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Preliminary in Vitro Assessment of the Potential of EST64454, a Sigma-1 Receptor Antagonist, for Pharmacokinetic Drug–Drug Interactions

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EST64454 is a selective sigma-1 receptor ligand intended for orally administered pain treatment that showed a promising profile in the lead optimization process. As part of the preliminary compound profiling, the potential for future drug–drug interactions was explored in vitro. Both direct and time-dependent CYP inhibition for CYP1A2, 2C9, 2C19, 2D6 and 3A4 was studied in human liver microsomes. EST64454 showed a low potential for CYP inhibition (IC50 between 100 and 1000 µM) and as time-dependent inhibitor (IC50 shift mainly around 1). CYP induction studies with HepaRG® cells revealed no CYP induction at concentrations ≤50 µM, as shown by the CYP1A2, 3A4 and 2B6 activities measured. Reaction phenotyping was assessed after incubation with recombinant human enzymes. Although a very low metabolism was observed, several enzymes catalyzed the formation of metabolites, including CYP3A4, 2C19 and flavin monooxygenases (FMO) 1 and 3. EST64454 was not a P-glycoprotein (P-gp) substrate and was highly permeable in Caco-2 cells. P-gp inhibition was only observed at 200 µM, the highest concentration studied. Preliminary studies suggest that neither CYP nor P-gp interaction of EST64454 would be of any concern for further development. At later stages, the interaction kinetics and the clinical relevance of these findings will be thoroughly evaluated.

Key words CYP inhibition; CYP induction; phenotyping; P-glycoprotein interaction

INTRODUCTION

During the early stages of Drug Discovery new compounds are synthesized and tested in several assays. The purpose of these assays is to screen new compounds in terms of their physical chemistry, activity, safety and absorption, distribution, metabolism and excretion (ADME) properties. Accordingly, most assays are performed with a high throughput format. The purpose of this initial screening is to narrow down the number of compounds according to the pre-set desired profile. At a later stage of Drug Discovery, the compounds with the best balanced profile are selected for further characterization, and finally for evaluation in clinical trials, providing all requirements are met.

The potential of new compounds for drug–drug interactions is assessed as part of the Drug Discovery process because these interactions can lead to therapeutic inefficacy or adverse events with varying degrees of severity. Pharmacokinetic drug–drug interactions are interactions that occur when one drug alters the pharmacokinetics of a coadministered drug. Drugs can be viewed as victims or perpetrators of the interaction, the final result depending on the characteristics of the coadministered drugs. This type of interactions can be based on either metabolism or transporters. When the test compound is evaluated as the perpetrator of the interaction, its potential as inhibitor and inducer of metabolic enzymes and transporters should be investigated. On the other hand, if the test compound is being evaluated as the victim of the interaction, assessment of its reaction phenotyping and transporters involved in its disposition is required.

EST64454 (1-(4-(2-((1-(3,4-difluorophenyl)-1H-pyrazol-3-yl)methoxy)ethyl)piperazine-1-yl)ethanone) is a selective sigma-1 receptor (σ1R) antagonist (Ki = 22 nM) synthesized by ESTEVE Pharmaceuticals (Barcelona, Spain) for orally administered pain treatment1) (Fig. 1).

Current preclinical evidence provides a strong rationale for the potential of σ1R antagonists as a new therapeutic approach to pain management, on account of the modulatory role of this receptor on nociception.2) E-52862, the leading compound in the field, has shown efficacy in a Phase II clinical trial on oxaliplatin-induced neuropathy.3)

During the lead optimization process of σ1R project, EST64454 was one of the compounds with a promising profile selected for further characterization. Among its profiling, the assessment for drug–drug interactions was especially relevant, as concomitant medication is expected in the clinical setting.

The purpose of the present study was to perform a pre-

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Fig. 1. Structure and Product Ions of the m/z Spectrum of EST64454, and Proposed Origin of Key Product Ions
liminary in vitro assessment of the potential of EST64454 for pharmacokinetic drug–drug interactions. We focused on CYPs, the enzymes most commonly involved in drug metabolism, and P-glycoprotein (P-gp), a well-known transporter. Accordingly, EST64454 was assessed as CYP inductor as well as CYP and P-gp inhibitor. The likelihood of drug–drug interaction where EST64454 disposition could be altered was also assessed by characterizing its reaction phenotyping and exploring whether or not it could be a substrate of an efflux transporter, such as P-gp.

MATERIALS AND METHODS

Chemicals and Reagents All probe substrates used in CYP interaction assays and their respective metabolites, as well as the reference compounds, were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), except 6β-hydroxytestosterone and tienilic acid which were purchased from BD Biosciences (San Jose, CA, U.S.A.). Other chemicals were obtained from the following sources: Sigma-Aldrich (sulfasalazine, glucose-6-phosphate, digoxin, verapamil, for-}

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Assay conditions are summarized in Supplementary data. In brief, after a 5-min pre-warming of the incubation mixture at 37°C, reactions were initiated by adding human liver microsomes (≤0.5 mg/mL). Upon completion of the incubation period, the reaction was terminated by adding an equal volume of ice-cold acetone/MeOH (70% (v/v)). The precipitated protein was removed by centrifugation (1500 g for 20 min at 4°C) and the resultant supernatant was diluted with 0.1% formic acid in water prior to analysis.

To evaluate EST64454 as a direct inhibitor, human liver microsomes were incubated with CYP probe substrates (at concentrations approximately equal to their reported K_{in}) in the presence of EST64454 (at concentrations ranging from 1500 to 0.7 μM) to determine its IC_{50} value.

For time-dependent inhibition assays, EST64454 was pre-incubated with human liver microsomes at 37°C for 30 min in the presence and absence of an NADPH-generating system. After pre-incubation, the probe substrate was added and incubation was continued for a CYP-dependent period of time to measure residual activity. Incubation time and EST64454 concentrations were equal to those used when evaluating direct CYP inhibition.

The IC_{50} for each CYP and type of inhibition assessed was calculated by non-linear regression of the metabolite formed versus the inhibitor concentration curves. Curves were fitted to the following equation using Sigma Plot software (v 11.0):

\[ y = \frac{a}{1 + \left(\frac{x_0}{x}\right)^b} \]

wherein, \(a\) is the upper-lower asymptote; \(b\) is the slope factor; \(x_0\) is the IC_{50}; \(y_0\) is the lower asymptote; \(x\) is the inhibitor concentration; and \(y\) is the peak area of the metabolite formed expressed as percentage of the response for the corresponding positive control. Standard error of IC_{50} so estimated is given.

CYP Induction Assay The CYP induction potential of EST64454 was assessed using cryopreserved HepaRG™, a human hepatoma cell line.b The procedures for thawing, plating and maintenance of HepaRG™ cells were carried out as described by the cell supplier. During the experiment, cell culture was maintained in an incubator at 37°C and an atmosphere of 5% CO₂ with 95% relative humidity. Cells were seeded (200 cells/mL) in collagen I-coated 24-well plates. After 3-d adaptation period, cells were treated in triplicate with 0.1% formic acid in water prior to analysis.

After 3-d incubation, the reaction was terminated by adding an equal volume of ice-cold MeOH containing sulfasalazine (0.1 μg/mL), added as internal standard. The precipitated pro-

The induction potential of CYP1A2, 2B6 and 3A4 was assessed by measuring the CYP substrate-based metabolite formation. On day 7, EST64454 or reference inducers were replaced by the substrates for each isoform assayed: phenacetin (CYP1A2; 100 μM), theophylline (CYP1A2; 100 μM), theophylline (CYP1A2; 100 μM), and rifampicin (CYP3A4; 10 μM). The induction potential of CYP1A2, 2B6 and 3A4 was assessed by measuring the CYP substrate-based metabolite formation. On day 7, EST64454 or reference inducers were replaced by the substrates for each isoform assayed: phenacetin (CYP1A2; 100 μM), theophylline (CYP1A2; 100 μM), theophylline (CYP1A2; 100 μM), and rifampicin (CYP3A4; 10 μM). After 45 min-incubation, the reaction was stopped in a 100-μL cell culture sample by adding an equal volume of ice-cold MeOH containing sulfasalazine (0.1 μg/mL), added as internal standard. The precipitated pro-

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After 3-d incubation, the reaction was terminated by adding an equal volume of ice-cold MeOH containing sulfasalazine (0.1 μg/mL), added as internal standard. The precipitated pro-
tein was removed by centrifugation (16090 × g for 10 min at 4°C) and the resultant supernatant was then diluted with 0.1% formic acid in water prior to analysis.

The induction potential of the compound was expressed as percentage of positive control and “the fold induction” versus the solvent vehicle control:

\[
\text{% positive control} = \frac{\text{Activity}_{TC} \times 100}{\text{Activity}_{positive control}}
\]

\[
\text{Fold induction} = \frac{\text{Activity}_{TC}}{\text{Activity}_{vehicle control}}
\]

wherein, Activity is the area of the chromatographic peak corresponding to the metabolite for each CYP isoform in the presence of the positive control (reference inducer), the test compound (TC) or the vehicle control (DMSO).

**Reaction Phenotyping Assay**

Reaction phenotyping of EST64454 was evaluated using several recombinant human enzymes individually (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4, FMO1, FMO3, MAO-A and MAO-B). 9) EST64454 was evaluated using several recombinant human enzymes individually (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4, FMO1, FMO3, MAO-A and MAO-B). 9) Furthermore, the dependence of metabolism on NADPH was assessed in human liver microsomes. Incubations with rat liver microsomes were carried out in parallel to support metabolism formation assessment.

The compound stock solution (10 mM) was prepared in DMSO and further diluted in acetonitrile (1/10), the test compound concentration in incubation medium being 10 μM (1% organic solvent (v/v)).

EST64454 was incubated in duplicate at 37°C under standard conditions (30 mM MgCl2, NADPH generating system, 0.5 mg protein/mL) using a robotic liquid handling system (Freedom Evoware 150, TECAN, Männedorf, Zürich, Switzerland) and gently shaking for 1 h. Incubation buffer was dependent on the enzyme tested (50 mM Na/K phosphate buffer pH 7.4 for CYP1A2, 2B6, 2C8, 2C19, 2D6, 2E1 and 3A4, MAO-A and MAO-B, 50 mM Tris buffer pH 7.4 for CYP2A6 and 2C9, and 100 mM glycine buffer pH 8.4 for FMO1 and FMO3). When incubating with human and rat liver microsomes, the total concentration of CYPs was 0.3 mM/mL. The microsomal protein concentration when incubating with CYPs was normalized to 0.5 mg/mL using control supersomes with no catalytically active enzyme. The final incubation volume was 500 μL. Known enzyme substrates were included in parallel as positive controls. Reactions were stopped at 0 and 60 min by adding 75-μL incubate to an equal volume of cold acetonitrile (50% organic solvent (v/v)). Samples were then diluted with 0.1% formic acid in water, mixed and centrifuged at 8°C for 30 min at 5682 × g prior to analysis.

Compound disappearance in media after 60-min incubation with microsomes or enzymes, as well as metabolite formation were measured. Results were expressed as percentage of test compound remaining after incubation with liver microsomes or each enzyme studied.

**Drug Transport Assays**

The interaction of EST64454 with P-gp either as a substrate or as an inhibitor was assessed in human colon adenocarcinoma (Caco-2) cells. 10) Cells were seeded as a monolayer in HTS Transwell 24-well plates with polycarbonate microporous filters (0.4-μm pore diameter, 0.33-cm² area). A ready-to-use kit (CacoReady) was used. Upon reception, cell maintenance was carried out for 1 week according to the procedure described by the supplier.

Transport assays were performed with 21-d confluent monolayer cells with passage number ranging from 55 to 58. Incubations were done in HBSS media supplemented with 10 mM glucose (pH 7.4). During maintenance and transport assays, cells were maintained at 37°C in an atmosphere of 5% CO2 with a relative humidity of 95%. Transport experiments were carried out in duplicate using digoxin (10 μM) as reference P-gp substrate. Compound stock solutions were prepared in DMSO and further diluted in incubation media, with final organic percentage being 1%.

Maintenance media was replaced by incubation media in both donor and receiver sides, followed by a 15-min pre-incubation period. After pre-incubation, the procedure carried out depended on the purpose of the assay. When assessing EST64454 as a potential P-gp substrate, the bidirectional permeability assay was started by adding EST64454 solution (10 μM) to either the apical (200 μL); for apical-to-basolateral transport assay; A-to-B) or basolateral (600 μL; for basolateral-to-apical transport assay; B-to-A) sides of the monolayer. However, when assessing EST64454 as potential P-gp inhibitor, digoxin solution (10 μM) was added to either the apical or basolateral sides of the monolayer in the absence and the presence of a known P-gp inhibitor (verapamil, 100 μM) or EST64454 (2, 20 and 200 μM). After 2-h incubation at 37°C with gentle shaking, samples from both sides of the monolayer were withdrawn for compound determination. After aspirating remaining media, the integrity of the Caco-2 cell monolayer was checked with Lucifer yellow (100 μM in incubation media). After 1-h incubation at 37°C and gentle shaking, the basolateral sample fluorescence was measured and the apparent permeability was calculated (cut-off value of Papp, A-to-B = 10 nm/s).

The apparent permeability of the compound (EST64454, digoxin or Lucifer yellow) was calculated using the equation:

\[
P_{app} = \frac{1}{A \cdot C_i} \frac{dQ}{dt}
\]

wherein, dQ/dt is the rate of appearance of compound at the receiver side, A is the surface area of the cell monolayer, and C_i is the initial compound concentration in the donor side.

The efflux ratio was estimated from the ratio between apparent permeabilities (P_{app, A-to-B}/P_{app, B-to-A}). The recovery was calculated in both transport assays.

A two-way ANOVA was performed on data from the P-gp inhibition study to determine statistically significant differences between treatment groups; p < 0.05 was considered to be statistically significant.

**Analytical Procedures**

All analyses of CYP activities in both induction and inhibition assays were performed with HPLC-MS/MS methods. The chromatographic system consisted of a triple quadrupole mass spectrometer 4000 QTRAP (AB Sciex, Framingham, MA, U.S.A.) coupled with a CTC PAL autosampler and a 1200 chromatographic pump (Agilent Technologies, Santa Clara, CA, U.S.A.). An XSelect HSS C18 (2.5 μm particle size, 3.0 × 50 mm) (Waters, Milford, MA, U.S.A.) HPLC column was used. Column temperature was kept at room temperature during analysis. The mobile phase consisted of solvent A (0.0155% formic acid in water) and solvent B (0.0155% formic acid in acetonitrile). The gradient followed was CYP-dependent and ranged from 90:10 (or 70:30)
to 0:100 (A:B solvents (v/v)) in 4 or 6 min. The mobile phase flow rate was set at 0.5 mL/min. The chromatographic peak areas of analytes were integrated with an Analyst data system (version 1.4.2; AB Sciex).

Samples from phenotyping and transport assays were analyzed with an ultra performance liquid chromatography (UPLC)-MS/MS system (Waters, Milford, MA, U.S.A.). The chromatographic system was composed of an Acquity® UPLC pump, and a sample and column manager coupled with a triple quadrupole mass spectrometer (TQD). An Acquity UPLC® BEH C18 (1.7 µm 2.1 × 50 mm) Waters UPLC column was used. Column temperature was maintained at 35°C during analysis. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The flow rate was set at 0.6 mL/min. In phenotyping studies, the sample was eluted with a gradient from 95:5 to 75:25% of solvents A:B (v/v) in 3 min, and 75:25 to 0:100% of solvents A:B in 0.5 min. The gradient in transport studies ranged from 95:5 to 0:100% of solvents A:B (v/v) in 1 min. Both gradients finished with 1-min column washing with 100% acetonitrile.

Chromatograms were acquired by MRM mode with an electrospray ionization source. EST64454 and digoxin were quantified in the positive ionization mode using transitions 365.2 > 193 and 781 > 651, respectively. As for EST64454 metabolites, incubation samples were acquired by both full and product ion scans in order to detect the metabolites formed. Their most intense MRM transitions were selected for further metabolite monitoring. Peak chromatographic areas of analytes were integrated with a MassLynx data system (version 4.1; Waters). For transport studies, a seven-point calibration curve was used for quantitation purposes with a concentration range from 0.02 to 12 µM of EST64454 (P-gp substrate assays) or digoxin (P-gp inhibition assays).

RESULTS

CYP Inhibition The CYP inhibition potential of EST64454 was studied in human liver microsomes with and without 30-min pre-incubation. The IC_{50} could be calculated for all CYPs except CYP1A2, 2C9 and 2C19, as shown in Fig. 2. An IC_{50} estimate was considered for these three CYPs, based on data observation. IC_{50} values and IC_{50} shifts of known reference inhibitors were consistent with data reported in the literature (results shown in Supplementary materials).11–14

EST64454 had high IC_{50} values, ranging materials 200 to 1000 µM in the absence of pre-incubation. As for CYP3A4, the IC_{50} observed was higher than 200 µM regardless of the substrate used, testosterone or midazolam. In addition, the IC_{50} shift observed in the time-dependent inhibition studies was ≤1-fold for all CYP isoforms except CYP3A4 which showed an IC_{50} shift of 2-fold.

CYP Induction The potential of EST64454 as CYP inducer was evaluated in HepaRG™ cells using CYP1A2, 2B6 and 3A4 activities as endpoints. As summarized in Table 1, EST64454 showed no relevant CYP induction in the concentration range of 1 to 50 µM. Regardless of the CYP isoform, EST64454 produced an inductive response in enzyme activity ≤20%. Additionally, the induced CYP activity was less than 2-fold versus DMSO baseline activity—a very low value as compared to that observed for the corresponding reference inducers (from 8 to 18-fold induction).

Phenotyping Reaction The enzymes involved in the metabolism of EST64454 were identified after incubation with recombinant human CYP, FMO and MAO enzymes. The enzyme activity observed for positive controls validated the assay (results shown in Supplementary materials).

The results shown in Table 2 suggest that EST64454 metabolism is very low regardless of the presence of NADPH (96–98% of compound remaining). As for recombinant enzymes, similar results as with human microsomes were observed for all enzymes except CYP3A4. For this CYP, the metabolism of EST64454 was slightly higher than for the other enzymes (83% vs. 93–100% of compound remaining).

In addition to EST64454 disappearance determination, metabolite formation in the incubation media was determined in rat liver microsomes to support phenotyping assessment. At the end of incubation, four metabolites formed by oxidation of EST64454 were detected: one N-oxide (M3, m/z 381) and four metabolites with unknown structure: one metabolite with m/z 381 (M1) and two metabolites with the same m/z but different retention times, (M2 and M4, m/z 379) (Fig. 3). Product ions of m/z mass spectrum for metabolites are shown in Fig. 4.

As shown in Fig. 5, CYP3A4 formed three out of the four metabolites monitored. An additional metabolite (M5) with m/z 367 and very low intensity was also observed. Unlike CYP3A4, CYP2C19, FMO1 and FMO3 only catalyzed the formation of the N-oxide metabolite (M3) (results shown in Supplementary materials).

Drug Transport Interactions The evaluation of transporter drug–drug interactions of EST64454 focused on efflux transporters. EST64454 was assessed both as P-gp substrate and inhibitor in a bidirectional transport assay using Caco-2 cells.

As summarized in Table 3, an efflux ratio of 0.7 was observed when EST64454 was evaluated as P-gp substrate as opposed to digoxin, a known P-gp substrate, with an efflux ratio of 15.2. At the end of incubation, the recovery was close to 100%.

The potential of EST64454 as P-gp inhibitor was studied at three concentrations comprised in a 100-fold range (Table 3). When EST64454 was added to the incubation media, the efflux ratio of digoxin decreased by more than half (15.2 vs. 6.1) at the highest concentration assayed (200 µM), whereas the efflux ratio values were similar at 20 and 2 µM (15.2 vs. 10.1 and 15.0, respectively). Differences between the efflux ratio of digoxin in the presence and absence of EST64454 were statistically significant only at the highest concentration (200 µM).

In addition, studies with verapamil, a known P-gp inhibitor, were run in parallel for comparison. Unlike EST64454, verapamil decreased the efflux ratio of digoxin to a value close to 1.

DISCUSSION

EST64454 was one of the compounds selected to advance into further profiling during the Lead Optimization process based on results from the preliminary screening of safety, pharmacology and ADME properties, among others. As part of compound profiling, an early evaluation of the victim
Fig. 2. Effect of EST64454 on Selected CYP Activities with and without 30-min Pre-incubation after Incubation with Human Liver Microsomes in the Presence and Absence of NADPH at 37°C

The inhibition of CYP3A4 was evaluated using both testosterone and midazolam as substrates.

Table 1. Effect of EST64454 and Reference Inducers on CYP Activity of HepaRG™ Cells

| Compound     | Concentration (µM) | Fold induction vs. DMSO | % Positive control inducer |
|--------------|--------------------|--------------------------|----------------------------|
|              |                    | CYP1A2       | CYP2B6       | CYP3A4       | CYP1A2 | CYP2B6 | CYP3A4 |
| EST64454     | 0                  | —            | —            | —            | 5.3 ± 0.3 | 12.0 ± 1.9 | 9.7 ± 0.0 |
|              | 1                  | 0.8 ± 0.2    | 1.2 ± 0.1    | 1.0 ± 0.1    | 4.4 ± 1.0 | 14.0 ± 0.8 | 9.5 ± 0.2 |
|              | 10                 | 0.7 ± 0.03   | 1.6 ± 0.1    | 1.3 ± 0.1    | 3.8 ± 0.2 | 19.7 ± 0.7 | 13.1 ± 0.5 |
|              | 50                 | 0.7 ± 0.02   | 1.7 ± 0.7    | 2.0 ± 0.5    | 3.9 ± 1.1 | 20.2 ± 2.1 | 19.3 ± 1.4 |
| Omeprazole   | 50                 | 18.8 ± 0.9   | —            | —            | —        | —        | —        |
| Phenobarbital| 1000               | —            | 8.4 ± 0.2    | —            | —        | —        | —        |
| Rifampicin   | 10                 | —            | —            | 10.3 ± 1.2   | —        | —        | —        |

Results are expressed as mean fold induction and mean percentage of positive control inducer response (± standard deviation (S.D.), n = 3) vs. the vehicle control (DMSO).
and perpetrator potential of EST64454 for pharmacokinetic drug–drug interactions was evaluated in vitro. When designing interaction studies, the corresponding guidelines provided by U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) were taken into account. However, due to the limited knowledge of the pharmacokinetics and pharmacodynamics of EST64454 and to the preliminary nature of this evaluation, these guidelines could not be used to their full extent.

Bearing this in mind, the potential of EST64454 as CYP inhibitor was evaluated in both a reversible (i.e., direct inhibition) and time-dependent manner. Regardless of the type of inhibition, the IC₅₀ values of EST64454 were determined in 5 out of the 7 CYPs recommended by FDA and EMA. As a preliminary assessment, the CYPs mostly involved in drug metabolism were selected, namely, CYP1A2, 2C9, 2C19, 2D6 and 3A4. The test compound concentration range was selected based only on its maximum solubility because the clinically relevant plasma concentration was unknown and could not be predicted from the information available at that time.

In the direct inhibition studies, EST64454 showed IC₅₀ values ranging from 200 to 1000 µM. The IC₅₀ could not be calculated by non-linear regression for CYP1A2, 2C9 and 2C19 due to the very low inhibition observed. Subsequent time-dependent inhibition studies confirmed these results.

A time-dependent shift in IC₅₀ was used as an initial assessment of the potential of EST64454 as time-dependent inhibitor. The 30-min pre-incubation period used in the present study has been reported to be long enough to allow interaction between the enzyme and the inhibitor, and to detect a potential for irreversible inhibition. Typically, compounds with IC₅₀ values higher than 10 µM are considered to be weak inhibitors in early Drug Discovery. Accordingly, the perpetrator potential of EST64454 as a direct CYP inhibitor will be very low. Regarding time-dependent inhibition, the interpretation of IC₅₀ shift is unclear. IC₅₀ shifts of 1.2 to 3-fold have been used in the industry as an indicative value of time-dependent inhibition. In this study, EST64454 showed IC₅₀ shifts =1-fold for all CYPs except CYP3A4 (2-fold IC₅₀ shift). The latter result could be interpreted as time-dependent inhibition. However, similar IC₅₀ values could be inferred from visual comparison of the sigmoidal curves found with and without pre-incubation. On account of the data variability found, at this stage the 2-fold IC₅₀ shift calculated for CYP3A4 was considered to be inconclusive. Therefore, these results suggest that perpetrator potential of EST64454 as time-dependent CYP inhibitor will be low for CYP1A2, 2C9, 2C19 and 2D6 whereas further studies will be needed to reach a conclusion on CYP3A4 time-dependent inhibition.

In addition to CYP inhibition, the potential of EST64454 as CYP inductor was assessed using HepaRG cells with enzyme activity as endpoint. Even though hepatocytes are the in vitro system preferred by Regulatory agencies, the lack of donor-to-donor variability makes HepaRG a useful tool in Drug Discovery for a preliminary assessment of the induction potential of compounds. Induction assessment with HepaRG cells does not exclude studies with human hepatocytes at later stages.

The concentration range of EST64454 assayed for CYP induction could not be based on human plasma concentrations, similarly to inhibition assessment. Instead, the information available on solubility, cytotoxicity and CYP inhibition was considered. Neither solubility nor cytotoxicity was a

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**Table 2. Metabolic Stability of EST64454 in Human Liver Microsomes (HLM) and Recombinant Enzymes at 10 µM after 1-h Incubation**

| Human enzyme       | Compound remaining (%) |
|--------------------|------------------------|
| HLM                | 96                     |
| HLM-NADPH          | 98                     |
| CYP1A2             | 102                    |
| CYP2A6             | 101                    |
| CYP2B6             | 104                    |
| CYP2C8             | 92                     |
| CYP2C9             | 101                    |
| CYP2C19            | 93                     |
| CYP2D6             | 94                     |
| CYP2E1             | 91                     |
| CYP3A4             | 83                     |
| FMO1               | 95                     |
| FMO3               | 101                    |
| MAO-A              | 102                    |
| MAO-B              | 96                     |

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**Fig. 3. Ion Chromatograms of EST64454 Metabolites Formed by Rat Liver Microsomes (RLM) at Times 0 and 60 min**

Incubation was performed at 37°C and 10 µM test compound.
limiting factor (unpublished results). As for CYP inhibition, concentrations <50 µM allowed studying CYP induction while minimizing CYP inhibition. Enzyme activities of 0.5 to 2-fold of vehicle control and ≤20% of positive controls suggest that EST64454 has no perpetrator potential as inductor of CYP1A2, 2B6 and 3A4 at concentrations ≤50 µM. The potential for CYP2D6 induction was not studied because CYP2D6 is not an inducible enzyme. As for CYP2C isoforms, they were not assessed because their induction is regulated by the activation of the same nuclear receptor involved in CYP3A4 induction, the pregnane X receptor (PXR). 7)

The ultimate goal of in vitro interaction studies is to ascertain whether or not drug–drug interaction studies with a probe drug should be part of the future clinical development of the
new drug. Regulatory agencies recommend an initial evaluation of direct inhibition based on the maximum unbound plasma concentration at the maximum recommended clinical dose, and on the inhibition constant of the perpetrator (i.e., EST64454). Both of them are used to predict the ratio of the area under the plasma concentration–time curve of the victim drug, $R_1$, in the presence and absence of the perpetrator. Likewise, for drugs and CYP3A4 inhibitors intended for oral use, it is also recommended to calculate the $R_{1,gut}$ ratio from the intestinal luminal concentration and inhibition constant of the perpetrator. Ratios $R_1 \geq 1.02$ and $R_{1,gut} \geq 11$ imply further investigation according to the guidelines. At this stage, we can only hypothesize that these ratios for victim drug would be found in humans at EST64454 unbound plasma concentrations $\geq 2.3 \mu M$ and oral dose $\geq 100 mg$ (assuming a volume of 250 mL and a reversible inhibition constant of $114 \mu M$ calculated from the lowest IC$50$ found). This prediction should be refined as part of the EST64454 development process.

A preliminary assessment of EST64454 reaction phenotyping was also performed with the purpose of evaluating its potential as victim of metabolism-mediated drug–drug interactions. A test compound concentration of $10\mu M$ was considered appropriate for assessing metabolism and detecting possible metabolites. At this early stage, reaction phenotyping was focused on phase I metabolism based on the test compound chemical structure. In addition to CYPs, FMO and MAO isoenzymes were also assayed as they can be involved in the metabolism of amine-containing compounds, such as EST64454.

Incubations with rat liver microsomes were carried out in parallel to support metabolite formation assessment. The selection of rat as opposed to human liver microsomes was based on the lower metabolic stability of EST64454 observed in the rat (67 vs. 96% of test compound remaining, unpublished results).

Due to the low metabolism of EST64454, metabolite formation in incubation media was helpful in identifying the enzymes involved in its metabolism. The presence of metabolites in incubation samples was only qualitative as metabolites were not synthetized at this stage. Accordingly, this approach did not allow us to ascertain the contribution of each enzyme to the overall metabolism of the compound or the contribution of individual enzymes to the formation of a specific metabolite.

The metabolism of EST64454 seems to be mainly mediated by CYP3A4 as it forms most of the metabolites detected in liver microsomes. Other enzymes involved in its metabolism were CYP2C19 and FMO isoenzymes. The participation of more than one enzyme in EST64454 metabolism is a positive characteristic for the compound because the saturation of a metabolic route when coadministered with drugs sharing the same route would be prevented. Further studies with chemical inhibitors or antibodies for specific enzymes should be conducted to confirm and complete these phenotyping results.

In addition, participation of other enzymes as well as interspecies differences in metabolite profiling should be studied at later stages.

The role of transporters in drug–drug interactions was also evaluated, specifically P-gp, one of the most studied transporters. A preliminary assessment of P-gp interaction for EST64454 was carried out in Caco-2 cells at a passage number which assured P-gp expression. EST64454 was evaluated as potential victim for P-gp interaction at $10\mu M$. This concentration is that used for routine screening of drug permeability in Drug Discovery and, as such, had no clinical relevance. Unlike the positive control, EST64454 showed an efflux ratio $<2$, thus suggesting that EST64454 is not a potential substrate of efflux transporters expressed in Caco-2 cells, including P-gp.

In view of these results, the confirmation of this finding with specific efflux transporter inhibitors was not performed. On the other hand, assuming that the permeability of EST64454 is independent of buffer pH in the apical side (pH 6.5 vs. 7.4), a good oral absorption of EST64454 can be predicted in humans ($P_{app} A\rightarrow B > 100\text{nm/s}$).

As with CYPs, the perpetrator potential of EST64454 as P-gp inhibitor was assessed in vitro using Caco-2 cells and three concentrations (2, 20 and $200\mu M$). The compound solubility in assay conditions was the only criterion used for selecting this concentration range because no information was available on its clinically relevant plasma concentrations.

When given orally, the in vivo relevance of the P-gp inhibition found at $200\mu M$ will depend on the gut luminal concentration of EST64454 and its IC$50$ for the transporter. Although the purpose of the present study was not to determine the IC$50$ for P-gp inhibition, a value of IC$50$ close to $200\mu M$ can be concluded from data observation. According to FDA guidance, when the intestinal concentration-to-IC$50$ ratio of the inhibitor is $\geq 10$, the potential for drug–drug interaction cannot be ruled out and further studies should be performed. Similarly to the preliminary estimation of in vivo relevance of CYP inhibition, an EST64454 threshold dose of $180 mg$ can be estimated for P-gp (assuming a volume of 250 mL). This prediction should be confirmed by further studies including proper IC$50$ estimation for P-gp as part of the EST64454 development process.

In conclusion, the results of this preliminary in vitro study suggest that EST64454 has a low potential to be a victim and

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Table 3. Apparent Permeabilities of EST64454 in Caco-2 Cell Monolayer after 2-h Incubation

| Substrate | Inhibitor   | Inhibitor concentration ($\mu M$) | $P_{app}$ (nm/s) | Recovery (%) | Efflux ratio |
|-----------|-------------|----------------------------------|-----------------|--------------|-------------|
|           |             |                                  | A-to-B | B-to-A | A-to-B | B-to-A |
| Digoxin   | —           | —                                | 20.4   | 292.5  | 88.0   | 104.0  | 15.2   |
| EST64454  | —           | —                                | 514.5  | 342.4  | 101.0  | 96.6   | 0.7    |
| Digoxin   | EST64454    | 2                                | 18.6   | 265.9  | 71.4   | 87.6   | 15.0   |
| Digoxin   | EST64454    | 20                               | 24.1   | 234.1  | 70.0   | 85.5   | 10.1   |
| Digoxin   | EST64454    | 200                              | 37.4   | 216.5  | 71.7   | 91.1   | 6.1*   |
| Digoxin   | Verapamil   | 100                              | 89.7   | 78.9   | 70.3   | 93.2   | 0.8    |

Probe P-gp substrate (digoxin) and inhibitor (verapamil) were included. *$p<0.05$ compared with digoxin without inhibitor. Abbreviations: A = apical, B = basolateral.
perpetrator of pharmacokinetic drug–drug interactions based on metabolism and P-gp interaction. Interaction processes should be thoroughly studied in vitro to assess their in vivo relevance and subsequent clinical trial planning.

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Conflict of Interest All authors are current or past full-time employees of ESTEVE Pharmaceuticals.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) García López M, Torrens Jover A, Díaz Fernández JL, Caamaño Moure AM, Patent WO. 2011147910A1 (2011). https://patentscope.wipo.int
2) Merlos M, Burgueño J, Portillo-Salido E, Plata-Salamán CR, Vela JM. Pharmacological modulation of the Sigma 1 receptor and the treatment of pain. Adv. Exp. Med. Biol., 964, 85–107 (2017).
3) Bruna J, Videlà S, Argyriou AA, Velasco R, Villora J, Santos C, Nadal C, Cavaletti G, Alberti P, Briani C, Kalofoños HP, Cortinovis D, Sust M, Vaqué A, Klein T, Plata-Salaman C. Efficacy of a novel Sigma-1 receptor antagonist for oxaliplatin-induced neuropathy: a randomized, double-blind, placebo-controlled phase IIa clinical trial. Neurotherapeutics, 15, 178–189 (2018).
4) Walsky RL, Obach RS. Validated assays for human cytochrome P450 activities. Drug Metab. Dispos., 32, 647–660 (2004).
5) Lahoz A, Donato MT, Picazo L, Gómez-Lechón MJ, Castell JV. Determination of major human cytochrome P450s activities in 96-well plates using liquid chromatography tandem mass spectrometry. Toxicol. In Vitro, 21, 1247–1252 (2007).
6) European Medicines Agency (EMA). Committee for medicinal products for human use (CHMP). “Draft guideline on the investigation of drug interactions, June 2012.” https://www.ema.europa.eu/en/human-regulatory/research-development/scientific-guidelines, accessed 1 May, 2019.
7) Food Drug Administration (FDA), Center for Drug Evaluation and Research (CDER). “Draft guideline for industry. In vitro metabolism- and transporter-mediated drug–drug interaction studies, October 2017.” https://www.fda.gov/drugs/guidances-drugs-all-guidances-drugs, accessed 1 May, 2019.
8) Kanebratt KP, Andersson TB. HepaRG cells as an in vitro model for evaluation of cytochrome P450 induction in humans. Drug Metab. Dispos., 36, 137–145 (2008).
9) Lu AYH, Wang RW, Lin JH. Cytochrome P450 in vitro reaction phenotyping: A re-evaluation of approaches used for P450 isoform identification. Drug Metab. Dispos., 31, 345–350 (2003).
10) Hu M, Ling J, Lin H, Chen J. Use of Caco-2 cell monolayers to study drug absorption and metabolism. Optimization in Drug Discovery. In vitro methods. (Yan Z, Caldwell GW eds.) Humana Press, Totowa, New Jersey, pp. 19–36 (2004).
11) Grimm SW, Einolf HJ, Hall SD, He K, Lim H-K, Ling K-H, Lu C, Nomeir AA, Seibert E, Skordos KW, Toon GR, Van Horn R, Wang RW, Wong YN, Yang TJ, Obach RS. The conduct of in vitro studies to address time-dependent inhibition of drug-metabolizing enzymes: a perspective of the pharmaceutical research and manufacturers of America. Drug Metab. Dispos., 37, 1355–1370 (2009).
12) Obach RS, Walsky RL, Venkatakrishnan K. Mechanism-based inactivation of human cytochrome P450 enzymes and the prediction of drug–drug interactions. Drug Metab. Dispos., 35, 246–255 (2007).
13) Ogilvie BW, Yerino P, Kazmi F, Buckley DB, Rostami-Hodjegan A, Paris BL, Joren P, Parkinson A. The proton pump inhibitor, omeprazole, but not lansoprazole or pantoprazole, is a metabolism-dependent inhibitor of CYP2C19: implications for coinadministration with clopidogrel. Drug Metab. Dispos., 39, 2020–2033 (2011).
14) Perloff ES, Mason AK, Dehal SS, Blanchard AP, Morgan L, Ho T, Dandeneau A, Crocker RM, Chandler CM, Boily N, Crespi CL, Stresser DM. Validation of cytochrome P450 time-dependent inhibition assays: a two-time point IC50 shift approach facilitates kinase assay design. Xenobiotica, 39, 99–112 (2009).
15) Guengerich FP. Cytochrome P450s and other enzymes in drug metabolism and toxicity. AAPS J., 8, E101–E111 (2006).
16) Hughes JP, Rees S, Kalindjian SB, Philpott KL. Principles of early Drug Discovery. Br. J. Pharmacol., 162, 1239–1249 (2011).
17) Yan Z, Caldwell GW. Metabolism profiling and cytochrome P450 inhibition & induction in drug discovery. Curr. Top. Med. Chem., 1, 403–425 (2001).
18) Chu V, Einolf HJ, Evers R, Kumar G, Moore D, Ripp S, Silva J, Sinha V, Sinz M, Skerjancie A. In vitro and in vivo induction of cytochrome P450: a survey of the current practices and recommendations: a pharmaceutical research and manufacturers of America perspective. Drug Metab. Dispos., 37, 1359–1354 (2009).
19) Parkinson A, Ogilvie BW. Biotransformation of xenobiotics. Casarett and Doull’s Toxicology. The basic science of poisons. (Klaassen CD ed.) McGraw-Hill Companies Inc., pp. 19–36 (2007).
20) Giacomini KM, Huang SM, Tweedie DJ, et al. Membrane transporters in drug development. Nat. Rev. Drug Discov., 9, 215–236 (2010).
21) Balimane PV, Han Y-H, Chong S. Current industrial practices of assessing permeability and P-glycoprotein interaction. AAPS J., 8, E1–E13 (2006).