Preclinical pharmacokinetics, CYP phenotyping, and tissue distribution study of novel anti-breast cancer candidate S-011-1559

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ABSTRACT

1. S-011-1559 is a tyrosine-derived novel benzoxazine CDRI molecule targeted to the oestrogen-related receptor (ER-α/β) modulator in breast cancer. To explore the pharmacokinetics of S-011-1559, a selective and sensitive bioanalytical method using LC-MS/MS was established and validated in different biological matrices of female rats.

2. Blood-to-plasma ratio and plasma protein binding (PPB) of S-011-1559 were found to be <1 and >97% in both rats and humans, respectively. The human serum albumin (HSA) and alpha-1-acid glycoprotein (AAG) binding was found in the range of 68 to 45% and 14% respectively. Half-life and intrinsic clearance by microsomal stability study were found to be 28.83 min and 0.05 mL/min/mg in rats, 78.35 min and 0.036 mL/min/mg in humans, respectively. The IC50 value of S-011-1559 against CYP isoforms was revealed to moderately inhibit CYP2D6 by a reversible non-competitive mechanism.

3. Tissue distribution of S-011-1559 on single intravenous injection at 2 mg/kg was found in the order of C lungs > C mammary gland > C spleen > C heart > C kidney > C liver > C brain.

4. The data from the present study provides crucial information about S-011-1559 for further development as a novel potential drug candidate in modulating ER-α/β receptors of lung and breast neoplasia.

Introduction

Breast cancer is the most common cancer and the first leading cause of incidence and death in women, while lung cancer is the world’s first leading cause of death according to the International Agency for Research on Cancer WHO report 2020 (Sung et al. 2021) (Momenimovahed and Salehiniya 2019).

Metastatic triple-negative breast cancer (TNBC) is a heterogeneous disease that accounts for 10–15% of all breast cancers and the standard treatment for TNBC is chemotherapy due to poor prognosis. Common target organs of metastatic TNBC are bone, lung, liver, and brain (Jin and Mu 2015). The metastatic characteristic of oncogenic cells is the leading cause of cancer-related morbidity and death (Guan 2015). Early hallmarks of the metastatic process include the loss of epithelial properties and the development of mesenchymal characteristics. In the breast cancer suffering population a haematogenous or lymphatic channel is used by breast cancer metastases to infiltrate into the lungs (Ghosh et al. 2018). Thus, current cancer treatment will be mainly reliant on medications that can prevent cancer from spreading.

Currently, medication research for the treatment of breast cancer is heavily centred on the development of pharmaceuticals that work against a specific biological target (mammary gland) with high potency, selectivity, as well as innovative therapies towards other metastatic organs (Masoud and Pagès 2017). The most notable target for breast cancer and lung cancer therapy is the oestrogen receptor (ER), which plays a key role in the treatment (Stabile et al. 2005). In prior research (Jana et al. 2021), S-011-1559 developed by CSIR-CDRI showed a larger significant impact on breast cancer than general chemotherapeutic medicines with some preliminary therapeutic activity in lung cancer when studied in vivo. The pharmacokinetics of pipeline drug S011-1559 in detail yet remains to be explored.

New chemical entities (NCEs) are well-known for their importance in the drug discovery process. Prior to clinical trials of NCEs, pre-clinical in vitro and in vivo pharmacokinetic
studies such as drug partitioning from the blood to plasma, plasma protein binding, and tissue distribution study are required to predict and reveal their efficacy and toxicity (Zhou et al. 2020). Furthermore, tissue-to-blood partition coefficient (Kp value) and biodistribution would aid in a better understanding of a drug behaviour, accumulation, and pharmacological action at the receptor site in tissue (Johanson and McQueen 2010).

In the present study, we have developed and used a sensitive, selective, and validated LC-MS/MS bioanalytical method to quantify the analyte levels of S-011-1559 in different biological matrices in female rats and in vitro PK studies. The outcomes of this study, such as validated bioanalytical method, blood to plasma partitioning, plasma protein, and human serum albumin binding, microsomal stability, reaction phenotyping, and tissue distribution study revealed that the maximum concentration of S-011-1559 was found in the lungs, with a maximum Kp value. Therefore, S-011-1559 can be further screened for activity against lung cancer and can be explored for new target-based drug delivery against oncogenic cells in the lungs.

**Materials and methods**

**Chemical and reagents**

S-011-1559 (purity > 99%) was synthesised in the Medicinal Chemistry Division of Central Drug Research Institute (CDRI), Lucknow, India. Carbamazepine (CBZ), amicon ultra filter device (Amicon® Ultra 0.5 mL; Batch number: UFC5010BK) with molecular weight cut-off (MWCO) of 10 kDa, human serum albumin (Product number: A9511) and alpha1-acid glycoprotein (Product number: G9885) were procured from Sigma Aldrich (Sigma Aldrich, MO, USA). The human liver microsome (HLM) was purchased from BD Gentest (Woburn, MA, USA). The β-Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH; Product code: 99197) was purchased from Sisco Research Laboratories Pvt. Ltd (Mumbai, India). TRIS (Trizma® base), MgCl2, phenacetin, paracetamol, coumarin, 7-hydroxycoumarin, omeprazole, diclofenac, 4'-hydroxy diclofenac, dextromethorphan, dextrophan, chlorzoxazone, 6-hydroxy chlorzoxazone, dapsone, midazolam, 1-hydroxy midazolam, rosuvastatin, bupropion hydrochloride, hydroxy bupropion, paclitaxel, 6x-hydroxy paclitaxel, 5-hydroxy omeprazole α-Naphthoflavone, pilocarpine hydrochloride, ticlopidine hydrochloride, chlorzoxazone, quercetin, sulphaphenazole, quinidine, ketoconazole, testosterone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tert-Butyl-methyl ether (TBME) and LCMS grade acetone were supplied from Merck (KGaA, Darmstadt, Germany). Heparin was purchased from the local store. Deionised water (DW) was obtained from the Merck Milli Q water purification system (18.2 MΩcm). The chemical structure of S-011-1559 is shown in Figure 1.

![Structure of S-011-1559.](image)

**Chromatographic and mass spectrometry conditions**

WATERS HPLC (Milford USA) system assembled with quaternary pump (Delta 600) and autosampler (WATERS 2707) coupled to API-3200 (applied Biosystems Sceix, Canada) triple quadrupole mass spectrometer were used for chromatographic separation and detection of S-011-1559.

Reverse phase Waters symmetry shield, C18 (150 × 4.6 mm id 5 μm) column coupled with the C-18 guard column was used for chromatographic separation of analyte and internal standard (Carbamazepine, IS). Elution of the analyte and IS was carried out in isocratic mode at a flow rate of 0.6 mL/min using acetonitrile: triple distal water (0.1% formic acid). The ratio of organic: aqueous mobile phase was kept as 68:32 (v/v) with a fixed sample injection volume of 10 μL and run time of 5 min.

Ionisation of the analyte was carried out in positive multiple reaction monitoring (MRM) mode. Electrospray ionisation (ESI) source-related parameters such as nebuliser gas (GS1), heater gas (GS2), curtain gas, collision gas, source temperature, and ion spray voltage were optimised at 45 psi, 45 psi, 16 psi, medium, 450°C, and 5500 V, respectively. Compound-dependent parameters that include de-clustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) were optimised and found to be 46, 10, 43, 2, for S-011-1559 and 40, 10, 25, 10 for CBZ, respectively. The precursor/product-ion of S-011-1559 and IS were observed at m/z 459.2 → 134.1 and m/z 237.1 → 194.1, respectively, under the above-optimised conditions. Quantification of the analyte from in vitro and in vivo samples was determined by using Analyst software, version 1.6.2 software (AB Sciex, Canada) of the LC-MS system.

**Preparation of working stock, calibration standard, and quality control samples**

The mother stock solution of 1 mg/mL was prepared in acetonitrile for S-011-1559 and IS respectively. Standard solution for calibration standard (CS) were further prepared by serial dilution of the S-011-1559 stock solution in acetonitrile with a dynamic calibration range of 0.98 – 500 ng/mL (0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 ng/mL), and IS was kept at a single concentration of 25 ng/mL. Samples of CS and QC's were prepared by spiking 5 μL of working stocks of CS and QC's into 95 μL of the matrix (tissue homogenate/plasma), further processed, and quantified. Quality
control samples (QCs) that include a lower limit of quantification (LLOQ; 1 ng/mL), low-quality control (LQC; 3 ng/mL), medium-quality control (MQC; 25 ng/mL), and high-quality control (HQC; 375 ng/mL) of S-011-1559 were prepared in acetonitrile by dilution of mother stock solution. Mother and working stock solutions of analytes were stored at 4 °C until analysis.

**Animals**

Female Sprague Dawley (SD) rats of weight 200 ± 20 g were procured from the CSIR-CDRI Laboratory Animal Division (Lucknow, India). Prior to the experiment, animals were acclimatised for one week at 23 ± 2 °C temperature under 50–70% relative humidity, and a 12/12-h light/dark cycle with an ad libitum diet and water. All animal experiments were performed according to laboratory-approved guidelines of the Institutional Animals Ethical Committee (IAEC//2020/94/Renew-1/SL.no.31) at CSIR-CDRI, Lucknow.

**Extraction optimisation**

S-011-1559 was extracted and processed from biological samples of in vitro study (human serum albumin, plasma protein binding, and blood to plasma ratio study) and in vivo plasma samples according to the previously reported protein precipitation (PPT) method (Jana et al. 2021). Various solvents including acetonitrile, methanol, and their combinations were employed for the precipitation of proteins in tissue samples, whereas, Hexane, Hexane: ethyl-acetate (80:20, v/v), TBME, and ethyl-acetate were tried for best recovery of the analyte along with least matrix effect from PPT processed tissue samples. However, to achieve maximum recovery and to overcome the matrix effect in tissue samples, PPT integrated with the liquid-liquid extraction (LLE) technique was employed. Finally, protein precipitation from tissue samples using acetonitrile followed by further liquid–liquid extraction of S-011-1559 using TBME yielded a cleaner sample for quantitative analysis.

**Tissue sample processing**

Briefly, 100 μL of tissue sample homogenate was taken and precipitated with 300 μL acetonitrile containing IS (25 ng/mL) followed by the addition of 3.5 mL tert-butyl-methyl ether (t-BME) and vortexed for 5 min. Thereafter, samples were centrifuged at 1500 g for 10 min and 2 mL supernatant was collected and dried under the nitrogen drier of Calliper Life Sciences Turbovap® LV Concentration Workstation (Canada-Mexico, USA) at 40 °C. The dried residues were reconstituted with a 100 μL acetonitrile, transferred to LC-MS/MS autosampler vial, and a 10 μL sample was injected into LC-MS/MS system.

**Validation of LC-MS/MS method**

The developed LC-MS/MS-based bioanalytical method of S-011-1559 was fully validated for selectivity, specificity, accuracy, precision, matrix effect, carryover, calibration range, and stability according to USFDA guidelines (Meesters and Voswinkel 2018).

**Plasma protein binding, non-specific binding, and human serum albumin binding study of S-011-1559 using ultrafiltration method**

A plasma protein binding study was performed in triplicate using female rat plasma and human plasma at two different concentrations (1 μM and 10 μM) as per previous reports (Taylor and Harker 2006). In brief, test samples (S-011-1559) and control samples were prepared in freshly collected plasma and incubated in a water bath at 37 °C for 30 min. Post incubation, 200 μL of the sample was withdrawn and added to the Amicon ultra reservoir of ultrafiltrate unit (UFU) related to a pre-weighted filtrate collection tube (FCT). After centrifugation of samples at 9000 g for 10 min, the UFU was inverted and exchanged among test and control samples FCT, and then recentrifuged for 2 min at 700 g. Thereafter, a 100 μL sample from the retentate of the FCT was collected, processed, and analysed for S-011-1559 quantification using LC-MS/MS.

Non-specific binding (NSB) was performed at 1 μM of S-011-1559 in triplicate. In each replicate, 200 μL of tris buffer (50 mM pH adjusted to 7.4) and plasma samples were incubated for 30 min into the FCT and UFU, respectively. After incubation, samples of FCT and UFU were diluted with an equal volume of blank plasma and blank tris buffer to ensure homogeneity of the matrix. Thereafter, a 100 μL sample was collected, processed as described above, and analysed. The percent of NSB binding was determined by using an equation.

\[
\% \text{NSB} = \left( \frac{\text{Peak area ratio of FCT}}{\text{Peak area ratio of FCT} + \text{Peak area ratio of UFU}} \right) \times 100
\]

HSA and AAG binding study of S-011-1559 was also conducted similar to the plasma protein binding ultrafiltration method. Here, HSA or albumin (ALB, 40 g/L) and AAG (1 g/L) were prepared in PBS (pH-7.4) and the study was carried out at three different concentration levels of S-011-1559 (1 μM, 5 μM, and 10 μM). The percent protein-bound (%P) of S-011-1559 was estimated by using the equation;

\[
\% P = \frac{(P_t - P_f)}{P_t} \times 100
\]

where, \( P_t \) is the total S-011-1559 concentration and \( P_f \) is the free fraction or unbound concentration of S-011-1559 in the filtrate region.

**Blood-to-plasma ratio**

Partitioning of S-011-1559 between blood and plasma was carried out at two different concentrations (1 μM and 10 μM) as per reports (Yu et al. 2005). Freshly, heparinised blood and plasma of rat and human were collected and incubated in glass vials (n = 3) at 37 °C in a water bath for 15 min.
Thereafter, 5 μL of freshly prepared working stock (prepared in acetonitrile 200X of 1 and 10 μM) of S-011-1559 were mixed into the 995 μL blood and plasma. At fixed time intervals of 0, 10, and 30 min, a sample volume of 300 μL blood, and 100 μL plasma were collected in a new microcentrifuge tube. Blood samples were centrifuged at 1000 g for 10 min, and finally, both the plasma samples (plasma collected post drug spiking in blood and drug spiked plasma) were processed according to the above-described protocol in the section followed by the S-011-1559 quantification equation

\[
\text{Blood to plasma ratio} = \frac{\text{Area ratio of analyte/IS in whole blood}}{\text{Area ratio of analyte/IS in plasma}}
\]

**Enzyme kinetics parameters (K_m and v_max) of S-011-1559 using human liver microsome**

The phase I metabolic reaction for enzyme kinetics of S-011-1559 was performed in the human liver microsome (HLM). The metabolic stability of S-011-1559 for the rate of reaction in linear range was optimised at 1 μM of S-011-1559 with various protein concentrations (0.125, 0.25, 0.5, 0.75 and 1 mg/mL) and time points (0, 5, 10, 20, 30, 45 and 60 min). Optimised protein concentration in the reaction mixture was used to determine the Michaelis-Menten constant (K_m) and reaction velocity (V_max).

Briefly, the reaction mixture of TRIS buffer (50 mM, pH 7.4) and MgCl_2 (10 mM), microsome (0.5 mg/mL) and various concentrations of S-011-1559 (0.5, 1, 2.5, 5, 10, 20, 30, 40 and 50 μM in 0.5% v/v acetonitrile) were prepared and pre-incubated for 10 min at 37°C. The reaction was initiated with 2 mM NADPH followed by a collection of samples at specified time points i.e. 0 and 30 min. The collected sample (100 μL) was quenched using ice-cold 300 μL acetonitrile containing IS (10 ng/mL). The samples were further processed and substrate consumption was quantified according to the above-described method using the calibration curve prepared with heat-inactivated microsomes. The velocity of reaction (v) at each substrate concentration (S) was evaluated from the utilised concentration/mg of protein/incubation time. The enzyme kinetic parameters (K_m; Michaelis-Menten constant, and maximum reaction velocity; V_max) were determined through the best fit curve using GraphPad Prism 5.0 software (San Diego, CA) using equation.

\[
v = \frac{V_{\text{max}} \times \langle S \rangle}{K_m + \langle S \rangle}
\]

**Microsomal stability, microsomal fraction unbound drug, and hepatic clearance of S-011-1559 in rat and human liver microsomes**

The reaction mixture for in vitro metabolic stability study of S-011-1559 was prepared similar to above-described method in 'Enzyme kinetics parameters (K_m and v_max) of S-011-1559 using human liver microsome' section and incubated in a 37°C heated water bath for 10 min. After pre-incubation, the enzymatic reactions were initiated by spiking 5 μL of S-011-1559 to make a final concentration of 1 μM (0.5%, v/v acetonitrile) in the reaction mixture and 100 μL sample was collected at specified time points (0, 5, 10, 20, 30, 45 and 60 min). The positive control reaction using testosterone and negative control reaction without NADPH was considered respectively. The samples were further processed and quantified for the determination of the percent remaining S-011-1559 at the specified time points relative to 0 min (considering to be 100%).

To determine the fraction of unbound drug in microsome by ultratfiltration method, the control mixture and reaction mixture (with 0.5 mg/mL microsome) consisted of TRIS buffer (50 mM, pH 7.4), MgCl_2 (10 mM), and 1 μM S-011-1559 incubated at 37°C in a water bath. After 30 min, 200 μL samples were withdrawn in a UFU integrated to FCT and centrifuged for 1 h at 800 g. From FCT, 100 μL of the sample was collected, processed, and analysed using LC-MS/MS. The fraction of unbound drug was calculated by using the area ratio (analyte/IS) in reaction samples to control samples (Giuliano et al. 2005).

The elimination rate constant "K_e" (1/min) of the compound was estimated through slope (time vs. log percent of remaining drug) and half-life (t_{1/2}) was calculated by using Equation (1). The in vitro microsomal intrinsic clearance (Cl_{int, m, in vitro}) was calculated using Equation (2) and the hepatic clearance (Cl_{int, H, in vitro}) for in vitro to in vivo extrapolation (IVIVE) was determined with the help of allometric scaling factor by using Equation (3). The microsomal protein per gram liver (MPPGL) was considered 50 for human and 70 for rat, respectively. In addition, the gram of liver per kilogram body weight was considered to be 21.43 for human and 40 for rat, respectively (Gabrielsson and Weiner 2001). For the prediction of in vivo hepatic clearance (Cl_{H, in vivo}), a correction factor (cf) such as fraction unbound drug in plasma (f_{up}) and microsome (f_{un}) along with the blood to plasma ratio (b/p) was calculated and used. The calculated in vivo hepatic clearance data with (Cl_{H, cf, in vivo}) and without correction factor (Cl_{H, in vivo}) were further extrapolated on the well stirred model by using Equation (4) for the final prediction of in vivo hepatic clearance (Obach et al. 1997).

\[
t_{1/2} = \frac{(0.693)}{K_e}
\]

\[
Cl_{int, m, in vitro} = \frac{(0.693)}{t_{1/2}} \times \frac{\text{Reaction mixture volume (mL)}}{\text{Protein concentration (mg)}}
\]

\[
Cl_{int, H, in vitro} = Cl_{int} \times \frac{\text{mg of protein}}{\text{gram of Liver}} \times \frac{\text{gm of liver}}{\text{kg of body weight}}
\]

\[
Cl_{H, in vivo} = \frac{\left[Q \times f_{un} \times (\text{Cl}_{H, cf, in vivo})\right]}{b/p}
\]

where, Q represents hepatic blood flow.
IC_{50} determination of S-011-1559 for CYP isoforms enzymes in HLM

The in vitro inhibition profile of S-011-1559 in HLM for major nine CYP enzymes (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and CYP3A4) was determined as recommended by U.S. FDA. Cocktail assay approach was followed for the comparison of metabolites that results from the interaction of S-011-1559 and standard inhibitor with specific CYP substrates and metabolites as represented in Supplementary (S) Table S1. For quantification of metabolites, metabolite-specific MRM transition in both positive and negative modes was used from a previously reported article to evaluate the IC_{50} (Kim et al. 2005) (Bhateria et al. 2016).

The reaction mixture of tris buffer (50 mM, pH 7.4), MgCl_{2} (20 mM), protein HLM (0.5 mg/mL), CYP substrate cocktail, and S-011-1559 (0.0001 to 100 μM) was preincubated for 15 min in water bath at 37°C. Initiation of the reaction was done by spiking NADPH (4 mM). After 20 min, 100 μL of reaction sample was quenched in 300 μL ACN containing IS (0.1 μM of CBZ for positive mode and chlorthalidone for negative mode). The samples were processed according to the optimised bioanalytical method.

A positive control (standard inhibitor) and control samples (without any inhibitor) were processed in parallel similar to the reaction samples. The data were then analysed by comparing the metabolite formation of the reaction sample to the control sample represented in the form of percent CYP activity using the following equation

\[
\% \text{ CYP Activity} = \frac{\text{Area ratio of metabolite in test sample}}{\text{Area ratio of metabolite in control sample}} \times 100
\]

The IC_{50} value was estimated by plotting of % CYP control activity versus log of inhibitor concentrations by using the non-linear regression model in GraphPad Prism 5.0 software (San Diego, CA).

Evaluation of inhibition rate constant (K_{i}) and the mechanism of inhibition in HLM

Determination of inhibition rate constant (K_{i}) and the mechanism of inhibition for S-011-1559 on specific CYP isoform was performed in HLM based on the IC_{50} value evaluated above. The K_{i} assay was performed for specific CYP2D6 enzyme by using substrate (dextromethorphan) concentration in the range of 0.93–14.88 μM and inhibitor (S-011-1559) concentrations (0.55 – 55 μM). The positive control assay was also conducted using a standard quinidine inhibitor at a concentration ranging from 0.001 to 1 μM. The following assay was carried out in the same manner as the IC_{50} inhibition assay in triplicate. The K_{i} and mechanism of inhibition were calculated by fitting the type of enzyme inhibitory model as nonlinear regression models (competitive, non-competitive, uncompetitive, and mixed type of inhibition) using Phoenix WinNonlin version 7.0 (Pharsight Corporation, Mountain View, USA). The mechanism of inhibition was visually described by using a double reciprocal Lineweaver-Burke plot.

IC_{50} shift of S-011-1559 using time-dependent CYP inhibition (TDI) assay

A time-dependent CYP inhibition (TDI) assay was performed in HLM to predict accurately in vivo drug–drug interaction and also to assess the decrease in enzyme activity by the irreversible inhibition of CYP2D6 with respect to incubation time. The TDI assay was performed on the various concentration of S-011-1559 in the presence and absence of NADPH and it was performed according to the procedure described above in IC_{50} determination.

Briefly, the reaction mixture includes tris buffer (50 mM, pH 7.4), MgCl_{2} (10 mM), HLM (0.5 mg/mL), and S-011-1559 (0.0001-100 μM concentration) with and without NADPH (4 mM). This was pre-incubated for 30 min at 37°C in a shaking water bath (Julabo, Germany). Consecutively, to determine IC_{50} shift in the second assay, a 10-μL aliquot of the pre-incubated reaction mixture was added to another pre-incubated reaction mixture of tris buffer (50 mM, pH 7.4), MgCl_{2} (10 mM), HLM (0.5 mg/mL), NADPH (4 mM), and dextromethorphan concentration of (5X K_{i}) as substrate in triplicate. After 15 min of incubation, 100 μL of reaction sample was quenched in 300 μL IS containing ACN. The samples were further processed and quantified using the bioanalytical method described above. The IC_{50} shift values for the TDI were evaluated by plotting the percent activity of enzyme versus log conc of S-011-1559 in the presence and absence of NADPH.

Pharmacokinetic and tissue distribution study in female SD rat

The plasma PK profile and tissue distribution profile of S-011-1559 were carried out in female SD rats (A total of 39 animals, n = 3 for each time point) on intravenous administration (IV) through the tail vein at a 2 mg/kg dose. S-011-1559 solution for IV administration was prepared as reported in previously published literature (Jana et al. 2021). Blood samples were collected terminally into the polypropylene-coated microcentrifuge tubes containing heparin (22 IU/mL) at pre-defined time points of 0.084, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16 and 24 h respectively. Plasma samples were separated and collected from the blood by centrifugation at 1500 g for 15 min. In addition, tissue samples (lung, liver, heart, brain, kidney, mammary gland, and spleen) were also collected simultaneously and rinsed thoroughly with normal saline to remove the blood and then blotted with filter paper. After weighing, each tissue sample was individually homogenised with deionised water (1:3, w/v) by using a homogeniser (IKA®, T18 digital Ultra TURRAX®). All samples were immediately stored at −70°C ± 10°C until analysis. S-011-1559 was further extracted from the plasma and tissue samples for bioanalytical quantification according to the procedure described in ‘Extraction optimisation’ section. To derive a more accurate tissue concentration of the analyte, a
blood volume correction factor was applied (Chandasana et al. 2016).

Corrected tissue concentration (ng/g) at a time = \{tissue concentration (ng/g) at a time – \{plasma concentration (ng/mL) at a time x tissue correction factor\}\}.

Pharmacokinetic parameters estimation

The PK parameters of S-011-1559 were evaluated by Phoenix WinNonlin software version 6.3 (Pharsight Corporation, Mountain View, CA, USA) using a non-compartmental analysis model. The PK parameters derived from the best fit NCA model included the area under the plasma concentration–time curve from the infinity (AUC\(_{0-\infty}\)), plasma concentration (C\(_0\)), time to reach the maximum concentration (T-max), half-life (t\(_{1/2}\)), clearance (CL) and volume of distribution (V\(_d\)).

Results

Method development

An API-3200 LC-MS/MS (Applied Biosystems system) coupled with an electrospray ionisation (ESI) source was successfully applied to quantify S-011-1559. Ionisation was carried out in both positive and negative polarity modes to confirm the mass spectrometer parameters for the detection of S-011-1559. Finally, the most intense precursor ion and product ion were obtained abundantly in positive mode as compared to negative mode, which was due to the presence of nitrogen atom in the analyte ring (Qian et al. 2001). Therefore, the precursor ion m/z 459.4 of S-011-1559 was operated on positive ion polarity and characterised due to the predominant generation of [M + H]\(^+\) ions in the full scan. The key MRM transitions of precursor/product-ion at m/z 459.4/134.2 for quantitative analysis of the analyte are shown in Figure 2 and the proposed schematic fragmentation pathway of S-011-1559 due to loss of -C\(_{13}\)H\(_{18}\)NO and -C\(_8\)H\(_{10}\)O fragments is shown in Figure 3.

The chromatographic conditions such as column, column temperature, and choice of solvent were optimised after several experimental runs to obtain better chromatographic resolution and sensitivity. Finally, Waters Symmetry shield, C18 (100 × 4.6 mm, ID 5 \(\mu\)m) column was found to be most suitable in the separation of the analyte under isocratic elution using ACN: DW (0.1% formic acid) at a flow rate of 0.6 mL/min within 5 min of retention time.

The use of structural analog or stable labelled isotope as an IS is well known and extensively reported in the literature. The ratio of blood-to-plasma concentration (\(C_p/C_d\)) was found to be less than 1 (\(n = 3\)), suggesting that the distribution of S-011-1559 is considerably higher towards plasma in comparison to blood cells. The average blood-to-plasma ratio for female SD rats and human at 1 \(\mu\)M and 10 \(\mu\)M was found to be 0.60 ± 0.03 and 0.49 ± 0.27, and 0.84 ± 0.07 and 0.87 ± 0.21, respectively.

Analyte extraction optimisation from plasma and tissue

A number of bioanalytical techniques for the extraction of analyte and IS using a combination of different solvents were trialled, of which PPT and PPT followed by LLE were found to generate satisfactory recovery overcoming matrix concerns (hydrophilic interference of plasma components). The simple PPT method is an easy single-step technique and it was applied for the extraction of analyte from plasma samples due to insignificant interference by the plasma components. However, extraction of the analyte from tissues on the application of PPT was found to be poor. LLE technique using a range of polar and nonpolar solvents was found to dramatically reduce the matrix effect. Although the liquid–liquid extraction method could minimise the matrix effect, a sound recovery remains a concern to resolve. Thus, PPT followed by LLE was finally chosen to obtain a good recovery with a minimum matrix effect. Finally, ACN was chosen as a protein precipitating agent breaking more complex matrices of the tissues followed by the extraction of an analyte using tert-butyl methyl ether(t-BME) as an LLE solvent yielding a maximum recovery of the analyte with minimum matrix effect.

Assay validation

Under the optimised conditions, S-011-1559 and IS were detected at the retention time of 1.7 and 2.86 min respectively. The developed bioanalytical method was found to be selective and specific for quantification of the analyte extracted from plasma and tissues without any significant interference of matrix at the retention time.

A calibration curve (0.98 to 500 ng/mL), correlation coefficients, intra-day and inter-day accuracy, precision, stability under different storage conditions, mean extraction recovery, carryover effect, dilution integrity of S-011-1559 have been represented in the Supplementary Materials in Tables S2-S5. No carry-over effect of the analyte was found post-ULOQ injection and the analyte maintained its integrity on 6X dilution with the same matrix.

The chromatograms of blank liver homogenate or spiked with S-011-1559 and IS along with a pharmacokinetic sample upon intravenous administration of S-011-1559 are shown in Figure 4.

Blood-to-plasma ratio

The ratio of blood-to-plasma concentration (\(C_p/C_d\)) was found to be less than 1 (\(n = 3\)), suggesting that the distribution of S-011-1559 is considerably higher towards plasma in comparison to blood cells. The average blood-to-plasma ratio for female SD rats and human at 1 \(\mu\)M and 10 \(\mu\)M was found to be 0.60 ± 0.03 and 0.49 ± 0.27, and 0.84 ± 0.07 and 0.87 ± 0.21, respectively.
Plasma protein binding

PPB is an indicative measurement of bound and unbound drugs that reflect drug distribution, clearance, pharmacodynamic activity and help in the safety evaluation of toxicokinetic studies (Bohnert and Gan 2013). The percent of PPB of S-011-1559 at 1 μM and 10 μM was found to be 99.63 ± 0.04 and 99.85 ± 0.11 in the plasma of female SD rats and 98.16 ± 2.52 and 97.15 ± 1.46 in human plasma, respectively. Non-specific binding of S-011-1559 was found to be less

**Plasma protein binding**

Figure 2. Represents, MS/MS spectra of S-011-1559 were shown as the most prominent product ion from the parent or precursor ion.

Figure 3. The Schematic proposed fragmentation pathway of S-011-1559 represented as parent ion (m/z 459.26) and its product-ion (m/z 134) by the loss of mass m/z 122.07 and m/z 204.14.
than 2.8% which indicated the negligible NSB of the compound.

S-011-1559 was found to be a lipophilic compound and slightly basic or neutral nature with an estimated ClogP-value of 5.7 as evaluated by Chemdraw professional (version 15.0) software. The binding of S-011-1559 in HSA and AAG was determined at physiological pH-7.4 at different concentrations.

The average binding of S-011-1559 at 1 μM, 5 μM, and 10 μM concentration in HSA and AAG was found to be 69.81 ± 4.95, 68.81 ± 2.80, 47.68 ± 6.11%, and 14.93 ± 1.81, 18.09 ± 1.08, 19.38 ± 4.26% respectively. The percent free form of S-011-1559 in human serum albumin is shown in Figure 5.

**Enzyme kinetics parameters (Km and Vmax) of S-011-1559 in human liver microsome**

To determine km and Vmax and to ensure that the reaction was in the linear phase, the microsomal protein concentration and incubation time were optimised. About 20 min post-incubation in HLM, the linearity of S-011-1559 in microsomal stability was found to be up to 1.0 mg/mL microsomal protein. For Michaelis–Menten (MM) calculation, the assays were performed at 0.5 mg/mL for a 20-min incubation time. The Km and maximal velocity rate (Vmax) were found to be 15.77 ± 1.78 μM and 0.32 ± 0.015 μmol/min/mg respectively. A graphical representation of the enzyme kinetic profiles (velocity vs. concentration of the S-011-1559) is shown in Figure 6.
In vitro microsomal stability and predicted hepatic clearance

Metabolic stability of S-011-1559 was performed in human and rat liver microsome (RLM) along with positive control (testosterone). The percent remaining drug at 60 min was found to be 55.68 ± 5.35% in HLM, and 24.25 ± 0.63% in RLM as represented in Figure 7. The fraction of unbound drug in microsome for human and rat was found to be 0.01 ± 0.00 and 0.02 ± 0.01 respectively. Half-life, K_e, \( C_{int,H}, \) \( C_{int,R}, \) in \( vitro \), \( C_{int,H}, \) \( H, \) in \( vitro \) and in vivo hepatic clearance (CLH) parameters such as \( C_{int,H}, \) \( cf \) in \( vivo \) and \( C_{H,cf \, wc}, \) \( wcf \) in \( vivo \) predicted by the well-stirred model for rat and human is shown in Table 1.

**IC\(_{50}\) determination of S-011-1559 in CYP isoforms**

The IC\(_{50}\) value of S-011-1559 was found to be 5.57 ± 0.1 \( \mu \)M against CYP 2D6 with moderate inhibition. In addition, the IC\(_{50}\) value of S-011-1559 against the remaining CYP isoforms is reported in Table 2.

Inhibition rate constant (K\(_i\)) and the mechanism of CYP2D6 inhibition by S-011-1559

Following enzymatic assays, the inhibition rate constant (K\(_i\)) was found to be 8.08 \( \mu \)M. This concentration was specific for CYP2D6 isoforms that followed a non-competitive inhibitor mechanism. The lineeweaver-Burk plot reveals S-011-1559 to exhibit non-competitive inhibition plotted as nonlinear regression and it is shown in Figure 8.

![Figure 6](image1.png)

**Figure 6.** The Michaelis-Menten kinetics of S-011-1559 in humans, data are represented in mean ± SD (n = 3) for the determination of \( K_m \) and \( V_{max} \).

![Figure 7](image2.png)

**Figure 7.** Microsomal stability of S-011-1559 with and without NADPH at 37 °C, with A) human and B) rat liver microsomes to Phase-I dependent metabolism. The percent remaining of S-011-1559 compared with control samples at time zero (100%).

| Liver microsomes | \( t_{1/2} \) (min) | \( K_e \) 1/min | \( C_{int,H}, \) \( \text{in vitro} \) (mL/mg/min) | \( C_{int,R}, \) \( \text{in vitro} \) (mL/min/kg) | \( C_{H,cf}, \) \( \text{in vivo} \) (mL/min/kg) | \( C_{H,wc}, \) \( \text{in vivo} \) (mL/min/kg) |
|------------------|---------------------|----------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Human            | 78.35 ± 15.28       | 0.009 ± 0.002  | 0.036 ± 0.006                   | 54.31 ± 9.53                   | 52.35                           | 329.50                          |
| Rat              | 28.83 ± 0.74        | 0.023 ± 0.0005 | 0.05 ± 0.001                    | 96.18 ± 2.51                   | 12.07                           | 21.46                           |

**Table 1.** The clearance parameter of S-011-1559 was estimated by microsomal stability in human and rat liver microsomes.

| Inhibitor                      | CYP 1A2 | CYP 2B6 | CYP 2A6 | CYP 2E1 | CYP 2D6 | CYP 2C9 | CYP 2C8 | CYP 2C19 | CYP 3A4 |
|--------------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| S-011-1559                     | >100    | >100    | >100    | >100    | 5.57 ± 0.1 | 40.5 ± 0.6 | 37.2 ± 17.6 | 45.1 ± 0.1 | >100 |
| \( \alpha \)-Naphtho flavone  | 0.31 ± 0.08 | >100    | >100    | >100    | 3.69 ± 1.1 | 14.03 ± 0.8 | 0.32 ± 0.26 | 0.64 ± 0.1 | 0.16 ± 0.1 |
| Ticlopidine                    | 0.5 ± 0.2 | 0.5 ± 0.2 | 3.69 ± 1.1 | 14.03 ± 0.8 | 0.32 ± 0.26 | 0.64 ± 0.1 | 0.16 ± 0.1 | 0.12 ± 0.06 | 0.14 ± 0.06 |
| Pilocarpine                    |         |         |         |         |         |         |         |         |         |
| Di-ethyl thio-carbamate (DETC) |         |         |         |         |         |         |         |         |         |
| Quinidine                      |         |         |         |         |         |         |         |         |         |
| Quercetin                      |         |         |         |         |         |         |         |         |         |
| Sulphaphena-zole               |         |         |         |         |         |         |         |         |         |
| N-benzyl nirvanol              |         |         |         |         |         |         |         |         |         |
| Ketoconazole                   |         |         |         |         |         |         |         |         |         |

**Table 2.** IC\(_{50}\) values of S-011-1559 and standard inhibitor against CYP isoforms in human liver microsome.
IC\textsubscript{50} shift of S-011-1559 using time-dependent CYP inhibition (TDI) assay

The TDI is represented by shifting of inhibition curve towards a lower IC\textsubscript{50} value in the presence of NADPH. From the assay, it was found to have no remarkable shift in the IC\textsubscript{50} values in the presence of NADPH. This demonstrates that S-011-1559 is not a time-dependent inhibitor and the IC\textsubscript{50} shift curve is shown in Figure 9.

Pharmacokinetic and tissue distribution

Pharmacokinetics and tissue distribution profile of S-011-1559 in female SD rat on IV bolus administration at 2 mg/kg were successfully determined using the developed and validated bioanalytical LC-MS/MS method. The mean plasma and tissue concentration-time profile of S011-1559 are shown in Figure 10, and the mean pharmacokinetic parameters deduced are listed in Table 3. S-011-1559 was found to be widely distributed across different tissues of which a major chunk was found in the lung tissue.

Discussion

Patients suffering from breast cancer for a prolonged duration results in metastasis of oncogenic cells to other healthy organs including the lungs even after mastectomy. S-011-1559 is a promising agent against triple-negative breast...
cancer and PK provides insight to optimise the dose, dosage regimen, and applicability of the compound.

To study PK, a bioanalytical method was developed using LC-MS/MS method. The mass spectrometric approach was chosen because of its high specificity, sensitivity, and selectivity as compared to HPLC. High cost and difficulty in the procurement of novel synthetic molecules create limitations in the initial stages of drug discovery with an emphasis on pharmacokinetic study.

This developed bioanalytical LC-MS/MS method of S-011-1559 is fully validated for application to in vitro and in vivo pharmacokinetic studies with high sensitivity as well as selectivity.

The blood-to-plasma ratio is a crucial parameter for predicting the whole-body pharmacokinetics of an NCE. The blood-to-plasma ratio study gives an indication of drug partitioning either in blood or plasma along with drug affinity towards erythrocytes and further concentration that may lead to hematotoxicity (Yu et al. 2005). From this study, the tendency towards erythrocytes and further concentration that may remain unsaturated at both concentrations. On contrary, S-011-1559 bound to HSA was found to reach saturation at 10 μM which results in a higher free form of S-011-1559. In addition, variability of AAG concentration under diseased and normal conditions may alter the free form of the drug. The amount of S-011-1559 bound to HSA and AAG may further exhibit drug-drug interaction. The results of this study affirm the balance between the free and bound form of the drug in the plasma of the target site to elicit a therapeutic effect.

Microsomal stability data of S-011-1559 reveal that its metabolism takes place mainly by the CYP-dependent Phase-I reaction. CYP2D6 was found to be moderately inhibited (IC_{50} value < 10 μM) in a non-competitive reversible mechanism by S-011-1559 with no inhibition against other CYP isoforms that may result in drug-drug interaction. CYP2D6 polymorphisms are expressed differently in each person for which pharmacological dosage studies can result in either an unfavourable effect or no drug response (Ingelman-Sundberg 2005).

The results of the tissue distribution study reveal that S-011-1559 spreads quickly and widely to all the tested tissues. Significant higher levels of S-011-1559 in lungs at 5 min could be affected by blood perfusion and lipophilicity of the compound which is critically related to tissue: plasma partition coefficient (Kp) value. The Kp value of S-011-1559 for tissues was found in order of lung > mammary gland > spleen > kidney > Heart > liver > brain, which was consistent with the trend of tissue concentration, demonstrating drug dispersion in tissues. In addition, S-011-1559 has also been demonstrated to exhibit pharmacological activity against metastasis of lung cancer in preclinical studies (Jana et al. 2021). Hence, in addition to the breast, the lungs may be considered a new target organ for S-011-1559 that opens up new avenues for drug development against oncogenic cells of the lungs.

Table 3. Pharmacokinetic parameters in the female Sprague Dawley rat of plasma and tissues by intra-venous administration of S-011-1559 (2 mg/kg dose). Values are represented as mean ± SD (n = 3).

| Matrix      | Kp (ng/g) | C0 (ng/mL) | AUC_{inf} (h^*ng/g) | Vz (L/kg) | CL (g/h/kg) | t1/2 (h) | Tmax (h) | MRT (h) |
|-------------|-----------|------------|---------------------|------------|-------------|----------|----------|---------|
| Lung        | 23.54 ± 1.65 | 1875.94 ± 17.39 | 10521.28 ± 737.59 | 1449.32 ± 353.00 | 190.56 ± 13.36 | 5.24 ± 0.92 | 0.08 ± 0.00 | 7.95 ± 1.20 |
| Liver       | 0.81 ± 0.11 | 65.28 ± 0.70 | 360.07 ± 50.01 | 42132.27 ± 1536.66 | 5608.49 ± 779.01 | 5.27 ± 0.92 | 0.5 ± 0.00 | 8.56 ± 1.19 |
| Spleen      | 3.42 ± 0.19 | 187.10 ± 5.58 | 1529.06 ± 502.86 | 8935.53 ± 789.4 | 1560.63 ± 506.28 | 3.52 ± 1.45 | 0.25 ± 0.00 | 7.35 ± 0.54 |
| Kidney      | 2.59 ± 0.41 | 433.68 ± 62.32 | 1157.62 ± 184.48 | 9312.30 ± 1786.17 | 1749.88 ± 278.86 | 3.79 ± 1.31 | 0.29 ± 0.19 | 5.58 ± 0.54 |
| Heart       | 3.45 ± 0.66 | 1073.8 ± 36.44 | 1544.32 ± 296.88 | 7255.32 ± 639.56 | 1319.44 ± 539.65 | 3.85 ± 0.44 | 0.08 ± 0.00 | 5.14 ± 0.45 |
| Brain       | 0.79 ± 0.07 | 194.32 ± 36.84 | 357.08 ± 30.34 | 26565.22 ± 8061.77 | 5621.35 ± 477.71 | 3.24 ± 0.72 | 0.03 ± 0.00 | 4.31 ± 0.22 |
| Mammary gland | 13.27 ± 2.10 | 514.3 ± 129.4 | 5930.75 ± 937.57 | 3376.37 ± 228.66 | 341.49 ± 53.99 | 6.90 ± 0.63 | 4 ± 2.83 | 8.21 ± 0.28 |
| Plasma      | 307.56 ± 61.52 | 446.91 ± 7.56 | 25.94 ± 6.98 | 4.52 ± 0.17 | 3.95 ± 0.91 | 0.08 ± 0.00 | 5.19 ± 0.32 |

Each value denotes mean ± SD, Kp represents the ratio of AUC tissue to plasma AUC.

In conclusion, before the conduct of clinical trials for a new chemical entity, in vivo preclinical pharmacokinetic studies should be checked extensively. This is the first report that presents the pharmacokinetic behaviour of S-011-1559 as an NCE which opens up further research avenues to conduct in vivo animal studies. The developed and fully validated bioanalytical method was applied successfully in the quantification of S-011-1559 in the female rat plasma and tissues.
within 5 min. The compound also has an affinity towards lungs, mammary gland, and spleen as compared to other highly perfused organs that favours further development of the molecule. With this bioanalytical method, data from in vitro and in vivo preclinical tissue distribution study can be utilised for further design and anticipation of S-011-1559 in the preclinical and clinical studies.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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