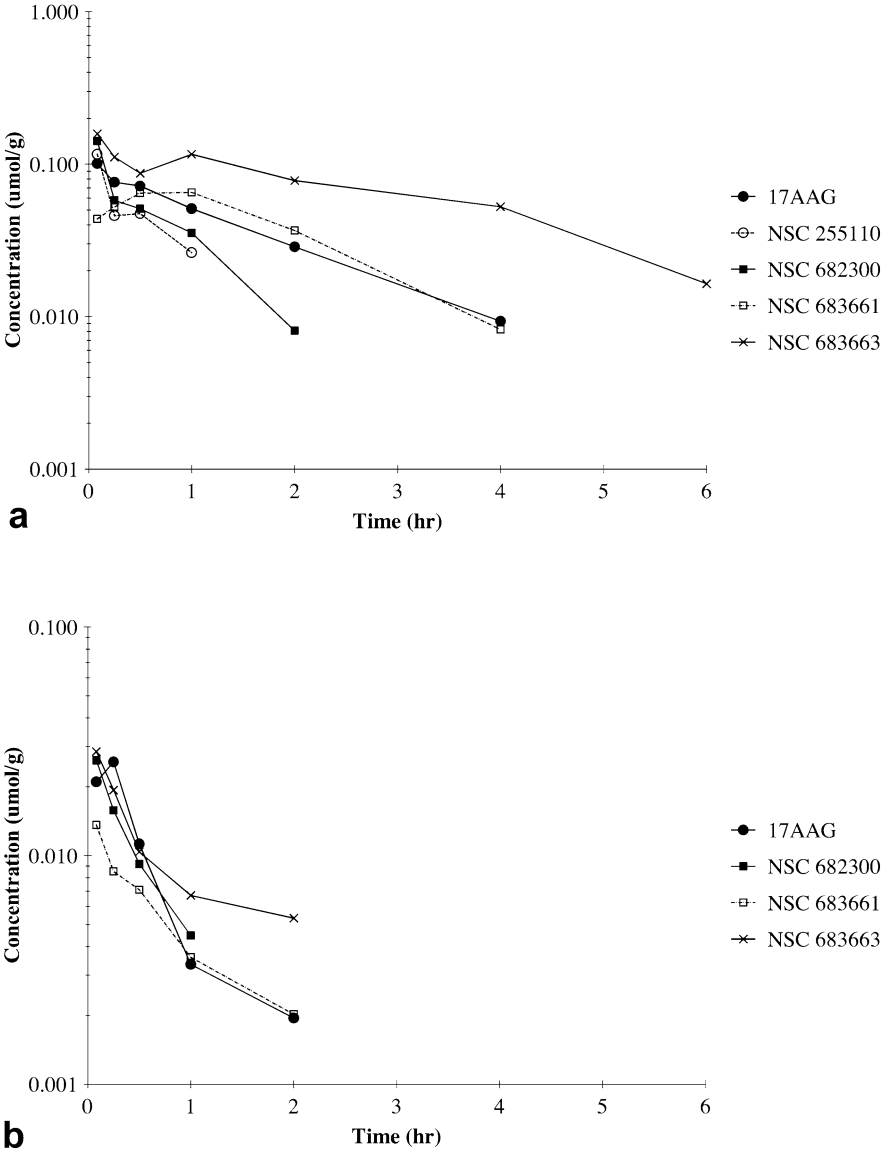

Fig. 6a, b Liver concentration-time curves of 17AAG, NSC 255110, NSC 682300, NSC 683661 and NSC 683663 following intravenous administration to mice. Compounds were administered individually (**a**) at 50 mg/kg with the exception of NSC 682300, which was administered at 12.5 mg/kg, or in combination (**b**) at 5 mg/kg each. The mean ($n = 3$) liver concentration at each time point is plotted

were formed by the mouse in vivo. Although the experiments were not designed for quantitative analysis, Table 4 summarizes the proposed metabolites of 17AAG, NSC 683661 and NSC 683663 formed in vitro and in vivo.

Discussion

There is currently much interest in developing novel inhibitors of Hsp90 for cancer therapy and one ap-

Table 3 Ranking of geldanamycin analogues from the highest (top) to the lowest (bottom) AUC_{last} in plasma, liver and kidney following single compound and cassette intravenous administration to mice. Compounds were administered in the cassette at 5 mg/kg each and individually at 50 mg/kg unless otherwise indicated

Plasma				Liver		Kidney	
Individual[a]	Individual[b]	Individual[c]	Cassette	Individual[b]	Cassette	Individual[b]	Cassette
NSC 682300	NSC 683663	17AAG	17AAG	NSC 683663	NSC 683663	NSC 682300	NSC 682300
17AAG	17AAG	NSC 683663	NSC 683663	17AAG	17AAG	NSC 683663	NSC 683663
NSC 683661	NSC 683661	NSC 255110	NSC 255110	NSC 683661	NSC 682300	17AAG	17AAG
NSC 683663	NSC 255110	NSC 683661	NSC 683661	NSC 682300	NSC 683661	NSC 683661	NSC 683661
NSC 255110[d]	NSC 682300[e]	NSC 682300[e]	NSC 682300	NSC 255110	NSC 255110	NSC 255110	NSC 255110

[a]Administered at 5 mg/kg
[b]AUC_{last} calculated using all measurable concentrations
[c]AUC_{last} calculated up to the time of the last measurable concentration following cassette dosing
[d]Not detectable.
[e]Administered at 12.5 mg/kg

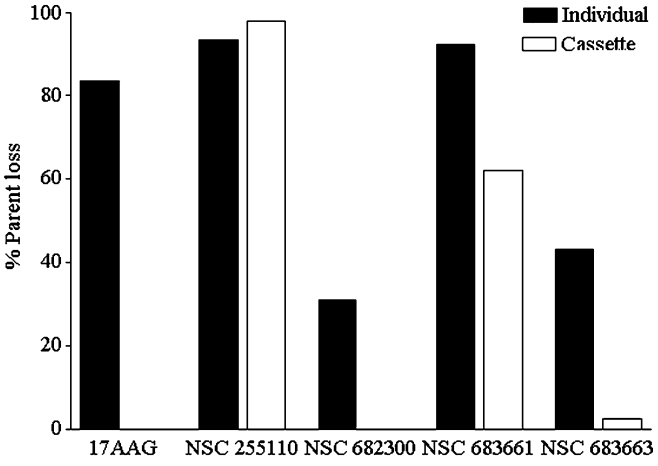

Fig. 7 Comparison of the extent of metabolism of geldanamycin analogues following incubation with mouse liver microsomes individually and in combination at 15 μ*M* each. Values represent mean parent loss following duplicate incubation

proach is the synthesis of structural analogues of existing inhibitors [3, 13, 19]. Modification of the geldanamycin structure may overcome the limitations of 17AAG, which is the first known inhibitor of Hsp90 to enter clinical trials. Given that pharmacokinetic prediction and evaluation is commonly a bottleneck in the drug discovery process, the current studies were performed to critically evaluate whether cassette dosing is a suitable approach to potentially identify further analogues of geldanamycin with improved pharmacokinetic properties in a relatively high throughput manner. To our knowledge, this is only the second published study to evaluate the potential of cassette dosing in oncology drug development, the first being our report on cyclin-dependent kinase inhibitors [18]. In addition, whereas cassette dosing studies normally measure drug levels only in the plasma or serum, this report describes the first assessment of tissue levels. Also of note is the fact that given the potential for drug–drug interactions to affect metabolism, we examined the microsomal metabolism of the geldanamycin analogues alone and in a cassette.

Cassette dosing requires a selective and sensitive method to detect multiple compounds simultaneously following their co-administration at low doses and LC/MS/MS with MRM is the method of choice [4]. With a limit of detection of 0.5 μ*M*, the current analytical method is between two-fold and five-fold less sensitive than those already published for the preclinical evaluation of geldanamycin and related compounds [8, 9, 22]. However, these methods use LC/UV rather than LC/MS/MS detection. Preliminary studies revealed that LC/UV did not provide adequate selectivity for cassette analysis (data not shown). In addition, it is unlikely that LC/UV would be able to distinguish between NSC 255110 (Fig. 2) and the co-eluting metabolite of 17AAG, NSC 683661 and NSC 683663 proposed to be 17AG (Fig. 8). Full analytical validation to GLP guidelines [21] would generate a considerable amount of additional work incompatible with the high throughput objectives of cassette dosing. Nevertheless, the assay was specific and the quality controls that were included demonstrated acceptable accuracy and precision (< 15% variation).

Prior to evaluating whether cassette dosing was suitable for this series, the pharmacokinetic properties of the five geldanamycin analogues following individual administration to mice were investigated. Despite the differences in assay sensitivity, the plasma pharmacokinetic parameters derived for 17AAG in the current study compare well with those determined previously [8]. Following administration at 50 mg/kg, the parameters of AUC, clearance and half-life in the current study were 34.9 h nmol/ml, 49.1 ml/h and 0.99 h, respectively, compared to 912 μg min/ml (26.0 h nmol/ml), 44 ml min/kg (52.8 ml/h) and 53 min (0.88 h) following administration of 17AAG at 40 mg/kg in a lyophilized formulation [8].

The AUC of one of the compounds investigated, namely NSC 683663, was higher than that of 17AAG when the two compounds were compared as single agents (Table 1). The compounds distributed well to tissues and, with the exception of NSC 682300, liver concentrations exceeded kidney levels (Table 2), as previously reported for 17AAG [8] and 17DMAG [9].

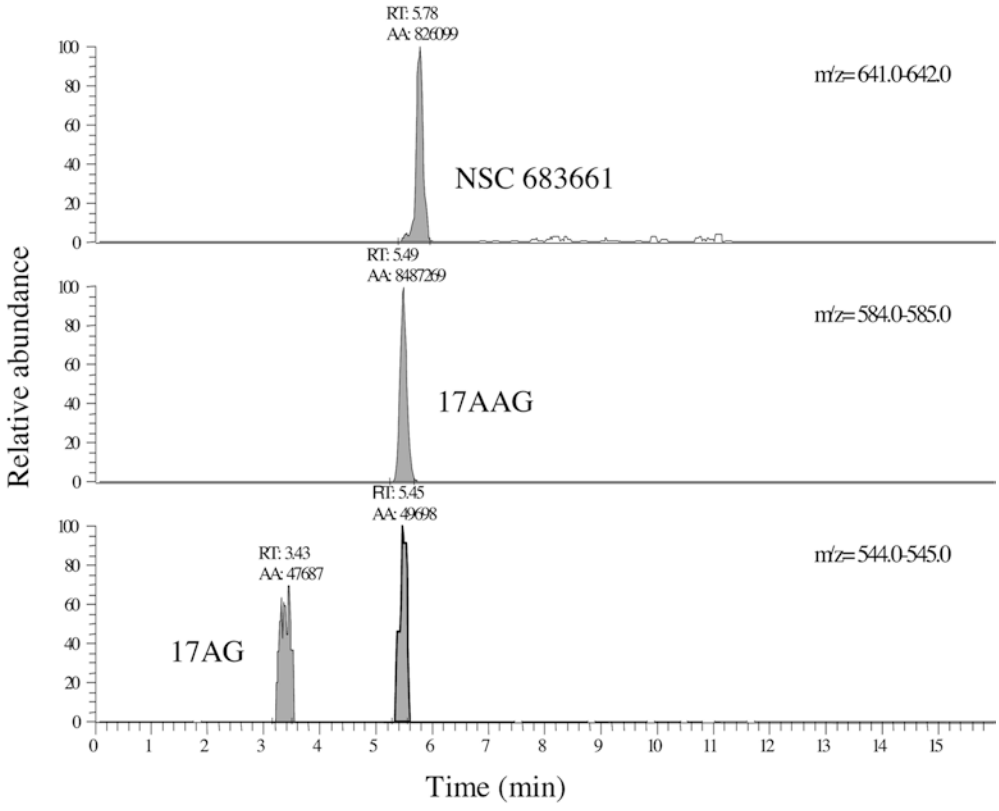

Fig. 8 LC/MS chromatogram of NSC 683661 and proposed metabolites following individual incubation with mouse liver microsomes

Table 4 Proposed metabolites of 17AAG, NSC 683661 and NSC 683663 formed in vitro and in vivo. In vitro data were obtained following incubation of individual compounds with mouse liver microsomes for 20 min. In vivo data were obtained from plasma sampled from mice 1 h following individual intravenous administration at 50 mg/kg. The presence and absence of a proposed metabolite are indicated by + and −, respectively

Proposed metabolite	17AAG		NSC 683661		NSC 683663	
	In vitro	In vivo	In vitro	In vivo	In vitro	In vivo
17AAG	+	+	+	+	+	+
17AG	+	+	+	+	–	–
Epoxide	+	+	–	–	–	–

Despite being administered at a lower dose, NSC 682300 displayed the highest kidney AUC of the five compounds but relatively low plasma and liver levels. Together with the fact that NSC 682300 was the most metabolically stable of the compounds following incubation with mouse liver microsomes (Fig. 7), this suggests that NSC 682300 may undergo renal excretion. The renal excretion of 17AAG has previously been reported to be minimal, with only 2.0–3.2% of the delivered dose accounted for in urine [8]. However, previous studies have shown that whilst the metabolism of 17DMAG is limited, 10.6–14.8% of the delivered dose is excreted in the urine [9]. With the exception of NSC 682300, the rank order from the least to the most metabolized compound following individual microsomal incubation (Fig. 7) was the same as that from the highest to the lowest plasma and liver AUC following in vivo administration (Table 3), suggesting that hepatic metabolism is a major clearance mechanism of these compounds.

The suitability of cassette dosing was assessed by comparing the pharmacokinetics of the five compounds following administration alone and in combination. There were considerable differences between the plasma pharmacokinetic parameters of the compounds following cassette and single compound dosing (Table 1). Nevertheless, the AUC rank order was the same whether the compounds were administered alone at 50 mg/kg, or 12.5 mg/kg for NSC 682300, or in combination at 5 mg/kg each, with 17AAG displaying the highest AUC, but only when AUCs calculated up to the same time point were compared (Table 3). This was observed despite the fact that one of the compounds (NSC 682300) was administered at a fourfold lower dose as a single agent as compared to the other analogues. However, when all measurable concentrations at the higher dose were included, NSC 683663 had a significantly higher AUC than the other compounds and a nine-fold longer half-life following individual compared to cassette administration (Table 1). Although cassette dosing is used as a screening tool, it is important that the compounds with the desired pharmacokinetic properties within a series are identified. If cassette dosing of the current compounds had been carried out in a drug discovery project that was seeking to identify a compound with greater exposure than 17AAG, then all compounds would have

been eliminated on the basis of having lower AUCs than 17AAG, including NSC 683663.

As observed in the current study, it has previously been demonstrated that members of the benzoquinone ansamycin series display nonlinear pharmacokinetics [8, 9], indicating that one or more of the clearance mechanisms of these compounds, which may involve hepatic metabolism [7], renal excretion [8, 9] or biliary excretion [9, 15], is saturated at high doses. This property is undesirable for cassette dosing. If the pharmacokinetics of five compounds in a cassette were not linear to the same extent, then this could lead to differences in ranking at the relatively low doses used in cassette dosing studies compared to higher, more pharmacologically relevant doses.

To determine whether metabolic drug-drug interaction occurred following cassette dosing, the five compounds were incubated with mouse liver microsomes alone and in combination. With the exception of NSC 255110, the extent of parent compound loss was greater following individual compared to cassette incubation, demonstrating that the compounds do interfere with each other's metabolism (Fig. 7). It has previously been shown that 17AAG is metabolized by CYP3A4 [7], which is highly susceptible to inhibition. Many agents that are metabolized by this enzyme are involved in drug-drug interactions when coadministered with other therapeutics. If 17AAG is involved in drug-drug interactions then this has implications for those patients taking part in clinical trials of the drug who might be receiving other CYP3A4-interactive agents. There may also be implications for the planned clinical combination studies. Interestingly, there was no difference in the extent of metabolism of NSC 255110 whether it was incubated alone or in combination. This compound differs from the others in that it lacks an alkylamino substituent at position 17 of the ansamycin ring and has a dimethyl hydrazone substituent at position 19 (Fig. 1). Thus it is possible that this analogue is metabolized differently from the other compounds.

Qualitative analysis of the microsomal incubations revealed that NSC 683661, and also NSC 683663 to a lesser extent, is metabolized to 17AAG (Fig. 8). This may explain why 17AAG did not appear to be metabolized when it was coincubated with the other compounds. Subsequent studies revealed that 17AAG is also formed from NSC 683661 and NSC 683663 in vivo (Table 4). The fact that two compounds in the cassette are converted to 17AAG might explain, in part, why 17AAG had the highest AUC following cassette but not single compound dosing. It is likely that NSC 683661 and NSC 683663 were synthesized as prodrugs designed to undergo hydrolysis by carboxylesterases to produce 17AAG. This was clearly observed both in vitro and in vivo. Although plasma stability studies were not performed, indirect evidence suggested that hydrolysis in plasma is unlikely to be rapid. The results obtained suggest that it may be best to avoid combining an agent within a cassette that may be metabolized to another agent present in the same cassette and also highlight the need to take particular care when selecting compounds to group together in cassettes.

In summary, the present studies showed that this series of geldanamycin analogues exhibits several undesirable features for cassette dosing. Firstly, members of this series display non-linear pharmacokinetics, which may lead to differences in compound ranking at low and high doses. Secondly, there is evidence of drug-drug interaction following cassette dosing. The likelihood of encountering a drug-drug interaction following cassette dosing increases as the dose and number of compounds is increased [23]. The cassette dose of 5 mg/kg per compound used in the current study was higher than those used typically (0.6–1 mg/kg). However, a dose reduction was not possible due to the limited sensitivity of the analytical assay, which was a major limitation in this study. We therefore conclude that cassette dosing is not recommended for the present chemical series. This study highlighted issues that need to be considered in the future with other classes of compound.

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References

1. Bailer AJ (1988) Testing for the equality of area under the curves when using destructive measurement techniques. J Pharmacokinet Biopharm 16:303
2. Banerji U, O'Donnell A, Scurr M, Benson C, Brock C, Hanwell J, Stapleton S, Raynaud F, Simmons L, Turner A, Walton M, Workman P, Judson I (2002) A pharmacokinetically (PK)–pharmacodynamically (PD) driven phase I trial of the HSP90 molecular chaperone inhibitor 17-allylamino 17-demethoxygeldanamycin (17AAG). Proc Am Assoc Cancer Res 43:1352
3. Banerji U, Judson I, Workman P (2003) The clinical applications of heat shock protein inhibitors in cancer—present and future. Curr Cancer Drug Targets 3:385
4. Bayliss MK, Frick LW (1999) High-throughput pharmacokinetics: cassette dosing. Curr Opin Drug Discov Devel 2:20
5. Berman J, Halm K, Adkison K, Shaffer J (1997) Simultaneous pharmacokinetic screening of a mixture of compounds in the dog using API LC/MS/MS analysis for increased throughput. J Med Chem 40(6):827
6. Brunton VG, Steele G, Lewis AD, Workman P (1998) Geldanamycin-induced cytotoxicity in human colon-cancer cell lines: evidence against the involvement of c-Src or DT-diaphorase. Cancer Chemother Pharmacol 41:417
7. Egorin MJ, Rosen DM, Wolff JH, Callery PS, Musser SM, Eiseman JL (1998) Metabolism of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) by murine and human hepatic preparations. Cancer Res 58:2385
8. Egorin MJ, Zuhowski EG, Rosen DM, Sentz DL, Covey JM, Eiseman JL (2001) Plasma pharmacokinetics and tissue distribution of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) in CD2F1 mice1. Cancer Chemother Pharmacol 47:291
9. Egorin MJ, Lagattuta TF, Hamburger DR, Covey JM, White KD, Musser SM, Eiseman JL (2002) Pharmacokinetics, tissue distribution, and metabolism of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (NSC 707545) in CD2F1 mice and Fischer 344 rats. Cancer Chemother Pharmacol 49:7

10. Erlichman C, Toft D, Reid J (2001) A phase I trial of 17-allylamino-geldanamycin in patients with advanced cancer. Proc Am Assoc Cancer Res 42:4474
11. Hostein I, Robertson D, DiStefano F, Workman P, Clarke PA (2001) Inhibition of signal transduction by the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin results in cytostasis and apoptosis. Cancer Res 61:4003
12. Kelland LR, Sharp SY, Rogers PM, Myers TG, Workman P (1999) DT-diaphorase expression and tumor cell sensitivity to 17-allylamino-17-demethoxygeldanamycin, an inhibitor of heat shock protein 90. J Natl Cancer Inst 91:1940
13. Maloney A, Workman P (2002) HSP90 as a new therapeutic target for cancer therapy: the story unfolds. Expert Opin Biol Ther 2:3
14. Munster PN, Tong W, Schwartz L, Larson S, Kenneson K, De La Cruz A, Rosen N, Scher H (2001) Phase I trial of 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) in patients with advanced solid malignancies. Proc Am Soc Clin Oncol 20:327
15. Musser SM, Egorin MJ, Zuhowski EG, Hamburger DR, Parise RA, Covey JM, White KD, Eiseman JL (2003) Biliary excretion of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) and metabolites by Fischer 344 rats. Cancer Chemother Pharmacol 52:139
16. Page J, Heath J, Fulton R, Yalkowsky E, Tabibi E, Tomaszewski JE (1997) Comparison of geldanamycin (NSC-122750) and 17-allylaminogeldanamycin (NSC-330507) toxicity in rats. Proc Am Assoc Cancer Res 38:308
17. Panaretou B, Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH (1998) ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. EMBO J 17:4829
18. Raynaud FI, Fischer PM, Nutley BP, Goddard PM, Lane DP, Workman P (2004) Cassette dosing pharmacokinetics of a library of 2,6,9-trisubstituted CDK2 inhibitors prepared by parallel synthesis. Mol Cancer Ther 3:353
19. Sausville EA, Tomaszewski JE, Ivy P (2003) Clinical development of 17-allylamino, 17-demethoxygeldanamycin. Curr Cancer Drug Targets 3:385
20. Schulte TW, Neckers LM (1998) The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. Cancer Chemother Pharmacol 42:273
21. Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, McGilveray IJ, McKay G, Miller KJ, Patnaik RN, Powell ML, Tonelli A, Viswanathan CT, Yacobi A (2000) Bioanalytical method validation—a revisit with a decade of progress. Pharm Res 17:1551
22. Supko JG, Hickman RL, Grever MR, Malspeis L (1995) Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. Cancer Chemother Pharmacol 36:305
23. White RE, Manitpisitkul P (2001) Pharmacokinetic theory of cassette dosing in drug discovery screening. Drug Metab Dispos 29:957
24. Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci U S A 91:8324
25. Wilson RH, Takimoto CH, Agnew EB, Morrison G, Grollman F, Thomas RR, Saif MW, Allegra C, Grochow L, Szabo E, Hamilton M, Monahan BP, Neckers L, Grem JL (2001) Phase I pharmacologic study of 17-(allylamino)-17-demethoxygeldanamycin (AAG) in adult patients with advanced solid tumours. Proc Am Soc Clin Oncol 20:325
26. Workman P (2003) The opportunities and challenges of personalized genome-based molecular therapies for cancer: targets, technologies and molecular chaperones. Cancer Chemother Pharmacol 52 [Suppl 1]:S45
27. Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double J, Embleton J, Newell D, Ramond R, Stables J, Stephens T (1998) United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (2nd edn). Br J Cancer 77:1
28. Yuan J (1993) Estimation of variance for AUC in animal studies. J Pharm Sci 82:761