RESEARCH PAPER

Accepted 25 July 2001
Revised 18 July 2001
Received 21 March 2001
Indian J. Pharm. Sci., 2001, 63(5) 401-407

Discovery Screening of the *In Vitro* Cytochrome P450 Inhibitory Potency of HIV-Protease Inhibitors: Comparison to Saquinavir, Indinavir, Nelfinavir, and PNU 140690

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HIV-1 protease inhibitors are an important part of the arsenal for the treatment of AIDS. Currently available protease inhibitors are known to interact with CYP3A4. Consequently, they are highly prone to drug-drug interactions with other coadministered drugs that are substrates or inhibitors of CYP3A4. Thus, development of newer HIV-1 protease inhibitors with a lower tendency for drug-drug interactions would be advantageous. We have compared the CYP450 inhibitory profiles of several discovery phase HIV-1 protease inhibitor candidates with saquinavir, indinavir, nelfinavir, and a structurally related compound, PNU 140690. The data indicate that discovery phase compounds have a different inhibitory profile than the other test compounds and show a lower potential for CYP3A4 and CYP2D6 based drug-drug interactions. However, they have a higher potential for CYP1A2 and CYP2C9 based drug-drug interactions. None of the compounds tested inhibited CYP2E1 or CYP2A6 to any significant extent.

HIV-1 protease (aspartic proteinase) inhibitors are promising agents for the treatment of AIDS[1]. Their combination with established reverse transcriptase inhibitors has been shown to maintain a high level of suppression of viral replication as well as reduction in the development of mutant strains[1,2]. Several protease inhibitors have been clinically evaluated for the treatment of AIDS, the salient examples being saquinavir, indinavir, nelfinavir and ritonavir[1-3]. One of the important considerations for clinical success of HIV-1 protease inhibitors is their performance as stand-alone therapy or as part of add-on therapy to conventional AIDS management protocols. In this regard, most of the HIV-1 protease inhibitors have been shown to be substrates and/or inhibitors of CYP3A4[4-6]. Therefore, the present protease inhibitors are highly prone to metabolic drug-drug interactions involving coadministered drugs whose clearance is also dependent on CYP3A4 mediated metabolism[4]. CYP3A4 is the most abundant of the CYP450 isoenzymes present in the human liver and can account for up to 50% of the total hepatic microsomal P450 content[7]. It thus seems prudent that criteria for selection of newer protease inhibitors coming out of discovery programs include the drug-drug interaction potential of the new chemical entity with respect to the CYP450 isoenzymes, especially CYP3A4[8,9].

There are multiple approaches to determine the drug-drug interaction potential of a new chemical entity with respect to CYP450 mediated metabolism[7, 10-15]. However most of them are based on prior knowledge of the metabolic pathways and the isoenzymes involved therein[16]. A unique approach that circumvents this requirement is to look at the ability of a new chemical entity to inhibit CYP isoenzyme specific catalytic activities of suitable probe substrates[16]. In addition, this approach identifies both catalytically specific and non-specific interactions[16]. We have utilized this approach to screen several discovery HIV-protease inhibitors based on their potential for CYP450-based drug-drug interaction potential, more specifically with regard to the inhibition

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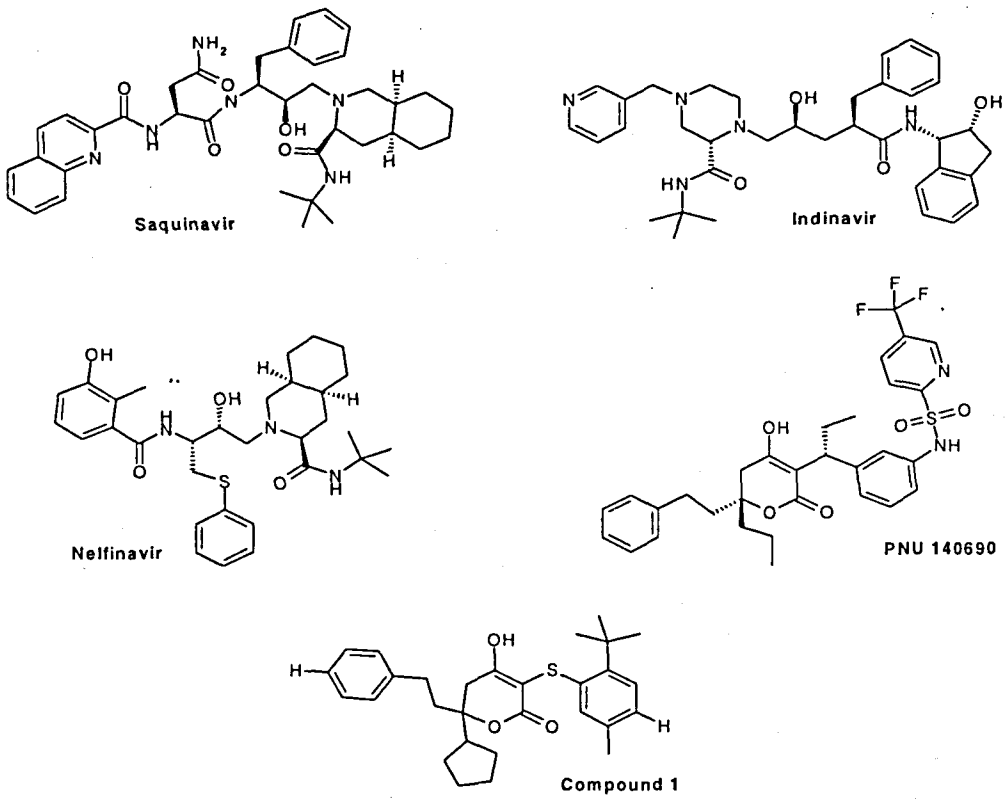


Fig. 1: Chemical structures of HIV protease inhibitors used in this study

of CYP3A4 isoenzyme specific activity. This report presents the results of the overall inhibitory profiles of these potential developmental lead compounds[17-22] (Table 1). Also presented is a comparison of their profiles both with marketed protease inhibitors (saquinavir, indinavir, and nelfinavir) as well as a structurally similar compound PNU 140690[23] (fig. 1).

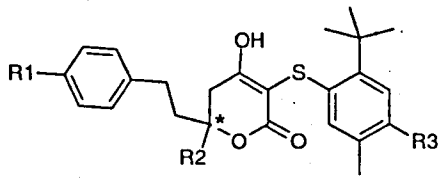
MATERIALS AND METHODS

Compounds 1-12 (Table 1) were synthesized at the chemistry department at Pfizer Global Research and Development, Ann Arbor Laboratories, Ann Arbor, MI[17-22]. Saquinavir mesylate was a gift from Hoffman-LaRoche (Nutley, NJ). Indinavir sulfate was a gift from Merck Research Laboratories (Rahway, NJ). AG-1343 (nelfinavir mesylate) was a gift from Agouron Pharmaceuticals Inc. (San Diego, CA). PNU 140690 was synthesized at the chemistry department at Pfizer Global Research and Development, Ann Arbor Laboratories, Ann Arbor, MI. Acetaminophen, 3-acetamidophenol, phenacetin, chlorzoxazone, coumarin, testosterone, 6β-hydroxytestosterone, 11β-hydroxytestosterone, tolbutamide, chlorpropamide, and β-nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH) were obtained from Sigma Chemical Co., St. Louis, MO. Umbelliferone (7-hydroxycoumarin) was obtained from Aldrich, Milwaukee, WI. 4-Hydroxymethyl tolbutamide, 6-hydroxy chlorzoxazone, racemic bufuralol, and hydroxy bufuralol were obtained from Gentest Corp., Woburn, MA. 5-Fluoro-2(3H)-benzoxzolone was a gift from Dr. M. J. Coon of the University of Michigan, Ann Arbor, MI. All other reagents and chemicals were of analytical grade.

Preparation of microsomes and incubation conditions:

Human liver tissue samples were obtained from either the International Institute for the Advancement of Science (IIAM) or the University of Chicago's Liver Tissue Procurement and Distribution Service (LTPADS). Microsomal fractions were obtained by standard differential centrifugation techniques from the frozen (-80°) samples[24]. The protein content of microsomes was

TABLE 1: STRUCTURES OF DISCOVERY COMPOUNDS TESTED FOR CYP450 INHIBITION PROPERTIES



Compound	R_1	R_2	R3
1	H	Cyclopentyl	H
2	OH	Methyl	OCH_2CH_2OH
3	OH	Methyl	$OSO_2NHCH_2CH_3$
4	OH	Isopropyl	OSO_2(piperazinyl)CH_3
5	OH	Isopropyl	OSO_2(benzyl)CN
6	OH	Isopropyl	NH_2
7	OH	Isopropyl	CH_2OH
7S	OH	Isopropyl	CH_2OH
7R	OH	Isopropyl	CH_2OH
8	OH	Isopropyl	NH_2
9	OH	Cyclopentyl	CH_2OH
9S	OH	Cyclopentyl	CH_2OH
10	NH2	Cyclopentyl	CH_2OH
11	NH2	Isopropyl	CH_2OH
11S	NH2	Isopropyl	CH_2OH
12	3-Thienyl	Isopropyl	CH_2OH

Compounds 1-12 represent different HIV protease inhibitors substituted at the R1, R2, and R3 positions. The chiral centre is represented by *.

determined by the Bradford assay, with the Bio-Rad protein assay kit[25].

Pooled human liver microsomal samples (n=6) were selected for the study. The ability of the discovery compounds 1-12, saquinavir, indinavir, nelfinavir, and PNU 140690 to inhibit the isoenzyme defining metabolic pathway of each probe substrate at its K_m was determined. Briefly, the probe substrates and their concentrations were as follows: Phenacetin – 10 µM (CYP1A2), coumarin - 4 µM (CYP2A6), tolbutamide – 100 µM (CYP2C9), bufuralol – 10 µM (CYP2D6), chlorzoxazone – 40 µM (CYP2E1), and testosterone – 50 µM (CYP3A4). Each incubation consisted of microsomal protein (0.1-1 mg/ml), NADPH (1 µM), probe substrate, and test compound (1, 10, 100 µM added in a 5 µl volume in DMSO) in a total volume of 0.5 ml 0.05 M potassium phosphate buffer, pH 7.4. The incubations were initiated by addition of NADPH and conducted at 37° for different time periods (10-30 min) depending on probe substrate used[26-32]. The experimental control consisted of a complete microsomal incubation with probe substrate containing the vehicle (DMSO). Determinations were performed in triplicate for the control and each of the different test compound concentrations.

Analytical methodology:

All the incubations were analyzed by reversed phase HPLC using 1) a Waters 710 WISP injector, Waters 600E pump, Lambda-Max Model 481 LC UV detector, and a

Hewlett-Packard 3390A integrator (CYP3A4, and CYP2E1 assays) or 2) a Perkin Elmer ISS200 injector, model 410 pump, Spectra-Physics Spectra 200 UV detector or Perkin Elmer LS40 fluorescence detector, and a Spectra-Physics Chrom Jet integrator (CYP1A2, CYP2D6, CYP2A6, and CYP2C9 assays). The analytical conditions were minor variations of published methods[26-31] and have been reported previously[32].

Calculations:

The IC_{50} values were calculated by plotting (Sigma Plot, v 3.0) the mean percent activity remaining (from triplicate samples) versus the inhibitor concentration, and subsequently extrapolating to yield the inhibitor concentration that corresponded to 50% activity remaining.

RESULTS AND DISCUSSION

Among the several qualities of a new developmental drug, the propensity for metabolism based drug-drug interactions, especially which related to the CYP450's, is an important consideration. Unfortunately, due to the ability of the CYP450's to metabolize a wide array of xenobiotics, it is fairly common to observe metabolic drug-drug interactions[8]. A drug-drug interaction is precipitated when two or more xenobiotics interact with the same enzyme and the metabolic pathway that is affected is a major route of elimination[8]. To this end, knowledge of the metabolic pathways, the CYP450's involved in the metabolism, and likely interaction potentials of the xenobiotics is essential for prediction of drug-drug interaction potential[8]. Several *in vitro* methods exist for the determination of the metabolic pathway and the enzymes involved therein[10-15]. This information, together with the knowledge of the mechanism of interaction and pharmacokinetic parameters of the interacting drugs, can allow one to predict, to an extent, the clinical significance of a drug-drug interaction[8]. Despite predictability of the existence or absence of a drug-drug interaction by *in vitro* methods, prospective evaluation of the exact magnitude of an interaction *in vivo* is inherently difficult[16]. In addition, at least from a drug discovery viewpoint, it is often impractical to conduct such extensive studies on a large number of compounds, especially in preclinical animal models that may not reflect the same type of interaction or degree of inhibition. Therefore, the more definitive drug-drug interaction methods are generally reserved for when a new chemical entity is further along in the development process, in particular, preclinical development or ultimately in clinical development. On the other hand, even with the aforementioned caveats, *in vitro* methods still have the potential for rapidly screening discovery compounds for CYP450 inhibition.

As an initial step in this process, a series of probe substrates were used to assess the inhibitory activity of compounds 1-12, saquinavir, indinavir, nelfinavir, and PNU 140690 on cytochrome P450 dependent, isoenzyme selective, metabolic pathways of probe substrates. The relative inhibitory profiles (IC_{50} values) of the two sets of compounds are presented in Table 2. Prior to analyzing the inhibitory potential of the compounds it is imperative that the experimental design be discussed. The efficacious plasma concentrations for compounds 1-12, in humans, to date are undetermined. Initial bioavailability studies in mice, rats, and dogs indicated that the maximal plasma concentrations of these compounds were in the range of 2-35 μM. Based on these initial studies, concentrations of 1, 10 and 100 μM were chosen for the drug-drug interaction studies. Whereas the study also involved profiling the interaction potential of other reported HIV-protease inhibitors it was necessary to select appropriate concentrations of these agents. In this regard, the maximal plasma concentrations observed in humans are 0.05-0.13 μM, 7-14 μM, and 6.5 μM for saquinavir (600 mg, po, tid), indinavir (800 mg, po, tid), and nelfinavir (600 mg, po, bid), respectively[3,4]. Based on these reported data, the concentrations of saquinavir, indinavir, nelfinavir and PNU 140690 were also selected to be 1, 10 and 100 μM. We realize that saquinavir plasma levels are 10-fold lower than the other agents and thus a lower range of concentrations would have been more relevant. Albeit, concentrations of 1, 10 and 100 μM saquinavir were chosen to allow for a better equimolar comparison of the compounds.

With this in mind, several characteristics of the pattern of inhibition are apparent. None of the tested compounds show any significant inhibition of CYP2E1 or CYP2A6 activity (IC_{50} >100 μM). An exception was found with Compound 1 that demonstrated broad inhibition for all CYP450 activities except CYP1A2. In relation to other CYP450 activities, compounds 1-12 show inhibitory profiles that are distinct from the marketed compounds, including PNU 140690. With regard to CYP2D6 inhibition, all of the marketed protease inhibitors, as well as PNU 140690 and compound 1, caused significant inhibition (IC_{50} 21.9 to 45.7 μM) whereas all of the remaining discovery compounds have little to no inhibitory effect

TABLE 2: CALCULATED IC_{50} (μM) VALUES FOR THE INHIBITION OF CYP450 ACTIVITIES BY HIV-1 PROTEASE INHIBITORS

Compound	CYP1A2	CYP2A6	CYP2C9	CYP2D6	CYP2E1	CYP3A4
1	>100	83	36	28	50	69
2	>100	>100	3	>100	>100	>100
3	>100	>100	<1.0	100	>100	76
4	-[a]	-	1.5	-	-	35
5	-	-	2.6	-	-	4.0
6	-	>100	35	>100	>100	76
7	>100	>100	12	>100	>100	100
7S	>100	>100	100	>100	>100	>100
7R	-	-	100	-	-	>100
8	24	>100	31.6	>100	>100	>100
9	-	>100	25	>100	>100	>100
9S	90.5	>100	50	91.2	>100	>100
10	-	-	48	-	-	84
11	-	>100	29	100	>100	66
11S	>100	>100	72.4	>100	>100	>100
12	45	-	9	>100	>100	>100
Saquinavir	>100	>100	100	33.9	>100	2.88
Indinavir	>100	>100	>100	45.7	>100	<1.0
Nelfinavir	>100	>100	>100	42.7	>100	3.20
PNU140690	32.6	>100	4.1	21.9	>100	3.16

Microsomal incubations were conducted in triplicate. Each incubation consisted of microsomal protein (0.1-1 mg/ml), NADPH (1 mM), CYP450 isoenzyme specific probe substrate, and test compound (0, 1, 10, 100 μM added in a 5 μl volume in DMSO) in a total volume of 0.5 ml 0.05 M potassium phosphate buffer, pH 7.4. Incubations with 0 μM test compound served as controls. [a] = activity not determined.

on CYP2D6 (IC_{50} 91.2 to >100 μM). Several of the discovery compounds show a higher propensity for drug-drug interaction potential involving CYP1A2 (8, 9S, 12) and CYP2C9 (1-7, 8-12) with IC_{50} 24 to 90.5 μM, and <1.0 to 72.4 μM, respectively. The three marketed compounds, on the other hand, show a higher propensity for drug-drug interaction potential involving CYP3A4 (<1.0 to 3.2 μM). PNU 140690 shows a significant drug-drug interaction potential with CYP1A2, CYP2C9, CYP2D6 and CYP3A4. Overall, several of the discovery compounds have a distinctively different inhibitory profile as compared to some of the protease inhibitors tested.

For compound 7, the inhibitory profiles between the racemic mixture and the enantiomers are similar, except for greater inhibition observed with CYP2C9. The inhibition of CYP2C9 was seen only with the racemic mixture (IC_{50} = 12 μM) and not with either the R- or S-enantiomers (IC_{50} >100 μM). Although the true reason for this discrepancy is unknown, it may be due to impurities found in the racemic mixture that are not present in the 'cleaner' isolated enantiomers. The inhibitory profile between the racemic compound 9 and the S-enantiomer of compound 9 were found to be very similar. The inhibitory profile between the racemic compound 11 and the S-enantiomer

of compound 11 were found to be similar, however the racemic mixture seems to be more inhibitory towards CYP2C9 and CYP3A4 than the S-enantiomer. The reason for evaluating S-enantiomers for their drug interaction potential was that previous data had shown that the S-enantiomers have better binding affinities to the HIV protease relative to the R-enantiomers[22].

The data obtained for the marketed compounds correlates well with previously published data in regard to their CYP450 inhibitory potential. Saquinavir has been shown to inhibit CYP2C9 and CYP3A4 activities (the same substrate probes as in our study) with IC_{50} values of 54 and 2.14 μM, respectively, with minimal inhibition of CYP1A2 or CYP2E1 activity[32]. In the same study, indinavir was shown to inhibit CYP3A4 activity with an IC_{50} value of 0.43 μM, with minimal inhibition of CYP1A2, CYP2C9 or CYP2E1 activity[33]. Other studies also indicate that indinavir inhibits CYP3A activity with a K_i of 0.17 to 0.5 μM[5,6,33]. Among the marketed protease inhibitors, however, ritonavir is the most potent CYP3A4 inhibitor (Ki = 0.02 μM)[33]. *In vitro* studies with human liver microsomes have shown that ritonavir potently inhibited the metabolism of saquinavir, indinavir, nelfinavir, and VX-478 with IC50 values between 0.25 to 2.2 μM suggestive of a significant drug-drug interaction potential[2]. Further *in vivo* studies in rats confirmed the qualitative *in vitro* observations. Co-administration of ritonavir with saquinavir, indinavir, nelfinavir, and VX-478 significantly increased the plasma levels of all four compounds while plasma levels of ritonavir remained unaltered[2].

We also realize the difficulties in comparing our data with reported IC_{50} values (that are substrate and concentration dependent) and K_i values for better assessment of the drug-drug interaction potential. However, the experimental design presented does allow for a cautious extrapolation of IC_{50} values to predicting K_i values. This is because the concentrations of the probe substrates chosen for the inhibition studies are equivalent to their apparent Michaelis-Menten constant (K_m) for the respective CYP450 isoenzyme mediated metabolic pathway. The choice of this substrate concentration allows for the simplification of some of the kinetic equations pertaining to reversible inhibition. The equations describing the different modes of reversible inhibition (the assumption being that reversible inhibition is the most common mechanism resulting in drug-drug interactions), namely, competitive, uncompetitive, and non-competitive have been previously described[34]. When the substrate concentration is equal to the K_m, and when the inhibitor concentration results in 50% inhibition of catalytic activity, the following is true: $[S] = K_m$, $[I] = IC_{50}$, $V = 0.25(V_{max})$[16,34]. Under these conditions, the equations can be simplified to show that for competitive and uncompetitive inhibition, $IC_{50} = 2\ K_i$, and for non-competitive inhibition, $IC_{50} = K_i$. With these simplifications, if one assumes reversible inhibition, the lower limit of the K_i equals one-half the determined IC_{50} value. Thus, the data obtained with this approach can be used effectively for screening of discovery compounds without resorting to extensive experimental protocols that are required for the determination of the K_i values.

In conclusion, we have used a simple *in vitro* inhibition screen to evaluate several discovery compounds in regards to their CYP450 interaction potential, and also compared them to other relevant compounds. The data indicate that the inhibitory profile of these discovery compounds is different from some of the other HIV-1 protease inhibitors. More importantly, the in-house discovery compounds have a favorable profile with respect to CYP3A4, the enzyme most prone to drug-drug interactions involving the presently available protease inhibitors.

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