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Lead Development: Validation and Application of High Throughput Screening for Determination of Pharmacokinetic Parameters for Enzyme Inhibitors†

Jasbir Singh,[a,*] Jim Soloweij,[b] Martin Allen,[a] Loran Killar[c] and Mark Ator[b]

Departments of [a]Medicinal Chemistry, [b]Enzyme and Receptor Biochemistry, and [c]Pharmacology, Sterling Winthrop Pharmaceutical Research Division, 1250 S. Collegeville Road, Collegeville, PA 19426-0900, U.S.A.

Abstract—An approach utilizing robotics (automation) for the rapid and reliable determination of protease inhibitor concentration in rat plasma samples is described. The bioassay protocol using an immobilized peptide substrate allows high sample throughput, compatible with parallel synthesis/SAR development strategy. Copyright © 1996 Elsevier Science Ltd

Introduction

Combinatorial chemistry and high throughput screening offer great promise to provide novel small molecule leads[1] which may subsequently be optimized through parallel synthesis.[2] Although these approaches have the potential to provide novel, potent, and selective compounds for a given enzyme or receptor target, one would still have to follow a serial approach to identify compounds with desirable in vivo profiles. It is obvious that employment of a parallel approach must be devised for lead development (Fig. 1) as well, if one is going to take full advantage of the combinatorial/parallel methodology to attain the ultimate goal—identification of novel therapeutic agents, suitable for clinical trials—in a more effective and timely manner. A closer look at the combinatorial synthesis and high throughput screening paradigm reveals that at the lead identification step one is dealing with far fewer biological test(s) compared with the number of compounds. Even at the lead optimization (in vitro) stage, one still performs relatively small number of biological tests per compound. However, at the lead development (in vivo) stage, the story is in fact very different. Not only does one require relatively large amounts (10^2–10^3 mg) of each compound for in vivo evaluation, but the number of biological tests per compound also increases at exponential scale (Fig. 2). Therefore, one would have to devise and employ a high throughput format that is suitable for parallel processing for lead development. In situ models are being increasingly employed to obtain pharmacokinetic, metabolism, and absorption related information at the earlier stage of drug discovery.[3–5] This information is subsequently used for the design of potent compounds with desired pharmacokinetic properties to rapidly gain access to the optimal therapeutic candidate(s) for animal safety studies and clinical trials. In this paper, we will describe an approach and validation studies for employing high throughput screening for rapid determination of protease inhibitor concentrations in plasma fluid, as a means to facilitate evaluation of various pharmacokinetic parameters.

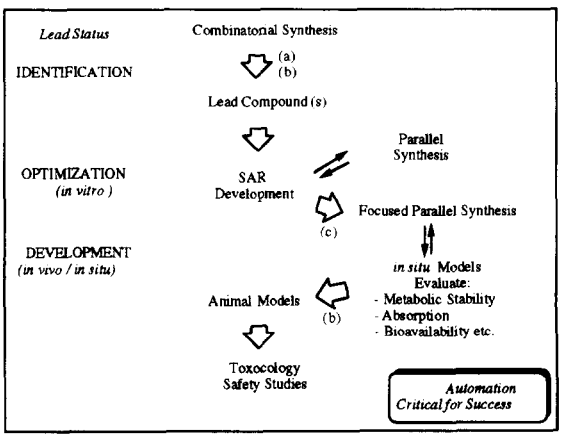


Figure 1. Parallel approach to discovery of therapeutically useful compounds. (a) High throughput chemistry (for the synthesis of mixtures of discrete compounds); (b) High throughput biology; (c) High throughput chemistry (to obtain hundreds of mg to g of *discrete* compounds.

†The work described here was carried out at Sterling Winthrop Pharmaceutical Research Division, before its divestiture to Sanofi Winthrop by Eastman Kodak Co. on 3 October, 1994.

*To whom all correspondence should be addressed at Nycomed Inc., 466 Devon Park Drive, P.O. Box 6630, Wayne, PA 19087-8630, U.S.A.

Abbreviations: CPG, controlled pore-glass; AMP, aminoprolyl-silyloxy; β-Ala, β-alanine; Acp, 6-amino caproic acid; COP, 7-hydroxycoumarin-4-propionic acid; FMOC, 9-fluorenylmethoxy-carbonyl; HOBt, *N*-hydroxy benzotriazole; mCl-t, a recombinant form of the human fibroblast collagenase.

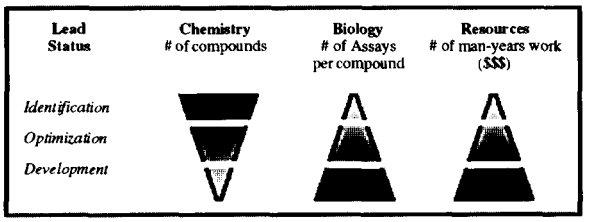


Figure 2. Rationale for employment of automation at *all* stages of drug discovery. Shading and width of the cones are intended to reflect relative effort required within each category.

Results and Discussions

Determination of pharmacokinetic parameters for a given compound essentially requires determination of its concentration in a variety of biological fluids or tissues at various time points following its administration to animals under study. There are a number of methods ranging from LC-MS, HPLC, or bioassays to gain this information.[3-5] Even though these various approaches may offer good sensitivity and reliability, they all have one thing in common—these are labor intensive, requiring appropriate method development either for a given series of compounds or on a compound by compound basis. Consequently, none of these methods are applicable to a high volume environment. In addition, HPLC based methods require the presence of a UV chromophore or a fluorophore, to enable detection and provide high sensitivity. For matrix metalloproteinase (MMP) based inhibitors **1**, we took advantage of the presence of an indole moiety (Fig. 3, compound **1**) and associated fluorescence to measure the inhibitor concentration in a variety of biological fluids using an HPLC based method, as described previously.[5] We wanted to expand this method to evaluate compounds that are not dependent on these special properties (UV or fluorescence).

To accomplish these goals, our recently reported immobilized peptide substrate approach was applied to determine protease inhibitor concentration in biological fluids at various time points. We chose a potent MMP inhibitor **2** (which lacks a UV chromophore/fluorophore) to validate this high throughput approach. The hallmark of our approach is based on generation of a *single* fluorescently tagged soluble hydrolysis product following proteolysis of an immobilized substrate and thus this format is amenable to robotic manipulation (automation), as summarized in Scheme 1.

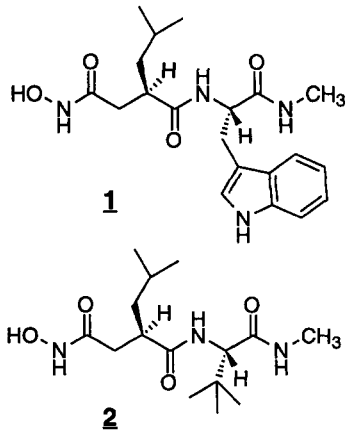
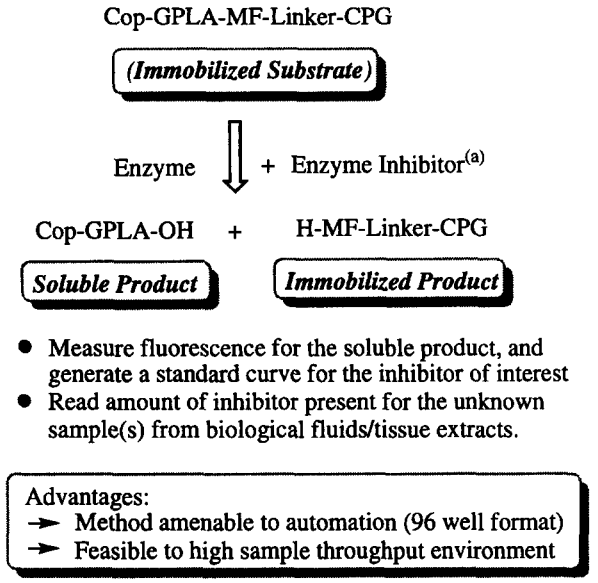


Figure 3. Structures of inhibitors **1** and **2**.



Scheme 1. Flow chart for validation of bioassay.

The hydrolysis of the immobilized substrate **3** (COP-GPLAMF-$(Acp)_5$-βAla-AMP-CPG) is linear with time at varying concentrations of the human fibroblast collagenase (Fig. 4) and stromelysin (data not shown) for over 60 min. As shown in Figure 5, the presence of up to 20% methanol does not interfere with the hydrolysis rate. A final concentration of methanol (15%), which is well within the tolerated limits, was used for our automated protocol.

All the method developments and generation of the standard curve for compound **2** were performed using robotic manipulations, employing a Packard MultiPROBE liquid handling system. The reliability of the results is evident from a low variance in the duplicate fluorescence values as shown in Figure 6 for the

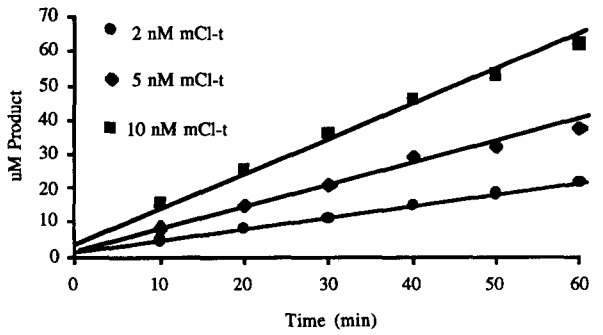


Figure 4. Product release from immobilized substrate vs time with mCl-t.

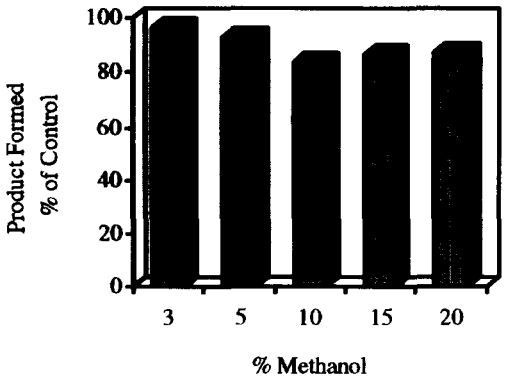


Figure 5. Effect of methanol on reaction with mCl-t.

standard curve and in Figure 7 for the high and low QC samples. The dose–response curve shown for compound **2** in rat serum is essentially identical with the curve generated in assay buffer (data not shown). The automated method gave lower variance than that compared with the collagen-based bioassay.

For our previous studies,[5] the HPLC method development was a major barrier to the number of compounds for which the desired pharmacokinetic parameter (plasma $t_{1/2}$ and biliary excretion) could be determined. By comparison, generation of the bioassay standard curve (over a concentration range of three log units), was completed in 1 day. In addition, since the samples for standard curve require only a small number of wells of the 96-well plate, it allows plenty of space to evaluate a number of unknown samples as well. Therefore, this robotics-based method should significantly increase high sample throughput, since sample collection (post-biology) would no longer be rate limiting. Inability to distinguish between the parent and an active metabolite(s) is an inherent limitation of all bioassay-based approaches. However, alternate

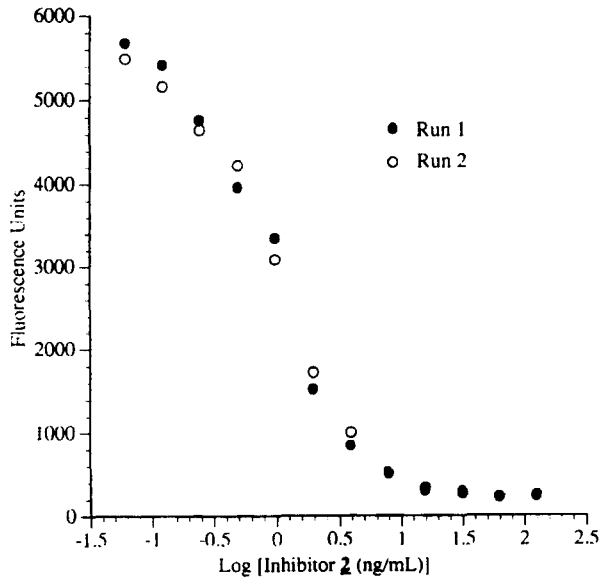


Figure 6. Plot of the fluorescence value (in duplicate) versus log of inhibitor **2** concentrations.

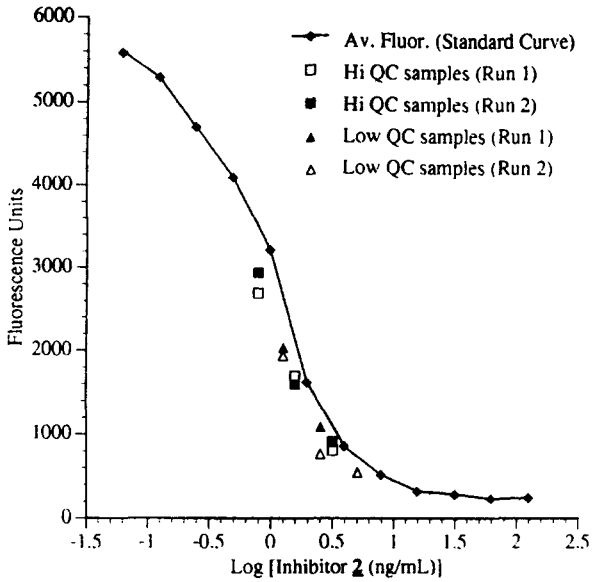


Figure 7. Standard curve, high and low QC data points. The graph shows the plot for the average fluorescence value for the standard curve, and values in duplicate for high and low QC samples in duplicates. (See Experimental for details).

methods like LC-MS (or MS-MS), could subsequently be employed to further answer specific questions.

We have previously reported[6] an excellent correlation between the immobilized and corresponding soluble substrates for mCl-t. We were delighted to find that, in fact, the assay with immobilized substrate **3** for inhibitor **2** gave an IC_{50} of 4.4 nM, which compares very favorably ($IC_{50} = 5$ nM) with the value obtained from assays using soluble substrate. This indicates that the assays using automation are equally applicable for the lead optimization step. Recently, several groups have reported on parallel synthesis of HIV protease inhibitors.[7] The assay format described here could potentially be tailored for other classes of proteases as well.[8] Finally, true application of this automation based approach to the combinatorial arena would require the ability to perform these in situ based studies with relatively small amounts (~ 100 mg) of compounds.[9] Preparation of these amounts of compounds utilizing automated parallel synthesis format is rapidly becoming a reality.

Conclusions

We have described an approach and the validation studies for the employment of automation for rapid and reliable determination of protease inhibitor concentration in plasma samples. Not only does this method allow automation and thus the opportunity to increase sample throughput, it also provided more reliable results. Even though we have utilized collagenase (mCl-t) for automated bioassays, this method is potentially applicable to other proteases or enzymes. One would simply need an appropriate immobilized substrate for the protease or the enzyme

of interest.[10] The fundamental strategy employed has potentially broad applications. We believe that the general concepts described could be extended for the determination of receptor antagonist concentrations as well.

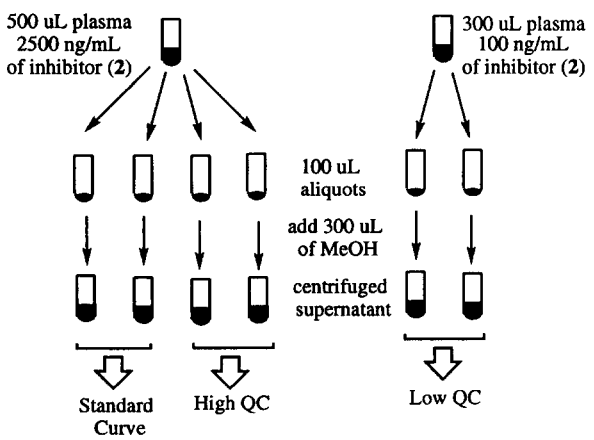
Experimental

Chemistry

A 20 g sample of CPG containing a homogenous population of linker [$(Acp)_5$-βAla][11] was coupled using our standard protocol of triple coupling with 10-fold excess of FMOC amino acid HOBt active esters (in situ activation method) in 96 reaction vessel format, employing Advanced ChemTech synthesizer model MPS350. Finally, each sample was capped with coumarin propionic acid (COP) as a fluorescent tag.[12] All samples were pooled together, washed with methylene chloride, and dried in vacuo. Amino acid analysis on the CPG bound peptide was carried out in triplicate to verify the ratio of G, P, L, A, M, F, Acp, and βAla as 1:1:1:1:1:1:5:1 (±3%), respectively, in order to verify the homogeneity of sample **3** (COP-GPLAMF-$(Acp)_5$-βAla-AMP-CPG). This sample (~20 g) was used for bioassays. The hydroxamate inhibitors, **1** and **2**, were prepared by the methods previously reported by us.[13]

Biological assays

Automated biological assays were performed on a small sample of the glass-bound peptides in a 96-well format. Typically, 4.0±0.3 mg glass-bound peptide sample **3** was weighed in individual cryo-tubes using a HP ORCA robot (customized for this purpose),[14] 175 μL of a buffer (containing 50 mM Tris pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$), 55 μL of plasma extract[15] containing inhibitor **2** (see next sections for details), followed by 44 μL of 2 nM mCl-t[16] (mature truncated human fibroblast collagenase) protease solution were added using the Packard Multi-PROBE. Samples were mixed on variable speed vortexer for 60 min. The samples were allowed to settle by gravity and a 125 μL aliquot of each sample was transferred to the appropriate position of a 96-well plate and fluorescence was read using a Fluoroskan plate reader (355 nm excitation and 460 nm emission).



Scheme 2. Protocol for determination of protease concentrations using immobilized substrate based bioassay. (a) See Experimental and Discussion for detail.

Generation of standard curve and sample preparation for validation of bioassay. The standard curve was generated using the inhibitor **2** in rat plasma. A solution of the inhibitor **2** in 500 μL of rat plasma (2500 ng/mL) was prepared and the sample was split into four 100 μL aliquots. Methanol (300 μL) was added to each tube and the mixture was spun on a table top centrifuge at 100 *g* for 10 min. The first two tubes were sequentially diluted with a mixture of 1 part rat serum and 3 parts methanol to generate dilutions ranging from 125 ng/mL to 61 pg/mL concentrations of inhibitor **2**, in duplicate. All these dilutions and sample preparations were carried out using Packard Multi-PROBE. These samples were used to generate a standard curve for the inhibitor **2**. The remaining two tubes were diluted 1:40, with a mixture of three parts methanol and one part rat plasma. These samples were diluted (2× and 4×) to provide samples for high QC. Samples for low QC were prepared from a 100 ng/mL solution of the inhibitor **2** in rat plasma, as shown in Scheme 2. All samples were generated in duplicate for bioassay.

Acknowledgment

We would like to thank Mr. David Whipple for carrying out amino acid analyses of the immobilized substrate **3**.

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4. We have routinely used a battery of in situ models for a number of protease inhibitor based projects. Development of elastase inhibitors is an example of the successful integration of this approach to drug discovery, see: Hlasta, D. J.; Subra-

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8. One should be able to employ a variety of continuous soluble substrates (including fluorescence energy transfer substrates) as long as one could efficiently automate the given assay protocol for the determination of the inhibitor concentration in various biological fluids, analogous to the description provided here.

9. We have recently reported the pharmacokinetic parameter determinations for a series of compounds with variations at the terminal N-methyl amide portion of inhibitor **1**, and all this work was performed with <100 mg of each of these compounds.

10. We have recently identified a number of substrates which are processed selectively by human fibroblast stromelysin, and are not cleaved by mCl-t. This work will be reported elsewhere.

11. Details for the preparation of homogenous linker will be published elsewhere, Singh, J. et al.

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14. Details of the modifications/customizations to the HP ORCA robot will be published elsewhere.

15. Since the plasma extract contains 75% methanol, the final concentration of methanol in the enzyme assays is 15%.

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