Cassette dosing pharmacokinetics of a library of 2,6,9-trisubstituted purine cyclin-dependent kinase 2 inhibitors prepared by parallel synthesis

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Abstract

Determination of pharmacokinetic properties in the intact animal remains a major bottleneck in drug discovery. Cassette dosing involves administration of a cocktail of drugs to individual animals. Here we describe the cassette dosing properties of a 107-membered library of 2,6,9-trisubstituted purine cyclin-dependent kinase 2 (CDK2) inhibitors. A three-step parallel synthesis approach produced compounds with purity ranging from 63% to 100%. Cassette dosing was validated by comparing the pharmacokinetic parameters obtained following i.v. administration of a mixture of olomoucine, R-roscovitine (CYC202), and bohemine, each at 16.6 mg/kg, with results for administration of single agents at 50 mg/kg. No significant difference was observed between the pharmacokinetic parameters of agents when dosed in combination compared with those of individual compounds. CYC202 showed the highest area under the curve (AUC) and the longest elimination half-life ($t_{1/2}$). Further cassettes evaluated the library of trisubstituted purines with CYC202 and purvalanol A included as pharmacokinetic standards in a validated limited sampling strategy. The ratios of pharmacokinetic parameters to that of CYC202 [AUC, maximum concentration (C_{max}), and $t_{1/2}$] remained similar when compounds were tested in two different cassettes or as individual compounds. Following dosing of the same cassette on three different days, there was less than 20% variation in pharmacokinetic parameters between days. The structure-pharmacokinetics relationship showed that the favored purine substituents are benzylamine and veratrylamine at position 6, amino-2 propanol at position 2, and methylpropyl or hydroxyethyl at position 9. Without cassette dosing, this study would have used 3 times as many animals and would have taken 4 times longer, illustrating the power of this method in lead optimization. [Mol Cancer Ther. 2004;3(3):353–362]

Introduction

The evolution of the drug discovery process over the last decade has been the result of the considerable progress in target validation, production of new chemical entities, and screening methodology (1, 2). Advances in the understanding of cell physiology, genomics, and genetic engineering techniques have facilitated the cloning and expression of newly discovered genes, creating novel therapeutic opportunities (3). The development of robotics has impacted on both screening and chemistry. Traditional medicinal chemistry has been complemented by combinatorial chemistry generating large libraries of compounds for evaluation by high-throughput screening and downstream test cascades.

This increase in the rate of compound production and screening has resulted in a bottleneck in *in vivo* evaluation at the whole animal level (4, 5). The difficulty in achieving appropriate drug levels in plasma is well known to be a limiting step in lead optimization. The traditional analytical tools used to monitor absorption, distribution, metabolism, and excretion (ADME) properties have proven inadequate in dealing with the large number of compounds that now require evaluation. Advances in liquid chromatography tandem mass spectrometry (LC-MS/MS), which is both selective and sensitive, have allowed, through multiple reaction monitoring (MRM), the simultaneous measurement of several compounds in the same sample (6–8). Different approaches have been taken to improve the throughput of preclinical pharmacokinetics (PK) (6, 9–13). In cassette dosing, also known as *n*-in-one or cocktail dosing, a combination of drugs is administered to a single animal (11–16). In the second method, samples are pooled after individual dosing (12). In the third approach, limited sampling strategies (even down to one time point) have been used as a predictor of area under the curve (AUC) in oral dosing studies (13). While the first approach has the inconvenience of potential drug-drug interactions, the second method still relies on extensive animal use, and the third approach would not be suitable for i.v. compounds with rapid clearance.

Although it is known that cocktail dosing is employed extensively in industry to increase throughput and hence overcome the ADME bottleneck, reducing costs and animal usage, there have been very few published examples of detailed validation of the methodology. To our knowledge, there are none in the cancer area. In the present study, we have used cassette dosing to evaluate the pharmacokinetics

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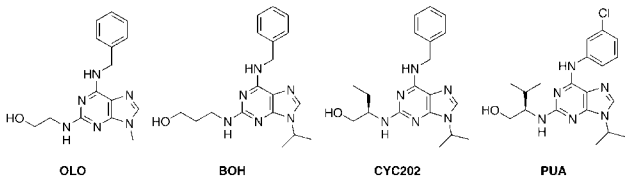

Figure 1. Chemical structures of lead 2,6,9-trisubstituted purine CDK inhibitor molecules. *OLO*, olomoucine; *BOH*, bohemine; *CYC202*, R-roscovitine; *PUA*, purvalanol A.

of a library of 2,6,9-trisubstituted purines. Olomoucine (Fig. 1) is the parent compound in this class of cyclin-dependent kinase (CDK)-specific ATP antagonists (14). Bohemine and R-roscovitine (CYC202) emerged as improved analogues of olomoucine from studies designed to probe the structure-activity relationship within this compound class (15, 16). Purvalanol A is the most potent trisubstituted purine in terms of antiproliferative activity known to date (17). Overall, several hundred purine analogues of this type have been reported over the past decade (18–20).

Preclinical evaluation of olomoucine, bohemine, and CYC202 in terms of pharmacokinetics in mice xenografted with human tumors revealed that while all three compounds are taken up rapidly in tumor, liver, spleen, and kidney, plasma clearance is also very rapid (21). This is due in part to cytochrome P450-mediated hepatic metabolism of the side chain alcohol to the corresponding carboxylic acid (22). Furthermore, the *in vitro* growth inhibitory effect of these compounds on tumor cells depends on the length of exposure and appears to be optimal after about 16 h (*i.e.*, approximately the duration of one cell cycle). The pharmacokinetic parameters observed, when considered in the light of the length of exposure needed for maximum activity, suggested that continuous or repeated administration might be necessary to achieve optimal *in vivo* antitumor activity. ADME properties are therefore of clear importance in the development of trisubstituted purine CDK2 inhibitors and overall our objective was to evaluate the potential of cassette dosing pharmacokinetics with these agents.

The compound set investigated (Fig. 2) was designed in the light of known requirements for 2,6,9-trisubstituted purine CDK inhibition with particular emphasis on the substituents that would confer the best pharmacokinetic properties. The following features were incorporated in the library design: (*a*) modest variation of the size and apolar nature of the aryl/alkyl group on the obligatory secondary amine in R^1; (*b*) narrow variation of R^2, where only small aliphatic substituents are tolerated; (*c*) modest variation and conformational constriction of the optimal hydroxyethylamino motif in R^3, as well as stabilization of the carbinol carbon in that substituent toward metabolic oxidation. The specific objectives of the work described in this paper were to evaluate the potential of cassette dosing pharmacokinetics with this class of compounds and to identify structural features that confer relatively favorable pharmacokinetic properties. Both these objectives were achieved. On the basis of the results reported here, future work will evaluate how this pharmacokinetic information can be integrated with structure-activity relationships for CDK inhibition, kinase selectivity, and cellular activity.

Materials and Methods

Synthesis

Olomoucine, bohemine (14, 16), CYC202 (23), and purvalanol A (18) were synthesized as previously described. The purine analogues shown in Fig. 2 were prepared by simultaneous parallel solution methods, the chemistry for which was adapted from known procedures (14, 16, 18, 24–30). In short: commercial 2,6-dichloropurine (minimum 95% purity; 100 mmol scale) was aminated in refluxing *n*-butanol with moderate excess of amines R^1-H (with auxiliary base triethylamine where necessary) for 4 h. The purine N9-H function in each of the carefully dried products (12.5 mmol scale) was then deprotonated by treatment with a slight excess of fresh sodium hydride in dry dimethylformamide, followed by reaction overnight at 75°C with the appropriate alkyl halides R^2-X, under anhydrous conditions. In those cases where R^2 is hydroxyethyl (analogues {XCX}), alkylation was performed with 2-bromoethoxy-tert-butyldimethylsilane (99%, Aldrich Chemical Co., Gillingham, Dorset, United Kindom) under similar conditions. The *O*-silyl protecting groups were removed by heating in methanolic HCl after the final amination step. The 2-chloro-6,9-disubstituted purine intermediates were then reacted (2 mmol scale) with large excess of amines R^3-H at 160°C for 3 h. Depending on the nature of the amines, sealed flask (no co-solvents) or open vessel (2,5-lutidine as solvent, including excess potassium carbonate addition) methods were used. Intermediates at each stage were isolated/purified by precipitation from suitable aqueous or organic solvents, extraction, or filtration through plugs of silica gel, depending on physical properties and appearance. Final products were purified by flash chromatography (31). Reaction progress was monitored using TLC. Where necessary, success of individual reactions was assessed by ^{1}H nuclear magnetic resonance (NMR) and/or mass spectrometry analysis. The purity of the final products was measured using analytical reversed-phase high-performance (high-pressure) liquid chromatography (HPLC) (Phenomenex IB-SIL 5 μM, C-18 BDS, 250 × 4.6 mm column; 1 ml/min; linear gradient 5–60% acetonitrile (MeCN) over 20 min, then isocratic at 60% acetonitrile in 0.1% aqueous trifluoroacetic acid; λ = 260 nm). Identity was ascertained by electrospray ionization mass spectrometry.

Preparation of Dosing Solutions

Stock solutions of compounds were made in DMSO (50 mM) and diluted to the required concentrations with 1% Tween 20 in saline. The final solution contained 10% DMSO. A few compounds were eliminated from the study on the basis of poor solubility. For quantification purposes, the stock solutions in DMSO were diluted in methanol (MeOH) and subsequently spiked in mouse plasma.

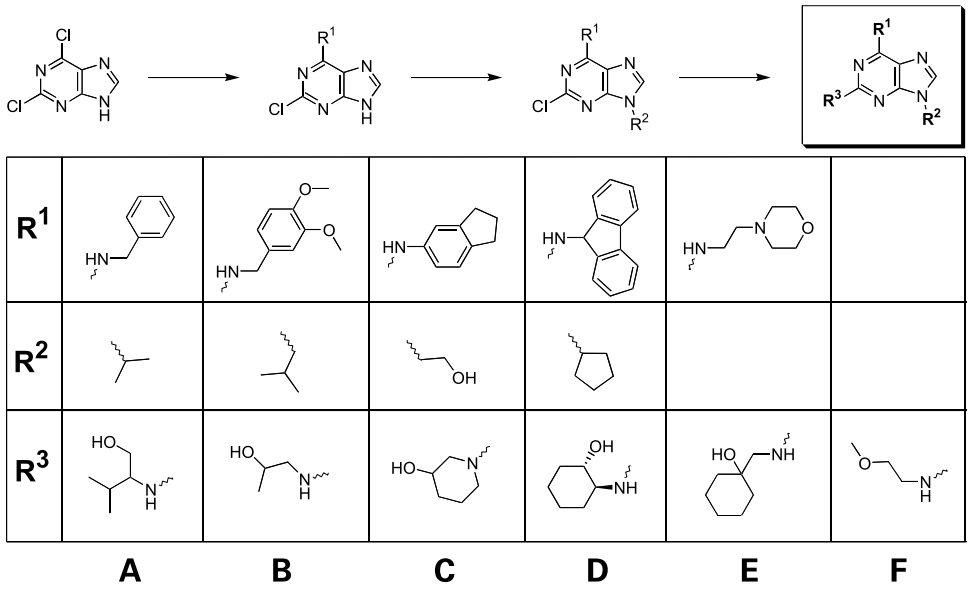

Figure 2. Synthesis and structures of novel 2,6,9-trisubstituted purine analogues used in this study. The stepwise introduction of 6-(R^1), 9-(R^2), and 2-(R^3) substituents used in the parallel synthesis of the analogue set starting from 2,6-dichloropurine is shown. Structures of compounds in the set are indicated as follows: {$R^1R^2R^3$}. For example, the compound 2-methoxyethylamino-6-benzylamino-9-cyclopentylpurine corresponds to member {ADF}.

Animals and Treatment Schedules

All experiments were carried out in accordance with the local institutional and national requirements (32). Female Balb C^- mice (6 weeks of age) were obtained from Charles River (Margate, Kent, United Kingdom) and were acclimatized to the laboratory conditions 2 weeks before the experiment. They were allowed food (Lillico, Betchwork, Surrey, United Kingdom) and water *ad libitum*. The animals weighed 20 g (±1.2 g) at the time of treatment. Following transient hyperthermia to induce vasodilation, animals were injected into the tail vein with a single dose of 50 mg/kg of olomoucine, CYC202, or bohemine in 10% DMSO, 0.5% Tween 20 in saline, or with a combination of the purines at a total dose of 50 mg/kg (16.6 mg/kg of each drug). Anesthesia was induced immediately before bleeding and maintained with 6% halothane in oxygen at a flow rate of 3 ml/min. Samples were taken at 0.25, 0.5, 1, 2, 4, 6, and 24 h post-dosing (n = 3 animals per time point). Blood was collected by cardiac puncture and was centrifuged for 10 min at 1500 × g. The plasma was decanted and frozen at −20°C until analysis within 2 weeks.

For cassette dosing of novel compounds, 166.6 nmol of each analogue (n = 5) were administered per mouse together with 166.6 nmol of either CYC202 or purvalanol A as the pharmacokinetics standard. This approximates to a total dose of approximately 20 mg/kg, chosen so that solubility of the agents would not limit the number of compounds that could be administered. Plasma was collected at 0.5, 2, 4, and 6 h post-administration (n = 2 animals per time point).

Choice of the Analogues Used in the Cassette

To avoid analytical interference, compounds administered together in the same cassette were selected so as to have at least 2–3 atomic mass unit (AMU) difference. In a given cassette, it was ensured that potential metabolites of the compounds (M + 16, M + 14, M − R^1, M − R^2) (33) would not have the same molecular weight as any other compound in the cassette. For example, Table 1 shows the compounds administered together in cassette A. CYC202, which has the slowest plasma clearance of the three originally studied analogues, was used as the internal pharmacokinetics standard. In cassettes where one of the compounds had the same molecular weight as that of CYC202, the latter was replaced by purvalanol A, which has superimposable plasma pharmacokinetics properties compared with that of CYC202.[3] Cassettes of six compounds, including the pharmacokinetics standard, were dosed at 166.6 nmol per mouse.

Analytical Methods

Compound measurements were performed by LC-MS/MS. The LC system used was a Waters 600 MS, connected to a Finnigan Mat TSQ 700 triple quadrupole mass spectrometer fitted with an electrospray ionization source (Thermoquest, Herts, United Kingdom). The capillary voltage was 4.5 kV and the capillary temperature was 250°C. Chromatography was performed using a 50 × 4.6 mm zwitterionic ABZ column (Supelco, Poole, Dorset, United Kingdom); elution was with a gradient from 80% 0.1% aq formic acid-20% MeOH to 100% MeOH over 5 min, followed by a 3-min isocratic run of 100% MeOH. Standard curves of the combined analytes were made in plasma at levels of 5, 10, 50, 250, 2,500, 25,000, and 125,000 nM. One standard curve included the combination of analytes. Plasma aliquots (100 μl) were added to 30 μl of MeOH solution containing 500 ng/ml internal analytical standard (6-benzylaminopurine for the validation experiment and olomoucine in the cassettes), incubated for 30 min, and then mixed with 3 volumes of 100% MeOH. Following centrifugation, the supernatant was transferred

[3] Unpublished observations.

Table 1. Example of compounds administered together in cassette A

Compound	FW	FW − 18	FW + 16	FW − R^1	FW + 14
CYC202	354.0		370	312	368
PUA	388–390		404–406	346–348	402–404
{EDE}	443.6	425.6	459.6	355.6	457.6
{BDE}	480.6	462.6	496.6	410.6	494.6
{BAA}	428.5	410.5	444.5	390.5	442.5
{CAD}	406.5	388.5	422.5	368.5	420.5
{CAB}	366.5	348.5	382.5	328.5	380.5

Note: For details of the compound coding system, see legend to Fig. 2. FW, formula weight.

to HPLC vials and 10 μl were injected onto the column. Detection was achieved with a triple sector mass spectrometer (TSQ700, Thermoquest) by MRM of selected daughter ions of the pseudomolecular ion $[M + H]^+$ through the first quadrupole and following the induced fragmentation in Q2, the appropriate product ion was selected on Q3. The collision gas was set at 1.2 Torr and the collision energy was optimized for each compound. For each cassette, MRM was performed and the analytical interference between compounds was assessed for each analyte in the cassette.

Relative peak areas (*versus* the internal analytical standard) were monitored and plotted against concentration (Graphpad Software, San Diego, CA) and shown to be linear ($r > 0.98$). Quality controls were used throughout at concentrations of 1,000 and 10,000 nM and had to be within 15% of nominal concentration.

Pharmacokinetic Calculations

Pharmacokinetics parameters were evaluated with the program WinNonLin Professional version 3.2 (non-compartmental analysis model 201, Pharsight, Mountain View, CA). In the different cassettes, the pharmacokinetics parameters, AUC (area under the concentration *versus* time curves to the last time point), C_{max} (maximum concentration extrapolated to t_0 by log-linear regression of the first two time points), $t_{1/2}$ (half-life), and V_{ss} (volume of distribution at steady state), were evaluated. The results are expressed as ratios to the value for the pharmacokinetics standard, allowing easy ranking and comparison of the analogues. Uniform weighting was used for the analysis.

Intra-Cassette and Inter-Cassette Variability

To evaluate the variability of cassette dosing, the analogues {ACC}, {BDF}, {CAE}, {DCD}, and {EDA}, which had previously been dosed in cassettes F, I, B, V, and E, respectively, were dosed again with CYC202 as internal standard (cassette Z). This cassette was dosed on three consecutive days and LC-MS/MS analysis performed on three different days. Two compounds that were dosed together in cassettes were also dosed individually for comparison ({ADB} and {CDE}).

Statistical Analysis

Comparison between all the pharmacokinetics parameters obtained following cassette or single dosing was carried out by Spearman's ranking test. The percentage of variation between C_{max}, AUC, V_{ss}, and $t_{1/2}$ in the two dosing schedules was evaluated. The differences between AUC across dosing methods and compounds were evaluated with a method used for destructive measurement techniques (34). The Bonferroni-adjusted critical value was $z = 1.96$ for two groups.

Results

Synthesis

Combinatorial solution synthesis of the target 2,6,9-trisubstituted purine analogues using the well-established synthetic route (14, 16, 18, 24–30) outlined in Fig. 2 afforded the desired compounds in overall isolated average chemical yields of 20–50% over the three synthetic steps. Following isolation by precipitation and short-column chromatography, compound purity ranged between 63% and 100%. Average purity as assessed by RP-HPLC analysis was 92% and compound identity was ascertained using LC-MS and ^{1}H NMR methods. The inclusion criteria for compounds in this study were >60% purity (by RP-HPLC), single impurities no more than 5% (by RP-HPLC), and confirmed identity of the main target component. These criteria are somewhat lower than would normally be expected of screening compounds but are consistent with library synthesis procedures where speed and throughput are the main objectives. The results are summarized in Table 2, together with pharmacokinetics data (see below).

Pharmacokinetics

Analytical Methodology. The standard curves for olomoucine, CYC202, and bohemine were linear ($r^2 > 0.98$) over the large dynamic range used (5 nM–125 μM), but this was not the case for some analogues, where linearity could only be obtained up to 10 μM. However, considering the relatively low doses used in the cassette (166 nmol), this dynamic range proved adequate for all compounds. The lowest limit of detection varied according to the compounds and was 5 nM in 10% of cases, 10 nM in 45%, 50 nM in 45%, and 250 nM in 10%. These levels are at least an order of magnitude below the concentration required to induce a 50% decrease in the cell number of A2780 human ovarian carcinoma cells after 96-h exposure as measured by the sulforhodamine B assay of the four standards (21). Of the 107 compounds analyzed, all but 4 gave a mass spectrometric signal at the correct pseudomolecular ion and all but 3 were soluble in the vehicle at the required concentration.

Validation of Cassette Dosing with Olomoucine, CYC202, and Bohemine. The plasma concentration *versus* time curves following administration of olomoucine, CYC202, and bohemine in combination or as separate agents were very similar (Fig. 3). The pharmacokinetics parameters of the compounds administered alone or in the cassette showed a maximum variation of 28% between the two dosing methods (Table 3). The relative ranking of each of the individual pharmacokinetics parameters was maintained with CYC202 showing the highest plasma levels and the longest $t_{1/2}$, being the only compound

Table 2. Summary analytical and pharmacokinetics results

Compound			Pharmacokinetic ratios relative to CYC202 or PUA			
Code[a]	FW	Purity (%)[b]	AUC	C_{max}	$t_{1/2}$	V_{ss}
{AAA}	368.5	89	0.2	0.4		
{AAB}	340.4	92	2	1	2	1.8
{AAC}	366.5	99	0.5	0.2	0.6	3.2
{AAE}	394.5	82	0.6	0.6		
{AAF}	340.4	92	0.6	1		
{ABA}	382.5	88	0.3	0.2		
{ABB}	354.5	91	NS			
{ABC}	380.5	95	1.6	3		
{ABE}	408.6	91	0.2	0.1	0.5	6.6
{ABF}	354.5	83	0.1	0.1		16.6
{ACA}	370.5	69	0	0.01		
{ACB}	342.4	100	1.4	2.1		
{ACC}	368.4	100	0.6	1.06	0.5	0.79
{ACD}	382.5	99	1TP			
{ACE}	396.5	100	1TP			
{ACF}	342.4	89	1TP			
{ADA}	394.5	81	0.5	0.4		
{ADB}	366.5	100	0.4	0.3	1.8	4.2
{ADC}	392.5	93	1.3	1.8		
{ADE}	420.6	84				
{ADF}	366.5	94	1.5	3.2		
{BAA}	428.5	92	0.7	1.8		
{BAB}	400.5	100	0.2	0.4		
{BAC}	426.5	95	0.3	0.3		
{BAD}	440.5	95	1.3	1.25	0.4	0.5
{BAF}	400.5	81	1.1	0.3	1.6	1.4
{BBA}	442.6	85	0.9	1.3		
{BBB}	414.5	84	0.1	0.1		
{BBC}	440.5	92	NS			
{BBD}	454.6	95	NSI			
{BBE}	468.6	63	1.6	0.3	1.8	2.7
{BBF}	414.5	85	1.4	2.2		
{BCA}	430.5	91	0.2	0.4		
{BCB}	402.5	84	0.2	0.1		
{BCC}	428.5	91	1.2	2		
{BCF}	402.5	95	1TP			
{BDA}	454.6	100	0.3			
{BDB}	426.5	96	0.1	0.1	0.5	5.9
{BDC}	452.6	96	0.1	0.2		
{BDD}	466.6	87	NSI			
{BDE}	480.6	100	0.4	0.3		
{BDF}	426.5	85	1.7	2	1	0.5
{CAA}	394.5	91	0.1	0.1		
{CAB}	366.5	89	1.3	2.3	0.7	2.9
{CAC}	392.5	92	NSI			
{CAD}	406.5	88	1	1	1	0.6
{CAE}	420.6	81	0.5	0.5	0.5	1.7
{CAF}	366.5	88	0.5	0.3	1	2.3
{CBA}	408.6	96	0.2	0.2		
{CBB}	380.5	89	0.4	1.2		
{CBC}	406.5	95	1	2		
{CBD}	420.6	87	0.5	0.5		
{CBE}	434.6	79	0.3	0.3		
{CBF}	380.5	95	0.4	0.3	1.3	1.2
{CCA}	396.5	97	1.4	1.4	0.9	0.6
{CCB}	368.4	91	NSI			
{CCC}	394.5	100	NSI			
{CCF}	368.4	92	0.6	0.6		
{CDA}	420.6	93				
{CDB}	392.5	84	0.3	0.4		
{CDC}	418.5	97	0.4	0.8		
{CDD}	432.6	84	NS			
{CDE}	446.6	76	0.6	0.6	0.6	2
{CDF}	392.5	97	0.1	0.1		
{DAA}	442.6	83	0.2	0.2		
{DAB}	414.5	99	1.1	1.3		0.8
{DAC}	440.6	94	NSI			
{DAE}	468.6	85				
{DAF}	414.5	94	0.5	0.23	1.7	3.3
{DBA}	456.6	90	0.3	0.2		
{DBB}	428.5	85	1	1.2		
{DBC}	454.6	90	NSI			
{DBE}	482.6	96				
{DBF}	428.5	86	0.8	0.8		
{DCA}	444.5	97	1.4	2.1		
{DCB}	416.5	99	1TP			
{DCC}	470.6	97	NSI			
{DCC}	442.5	99	NS			
{DCD}	456.6	100	2	2.1	0.5	4.1
{DCF}	416.5	94	0.5	0.3		
{DDA}	468.6	94	0.1	0.4		
{DDB}	440.6	99	0.8	0.6	1.7	2.1
{DDC}	466.6	99	NSI			
{DDD}	480.6	94	0.5	0.3		
{DDF}	440.6	99	NS			
{EAA}	391.5	91	0.1	0.1		
{EAB}	363.5	76	0.4	0.2		
{EAC}	389.5	98	NSI			
{EAD}	403.5	80	0.1	0.1		
{EAE}	417.6	94	0.4	0.7	0.9	1.4
{EAF}	363.5	91	0.8	0.8		
{EBA}	405.5	92	0.3	0.3		
{EBB}	377.5	72	2.4	3		
{EBC}	403.5	94	2.4	5		
{EBE}	431.6	82	0.7	0.7		
{EBF}	377.5	81	1TP			
{ECA}	393.5	99	0.5	0.8		
{ECB}	365.4	81	NSI			
{ECC}	391.5	100	0.7	0.7		
{ECD}	405.5	77	NSI			
{ECE}	419.5	92	NSI			
{ECF}	365.4	96	1	1		
{EDA}	417.6	85	0.8	1.7	1.7	1.7
{EDB}	389.5	81	NSI			
{EDC}	415.5	87	0.2	0.3		
{EDE}	443.6	93	1.3	2.2	0.7	0.3
{EDF}	389.5	88	NSI			

Note: PUA, purvalanol A; FW, formula weight; NS, not soluble; NSI, no signal; 1TP, one time point detectable. No value indicates that parameter cannot be calculated.
[a]Refer to Fig. 2 for explanation of compound coding system.
[b]Derived from integration (λ = 210 nm) of RP-HPLC chromatograms.

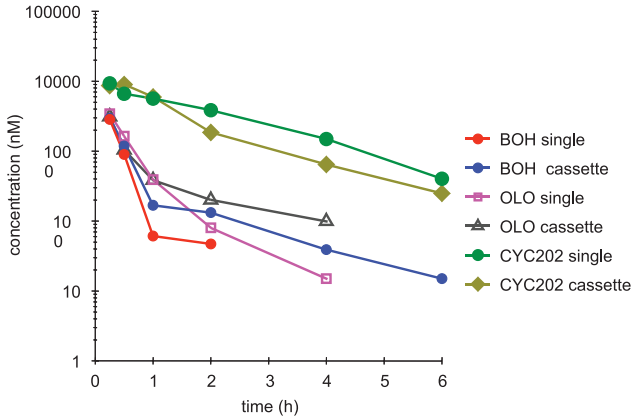

Figure 3. Plasma concentration *versus* time curves following administration of 50 mg/kg olomoucine, CYC202, and bohemine as single agents or 16.66 mg/kg of each in a cassette. Cassette dosing concentrations have been adjusted to 50 mg/kg assuming linear pharmacokinetics.

still detected 6 h post-administration. The pharmacokinetics parameters of the compounds alone or in the cassette were significantly correlated (Spearman's ranking test $r = 0.99$). In comparison to single dosing administration, cassette dosing was found to underestimate the CYC202 AUC and to overestimate the olomoucine and bohemine AUCs, while underestimating the C_{max} of CYC202 and bohemine and underestimating that for olomoucine. However, the ratios of the pharmacokinetics parameters olomoucine and bohemine to that of CYC202 in the cassettes *versus* a single agent were very similar, the C_{max} ratio of olomoucine showing the highest variations (0.6 as a single agent *versus* 0.9 in a cassette). Comparing the AUC of each compound as a single agent or following cassette dosing with the Bonferroni-adjusted test showed no significant differences. However, there were significant differences between the AUC values of CYC202 and bohemine and olomoucine, $P < 0.05$.

Determination of Limited Sampling Times. The pharmacokinetics parameters derived from sampling at 0.5, 2, 4, and 6 h with the more extensive data set described above are shown in Table 4. The number of time points represents a balance between having sufficient points for accurate pharmacokinetic estimates ($n > 3$) while minimizing animal use ($n < 5$). This was validated in experiments where $n = 8$. The pharmacokinetics parameters derived from this limited sampling strategy were very closely related to those derived from the full sampling set for both single and cassette dosing. The greatest difference was observed for the $t_{1/2}$ of bohemine (1.27 *versus* 0.97 h). However, the ranking of the compounds was maintained, with CYC202 showing the highest AUC, the highest C_{max}, and the longest $t_{1/2}$.

Reproducibility of Pharmacokinetics Standards in Cassette Dosing. The variability of the pharmacokinetics standards was assessed. In 17 cassettes, CYC202 was used as internal pharmacokinetics standard, while in 5 cassettes, which included compounds of $m/z = 354$ (the pseudomolecular ion for CYC202) or where interference with potential metabolites could be anticipated, purvalanol A was used as the internal pharmacokinetics standard. purvalanol A has a pharmacokinetics profile very similar to that of CYC202 (data not shown). The concentration *versus* time profiles of the pharmacokinetics standard CYC202 in all of the 17 cassettes in which it was included were determined, and the derived pharmacokinetics parameters are shown in Table 5. Up to 4-fold variation could be observed in the pharmacokinetics of CYC202 in different cassettes. For example, in cassette F, the AUC was 1,635 nM h compared with 6,475 nM h in cassette N. The $t_{1/2}$ of CYC202 was shorter than that observed in the single dose study and in some cases could not be evaluated due to the limited number of samples detectable. The pharmacokinetics parameters observed for purvalanol A when used as the internal pharmacokinetics standard also showed some variation, with the AUC ranging from 9,077 nM h in cassette E to 3,353 nM h in cassette L. Although the AUC and C_{max} appear lower and the V_{ss} higher with CYC202 used as internal standard, when compared with purvalanol A, there was no statistically significant difference in the AUCs between CYC202 and purvalanol A. The pharmacokinetics of CYC202 were linear with mean AUC after cassette dosing at 5 mg/kg of 3,763 nM, 6,741 nM after 16.6 mg/kg, and 20,225 nM after 50 mg/kg.

Table 3. Pharmacokinetic parameters for bohemine, olomoucine, and CYC202 dosed as single agents at 50 mg/kg or in the cassette at 16.66 mg/kg

Compound	Administration	C_{max}	Cl_{obs}	$V_{ss(obs)}$	MRT_{last}	$AUC_{inf(obs)}$	$t_{1/2\lambda z}$
		(nM)	(l/h)	(l)	(h)	(nM h)	(h)
Bohemine	cassette	7,353 (0.7)	1.15 (6.8)	0.70 (2.9)	0.53 (0.4)	2,542 (0.2)	0.97 (0.7)
	single	8,999 (0.7)	1.27 (9.0)	0.64 (2.9)	0.50 (0.3)	2,306 (0.1)	0.90 (0.7)
Olomoucine	cassette	9,208 (0.9)	1.10 (6.5)	0.67 (2.8)	0.56 (0.4)	3,030 (0.2)	1.03 (0.7)
	single	7,194 (0.6)	1.18 (8.4)	0.52 (2.1)	0.40 (0.3)	2,831 (0.1)	0.98 (0.7)
CYC202	cassette	10,471	0.17	0.24	1.23	16,368	1.38
	single	13,103	0.14	0.25	1.61	20,616	1.35

Note: Numbers in parens represent the ratios relative to CYC202. Cassette dosing concentrations have been adjusted to 50 mg/kg (multiplied by 3) for comparison.

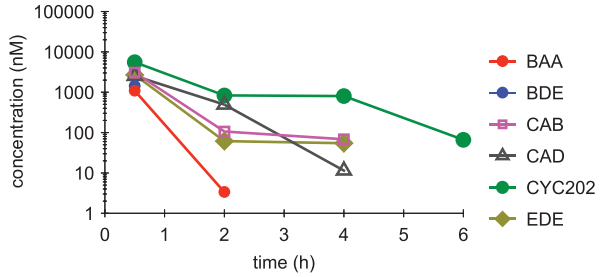

Figure 4. Plasma concentration *versus* time curves following administration of cassette A. 166.6 nmol of each compound were administered per mouse.

Pharmacokinetics of Library Compounds Dosed in Cassettes. Of the compounds dosed, 15 were undetectable after dosing (Table 2, NSI), although they were stable in plasma following spiking. Another 5 compounds were only detectable at 15 min post-dosing: {ACD}, {ACE), {ACF}, {BCF}, and {EBF}. Some compounds (n = 23) showed an AUC ratio ($AUC/AUC_{CYC202 \text{ or purvalanol A}}$) greater than 1: {BBE}, {EDE} {CAD}, {BAD}, {CAB}, {DAB}, {AAB}, {EBB}, {DBB}, {BAF}, {BBF}, {BDF}, {ADF}, {CBC}, {EBC}, {ABC}, {ADC}, {CCA}, {ECF}, {BCC}, {DCA}, {DCD}, and {ACB}. Of these compounds, only {BBE}, {AAB}, and {BAF} exhibited increased half-lives compared with CYC202 and all the others had an increased C_{max}. Overall, substitution in position 2 of the purine template with a saturated ring, such as *trans*-2 aminocyclohexanol or aminomethylcyclohexanol, did not increase the plasma AUC values when compared with CYC202. On the contrary, 4 out of the 20 compounds with an amino-2 propanol substituent had an AUC ratio greater than 1. A methoxyethylamino or a piperidinol substituent led to greater exposure compared to an ethyl-2-hydroxyethylamino group in 3 of 20 cases. At position 6, aromatic rings such as benzylamine and veratrylamine showed the highest AUC ratios (4 of 21 and 4 of 23 compounds with AUC ratios greater than 1), followed by aminoethylmorpholine substitution. Most of the aminoindane and aminofluorene compounds had lower AUC than the pharmacokinetics standard (20 of 22 and 19 of 21, respectively). Finally, hydroxyethyl and methylpropyl substituents at position 9 gave higher AUC ratios than isopropyl or cyclopentyl. An example of plasma concentration *versus* time curves (cassette A) is shown in Fig. 4.

Reproducibility of Cassette Dosing. Table 6 shows the reproducibility of dosing the same cassette (Z) on three different days. The pharmacokinetics behavior showed a high degree of reproducibility with a maximum of 20% variation in any parameter between days. The ranking was highly maintained for all the compounds. Table 7 shows the reproducibility of the pharmacokinetics ratios for compounds dosed in different cassettes. Compound {DCD} was not detected in any samples following dosing in cassette Z, while previously in cassette V, there was one detectable time point. This was also the case for {CDA}. The AUC ratios of compound {CAE} were quite different between cassettes F and Z but they were both below 1. Compound {BDF} dosed in the cassettes F and Z showed exactly the same ratios. In summary, whether compounds showed a higher or lower AUC, C_{max}, and $t_{1/2}$ values than CYC202 in one cassette, they behaved similarly when dosed in a different cassette.

Discussion

Cassette Dosing Pharmacokinetics

The requirement for early pharmacokinetics and metabolism information as part of the modern discovery process is now well documented (35). It is recognized that the likely success of a drug candidate in preclinical and subsequent clinical and commercial phases of development depends very heavily on appropriate ADME/pharmacokinetics properties. In recent years, various *in vitro* and *in vivo* methodologies with improved throughput have accelerated ADME/pharmacokinetics studies, but there still remains a bottleneck, especially as a result of high-throughput screening and combinatorial chemistry. There are several ways to accelerate the pharmacokinetics throughput: decreasing the analytical time (6, 8, 36); reducing the animal numbers, either by dosing several compounds together, or by limiting the number of sampling points, or both (9, 10); using *in silico* prediction methods (37); using Caco-2 cells for absorbability predictions (38); and employing high-throughput metabolism studies employing

Table 4. Pharmacokinetic parameters derived from the full data set and the limited sampling strategy (0.5, 2, 4, 6 h)

Compound	Administration	C_{max} (nM)	AUC_{last} (nM h)	$V_{ss(obs)}$ (l)	Cl_{obs} (h)	$AUC_{inf(obs)}$ (nM h)	$t_{1/2}\lambda_z$ (h)
Bohemine	all samples	7,675	2,819	0.54	1.01	2,897	1.27
	LSS	10,246	2,835	0.23	1.03	2,844	0.92
Olomoucine	all samples	6,232	3,018	0.67	1.08	3,089	1.01
	LSS	7,088	3,091	0.43	1.07	3,119	0.8
CYC202	all samples	11,371	15,788	0.24	0.17	16,285	1.38
	LSS	11,015	20,120	0.25	0.13	20,884	1.31

Note: LSS, limited sampling strategy.

Table 5. Pharmacokinetic parameters of CYC202 and Purvalanol A in the different cassettes

Compound	Cassette	AUC_{last}	Cl_{obs}	C_{max}	$t_{1/2}l_z$	t_{max}	$V_{ss(obs)}$
		(nM h)	(ml/h)	(nM)	(h)	(h)	(ml)
CYC202	A	4,508	37	4,166	0.97	0	43
CYC202	B	1,957	85	1,907	0.89	0	68
CYC202	C	4,719	35	3,583	0.78	0	37
CYC202	F	1,635	102	2,272	0.6	0	50
CYC202	G	4,530	36	6,047	1.53	0	25
CYC202	H	2,777	60	3,706	0.55	0	30
CYC202	I	2,198	76	2,194	0.51	0	51
CYC202	K	4,182	40	6,182	1.33	0	20
CYC202	M	5,070	33	6,664	0.57	0	17
CYC202	N	6,475	25	8,013	0.71	0	16
CYC202	Q	4,157	40	6,808	NE[a]	0	NE
CYC202	R	4,424	38	6,323	NE	0	NE
CYC202	S	3,704	45	5,107	NE	0	NE
CYC202	T	3,670	45	5,382	NE	0	NE
CYC202	W	2,994	54	4,165	NE	0	NE
CYC202	Z	3,222	51	3,035	0.83	0	34
PUA[b]	D	5,789	29	9,944	0.42	0	10
PUA	E	9,077	18	6,646	0.83	0	19
PUA	L	3,353	49	3,307	1.01	0	41
PUA	O	4,568	37	4,909	0.52	0	23
PUA	V	4,723	35	6,354	1.22	0	21

[a]NE, not estimated as too few time points were detectable.
[b]PUA, purvalanol A.

microsomal incubations (39). Decreases in analytical time have been achieved through the use of LC-MS/MS with MRM and short columns. MRM allows simultaneous detection of several analytes in the same sample. This can be used either for the analysis of pooled samples from different experiments or alternatively as part of cassette dosing.

Cassette dosing has been introduced in recent years as an efficient way of increasing the pharmacokinetics throughput (6, 9–13). It involves administration of a combination of compounds, usually 5–10, to the same animal using the same dosing solution. Although there are a number of theoretical and practical challenges, cassette dosing is now used quite widely as part of the contemporary drug discovery process, especially in the pharmaceutical industry. Some examples of cassette dosing have been reported in dogs, rats, and mice (8, 40, 41). There are, however, relatively few detailed studies of its application in the scientific literature and to our knowledge, there is no published study of cassette dosing describing the pharmacokinetics screening of potential anticancer agents. In the present study, we have used the approach of cassette dosing in mice to study the pharmacokinetic properties of a series of trisubstituted purine compounds. These were designed as part of a project to develop inhibitors of CDK2. However, purine compounds have potentially broad therapeutic potential (26). One example of this series, CYC202, has recently entered into phase I and II clinical trials as a potential anticancer drug (20). In addition, a large number of analogues have been synthesized, by ourselves and other groups, providing an important resource for identification of additional drug candidates. The objectives of the study were first to validate the cassette dosing methodology with known analogues and second to apply the validated method to a library of compounds. In all, a total of 107 trisubstituted purine derivatives synthesized by parallel synthesis were investigated and structure-pharmacokinetic relationships were derived from the results.

Initially, single *versus* cassette dosing was compared for olomoucine, bohemine, and CYC202. Up to 28% variation in the pharmacokinetics parameters was recorded between single and cassette dosing. CYC202 showed consistently the highest AUC with the longest $t_{1/2}$ and the highest C_{max}. This was true when the three compounds were dosed individually or in combination. Further analysis of the data set showed that a limited sampling strategy

Table 6. Reproducibility of cassette dosing on three different days

Compound	Day	C_{max} (nM)	AUC_{last} (h nM)	$t_{1/2}$ (h)
CYC202	1	2,944	2,769	1.04
	2	2,708	3,108	0.79
	3	3,035	3,222	0.83
{CAE}	1	617	443	NE[a]
	2	617	497	NE
	3	594	532	NE
{BDF}	1	4,416	4,253	0.83
	2	4,062	4,660	0.71
	3	2,831	4,830	0.63
{ACC}	1	2,649	1,662	0.6
	2	2,437	1,800	0.5
	3	2,731	1,868	0.46

[a]NE, not estimated as too few time points were detectable.

Table 7. Reproducibility between cassettes or as single agents

Compound	Cassette or single	AUC ratio	C_{max} ratio	$t_{1/2}$ ratio
{DCD}	Z	0	0	0
	V	0	0	0
{CAE}	F	0.5	0.5	0.5
	Z	0.3	0.2	NE
{BDF}	F	1.6	2	1
	Z	1.5	1.5	0.9
{ACC}	E	0.6	1.1	0.5
	Z	0.6	0.9	0.6
{CDA}	I	NE	1TP	NE
	Z	NE	1TP	NE
{ADB}	single	0.2	1.5	0.6
	M	0.4	1.8	0.3
{CDE}	single	0.3	0.9	0.3
	B	0.6	0.6	0.6

Note: Values given are expressed as a ratio to those for CYC202 or purvalanol A. NE, not evaluated; 1TP, only one time point detected.

consisting of 0.5, 2, 4 and 6 h sampling gave an accurate measure and ranking of the pharmacokinetics parameters for the compounds. This strategy was therefore used in subsequent studies.

Initially, it was thought that compounds could be administered at an approximate total dose of 50 mg/kg. However, due to limited solubility of some compounds, the cassettes had to be administered at a total dose of around 20 mg/kg (166.6 nmol of each compound per mouse). Five unknown compounds, together with a pharmacokinetics standard, were dosed simultaneously. There is no general consensus as to how many agents should be given together in cassette dosing. For example, up to 64 compounds have been administered together (9). However, the use of 5–10 compounds seems to be more common (42). In our study, increasing the number of compounds beyond 5 would have meant decreasing the dose administered for solubility reasons and would have required a more sensitive analysis with sample purification, thus increasing time and analytical cost.

The use of a pharmacokinetics standard has two goals. The first is to allow the pharmacokinetics properties for the compounds to be expressed as a ratio relative to the pharmacokinetics standard, providing a normalization that allows easy comparison of compounds between different cassettes. The second is to measure variation in the pharmacokinetics standard, for example due to drug-drug interactions in a cassette. There are no real guidelines as to how much variation is indicative of drug-drug interactions (4, 42). Also, in many studies, there is no assessment of the variability of the pharmacokinetics standard. We observed up to 4-fold variability in the AUC of our pharmacokinetics standard CYC202. Other investigators (41) also reported significant variability (5-fold) in the AUC of the pharmacokinetics standard. In addition, we observed a shorter $t_{1/2}$ in the standard when compared with the compound dosed alone, which has also been reported in other studies (10). This is possibly the result of administration in the cassette at a lower dose and limitations imposed by the sensitivity of the analytical method.

The observed variability of the pharmacokinetics standard could have several causes. Firstly, it could be explained by the variability at any point of the whole process from animal dosing to analytical measurement. When cassette (Z) was dosed on three consecutive days, there was less than 20% variation between days for any compound. This suggests that the 4-fold variation in AUC observed with CYC202 in different cassettes could be due to drug-drug interactions. The fact that a compound could be eliminated from selection for further studies when it actually has better pharmacokinetics than CYC202 or purvalanol A (false negative) would be a cause of major concern. In reality, all the compounds that were dosed in cassette Z and had previously been dosed in other cassettes, behaved similarly (*i.e.*, had similar AUC ratios and C_{max} ratios), suggesting that the test compounds were affected similarly to the pharmacokinetics standard. Of course, the possibility that compounds other than the pharmacokinetics standard could interact together cannot be totally eliminated. However, two other compounds that were dosed both alone and in different cassettes ({ADB} and {CDE}) ranked similarly to CYC202. This added to our confidence in using cassette dosing for ranking purposes.

In total, 10 compounds ranked similarly to the pharmacokinetics standard as single agents or in a different cassette and none of the compounds tested ranked differently. Although cassette dosing has been subject to a recent critical theoretical analysis (42), our studies have satisfied us that the approach can be used to give sufficiently reproducible pharmacokinetics behavior to provide an overall ranking of compounds for screening purposes. It is of course not the intention of cassette dosing to generate highly accurate pharmacokinetics analysis of individual compounds. The goal is efficient prioritization of compounds for further analysis (*e.g.*, testing in a disease model). In addition, rapid feedback can be provided to the medicinal chemists as to which chemical features confer beneficial pharmacokinetics properties.

Structure-Pharmacokinetic Relationships

On the basis of an assessment of the pharmacokinetics properties of the 2,6,9-trisubstituted purine analogues by cassette dosing (Table 1), certain conclusions can be drawn regarding the structure-pharmacokinetic relationships. Of the R^1 substituents (Fig. 2): all five R^1 substituents **A-D** were found in compounds with higher drug exposures as measured by AUC. At position R^2, there were no particular preferences amongst the four R^2 substituents while R^3 showed most of the variability in pharmacokinetics.

- R^1: **B** > **A** > **E** > **D** > **C**
- R^2: **C** > **A** = **B** > **D**
- R^3: **B** = **F** > **C** > **A** = **D** = **E**

Prevention of carbinol oxidation, the major metabolic pathway of CYC202 (22), did not result in the expected increased exposure.

With respect to carbinol oxidation, comparative studies with seven analogues have shown a correlation between plasma clearance and percentage compound metabolized in microsomal incubations (data not shown).

Conclusions

We have validated the use of cassette dosing for trisubstituted purine derivatives and have used it successfully to compare the pharmacokinetics properties of a library of 2,6,9-trisubstituted purine derivatives prepared by parallel synthesis. The combination of parallel synthesis and cassette dosing proved to be a valuable way of gaining rapid information on the comparative pharmacokinetics properties of this series. This resulted in a significant 4-fold gain of time and has reduced animal usage and costs by 3-fold. To our knowledge, this is the first report using cassette dosing in drug development for oncology. Pharmacokinetics information of this type, when used alongside other pharmacological properties such as enzyme inhibitory

activity and antiproliferative effect, should provide the basis for the development of novel trisubstituted purine derivatives as inhibitors of CDK2 or for other mechanisms of action. These studies are in progress. On the basis of our experience, we recommend the use of cassette dosing to evaluate the pharmacokinetic properties of novel classes of anticancer agents (43).

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