Review

The application of cassette dosing for pharmacokinetic screening in small-molecule cancer drug discovery

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Abstract

Pharmacokinetic evaluation is an essential component of drug discovery and should be conducted early in the process so that those compounds with the best chance of success are prioritized and progressed. However, pharmacokinetic analysis has become a serious bottleneck during the 'hit-to-lead' and lead optimization phases due to the availability of new targets and the large numbers of compounds resulting from advances in synthesis and screening technologies. Cassette dosing, which involves the simultaneous administration of several compounds to a single animal followed by rapid sample analysis by liquid chromatography/tandem mass spectrometry, was developed to increase the throughput of *in vivo* pharmacokinetic screening. Although cassette dosing is advantageous in terms of resources and throughput, there are possible complications associated with this approach, such as the potential for compound interactions. Following an overview of the cassette dosing literature, this article focuses on the application of the technique in anticancer drug discovery. Specific examples are discussed, including the evaluation of cassette dosing to assess pharmacokinetic properties in the development of cyclin-dependent kinase and heat shock protein 90 inhibitors. Subject to critical analysis and validation in each case, the use of cassette dosing is recommended in appropriate chemical series to enhance the efficiency of drug discovery and reduce animal usage. [Mol Cancer Ther 2007;6(2):428–40]

Introduction

Anticancer drug discovery and development has changed considerably over the last decade due to the identification of molecular abnormalities that drive cancer progression and advances in modern technologies (1–3). Despite the emergence of many new therapeutic targets, the discovery and development of novel anticancer drugs remains a long and expensive process. Typically, it takes 10 to 15 years and costs in excess of U.S.$800 to $900 million to bring a drug to market (4–6). In addition, only 1 in 9 or 10 agents entering clinical trials is approved (5, 6). Furthermore, the success rate for oncology drugs is only about half that of the average across all disease areas, at ~5% (5). Genomic and proteomic technologies, combinatorial chemistry, structure-based drug design, and high-throughput screening against large diverse or focused compound libraries are among the tools that are being applied to improve efficiency and accelerate the process of anticancer drug discovery (7–9).

A common challenge encountered in small-molecule drug discovery is the conversion of a potent and selective compound, with activity by the desired mechanism against tumor cells *in vitro*, into a compound that has 'drug-like' properties and reaches active levels in the circulation and ultimately the tumor. Lack of *in vivo* activity in animal models is usually attributed to suboptimal drug metabolism and pharmacokinetic (DMPK) properties (10). Furthermore, failure in the clinic has frequently been due to the same limitations (5, 11). A greater focus on DMPK and bioavailability properties in preclinical development is most likely responsible for the decrease in attrition in the clinic due to these issues (see next section). Therefore, it is essential that, in addition to potency and selectivity, the DMPK properties of lead compounds are optimized so that the best candidate can be selected for clinical development.

In the past, the initial assessment and optimization of compounds has focused on potency and selectivity. Increasingly, because pharmacokinetic properties are often limiting, assessment of DMPK properties features very prominently and at a much earlier stage. This allows selection of the compounds with the greater likelihood of success to be prioritized and progressed, whereas compounds with inherent pharmacokinetic and metabolic

Received 9/12/06; revised 12/1/06; accepted 12/21/06.

Grant support: Cancer Research UK [CUK] Programme grant C309/A2187 [the authors' work in the Centre for Cancer Therapeutics (http://www.icr.ac.uk/).

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N.F. Smith was a recipient of a Cancer Research UK Ph.D. Studentship and P. Workman is a Cancer Research UK Life Fellow.

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doi:10.1158/1535-7163.MCT-06-0324

liabilities are deprioritized or abandoned. However, the availability of new targets, together with advances in biological screening and chemical synthesis, have dramatically increased the number of compounds requiring DMPK evaluation, thus creating a major bottleneck in preclinical discovery (12). In response to these demands, a variety of methods have been developed to increase the throughput of DMPK evaluation. This article will briefly review these methods and then focus in particular on cassette dosing, which is used to increase the throughput of *in vivo* pharmacokinetic screening. Following a review of the cassette dosing literature, specific examples and potential applications of this technique in anticancer drug discovery are discussed. Finally, based on the experience of ourselves and others, some recommendations are provided.

DMPK in Drug Discovery

The starting point for a modern small-molecule drug discovery project commonly involves high-throughput or focused screening of a library of compounds against the target of interest (7, 8). The hits identified are assessed in a process known as 'hit exploration' followed by more detailed evaluation of the potential of a smaller number of chemical series in the 'hit-to-lead' phase. Leads may be assessed during a 'lead profiling' phase and selected chemical series are then improved during 'lead optimization' so that a compound for preclinical development can be selected (Fig. 1). As mentioned in Introduction, early assessment and optimization was initially focused heavily on the potency and selectivity of compounds against the target and on activity in cellular assays. More recently, as referred to above, it has become clear that the behavior of a compound in a whole organism needs to be taken into consideration at an early stage as part of the contemporary approach to mutivariable optimization (13).

Pharmacokinetics is the study of the time course of a drug within the body and incorporates the processes of absorption, distribution, metabolism, and excretion (ADME). Good, or at least reasonable, DMPK properties are normally required for a compound to show proof of principle activity in an animal model. Furthermore, suitable DMPK features are essential to the selection of an effective clinical development candidate and its successful progression through clinical evaluation. Indeed, analysis of the major reasons for withdrawal of drugs from development in the 1980s revealed that 39% of failures could be attributed to inappropriate pharmacokinetics in man (14, 15). Between 1991 and 2000, the rate of failure of drugs in clinical trials that could be attributed to poor DMPK/bioavailability fell from ~40% to just <10% (5). This reduction can be linked to incorporation of DMPK assessments into the preclinical drug discovery phase. Consequently, the importance of evaluating DMPK properties very early in the drug discovery process to reduce attrition during development is now well recognized, and there has been a considerable effort to develop suitable *in silico, in vitro*, and *in vivo* methods and models with which to do this (16).

For several years, *in silico* methods based on chemical structure have been used successfully for the prediction of simple physicochemical properties, such as lipophilicity, solubility, and hydrogen-bonding capacity. The 'rule of five', developed by Lipinski et al. (17), has had a major

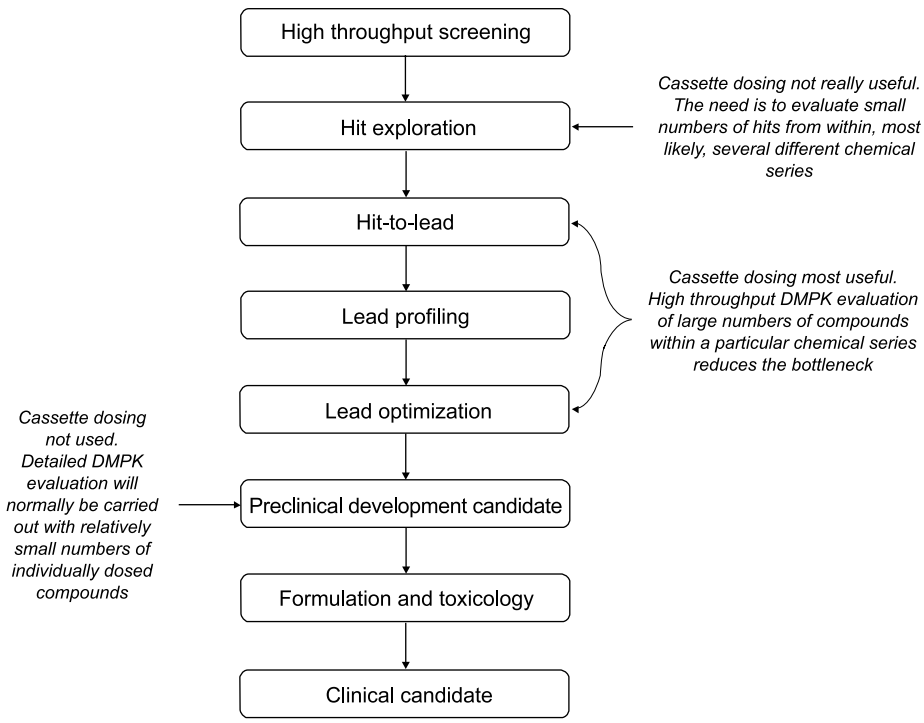

Figure 1. Schematic of the modern drug discovery process. Different phases of a typical modern drug discovery project. Cassette dosing and other high-throughput DMPK methodologies are most useful in hit-to-lead and especially lead optimization, during which large numbers of compounds from the same chemical series need to be evaluated and prioritized.

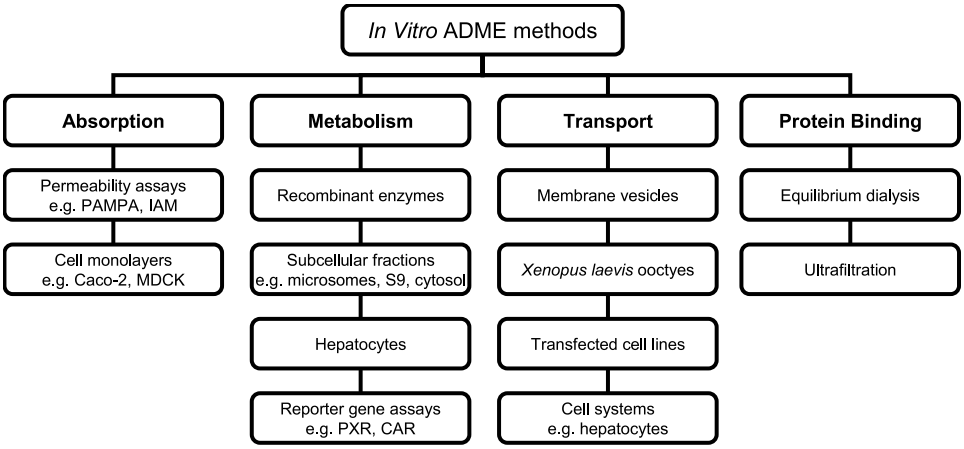

Figure 2. Summary of *in vitro* ADME methods.

effect by delineating the physicochemical properties exhibited by orally bioavailable drugs. However, the accurate prediction of ADME behavior has proved much more challenging (18). Encouragingly, there have been considerable advances in the development of models to predict pharmacokinetic properties, such as oral bioavailability, volume of distribution, elimination half-life, rate of absorption, and protein binding (19). The accuracy and value of *in silico* models is continually improving due to the availability of higher quality and more diverse data sets, better molecular descriptors, and increased computational power (19). With continued progress, *in silico* methods may eventually be used routinely and with greater confidence to influence both the design of compounds before synthesis and also the prioritization of compounds for screening and testing. Until then, *in vitro* and/or *in vivo* studies must be done in the laboratory to determine ADME properties.

Some of the *in vitro* methods that are commonly used to evaluate ADME properties during hit-to-lead and lead optimization, often in medium- or high-throughput, are summarized in Fig. 2. Caco-2 cell monolayers and the parallel artificial membrane permeability assay are among the methods used to predict intestinal permeability (for recent reviews of these technologies, see refs. 20 and 21, respectively). *In vitro* metabolic screening includes the assessment of compound stability in the presence of drug-metabolizing enzymes, the structural identification of metabolites, and determination of the enzyme(s) responsible for metabolite formation (22). In addition, compounds are routinely screened for their ability to inhibit or induce drug-metabolizing enzyme activity, which may result in clinically important drug-drug interactions. These properties are commonly assessed using recombinant enzymes and liver microsomes or other subcellular fractions. However, due to increased awareness of the important role played by hepatic uptake transporters (23), it has been suggested that hepatocytes should be the model system of choice for metabolism studies (24). In addition to the liver, uptake and efflux membrane transporters belonging to the solute carrier or ATP binding cassette transporter superfamilies are expressed in other organs of importance for drug disposition, such as the gastrointestinal tract and the kidneys. Methods developed to assess transport by hepatic and renal transporters have been reviewed recently (25).

Using the approaches outlined above, it is possible to study the processes of drug absorption, metabolism, and transport specifically. However, none of the currently available *in silico* or *in vitro* methods can predict the combined effect of these physiologic processes acting together on a given compound. For this reason, *in vivo* pharmacokinetic studies in animal models are still an essential component of lead candidate evaluation, lead optimization, and candidate selection.

In vivo Pharmacokinetic Screening

Conventional preclinical pharmacokinetic studies involve the administration of a single compound to several animals, which is followed by the analysis of plasma or tissue samples collected at specific time points across a time course. Inevitably, such studies are resource intensive, requiring a large amount of compound and many animals. It also takes considerable time to develop a specific and sensitive analytic method for each compound and for subsequent sample analysis. In recent years, several approaches have been developed to accelerate the process of *in vivo* pharmacokinetic screening based on advances in instrumentation and sample reduction (26).

The coupling of liquid chromatography to atmospheric pressure ionization mass spectrometry (LC/MS) and tandem mass spectrometry (LC/MS/MS) has had a huge effect on DMPK analysis throughout the entire drug discovery process (27). The sensitivity and selectivity of LC/MS, in addition to the opportunity it provides for rapid method development, have made it a particularly powerful technique to support high-throughput DMPK approaches (28, 29). LC/MS is now the method of choice to provide both qualitative and quantitative information in place of the more traditional high-pressure LC methods with UV or fluorescence detection (30). Multiple reaction monitoring done on a triple quadrupole instrument allows the detection of several compounds simultaneously and is the

enabling technology for the multiple compound pharmacokinetic approaches that will be discussed below.

Complementary to advances in LC/MS technology, several approaches have been used to reduce the number of samples arising from *in vivo* pharmacokinetic experiments in an attempt to increase throughput. The first of these techniques, known as 'sample pooling', involves combining equal aliquots of samples collected at a particular time point from several animals each dosed individually with a different compound (31–33). Pharmacokinetic parameters can be derived for each compound from the resulting plasma concentration-time profiles. A disadvantage of this procedure is that the plasma concentration of each compound is diluted when the samples are combined before simultaneous compound analysis by LC/MS/MS.

An alternative strategy is to pool the samples collected at each consecutive time point from a single animal dosed with an individual compound to produce a single sample for analysis (34, 35). The concentration of this pooled sample is then multiplied by the time period of collection to give an estimation of the area under the concentration versus time curve (AUC). Cox et al. (34) applied this procedure to rapidly screen compounds following their p.o. administration to rats (n = 2 per compound). Plasma samples collected at regular intervals up to 6 h were pooled and assayed to yield a truncated AUC. Measuring the plasma concentration at 6 h gave additional insight into the elimination half-life of the compounds. A limitation of this pooling approach is that, other than obtaining an estimated AUC value, pharmacokinetic parameters cannot be determined due to the lack of a plasma concentration-time profile.

The same group developed the 'cassette-accelerated rapid rat screen' (36). This approach involves the administration of each compound individually to two rats. Duplicate samples collected at each time point are then pooled and run alongside a limited standard curve. Using this approach, batches of six test compounds can be prepared by semiautomated sample preparation and assayed using a single 96-well plate.

Although the procedures described above increase the throughput of pharmacokinetic analysis after completion of the *in vivo* live phase due to sample reduction, none of them decreases the number of experimental animals used.

Cassette Dosing

Cassette dosing, which is also referred to as 'cocktail' or 'N-in-one' dosing, was first developed in the 1990s by scientists at Glaxo Wellcome (now GlaxoSmithKline; ref. 37). This approach involves the simultaneous administration of a mixture of several compounds (typically belonging to the same structural class) at relatively low doses to a single animal (38, 39). It should be emphasized that cassette dosing is not intended to accurately define pharmacokinetic parameters for each compound in a given mixture; rather, it is regarded as a screening tool to rapidly rank order compounds, eliminate those that exhibit poor pharmacokinetic properties, and identify those that should be prioritized for further evaluation (including subsequent discrete compound dosing, pharmacokinetic analysis, pharmacokinetic-pharmacodynamic studies, or efficacy determination). A very important aspect of cassette dosing is that rapid feedback is provided to medicinal chemists, thus guiding future synthetic efforts to optimize ADME properties alongside other key features, such as potency and selectivity (13).

Compared with conventional single compound administration and particularly compared with direct evaluation of all *in vitro* active compounds in animal tumor models, cassette dosing uses much fewer experimental animals. This has advantages from an ethical point of view because animal welfare guidelines call for a reduction in animal usage where possible (40, 41). The amount of compound required is also reduced, which is important at the early stages of a discovery project when compound supply is limited and the medicinal chemists' time is better spent designing and synthesizing new compounds rather than remaking larger amounts of known and potentially unpromising analogues. In addition, fewer samples are generated and the time taken for animal handling, sample preparation, and sample analysis is minimized, hence increasing efficiency and throughput.

However, in addition to the obvious advantages, there are potential complications associated with cassette dosing, which have resulted in some controversy surrounding this technique. A major concern is the risk of pharmacokinetic compound-compound interactions following the coadministration of multiple chemical entities (39, 42). These interactions may occur as a result of competitive inhibition of drug-metabolizing enzymes, transporter proteins, or plasma protein binding and may lead to false-negative as well as false-positive results (42). These issues were addressed in a discussion of the pharmacokinetic theory of cassette dosing by White and Manitpisitkul (42). Following several theoretical predictions, the authors made recommendations to minimize the risk of compound-compound interactions, which included keeping the number of coadministered compounds below five and administering the lowest doses detectable. The inclusion within each cassette of a pharmacokinetic standard (a structurally related compound that displays comparable pharmacokinetics following cassette and discrete dosing) to assess whether compound-compound interactions have occurred was also encouraged. However, this assumes that the pharmacokinetic standard is cleared by the same mechanism(s) as the other compounds in the cassette.

In addition to biological considerations, cassette dosing can be analytically challenging (38). A highly selective method is required to detect multiple compounds in a single sample simultaneously. Sensitivity is important because of the need to administer relatively low doses to minimize the risk of compound-compound interaction and avoid toxicity. Sources of analytical interference should also be considered, including collision cell cross-talk between structurally related compounds with similar

fragmentation patterns, in-source fragmentation of metabolites to produce the parent compounds, and ion suppression as a result of competition between the analyte and co-eluting analytes, residual matrix components, or mobile phase constituents. If analytic interference occurs, then chromatographic conditions should be optimized to ensure the resolution of interfering compounds. Clearly, the time taken for bioanalytical method development can be considerable, and, in some instances, it may defeat the purpose of cassette dosing. In our experience, the time required for assay development can vary from a couple of days to a few weeks. It should be emphasized that it is not necessary to fully validate the bioanalytical method used for cassette analysis to the most demanding level. In our experience, the inclusion of three sets of quality control standards (low, medium, and high) alongside the calibration curve is sufficient. These must be within 20% of nominal concentrations in order for a run to pass. Although a method capable of measuring all of the cassette compounds simultaneously is preferred, it may be easier to analyze the compounds individually with different methods, if the volume of plasma obtained allows. However, this should not be necessary if there is a sufficient degree of structural similarity between the compounds grouped together in a cassette.

Lastly, the formulation of several compounds in the same dosing solution can be challenging. Cassette dosing solutions should be checked for compound precipitation, particularly if the compounds are poorly soluble.

Despite these potential issues, cassette dosing was widely adopted by the pharmaceutical industry following the first publication on this technique in 1997 (37). The results of two surveys that were conducted to assess the popularity of cassette dosing were published several years later in 2004 (43, 44). The first survey, carried out by Ackerman (43), revealed that 75% of respondents (including representatives from small and large pharmaceutical companies, biotechnology companies, and contract research organizations) used cassette dosing, ranging from 'upon request' to their 'default method' for pharmaceutical screening. The second survey conducted by Manitpisitkul and White (44) showed that of the several participating pharmaceutical companies, 88% had used cassette dosing at some point and 44% of these continued to do so, with the frequency ranging from rarely to six times weekly.

Table 1 summarizes the cassette dosing studies that have been published in the literature to date. Several studies have focused mainly on the methodologic aspects of cassette analysis. Others have assessed whether the approach is suitable for a particular compound series by

Table 1. Summary of published cassette dosing studies

Study	Species	Compound class	Cassette size	Dose (mg/kg)*	Route
Berman et al., 1997 (37)	Dog	α_{1a} Receptor antagonists	5	Not disclosed	i.v.
McLoughlin et al., 1997 (67)	Dog	Not disclosed	10	0.5–1	i.v. and p.o.
Olah et al., 1997 (68)	Rat and dog	Not disclosed	10–12	1	p.o.
Allen et al., 1998 (69)	Rat	Not disclosed	5	1	p.o.
Shaffer et al., 1999 (70)	Dog	α_{1a} Receptor antagonists	5–22	0.25–0.3	i.v.
Gao et al., 1998 (71)	Rat and dog	Not disclosed	2	0.5	i.v. and p.o.
Frick et al., 1998 (39)	Dog	α_{1a} Receptor antagonists	12–22, 90	Not disclosed	i.v.
Bayliss and Frick, 1999 (38)	Mouse	Not disclosed	3, 9, 27, 89	1–3 (total)	i.v.
Rano et al., 2000 (72)	Dog	Indinavir derivatives	20	0.5	p.o.
Tamvakopoulos et al., 2000 (45)	Rat	Not disclosed	4	1	i.v.
Wu et al., 2000 (73)	Dog	Not disclosed	14	0.5	i.v.
Rajanikanth and Gupta, 2001 (74)	Rat	Aryloxy-substituted aryl-piperazinyls	3	3	i.v.
Zeng et al., 2002 (75)	Dog	Not disclosed	10	0.5	i.v.
Hasegawa et al., 2002 (76)	Rat	*N*-myristoltransferase inhibitors	5	2	i.v.
Macdonald et al., 2002 (77)	Dog	Pyrrolidine *trans*-lactams	8	2	i.v.
Andrews et al., 2003 (78)	Dog	Pyrrolidine *trans*-lactams	6	0.2	i.v.
Ohkawa et al., 2003 (79)	Mouse and rat	Not disclosed	2–7	0.5 and 1	i.v.
Mallis et al., 2003 (80)	Rat	Phytoestrogens	5	3	s.c. and p.o.
Zhang et al., 2004 (46)	Rat	Not disclosed	3–4	3	i.p.
Tong et al., 2004 (81)	Rat	VLA-4 antagonists	4	1–2	i.v. and p.o.
Raynaud et al., 2004 (47)	Mouse	2,6,9-Trisubstitued purines	6	20 (total)	i.v.
Smith et al., 2004 (49)	Mouse	Geldanamycin derivatives	5	5	i.v.
Sadagopan et al., 2005 (82)	Rat	Not disclosed	4	1 and 5	i.v. and p.o.
Jia et al., 2005 (83)	Mouse	Ethambutol derivatives	3	3–25	i.v., i.p., and p.o.
Janser et el., 2006 (84)	Rat	TACE/MMP inhibitors	5	1 and 3	i.v. and p.o.
Smith et al., 2006 (48)	Mouse	Diarylpyrazole resorcinols	5	4	i.v. and p.o.

Abbreviations: TACE, tumor necrosis factor–α-converting enzyme; MMP, matrix metalloproteinase.
*Per compound unless otherwise indicated.

comparing the plasma pharmacokinetics of several compounds following cassette and single compound administration. However, in some of the published studies, there was no attempt to validate the approach in this manner and only cassette dosing pharmacokinetic data are presented. Some groups have extended the cassette dosing approach to assess brain uptake in addition to plasma exposure (39, 45, 46). Very few publications have described the experience of the implementation of cassette dosing in the context of a particular drug discovery project or explored the problems associated with this approach. Indeed, it is very unlikely that results will have been published in instances where cassette dosing proved to be unsuccessful. Furthermore, the structural classes of the compounds investigated are not disclosed in many of the published studies, most likely because the information will be viewed as proprietary to the companies concerned. Nonetheless, it is evident from Table 1 that cassette dosing has been applied to a range of compound structures and animal species. Most of the published studies were done in the dog or the rat, although the mouse has also been used. Some studies have investigated cassettes containing large numbers of compounds but typically cassettes of 10 or less compounds have been dosed. The i.v., i.p., and p.o. routes of administration have been used. However, Table 1 shows that of these routes of cassette administration, the p.o. route is the least used. This may be due, at least in part, to the fact that other high-throughput methods are often used to determine potential liabilities for p.o. absorption or metabolism of compounds. Indeed, *in vitro* assays to assess absorption and metabolism (Fig. 2) are often done in addition to cassette dosing by the i.v. or i.p. routes. However, there is clearly the potential for drug-drug interactions at the levels of intestinal absorption and first-pass metabolism following p.o. cassette dosing. Nevertheless, it would be worth investing in more studies to investigate the potential of cassette dosing by the p.o. route, especially given that chronic p.o. administration is increasingly favored with the new generation of targeted molecular cancer therapeutics.

Although cassette dosing can obviously be applied to a variety of therapeutic areas, the remainder of this review will focus on cancer-specific applications of this technique.

Cassette Dosing in Anticancer Drug Discovery

Therapeutically meaningful inhibition of many of the novel molecular cancer targets that are under current investigation generally requires prolonged compound exposure, thus emphasizing the importance of evaluating the pharmacokinetic properties of promising compounds during early development. Furthermore, p.o. bioavailability is increasingly seen as desirable in the development of new molecular cancer therapeutics. Within our own Center, we have assessed the usefulness of cassette dosing in three studies related to drug discovery projects (47–49). The outcome was successful with two distinct chemical series acting on two different molecular targets. By successful, we mean that the technology allowed rapid analysis of the pharmacokinetics of large numbers of compounds and provided information and prioritization that was useful in the drug discovery project. Problems were identified with the third series, which prevented the implementation of cassette dosing. All three studies are discussed in the following paragraphs. As far as we are aware, these are the only published applications of cassette dosing in oncology drug development to date. In each case, we chose to conduct our studies in mice because this is the species that is normally used for most antitumor testing.

Our first cassette dosing experience was with a series of 2,6,9-trisubstituted aminopurine inhibitors of cyclin-dependent kinases. These are structurally related to seliciclib (*R*-roscovitine, CYC202; Fig. 3), which is in clinical development. This paper exemplified how the technique can be implemented successfully and usefully in a drug discovery program (47). The objective was to use cassette dosing to evaluate the pharmacokinetic properties of a library of 107 trisubstituted aminopurine compounds derived from the parent compound olomoucine, which undergoes extensive oxidative metabolism and rapid clearance (50, 51). To determine whether cassette dosing was suitable for this particular series of compounds, an initial validation exercise was carried out to compare the pharmacokinetics of olomoucine, bohemine, and seliciclib, following i.v. administration alone at 50 mg/kg and in combination at 16.6 mg/kg each. Reassuringly, the rank order of the compounds in terms of their pharmacokinetic parameters was maintained between discrete and cassette dosing. Furthermore, the actual pharmacokinetic parameter values (including clearance, AUC, maximum concentration, elimination half-life, and volume of distribution) were similar following administration by the two dosing methods. The plasma concentration-time curves following cassette and single dosing of the compounds are shown in Fig. 4A. Following the successful validation phase, members of the series were administered i.v. in cassettes of five compounds (166.6 nmol each) along with the pharmacokinetic standard (seliciclib or purvalanol A). Two mice per time point were used. Up to 4-fold variation was observed in the pharmacokinetic parameters of seliciclib, which was included in 17 cassettes as the pharmacokinetic standard. To allow the comparison of compounds across cassettes, the ratios of the pharmacokinetic parameters of each compound to that of the internal standard were calculated and structure-pharmacokinetic relationships were established. Reproducibility of cassette dosing was assessed by administering the same compounds in two different cassettes and also in the same cassette of compounds on three different days. The ratios of the pharmacokinetic parameters to that of the internal standard remained similar when compounds were administered in different cassettes or individually, and <20% variation in pharmacokinetic parameters was observed between days. Overall, the use of cassette dosing in this project was extremely valuable and allowed the rapid identification of the structural features within this particular aminopurine compound series that confer favorable pharmacokinetic properties. The structures of analogues 'EBC' and 'CDF', which exhibited a 2.4-fold

higher and a 10-fold lower AUC than seliciclib, respectively, are shown in Fig. 3.

Following the successful application of cassette dosing in the evaluation of trisubstituted aminopurine cyclin-dependent kinase inhibitors, we next investigated its potential to assess the pharmacokinetics of compounds belonging to two distinct chemical series of heat shock protein 90 (Hsp90) molecular chaperone inhibitors (48, 49). Based on evidence of promising biological and clinical activity (52, 53), phase II studies of the geldanamycin analogue 17-allylamino-17-demethoxygeldanamycin, which was the first known Hsp90 inhibitor to enter clinical trials, are currently under way. However, 17-allylamino-17-demethoxygeldanamycin has several limitations, such as poor solubility, low p.o. bioavailability in mice (54), and metabolism by polymorphic enzymes (55, 56). Approaches to discover Hsp90 inhibitors with improved pharmacologic properties include the synthesis of structural analogues of existing inhibitors and the search for novel chemotypes (57, 58).

The first series of Hsp90 inhibitors for which the cassette dosing approach was evaluated consisted of additional analogues of the benzoquinone ansamycin, geldanamycin (49). This example showed that cassette dosing is not appropriate for all chemical series and identified some key issues that should be considered when assessing the suitability of this technique for other classes of compound. Five compounds (Fig. 5), including 17-allylamino-17-demethoxygeldanamycin, were administered i.v. in combination and individually at the same dose (5 mg/kg) and a higher dose (12.5 mg/kg for NSC 682300; 50 mg/kg for all others) followed by the analysis of plasma, liver, and kidney concentrations. Three mice per time point were used. There were considerable differences in both the pharmacokinetic parameter values and the rank order of the compounds between cassette and discrete administration. The AUC and the half-life of some of the compounds were greater following cassette administration versus individual administration at the same dose. When all measurable concentrations at the higher dose were included in the calculation of pharmacokinetic parameters, the half-life of one of the compounds was 9-fold longer following individual compared with cassette administration and there was a disproportionate increase in AUC. If cassette dosing of these compounds had been conducted without prior validation of the approach, then this compound would have been eliminated from further evaluation based on having a lower AUC than 17-allylamino-17-demethoxygeldanamycin, when the AUC is in fact greater (Fig. 4B). The pharmacokinetic differences observed between cassette and single dosing were partly due to limited assay sensitivity and, therefore, the inability to detect the compounds beyond the early time points at the lower dose. More importantly, however, two of the three compounds that were detectable at the lowest dose exhibited nonlinear pharmacokinetics, which is an undesirable feature for cassette dosing. If the pharmacokinetics of the compounds in a cassette are not linear to the same extent, this could lead to differences in ranking at the relatively low administered amounts used in cassette dosing studies compared with higher, more pharmacologically relevant doses.

In vitro metabolism studies using liver microsomes provided further insight into the differences observed in cassette versus single dosing of the geldanamycin

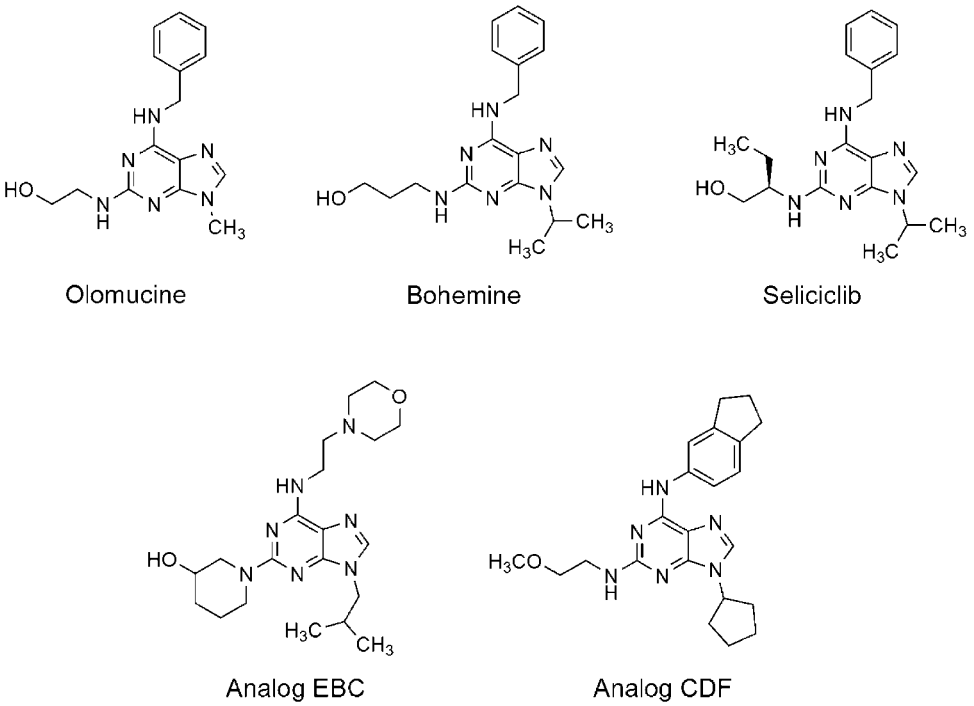

Figure 3. Structures of 2,6,9-trisubstituted aminopurines administered by cassette dosing in ref. 47.

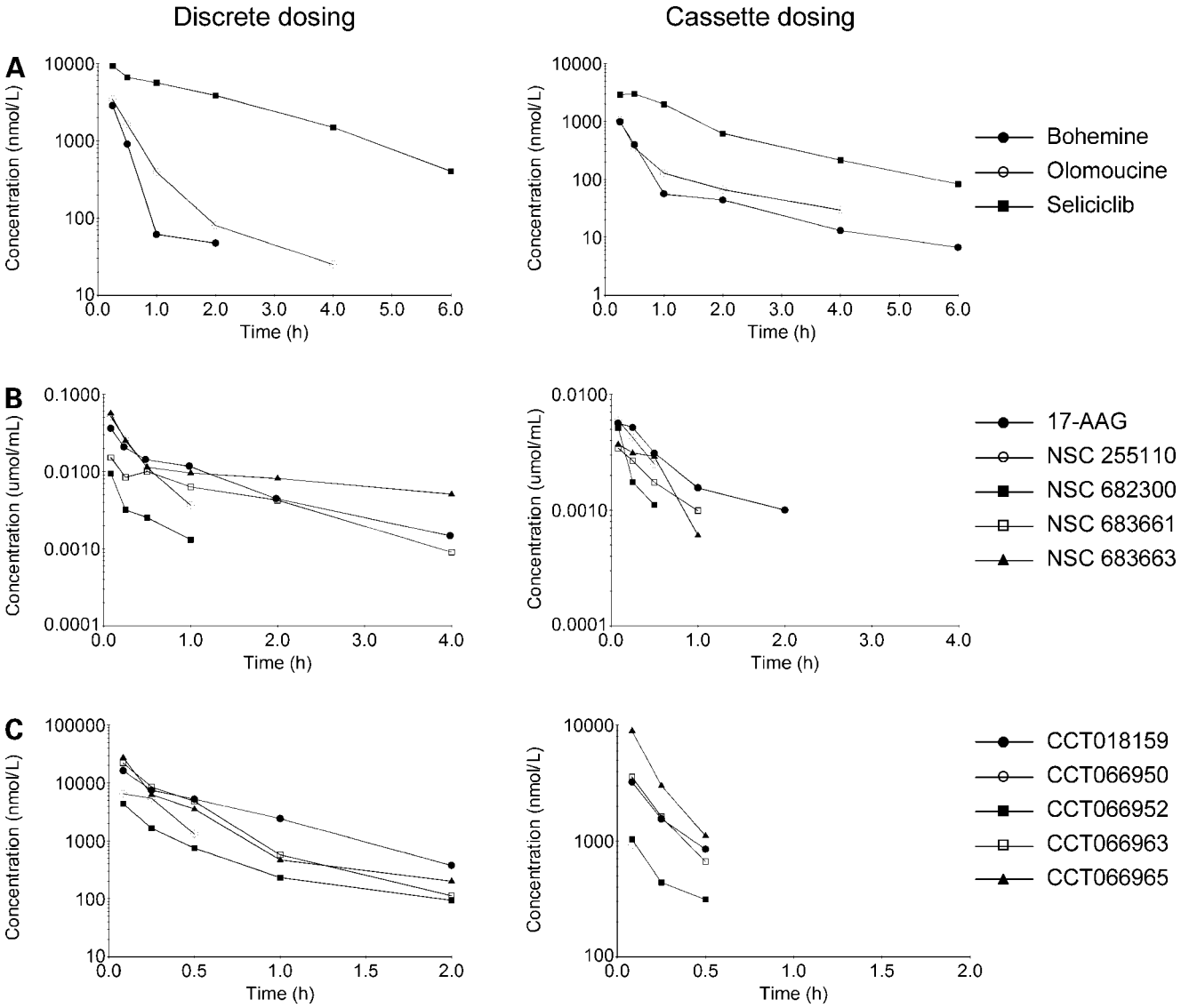

Figure 4. Plasma concentration-time curves in mice following discrete and cassette dosing of 2,6,9-trisubstituted aminopurines (**A**; ref. 47), benzoquinone ansamycins (**B**; ref. 49), and diarylpyrazole resorcinols (**C**; ref. 48).

analogues. The extent of metabolism of four of the five compounds was lower following incubation in combination compared with incubation alone, thus showing that one or more of the compounds inhibited the metabolism of the others. This could potentially result in compound-compound interactions following cassette administration. In addition, it was discovered that two of the compounds can be converted metabolically to another compound present in the cassette, highlighting the need to take particular care when grouping compounds together in cassettes. Because hepatic metabolism seems to be the major mechanism of elimination of these compounds, the assessment of *in vitro* metabolic stability may be a more appropriate predictor of pharmacokinetics than cassette dosing in this particular case.

The third example of cassette dosing in cancer drug discovery involves a series of compounds derived from the diarylpyrazole resorcinol CCT018159 (Fig. 6), a novel and quite potent inhibitor of Hsp90 ATPase activity that was identified by high-throughput screening (59, 60). Following some initial studies to characterize the pharmacokinetics and metabolism of these compounds individually, the suitability of cassette dosing for the pharmacokinetic assessment of a larger number of compounds was evaluated (48). Despite complications of rapid plasma clearance, extensive metabolism by glucuronidation, and RBC binding, cassette dosing proved to be a suitable approach to evaluate the pharmacokinetics of this compound series. Following i.v. administration, the rank order of the five compounds included in the validation from the

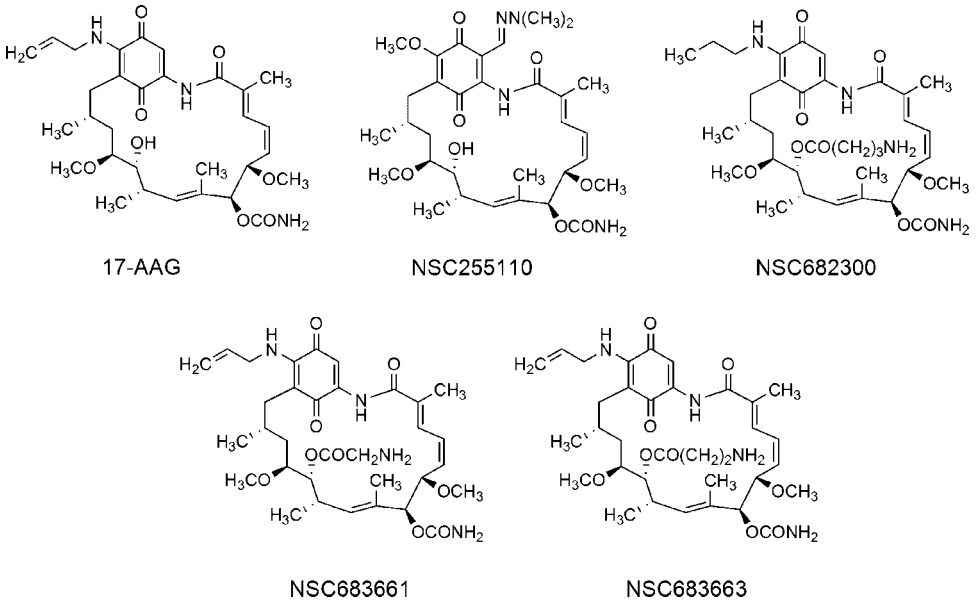

Figure 5. Structures of benzoquinone ansamycins administered by cassette dosing in ref. 49.

highest to lowest AUC was the same following discrete and cassette dosing (Fig. 4C). The compounds displayed linear increases in AUC as the dose was increased 5-fold from 4 mg/kg for cassette dosing to 20 mg/kg for discrete dosing. Furthermore, all of the calculated pharmacokinetic parameters were similar whether the compounds were dosed alone or in combination. Statistical analysis, using the method of Bailer (61, 62), revealed that there were no significant differences between the dose-normalized AUCs of the compounds following cassette and single compound administration. In this case, the results obtained from *in vitro* metabolic stability studies did not reflect the *in vivo* pharmacokinetics of these compounds. In fact, the compound that was metabolized to the greatest extent *in vitro* exhibited the slowest clearance *in vivo*. Cassette dosing was subsequently applied in this project to identify compounds with optimal pharmacokinetic properties and, along with other assays, helped in lead optimization and progression toward compounds with appropriate properties for a preclinical development candidate (63).

Conclusions

Cassette dosing is widely used as a drug discovery tool (43, 44). However, the published literature is limited due the predominant use of the technique in corporate programs. The main purpose of this review is to highlight the potential of cassette dosing and to encourage further evaluation and use of the technology in cancer drug development but at the same time to point out its limitations and pitfalls.

In our own Center, cassette dosing has been implemented successfully in two oncology drug discovery projects (in which the targets were cyclin-dependent kinases and

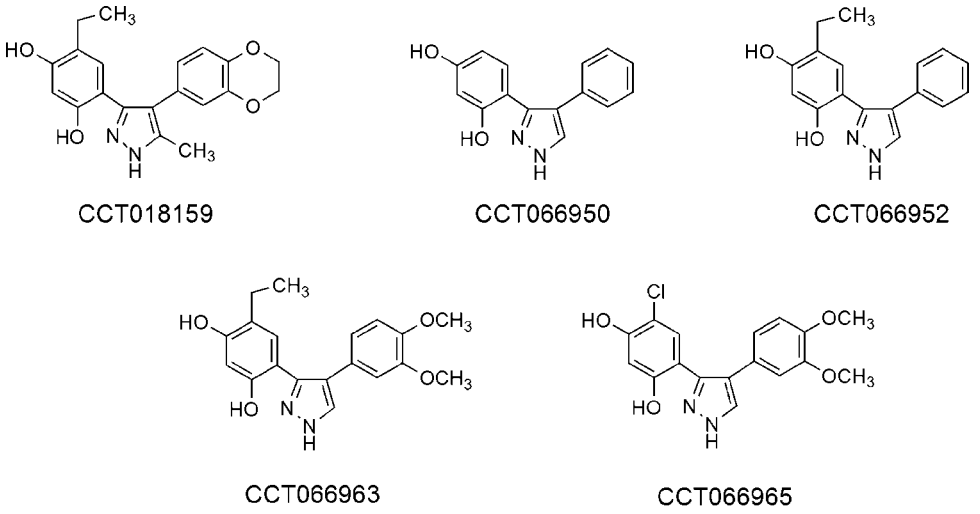

Figure 6. Structures of diarylpyrazole resorcinols administered by cassette dosing in ref. 48.

Hsp90) to screen and prioritize compounds based on their pharmacokinetic properties in a timely manner. Following a successful validation phase, we were able to use the technique to assess the pharmacokinetics of a large number of compounds and to use the information in compound prioritization. Table 1 gives several examples of successful use in other therapeutic areas. However, it is evident that the applicability of this technique is entirely dependent on the compound series under investigation. In our own experience, reviewed here, cassette dosing was effective with trisubstituted aminopurines and diarylpyrazole resorcinols but not with geldanamycin analogues. We suggest that the biological and analytical issues outlined in this review should be given strong consideration and precautions should be taken to minimize the risks associated with cassette dosing and to further advance the successful application of the approach. Based on the experience of ourselves and others, we recommend the following guidelines when considering the implementation of cassette dosing:

(1) During development of a LC/MS/MS method for the analysis of cassette dosing samples, interference arising from cross-talk, ion suppression, or the in-source fragmentation of metabolites should be evaluated. Chromatographic conditions should be optimized to ensure the resolution of interfering compounds if necessary.

(2) Extreme care should be taken when grouping compounds together in cassettes. In addition to the likelihood of common precursor and product ions between structurally similar compounds, the potential for metabolism-derived molecular weight clashes should be considered and avoided.

(3) Cassette dosing solutions should be thoroughly checked for compound precipitation, particularly if the compounds are poorly soluble. In our experience, a formulation containing up to 10% DMSO and 5% Tween 20 in saline has worked well in most cases.

(4) To minimize the potential for compound-compound interactions, the lowest doses that give detectable concentrations should be used and the total number of coadministered compounds should be small (no greater than five). It may be argued that greater numbers could be used to increase throughput and efficiency and that this approach could identify compounds with the 'best' ADME attributes, particularly those with a low affinity for drug-metabolizing enzymes and transporters. However, the use of higher compound numbers in cassettes has not been validated in the published literature. Without such validation, the choice of numbers in the cassette comes down to a judgment based on the balance of throughput versus risk. It is important that a pharmacokinetic standard should be included in each cassette.

(5) Prescreening for potent cytochrome P450 inhibitors and their exclusion from cassettes may avoid serious compound-compound interactions resulting from the competitive inhibition of drug-metabolizing enzymes. However, pharmacokinetic theory predicts that the intrinsic clearance of a compound will be reduced to the same extent whether one of the compounds in the cassette is a potent inhibitor or several of the compounds are weak enzyme inhibitors (42).

(6) The use of cassette dosing in a given project should always be preceded by a validation experiment to show that the method is suitable for the particular chemical series. Furthermore, it is critical that cassette and single dosing data are compared at regular intervals as the project progresses and structural diversity increases.

(7) Although there is significant potential for compound-compound interactions, it would be worth investing effort to determine the broader usefulness of cassette dosing for p.o. administration, particularly as this is usually the preferred route for new molecular cancer therapeutics.

It should be emphasized that cassette dosing should not be used to determine accurate pharmacokinetic parameters of compounds. Rather, its value is to be used as a method to rapidly and efficiently compare different compounds during drug discovery and to prioritize them for subsequent *in vivo* testing, such as antitumor efficacy studies. Compounds can be placed in rank order, or "right box" analysis can be used in which compounds are allocated to quantitatively defined categories, (e.g., poor, moderate, and good), and high bioavailability (8, 44). It can also provide rapid feedback to medicinal chemists during the iterative process of synthesis and evaluation, alongside other criteria, such as potency, selectivity, efficacy, and *in vitro* ADME properties. The main use of cassette dosing is during the hit-to-lead, lead profiling, and most especially the lead optimization phase, in which there is a need to prioritize within a large number of compounds from the same chemical series (Fig. 1). In the earlier hit exploration phase, cassette dosing has little value because only a few examples are evaluated within each of a large number of chemical series. In the later phase, in which the preclinical candidate is selected, it is more likely that accurate measurement of DMPK parameters will be determined after dosage of individual compounds. However, particularly in lead optimization, the value of cassette dosing and other high-throughput ADME methods can be very considerable.

Despite the potential advantages, it is very important to be careful to assess the issues associated with the implementation of cassette dosing in each particular context and specifically with each chemical series. The importance of achieving appropriate pharmacokinetic properties to the success of drug discovery projects is now very clear and the value of considering ADME properties quite early in the process is compelling. It is not cost effective to carry out detailed pharmacokinetic studies of very large numbers of compounds on an individual basis, hence the attraction of high-throughput methods. However, it is appropriate to take care to select the most appropriate high-throughput method and to validate the use of the chosen method. This may be cassette dosing for a given compound series, but in our experience,

this could involve alternative methods, such as metabolic stability screening using, for example, liver microsomes. Ultimately, it is the responsibility of the individual investigator or the drug discovery project team to weigh the advantages of cassette dosing, particularly in terms of compound throughput, against the risks associated with the approach and the time that may be needed for careful initial validation and the ongoing critical assessment that may be required as structural diversity increases during the life of a project.

Use of cassette dosing as a filter or prioritization tool can, in our experience, significantly reduce the number of compounds that need to be evaluated in downstream pharmacology and tumor model efficacy studies. Subsequent filters that can be applied include tissue uptake studies and the use of biomarker determination in a tumor or a surrogate normal tissue to show target inhibition. We frequently carry out tumor compound uptake and biomarker analysis on the same samples to improve efficiency and reduce animal usage. Only compounds that exhibit an appropriate level of tumor uptake and target modulation are then taken into tumor model efficacy studies. Such studies also allow pharmacokinetic-pharmacodynamic relationships to be established. Knowledge of these relationships is essential for an approach we have described as the pharmacologic audit trail (64, 65, 85). Sequential measurement of pharmacokinetic and pharmacodynamic end points informs lead optimization, compound profiling, candidate selection, and subsequent clinical development. Predictive efficacy determination in animal models, such as human tumor xenografts or transgenic systems, remains a significant challenge (66). However, the extent to which this acts as a bottleneck can be reduced by cassette dosing and the other prioritization filters discussed above.

Given the potential value to aid the development of drugs for the treatment of cancer and other diseases, we encourage the publication and dissemination not only of the methodology but also of the detailed results obtained with cassette dosing pharmacokinetic analysis (as in refs. 47–49). This will allow further critical analysis and the development of improved methodologies and processes. The benefit of this to the oncology community will be improvements in the efficiency of the drug discovery process, particularly in the lead optimization phase when pharmacokinetic-pharmacodynamic properties become especially critical. A further benefit of cassette dosing is the decrease in animal usage.

Acknowledgments

We thank our colleagues in the Center who contributed to the projects reviewed here and those at Cyclacel and Vernalis for excellent collaboration and discussion; Prof. Keith Jones for assistance with figures 3, 5, and 6; and Dr. Ian Collins for his review of the manuscript and helpful suggestions.

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Mol Cancer Ther 2007;6:428-440.